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Edward K.L. Chan
Marvin J. Fritzler *Editors*

Ten Years of Progress in GW/P Body Research

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Preface

The field of RNA degradation has attained significant recognition over the past decade for many reasons. One is that new technologies and methods have been devised that allow detailed molecular mechanisms to be elucidated and another is the success of using the genetically tractable budding yeast. During this period, investigators have noted the differences as well as the similarities between yeast and mammalian mechanisms of RNA turnover. One common feature is that the RNA processing machinery is localized to cytoplasmic particles that regulate mRNA translation and decay. These include most prominently the cytoplasmic glycine-tryptophan-motif-containing "GW Bodies," also called "Processing Bodies," and the stress granules. As was true of nuclear foci called "nuclear speckles" that were studied in the 1980s and 1990s, the discovery of GW/P bodies and stress granules is driving much biological research in the first decades of the twentieth century. Therefore, the publication of the book entitled: "Ten Years of Progress in GW/P Body Research" is very timely.

Both nuclear and cytoplasmic ribonucleoprotein foci are biologically intriguing, in part, because of the mere beauty of the microscopic images that when combined with molecular tools can be surmised to carry out essential cellular functions. Therefore, while the internal molecular workings indicate key roles for GW/P bodies and stress granules in gene expression, both have been challenging to dissect. The discovery of GW/P bodies described in this book occurred as a co-discovery of the GW bodies and P bodies by Fritzler and Chan at the University of Calgary and the Scripps Research Institute and Sheth and Parker at the University of Arizona, respectively. There had been earlier indications of localized RNA decay/translation granules by Wolf-Dietrich Heyer and by Satoru Kobayashi as well as others, but the concepts solidified in 2003 with the combined biochemical and visual data are described in this book.

When the first images of GW bodies in mammalian cells and P bodies in yeast cells were observed, the experience must have been aesthetically quite pleasing. Yet, few could have imagined at the time that these cell foci held the core RNA processing machinery central to gene expression that would go on to occupy the efforts of hundreds of biologists. The GW/P bodies contain RNA regulatory factors,

including RNA-binding proteins, enzymes and small noncoding RNAs of which the microRNAs are best known. Other molecules are found in the cytoplasmic foci such as Argonaut and PIWI of the RNA Interference Silencing Complex (RISC) as well as the expanded GW protein family to which the autoantibody of Chan and Fritzler was derived from the serum of a neurology patient. These co-discoveries were serendipitous as the Chan/Fritzler group came from the field of autoantibodies and autoimmunity that has led many important discoveries in RNP biology, and the Sheth/Parker group was investigating the underlying mechanisms of RNA decay. This book richly explores these original scientific discoveries and many of the subsequent detailed studies of the components and functions of RNA processing that take place in GW/P bodies. The author list is truly impressive and reading the history will pique the interests of students and senior investigators, and hopefully, provide insights into future directions of the field of RNA biology.

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Chapter 1
Introduction: The GW Body Story as an Example of Autoantibodies with Significant Impacts to Molecular Cell Biology

Edward K.L. Chan and Marvin J. Fritzler

Historically many antibodies, known as autoantibodies, directed to self-proteins have been identified as diagnostic biomarkers for systemic rheumatic diseases. For example, anti-double-stranded DNA and anti-Sm antibodies are markers for the classical autoimmune immune disease systemic lupus erythematosus (SLE) and have become components of the criteria for the disease classification (Tan et al. 1982). During the early years as more and more autoantibodies became known, the characterization of autoantibody specificity shed light on disease pathogenesis. For example, the level of anti-double-stranded DNA antibodies is often correlated to the severity of glomerular nephritis and is thought to participate in immune-mediated injury to tissues. Since the 1970s, the more modern era of autoantibody research, there have been several major developments. Some of the earlier techniques included double immunodiffusion, also known as the Ochterlony test, was the leading technique used in the identification and characterization of anti-Sm (Tan and Kunkel 1966), anti-RNP (Sharp et al. 1972), anti-SS-A/Ro, and anti-SS-B/La antibodies (Alspaugh et al. 1976), all of which helped support the clinical diagnosis of SLE, mixed connective tissue disorders (MCTD), and Sjögren's syndrome, respectively. This was followed by a new technology using indirect immunofluorescence on HEp-2 cells and this led to an enduring connection with cell biology because one could more readily appreciate distinct intracellular structures identified by many autoantibodies. Prior to immunofluorescence, the nature of most target autoantigens was not clear. The emergence of indirect immunofluorescence has advanced the discovery

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of many other autoantibodies that were not readily detected by the Ochterlony and other related immunoassays.

Many years ago, we both were trainees in the laboratory of Eng M. Tan, a pioneer in the discovery of many autoantibodies in systemic rheumatic diseases, at what was then named Scripps Clinic and Research Foundation in La Jolla, California (later renamed The Scripps Research Institute). During the postdoctoral training from November 1976 to June 1978, MJF was an active participant in the discovery of PCNA (Miyachi et al. 1978), proliferating cell nuclear antigen, a protein that is known to many investigators in different disciplines, including pathology, cell biology, and rheumatology to name just a few, but probably very few investigators appreciate the history of the discovery and naming of this interesting protein antigen from the study of autoantibodies exhibited in a few interesting SLE patients. The distinctive S-phase-specific nuclear indirect immunofluorescence pattern produced by anti-PCNA antibodies in HeLa and other tissue culture cells is a classical illustration of the beginning of rapid appreciation of how human autoantibodies could be important tools for cell and molecular biology (Tan 1989). This was an era when genome sequencing and the mechanisms of DNA replication were still quite underdeveloped. To date, more than 15,000 publications catalogued in PubMed are associated with PCNA.

In the same time period, another equally important piece of work related to cell and molecular biology was the identification of autoantibodies to centromere/kinetochore in sera of a subset of patients with scleroderma (Moroi et al. 1980), the CREST syndrome, or the limited cutaneous subset of systemic sclerosis (Fritzler and Kinsella 1980). The identification of anti-centromere allowed the subsequent characterization of the CENP proteins A, B, and C, which are major target autoantigens and important components of centromere/kinetochore. This was the beginning of the discovery of a whole series of CENP proteins and opened many new research avenues. According to PubMed, there are more than 950 and 600 publications on CENP proteins and anti-centromere antibodies, respectively. Thus the discovery of anti-centromere autoantibodies was important for both the clinical diagnosis of scleroderma and as an approach to the identification of new markers for the study of the molecular and cell biology of the centromere/kinetochore.

When EKLC joined the Tan laboratory in La Jolla as a postdoctoral fellow in November 1983, there were many exciting studies on the biochemical and immunochemical characterization of Sm/RNP, SS-B/La, SS-A/Ro, PCNA, and a number of nucleolar-specific autoantibodies. The nucleolar targets were identified as RNA polymerase I (Reimer et al. 1987b), the U3 ribonucleoprotein (Reimer et al. 1987a), and PM-Scl complex/exosome (Reimer et al. 1986). One study worthy of special mention was the identification of autoantibodies to the coiled body and the cloning of the marker protein p80-coilin (Raska et al. 1990; Andrade et al. 1991). The availability of p80-coilin as a marker for this nuclear body generated tremendous interest in this structure, which was first identified by the famous Spanish cytologist Ramón y Cajal some 100 years ago and the structure was later renamed Cajal body (Gall 2000). PubMed lists 680 articles on Cajal bodies and >200 reports on the protein coilin.

As a continuation of the celebration of the success in autoantibodies as useful probes in molecular and cell biology, the current volume is dedicated to the identification of the cytoplasmic bodies identified as GW bodies, once again using human autoantibodies (Eystathioy et al. 2002). The history of how GW bodies were identified using human autoantibodies is outlined in Chap. 2. As is often the case in scientific research, practically the same structure was identified independently as processing bodies or P bodies. The history of the discovery to P bodies is addressed in Chap. 3 by Roy Parker and colleagues. The linkage of GW bodies to the RNA interference pathway in part coincided with the move of the EKLC laboratory to the University of Florida, where he linked projects with the laboratory of Minoru Satoh, who has a long history in the study of a little known autoantibody known as Su. It was through this collaboration that Su was identified as an Ago protein, which is highly enriched in GW bodies. The discovery of anti-Su/Ago2 is summarized in Chap. 4 by Minoru Satoh and colleagues. Another dominant self-antigens in these GW/P bodies is Ge-1, a target autoantigen in primary biliary cirrhosis, which was discovered independently by Donald Bloch and colleagues and this is described in Chap. 5.

Chapters 6, 7, 8, 9 and 10 are contributions by laboratories that have lead studies on the role of GW182 and Ago2 in the miRNA pathway. Chapter 6 summarizes the work on functional domains required for GW182 binding to Ago2 and translational repression. Chapter 7 from the laboratory of Shobha Vasudevan outlines the current status of miRNA-mediated stimulation of gene expression. Chapter 8, contributed by Andrew Simmonds and colleagues, describes their study of Drosophila homologue of GW182, which they have named Gawky. Chapter 9 from the laboratory of Elisa Izaurralde summarizes their extensive work on the characterization of GW182 function in miRNA-medicated gene silencing. Chapter 10 authored by Anthony Leung and Philip Sharp discusses their findings on Ago proteins with respect to GW/P bodies.

Two GW182-dependent events mediated by miRNA targeting of mRNA is the relative contribution to translation repression, a process that theoretically is reversible and derepressed mRNA can return to active translation, or deadenylation, bringing target mRNA toward the decay pathway that likely takes place in GW/P bodies. Chapter 10 from Ann-Bin Shyu's laboratory addresses the issue of deadenylation associated with P bodies. Chapter 12 from Georg Stoecklin and Nancy Kedersha discusses the relationship of GW/P bodies with stress granules, which are well-established participants in the storage of mRNA during stress responses. Chapter 13 further discusses the possible relationship of GW/P bodies with other cytoplasmic ribonucleoprotein-containing structures. Chapter 14 is an interesting study of single nucleotide polymorphisms (SNP) in the trinucleotide repeat region of the GW182/TNRC6A gene identified in certain patients bearing anti-GW182 autoantibodies.

These chapters represent only a cross section of the exciting working in GW182 and its role in miRNA-mediated functional activities. Chapter 15 is a summary of other exciting developments, currently unresolved issues, and some future prospects in this new field.

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Chapter 2
The Discovery of GW Bodies

Marvin J. Fritzler and Edward K.L. Chan

Abstract Human autoantibodies were a key to the discovery of GW bodies and their integral protein, GW182. This publication marks the tenth anniversary of the discovery of GW182. As it turns out, the discovery of GW182 was quite timely because it coincided with the elucidation of the RNA interference (RNAi) pathway, which is now known to have a major role in post-transcriptional gene regulation. Following our publication of the essential features of GW182 in 2002, laboratories from around the world began investigations that led to the elucidation of the role of GW182 in RNAi and other pathways of mRNA processing and degradation. This chapter reviews the discovery of GW182 and the description of GWB and some of the observations that followed that still remain to be elucidated.

2.1 Introduction

Human autoantibodies have been key reagents in the identification and characterization of novel components and functions of cellular organelles and macromolecules. In turn, the elucidation of novel autoantibodies has led to new tools and diagnostic approaches in a variety of autoimmune conditions, providing the clinician with tools to make an earlier and more accurate diagnosis, predict prognosis and, in some cases, monitor disease activity (Table 2.1). For example, seminal

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Table 2.1 Some key proteins discovered and/or elucidated by use of human autoantibodies

Organelle	Target antigens	Molecular features/ functions	References
Spliceosome	snRNPs (U1-U6 RNP)	Splicing hnRNA	Lerner and Steitz (1979, 1981); Hardin et al. (1982)
Centromere/ kinetochore	CENP-A; -B, -C, -E, -F	Structure of metaphase chromosome, binding metaphase microtubules	Fritzler and Kinsella (1980); Moroi et al. (1980); Rattner et al. (1991, 1996); Earnshaw and Rothfield (1985); Earnshaw et al. (1987); Saitoh et al. (1992)
Small cytoplasmic RNP	SS-A/Ro60; SS-B/La; Ro52/ TRIM21	RNA quality control; RNA molecular chaperones; E3 ubiquitin ligase	Sim et al. (2009); Espinosa et al. (2006); Wada and Kamitani (2006)
Mitotic spindle apparatus	NuMA HsEg5	Movement of metaphase chromosomes	McCarty et al. (1981); Price et al. (1984); Whitehead et al. (1996)
Golgi complex	Golgins-95, -97, 160, 245 Macrogolgin/ giantin	Post-translational processing and transport of newly synthesized proteins	Kooy et al. (1992); Seelig et al. (1994); Lindstedt and Hauri (1993); Fritzler et al. (1984, 1992, 1993, 1995); Griffith et al. (1997); Eystathioy et al. (1999)
Nuclear envelope	gp210, Tpr	Components of nuclear pore complex	Courvalin et al. (1990); Dagenais et al. (1988); Wesierska-Gadek et al. (1995); Enarson et al. (2004); Ou et al. (2004)
Nucleolus	Fibrillarin (U3-RNP) 7-2, 8-2 RNP NOR-90 (hUBF)	Processing ribosomal RNA	Busch et al. (1985); Reimer (1990); Reddy et al. (1983); Okano et al. (1992); Rodriguez-Sanchez et al. (1987)
Exosome	PM/Scl-75 PM/Scl-100	Degradation of selected mRNA	Brouwer et al. (2001, 2002)
Coiled bodies	p80 coilin	Multifunctional: RNA-related metabolic processes such as snRNPs biogenesis, maturation and recycling, histone mRNA processing and telomere maintenance	Andrade et al. (1991); Raska et al. (1990)
Ku	DNA-PK	Repair damaged DNA	Reeves and Sthoeger (1989); Francoeur et al. (1986); Chan et al. (1989)

studies of Lerner and Steitz used human antibodies from a SLE patient to first identify key components of the spliceosome (U1-, U2-ribonucleoprotein (RNP)) (Lerner and Steitz 1979; Lerner et al. 1982). Antibodies from scleroderma patients were used to identify key components of the centromere/kinetochore (Fritzler and Kinsella 1980; Ren et al. 1998; Earnshaw et al. 1987; Fritzler et al. 2010) and nucleolus (Reimer et al. 1987). Human autoantibodies were also used to identify novel components of cytoplasmic targets in the mitotic spindle apparatus (McCarty et al. 1981; Price et al. 1984; Rattner et al. 1998), Golgi complex (Fritzler et al. 1984, 1993, 1995) and endosomes (Mu et al. 1995; Selak et al. 1999; Stinton et al. 2005).

The foundation for some of these discoveries were initially based on indirect immunofluorescence (IIF) analysis of tissue substrates but the use of tissue culture cells (i.e. HeLa, HEp-2) as superior diagnostic substrates became a turning point in the description and discovery of novel proteins (Nakamura et al. 1984; Fritzler 1986). Thereafter, there was a progression from IIF studies that suggested which cytoplasmic organelle was the target, to western immunoblot to determine some essential molecular features of the targets, to cloning that used immunoscreening of expression libraries, followed by sequencing and characterization of the DNA and the expressed protein. In more recent times, spectroscopic analysis and identification of immunoprecipitated proteins of interest in polyacrylamide gels derived from one or two dimensional electrophoresis as well as immunoscreening of protein microarrays have not only added important dimensions to understanding both the B cell repertoire in autoimmune diseases but also provided insight into the cell and molecular biology of target organelles and their cognate proteins, nucleic acids and lipids.

2.2 A Short History of the Discovery of GWB

The investigational approaches described above were keys to the discovery of GW182, its paralogs and ligands, all now known to be components of GW bodies (GWB). In the 1990s and early 2000s, our laboratories at the University of Calgary and The Scripps Research Institute in La Jolla, California turned their attention to human autoimmune sera that reacted with cytoplasmic components. Out of these investigations emerged the identification of unique targets in the mitotic spindle apparatus (Whitehead et al. 1997; Rattner et al. 1998), the Golgi complex (Fritzler et al. 1995, 2007) and endosomes (Selak et al. 1999; Stinton et al. 2005), which complemented much earlier studies of mitochondria (Fritzler and Manns 2002), cytoskeletal (Senécal et al. 1985), ribosome (Gordon et al. 1982; Meroni et al. 1984; Elkon et al. 1985), exosome/PM-Scl (Brouwer et al. 2002; Raijmakers et al. 2004; Mahler and Raijmakers 2007) and the neutrophil cytoplasmic (Bosch et al. 2006; Wiik 2009) autoantigens.

Since it was becoming apparent that antigens in virtually every cytoplasmic organelle were autoantibody targets, our attention turned to lysosomes because, for reasons that are still not clear, very little was known about autoantigen targets in this organelle (reviewed in (Stinton et al. 2004)). Thus, we were interested in sera that

produced staining patterns that suggested autoantigen targets in the lysosome. One such serum was from a 52-year-old female (pseudonym "Amy") from Fort McMurray, Alberta who was referred to a neurologist at the University of Calgary Medical Centre because of progressive severe ataxia (loss of balance and fine coordinated movements of limbs) and was found to have a mixed motor and sensory neuropathy. The IIF pattern produced by her serum autoantibodies produced a cytoplasmic staining pattern (Fig. 2.1) and based on the size and distribution of these "dots", we postulated that her autoantibodies targeted lysosomes. Shortly before Christmas 2000, a former graduate student who had identified the cytoplasmic linker protein, CLIP-170, as a target autoantigen (Griffith et al. 2002), returned to the University of Calgary lab looking for a short-term project and asked if he could attempt to identify the "Amy" target by immunoscreening a cDNA expression library. He set about the task working part time in evenings and weekends and shortly before Christmas, showed me the X-ray films of the first expression cloning experiments that revealed three positive signals that were thought to merit further analysis. Being quite excited about the possibility of discovering a unique lysosomal autoantigen target, the reactive phages were isolated when a strong signal at the very edge of the membrane was noticed (Fig. 2.2). In our hands, positive signals at the edge of the membrane "lifts" were fairly common in expression immunoscreening but, based on several years of experience, the rule of thumb was to "never pursue signals located at the edge of a filter because they inevitably turned out to be artefacts or false positive signals". However, this particular signal was notable because its complementary signal on the duplicate X-ray film was particularly strong. So, four agar plugs (Fig. 2.2: identified as E.1, E.2, E.3 and E.4) were pulled from the cDNA expression plates and replated on smaller agar plates to make sure that the reactivity persisted (i.e. was not a false positive) and to eventually achieve 100% purity of the isolated phages. The first 3–4 h filter lift was performed and the next day overnight nitrocellulose membrane lift was processed using a conventional immunoblot protocol and the index serum "Amy" as the antibody source. As the first processed X-ray films were scanned the results were disappointing : E.1 negative; E.2 negative; E.3 negative (these three lifts were on one film because four filters did not fit into a single film). So, discouragement was palpable and it was thought that this particular adventure was certainly a dead end until the second X-ray film was processed and a high intensity and high density positive signals of the E.4 phage plaques were observed. Taking into consideration the lab rule about false positive clones at the edge of a plate, there was understandable scepticism that this represented a valid signal, so three E.4 plaques were isolated, replated and the series of filter lifts were repeated. To our amazement the signals of all three E.4 subclones remained highly positive. The date was December 24 and it seemed like an early Christmas gift: a cDNA clone that was anticipated to reveal a novel lysosomal autoantigen. The day after Boxing Day, the process of isolating pure phage, preparing the cDNA, expressing recombinant protein and testing it against the index serum "Amy" and other control sera began. By now, there was some time constraints because one of us (MJF) was scheduled to go on a sabbatical leave on January 5 at The Scripps Research Institute in La Jolla, California joining up with

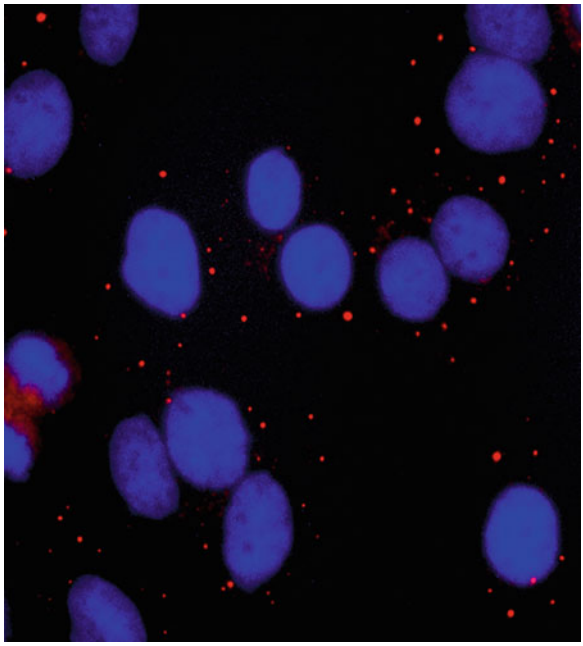

Fig. 2.1 Anti-GWB antibodies were characterized as a cytoplasmic discrete speckled indirect immunofluorescence pattern (*red*) on HEp-2 as well as a variety of other tissue culture cells. Nuclei are counterstained with DAPI. Original magnification 400×

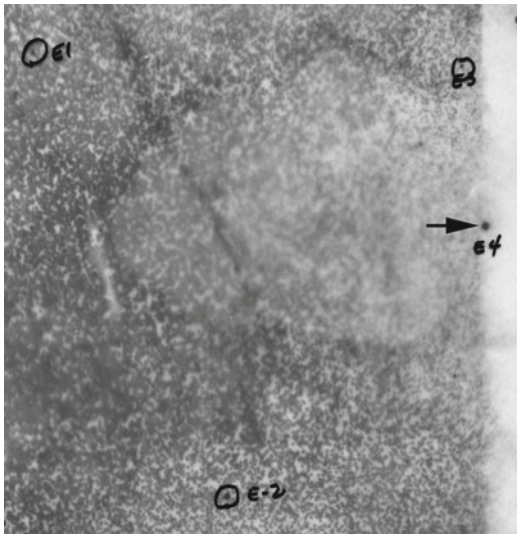

Fig. 2.2 Immunoscreening of a HeLa cDNA library (Stratagene, La Jolla, CA) with the index serum Amy identified four positive signals, originally identified as E1, E2, E3 and E4, on the X-ray film. On subsequent screening, only the clone represented as E4 (*black arrow*) retained reactivity and this was the source of the first cDNA sequence of GW182

my former mentor Dr. Eng Tan and working with a former University of Calgary graduate student, Dr. Ed Chan, the co-author of this chapter. As had been done with some earlier clones (Golgi, endosomes), the purified cDNA extracted from the E4 clone was sent to Dr. Ed Chan for sequencing. Several days later, Dr. Chan sent an email that had a guarded tone: there was confidence, based on a Kozak start sequence near the 5′ terminus and a putative open reading frame and a 3′ termination signal,

that the isolated cDNA was indeed coding a protein of interest. However, the deduced protein sequence indicated numerous GW (glycine-tryptophan) dipeptide repeats throughout much of the protein, a feature that was perplexing at the time. We wondered if this might be a sequencing error or an inherent cDNA artefact and decided to resequence that cDNA as well as other positive clones to determine if the same sequence was found.

By then, MJF was due to depart for the long drive from snow bound Calgary to sunny San Diego. About 5 days later, I had settled in the apartment in Solana Beach, California and was in Dr. Tan and Chan's laboratory ready to embark on a completely unrelated sabbatical project. However, at the first meeting Dr. Chan showed me the most recent sequence data that had validated the initial data and he thought we ought to give particular attention to this putative ~185 kDa target despite the fact there were no proteins with similar sequences in the GenBank and, in particular, no homologues with numerous GW dipeptide motifs (more on this later) as expressed in the cDNA of interest.

The next efforts concentrated on characterizing the reactivity of the Amy serum to determine the expression of GW182 in a variety of cells, tissues and organs, as well as candidate organelle targets by colocalization experiments using index antibodies that reacted with lysosomes, peroxisomes, endosomes and the Golgi complex (still believing this was a lysosomal target) only to find that the cytoplasmic structures reacting with the index serum did not co-localize with lysosomes or other known cytoplasmic organelles (Eystathioy et al. 2002). These puzzling observations prompted us to seek collaboration with electron microscopist Malcolm Wood in the Core Microscopy Facility at The Scripps Research Institute to see if we could validate these findings and learn more about the target organelle by immunoelectron microscopy. The technical expertise and experience of Malcolm Wood was pivotal to determining a key feature of the target since, much to our amazement, the gold labels were localized to 100–300 nm electron dense cytoplasmic structures that, unlike nearby mitochondria, lysosomes/multivesicular bodies or other cytoplasmic organelles, did not have a limiting bilayer membrane (Fig. 2.3). After some debate and review of the literature, we decided we would tentatively refer to these apparently unique cytoplasmic structures as GWB based on their apparent marker protein GW182. Shortly after we published our initial findings (Eystathioy et al. 2002), we became aware that distinct cytoplasmic foci similar to GWBs had been reported in 1997 by Bashkirov and colleagues who reported the cellular localization of mXRN1p in mouse E10 cells by IIF as distinct cytoplasmic domains (Bashkirov et al. 1997); these structures were later named P bodies, which are practically the same structures as they are labelled by both markers (Jakymiw et al. 2007).

At this point, we realized that the future of further studies relied heavily on a supply of "Amy" and/or other sera with identical reactivity. That led to an urgent email to Dr. Zochodne at the University of Calgary suggesting that when he saw the "Amy" patient at future visits to his outpatient clinic, it would be appreciated if he would obtain informed consent and additional sera to secure a source of human anti-GWB/GW182. Unfortunately, that never transpired because the patient "unexpectedly" passed away from heart failure. However, at about the same time, two other

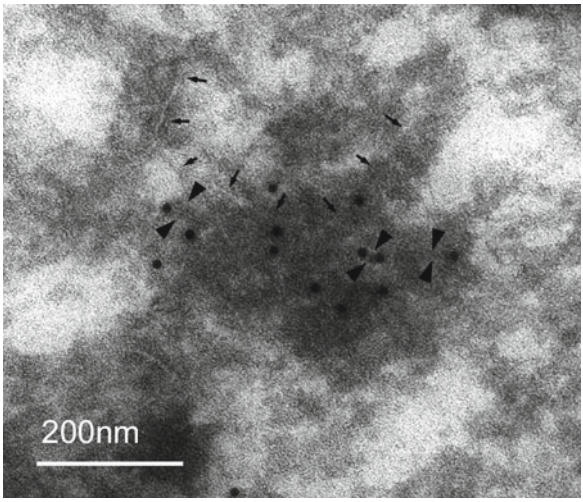

Fig. 2.3 Immunogold electron microscopy localization of GWBs in the cytoplasm of HeLa cells during interphase. Frozen sections of fixed and gelatin-embedded HeLa cells were incubated with the index human anti-GW182 serum diluted 1:400 and then post-immunolabelled with protein A-gold (10 nm). Representative gold-labelled cytoplasmic structures with diameters which vary from 100 to 300 nm. The gold labels are clustered on electron dense fibrils or strands, 8–10 nm in diameter (*arrowheads*). These fibrils appear to form the matrix that the gold decorates. Sometimes filaments are observed right through GWBs (*arrows*)

sera were referred to the Mitogen Advanced Diagnostics Laboratory for analysis. One we labelled IC-6 and it also had fairly typical GWB staining in combination with staining of the nuclear pore complex. Later studies would identify the nuclear pore complex targets as Tpr and gp210 (reviewed in (Ou et al. 2004; Enarson et al. 2004)). The second serum (18033) was referred by Dr. Zochodne's colleague, a neurologist in Barrie, Ontario from a female patient (identified as 18033) who was a clinical "carbon copy" of the index patient Amy and whose serum had high titer and virtual identical autoantibody reactivity. Subsequent to our first (Eystathioy et al. 2003b) and subsequent publication (Bhanji et al. 2007) of the clinical features of patients with GWB autoantibodies, we have identified over 300 sera with anti-GWB referred to us by colleagues in Japan (Dr. K. Miyachi), Brazil (Drs. L. Andrade and C. A. von Muhlen) and Australia (Dr. R. Wilson and W. Pollock), to name a few.

Shortly after the cDNA sequencing was virtually completed, Dr. Jack Keene from Duke University Medical Centre, gave an invited seminar at The Scripps Research Institute on his studies and mounting evidence for post-transcriptional gene regulation with a focus on key mRNA binding proteins such as HuR/Elav (Antic and Keene 1998; Keene 2001; Keene and Tenenbaum 2002); this at a time when evidence about the microRNA pathway was just starting to emerge. After his seminar, we patiently waited for him as he fielded questions and comments from the Scripps faculty and postdoctoral fellows. He kindly agreed to come to the lab so that

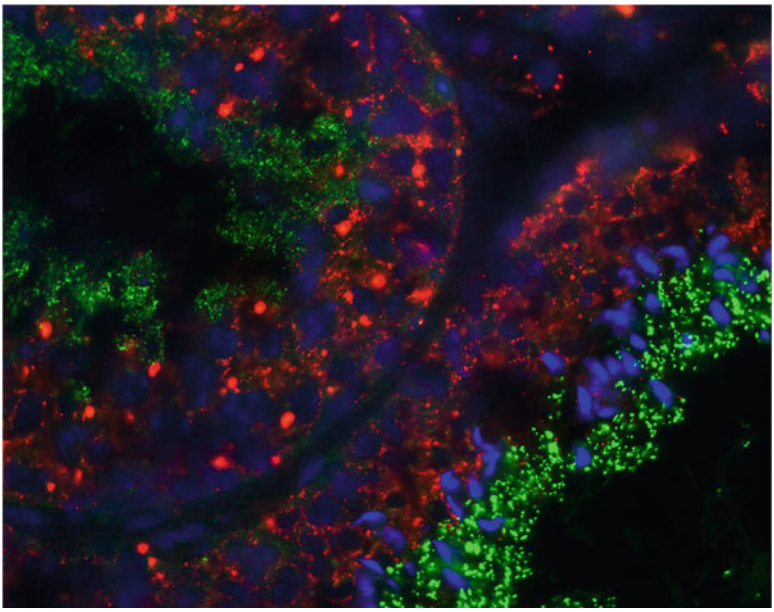

Fig. 2.4 Indirect immunofluorescence using the index anti-GWB serum Amy showed intense staining of mouse testis, particularly cells of the basal/germinative layers of the seminiferous tubules (*red*) compared to staining with a monoclonal antibody directed to golgin-97, a Golgi complex protein (*green*). Original magnification 400×

we could show him the sequence of an "interesting" protein and data on GWB; he took about 10 s to scan the sequence and noting the candidate RNA recognition motif (RRM) near the carboxy terminus (see (Li et al. 2008) and also Fig. 6.5 in Chap. 6) proclaimed "you likely have a mRNA binding protein". This serendipitous meeting with Dr. Keene was the beginning of productive collaboration that included his postdoctoral fellow at the time, Dr. S. Tenenbaum, who had developed expertise in what became known as the RIP-Chip analysis of mRNAs bound to proteins of interest (Tenenbaum et al. 2000, 2002). This collaboration eventually led to determining the spectrum of mRNAs bound by GW182 in HeLa (Eystathioy et al. 2002) and breast cancer (Luft 2005) cell lines.

Still on sabbatical at Scripps and assisted by an MSc student, LeeAnne Luft, other studies focussed on determining the tissue distribution of GW182 and GWB in mouse tissues by indirect immunofluorescence. While GWB could be identified in virtually every tissue, by far the most remarkable was testis (Fig. 2.4) and certain regions of the brain. The latter observation would eventually spark the interest of Joanna Moser, a Ph.D. student who, based on the earlier studies of LeeAnne Luft in breast cancer (Luft 2005), initiated studies of GWB in astrocytes and astrocytoma cells (Moser et al. 2007; Moser and Fritzler 2010b). Based on observations that GWB were remarkably over-expressed in rapidly dividing cells such as HEp-2 (Eystathioy et al. 2002), HeLa (Yang et al. 2004) and breast cancer (Luft 2005), and

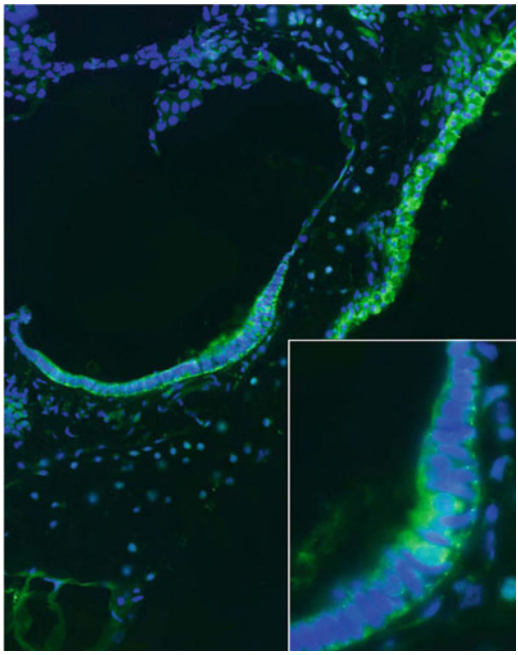

Fig. 2.5 Indirect immunofluorescence using the index anti-GWB serum Amy showed intense staining of the epithelium of Xenopus sp. embryos including the ocular cup (inset). Nuclei are stained blue with DAPI. Original magnification 200×

in collaboration with Dr. Leon Browder at the University of Calgary studies of Xenopus embryos were started, providing evidence that GWB were particularly highly expressed in the developing eye (Fig. 2.5).

After returning to Calgary from sabbatical leave and armed with a long list of potential projects, some of which would be taken up by Dr. Ed Chan at Scripps and then the University of Florida (Gainesville) and also a former laboratory assistant of Dr. Chan's, Theophany Eystathioy (aka Theo), who had several years earlier completed an MSc degree at the University of Calgary and in 2001 returned to complete a PhD in my laboratory. Keeping virtually weekly contact with Dr. Chan's laboratory, Theo initiated much of the early work that characterized GWB. A key observation at the time was that GW182 was a phosphoprotein that bound a unique subset of mRNAs (Eystathioy et al. 2002) and, in collaboration with Drs. B. Séraphin and N. Cougot (CNRS, Cedex, France) showed that GW182 co-localized with hDCp1 and hLSm4 (Eystathioy et al. 2003c). We also spent considerable effort attempting to purify GWB from cell lysates but the remarkable heterogeneity of size and their apparent tethering by a connecting filament (Fig. 2.6) made that approach less than ideal. In addition, the human index sera became helpful in studies by Dr. Nancy Kedersha who was interested in the association of GWB with stress granules (Kedersha et al. 2005) (see Chap. 12).

In appreciation that the human sera were not ideal reagents for the cell biology and biochemical studies, attempts were made to raise antibodies to GW182 in rabbits and murine monoclonal antibodies. While a number of rabbit sera were shown to have

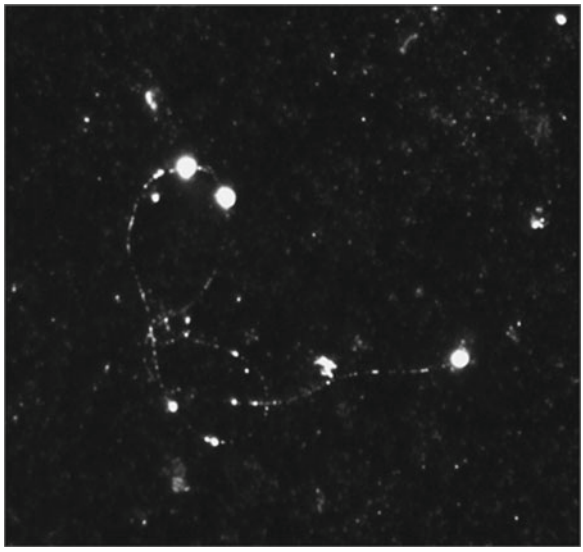

Fig. 2.6 HeLa cells were gently lysed with detergent and then layered onto a discontinuous sucrose gradient and then centrifuged at 100,000 × *g* for 2 h at 4 °C. Purified GWB, characterized as variable sized granules dispersed along a filament were stained with the 18033 human serum were found primarily at the 15% sucrose layer. Original magnification 600×

significant anti-GW182 activity, these sera were not particularly helpful for cell biology studies because they contained antibodies to other cellular components resulting in unsatisfactory IIF staining. At about the same time, with the assistance of the Southern Alberta Cancer Research Institute monoclonal laboratory, we successfully generated four monoclonal antibodies identified as 4B6, 2D6, 5C6 and 6D7 that were patented in 2008. Of these, 4B6 seemed to be the most useful because it was an IgG1 antibody and it replicated IIF staining that co-localized with the index human sera (Eystathioy et al. 2003a). Curiously, by IIF 5C6 showed that, in addition to cytoplasmic dot staining that co-localized with the index human sera, it also stained the nuclear envelope, a feature that was similar to another human serum, IC-6, a feature that became useful for a number of knock-down and cell biology experiments (Gavanescu et al. 2004; Pauley et al. 2006). These monoclonal antibodies quickly drew the interest of several companies such as Abcam and Santa Cruz Biologicals who eventually marketed them. However, it soon became clear that many investigators who purchased these antibodies had difficulties replicating their staining properties. In discussion with the companies and some of the researchers, it became clear that many investigators assumed they could dilute the monoclonal like monoclonals of different specificities. This notion despite clear statements in the legend to Fig. 2.1 of our related publication "All MAbs were added undiluted, except for the 2D6 MAb, which was added at a 1/25 dilution" (Eystathioy et al. 2003a). This misunderstanding eventually led us to develop a protocol for the use of 4B6 that we freely distribute to all users of the antibody. Another problem referred to our laboratory was the difficulty detecting GW182 by western blotting and/or immunoprecipitation. It became apparent that the most common problem was failure to load sufficient protein on the gels to allow the detection of GW182 and to address this concern, we published a protocol for the detection of GW182 and related proteins in cell lysates (Moser et al. 2009) (Fig. 2.7).

Indirect Immunofluorescence (IIF)
Anti-GW182 mouse monoclonal antibody (4B6)

Overview:
Successful use of the monoclonal antibody 4B6 is dependent on three critical considerations:

- **Cells and tissues:** In our laboratory and in the literature a wide variety of cells have been probed successfully with the monoclonal antibody. These include HeLa, HEp-2, HepG2, MDCK, astrocytoma, breast cancer cell lines, pig skin cell lines and many others. Not widely tested on tissues but excellent staining obtained of testing on skin.
- **Fixation:** A number of fixatives are effective but for routine staining fixation in two changes of ice cold acetone:methanol (3:1, v/v) for 15 minutes works very well. In addition, fixation with buffered 3% Paraformaldehyde followed by permeabilization with 0.5% Triton X for 10min also works well. Other fixatives have been tried with varying success.
- **Incubation time of monoclonal antibody on cell substrates**: Incubation must be a minimum of 2 hours at room temperature for any effective staining. Incubation overnight at 4C taking care to avoid dehydration gives the best results.

Protocol:

1. Dilute stock monoclonal antibody 1/10 – 1/100 with phosphate buffered saline (pH 7.1 - 7.3). On most substrates, best results are obtained at lower antibody dilutions (i.e. 1/10). It is recommended that three or four dilutions starting at neat (undiluted) and proceeding to 1/100 be tested to determine optimal conditions for your cells, tissues and general protocols.
2. Add 30 µl of the diluted antibody to fixed cell monolayers such as HEp-2 cell substrates (ImmunoConcepts Inc., Sacramento, CA).
3. Incubate for a minimum of 2hr at room temperature. For optimal results incubate overnight at 4ºC in a humid chamber (in sealed dish on top of moist paper towel).
4. Rinse away excess antibody solution with a gentle stream of 1xPBS (always direct the stream to the edge of the slide or coverslip or wells, never directly onto the cells) and then place the slide/cover slips in a glass jar (Coplin jar) containing 1xPBS and gently agitate on a shaker for 5 minutes; Repeat this wash step 3 times.
5. Taking care to ensure that the wells, slides or cover slips do not dry out, overlay the solution containing the secondary antibody (i.e. Alexa 488 conjugated anti-mouse IgG works well in our laboratory).
6. Incubate in a humid chamber in the dark (cover with foil or place in the cupboard) for 1 hr at room temperature.
7. Repeat step (4).
8. Overlay the moist cells with mounting medium (i.e. VectaShield containing DAPI: Vector Laboratories, Burlingame, CA) and then add a cover slip. Avoid bubbles under the cover slip.
9. If the intention is to keep the slides for a week or more, seal and adhere the cover slips to the slides with nail polish. After viewing with an appropriate UV microscope, slides are stored in a light tight holder at 4C.ensure the slides are stored in the fridge.

Fig. 2.7 Protocol for use of anti-GW182 monoclonal antibodies

As interest in GWB and GW182 paralogs (GW182, GW2 and GW3, named provisionally) increased, it became known that the original GW182 sequence that we submitted to GenBank based on our cDNA represented only the shorter of the two isoforms of GW182. In examining the genomic loci for GW182, a "TNRC6A" gene encoding a short transcript was identified ~50 kb upstream of GW182; this was reported in a study by investigators interested in identifying trinucleotide repeat containing gene in mammalian cells (Margolis et al. 1997). At the genetic level, the TNR region of the trinucleotide repeat containing the TNRC6A gene is encoded on exon 5 of chromosome 16p11.2 and is rich in CAG/CCA/G codons (reviewed in (Li et al. 2008)). We later showed that the TRNC6A gene can indeed be transcribed in the same transcript encoding the long isoform TNGW1 with an extra N-terminal QP-rich 253 amino acid sequence

upstream of the GW182 isoform (Li et al. 2008). In the meantime, GenBank had renamed the cognate genes as TNRC6A (GW182/TNGW1), TNRC6B (GW2) and TNRC6C (GW3) (see (Li et al. 2008) and also Fig. 6.5 in Chap. 6). It is noted that the TNRC6B and TNRC6C names can be distracting as they do not have a trinucleotide repeat domain as in TNRC6A; rather the most important similarity with these two proteins are their GW-rich domains and putative function in translational repression. Although the function of the TNR domain of the long form of GW182 proteins is not clearly understood, genetic aberrations of this type of TNR region have been associated with a number of neurological diseases including Huntington's disease, fragile X syndrome and spinocerebellar ataxia type 2 (SCA2) (Xuereb et al. 1996; Batra et al. 2010; Di Prospero and Fischbeck 2005). This is curious information in the context of the index patient Amy, patient 18033 and approximately one third of patients with anti-GWB who have a neurological disease (Eystathioy et al. 2003b; Bhanji et al. 2007). To that date, no studies were published determining if the TNR region of TNGW1 is the target of autoantibodies or if patients that produce anti-TNGW1 have a mutation of the TNR region, a feature that could conceivably render it immunogenic. To address this, we initiated a study of the TNR region, the key results of which are presented in Chap. 14.

During the course of our studies it became obvious that a number of cytoplasmic structures had one or more features of GWB (Moser and Fritzler 2010a). In addition, we have been intrigued that very few mammalian proteins contain repeat GW motifs, although a notable example is the GWG octapeptide repeat of the prion protein (Zahn 2003). This is also intriguing, since prion-related conditions in animals (i.e. "mad cow" disease, chronic wasting disease) and man (i.e. variant Creutzfeld Jakob syndrome, Gerstmann-Straussler-Scheinker Disease) have some neurological features that were seen in some of the anti-GWB patients.

2.3 Summary and Conclusions

Human autoantibodies were a key to the discovery of GWB and their integral protein, GW182. This book marks the tenth anniversary of the discovery of GW182 and GWB. Serendipitously, the discovery of GW182 was quite timely because it coincided with the elucidation of the RNA interference (RNAi) pathway, which is now known to have a major role in post-transcriptional gene regulation. Following our publication of the essential features of GW182 in 2002, laboratories from around the world began investigations that led to the elucidation of the role of GW182 in RNAi and other pathways of mRNA processing and degradation. A review of the history of the discovery of GWB and GW182 paralogs and the subsequent plethora of research studies on these structures can be attributed to a clinician scientist, Dr. Doug Zochodne at the University of Calgary, who is very interested in patients with autoimmune neurological syndromes. This was followed by a good fortune, remarkable expertise and advice of collaborators in a number of centres, and the coincidental elucidation of the miRNA/RNAi pathways. The study of GWB has taken on broad implications in the post-translational control of gene expression leading to numerous avenues of fruitful investigation still to be explored.

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Chapter 3
The Discovery and Analysis of P Bodies

Saumya Jain and Roy Parker

3.1 Introduction

The last decade has seen the discovery of a conserved class of cytoplasmic mRNP (messenger Ribonucleic Acid—Protein complexes) aggregates called *P*rocessing *Bodies* or P Bodies (Sheth and Parker 2003). They belong to a growing list of cytoplasmic mRNP aggregates, many of which are compositionally similar to P Bodies and consist of a host of translational repressors and mRNA decay factors. These aggregates have been suggested to play important roles in the regulation of gene expression through the control of translation and mRNA decay (Eulalio et al. 2007a; Parker and Sheth 2007). In this chapter, we review the experiments that led up to the discovery of P Bodies, their composition, relationship with other cellular structures and processes, and possible functions. A key theme is that P Bodies are composed of proteins functioning in translational control and mRNA degradation and thus play roles in the control of cytoplasmic mRNA.

3.2 The Mechanisms of mRNA Decay

An understanding of P Bodies requires knowledge of the pathways of mRNA decay in eukaryotic cells. Two general and highly conserved pathways of mRNA degradation exist (Parker and Song 2004) (Fig. 3.1). Both general decay pathways begin with the shortening of the poly-adenosine tail (poly-A tail), a process known as deadenylation. The predominant deadenylase is the Ccr4/Pop2(Caf1)/Not complex, which is a nine subunit complex with two core deadenylases CCR4 and POP2 (or CAF1), although the

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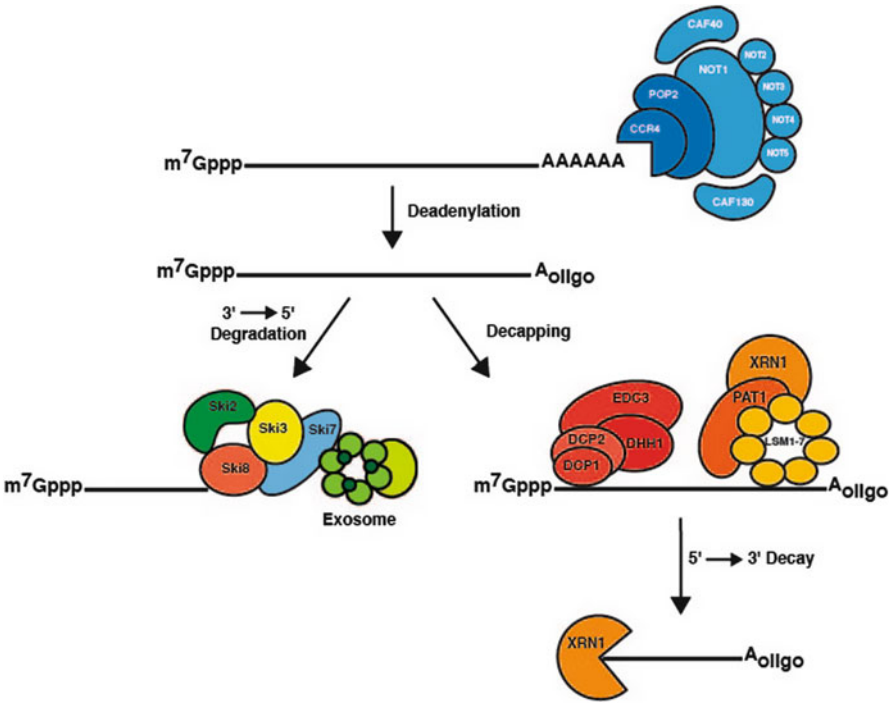

Fig. 3.1 mRNA decay pathways and the associated enzymes: mRNA are deadenylated followed by either 3′-5′ exonucleolytic cleavage as shown on the *left* or decapping and 5′-3′ decay as shown on the *right*

Pan2/Pan3 deadenylase also functions in poly-A shortening in both yeast and mammals (Tucker et al. 2001; Wiederhold and Passmore 2010; Yamashita et al. 2005). The shortening of the poly-A tail can lead to 3′ to 5′ degradation by the exosome, which utilizes the Ski2/Ski3/Ski8 complex and Ski7 proteins as cofactors (Anderson and Parker 1998). More commonly, at least in yeast, mRNA are decapped following deadenylation by the decapping enzyme Dcp2 and its coenzyme Dcp1 (Muhlrad et al. 1994). The removal of the cap structure leads to 5′ to 3′ degradation by the 5′-3′ exonuclease, Xrn1 (Larimer et al. 1992). Decapping is in competition with translation initiation (Schwartz and Parker 1999, 2000), and is promoted by several conserved decapping activators including Pat1, Dhh1, the Lsm1-7 complex, and Edc3, which can function to directly promote decapping and/or to inhibit translation initiation (Coller and Parker 2005; Nissan et al. 2010).

3.3 The Discovery of P Bodies

The key observation that suggested the existence of P Bodies in yeast was the involvement of homologs of yeast decapping activators in maternal mRNA storage during development in higher eukaryotes (Coller et al. 2001). During development

and in the germ line, maternal mRNA was known to be stored as translationally repressed mRNPs, mostly based on studies with *C. elegans* and *Drosophila* (Anderson and Kedersha 2006; Kloc and Etkin 2005). When Dhh1 was identified as an activator of decapping in yeast, it was notable that Dhh1 orthologs in *C. elegans* (CGH-1), *Drosophila* (Me31B), and *Xenopus* (p54) were known to be important for the translation repression of maternal mRNA (Coller et al. 2001; Ladomery et al. 1997; Nakamura et al. 2001; Navarro et al. 2001). Similarly, the yeast decapping activator Pat1, had an ortholog in *Xenopus* (called P100), that was also implicated in maternal mRNA storage (Rother et al. 1992). The dual role of these proteins in translation repression and in promoting decapping suggested that there was a common type of mRNP structure that might either be translationally repressed and stored for later reuse, or decapped and degraded. Moreover, since stored maternal mRNPs often aggregated into larger cytoplasmic mRNPs, this similarity of function raised the possibility that the decapping machinery and translation repressors/decapping activators might be seen in mRNP granules in the cytoplasm of somatic cells.

Various factors implicated in mRNA decapping were, in fact, observed to form cytoplasmic foci, which were eventually named cytoplasmic Processing Bodies (P Bodies) for their possible role in mRNA degradation. In 1999, Bashkirov et al. showed that the mouse ortholog of Xrn1 (named mXrn1) was found in discrete cytoplasmic foci (Bashkirov et al. 1997). Similarly, Pat1 was observed to be in cytoplasmic foci in yeast by immunofluorescence in a paper describing the role of Pat1 and the Lsm1-7 complex in mRNA decapping, though the brevity of the manuscript prevented commentary on this finding (Tharun et al. 2000). Then in 2002 and 2003, it was shown both in yeast and mammalian cells that the decapping enzyme Dcp2 and its associated coenzyme Dcp1, the Lsm1-7 complex, and Xrn1 were found in cytoplasmic foci (Ingelfinger et al. 2002; Lykke-Andersen 2002; Sheth and Parker 2003; Van Dijk 2002). Moreover, in HeLa cells and yeast cells, these components were shown to colocalize into a common particle (Ingelfinger et al. 2002; Sheth and Parker 2003). These results suggested that the mRNA decapping machinery could be concentrated in cytoplasmic structures.

An initial understanding of the composition, dynamics, and possible functions of these cytoplasmic foci came from additional experiments done in yeast cells (Sheth and Parker 2003). First, these foci contained not only decay factors but also mRNA, and particularly mRNA that were in the process of decay. This suggested that these foci could function as centers of mRNA decay. Second, the assembly of these mRNP aggregates was promoted when mRNA decay was inhibited after or at the stage of decapping, further implying a relationship between these foci and mRNA decay. Third, members of the exosome were not found in these granules, suggesting that any role of P Bodies in decay was limited to deadenylation and decapping. And last, these aggregates had an inverse relationship with translation. Trapping mRNA in polysomes by the use of drugs such as Cycloheximide, which blocks translation elongation, led to the absence of P Bodies while inhibiting translation initiation stimulated assembly (Teixeira et al. 2005). Together these observations suggested that P Bodies are mRNP aggregates linked to the processes of mRNA decay and translation. Moreover, because several

of the P Body components were known to function in translation repression and mRNA storage it was likely that mRNA might also be able to exit P Bodies and return to translation as was later demonstrated (see below).

3.4 Composition of P Bodies

P Bodies are generally thought to be aggregates of translationally repressed mRNPs. Two lines of evidence suggest that the mRNA in P Bodies are not engaged in translation: First, trapping mRNA in polysomes by inhibition of translation elongation decreases P Body formation, whereas inhibiting translation initiation and decreasing polysomes increases P Body formation (Teixeira et al. 2005; Liu et al. 2005). Second, translation initiation factors and ribosomal proteins are generally excluded from P Bodies with the exception of eIF4E. However, as P Bodies also contain the inhibitor of eIF4E function, the eIF4E binding protein eIF4E-T, this suggests that eIF4E in P Bodies is most likely associated with repressed and not translating mRNA (Andrei et al. 2005; Ferraiuolo et al. 2005).

mRNA in P Bodies are joined by a host of protein factors that localize to P Bodies and often have roles in mRNA decapping or translation repression (Table 3.1). Several such factors are: the decapping enzyme complex Dcp1-Dcp2; decapping activators Edc3 and the Lsm1-7 complex; and factors that function as both translation repressors and decapping activators, Scd6, Dhh1, and Pat1 (Buchan et al. 2010). Other decay factors such as the 5′-3′ exonuclease Xrn1 and subunits of the deadenylase complex (CCR4/POP2/NOT complex) also localize to P Bodies (Cougot et al. 2004; Teixeira and Parker 2007). A reasonable working model is that P Bodies are aggregates of mRNA associated with a core set of proteins consisting of the decapping and translation repression machinery.

Other factors have been reported in P Bodies only in mammalian cells or in certain conditions or mutants. One such important set of factors is that of the members of the miRNA pathway that are completely lacking in yeast and can form the related GW-bodies, which can either overlap with or be separate from P Bodies in mammalian cells (See Chap. 6 by Yao, Li, and Chan, and Chap. 9 by Izaurralde et al.) (Eulalio et al. 2008; Lian et al. 2009). Similarly, under some conditions or mutant genetic backgrounds proteins involved in nonsense-mediated decay (NMD) can localize to P Bodies (see below).

3.5 Assembly of P Bodies in Yeast

Several sets of experiments have suggested a model for how P Bodies assemble in yeast with the following key points (Fig. 3.2). First, via an intertwined set of mRNP remodeling events and ribosome run-off, mRNA exit polysomes and associate with the translation repression/P Body components. Second, various protein factors

Table 3.1 List of P Body factors (Ce: *C. elegans*, Sc: *S. cerevisiae*, Dm: *D. melanogaster*)

Factor	Function	Organism	Reference
Ago2	miRNA function	Mammals	Leung et al. (2006)
ALG-1	miRNA function	Ce	Ding et al. (2005)
APOBEC3G, APOBEC3F	Deaminase with antiviral activity	Mammals	Wichroski et al. (2006)
BRF1	mRNA decay	Mammals	Kedersha et al. (2005)
CCR4-NOT complex	Deadenylation	Mammals, Sc	Andrei et al. (2005); Sheth and Parker (2003)
CPEB	Translation regulator	Mammals	Wilczynska et al. (2005)
DCP1/DCAP-1	Decapping enzyme subunit	Mammals, Sc, Ce, Dm	Cougot et al. (2004); Gallo et al. (2008); Sheth and Parker (2003); Tritschler et al. (2007); Wilczynska et al. (2005)
DCP2/DCAP-2	Decapping enzyme	Mammals, Sc, Ce, Dm	Cougot et al. (2004); Gallo et al. (2008); Sheth and Parker (2003); Tritschler et al. (2007); Wilczynska et al. (2005)
Dcs2	Stress-induced regulatory protein; modulates m7G-oligoribonucleotide metabolism	Sc	Malys and McCarthy (2006)
Ded1	Translation regulator, mRNA export	Sc	Beckham et al. (2008)
Eap1	eIF4E binding protein	Sc	Buchan et al. (2008)
Edc1-2	Decapping enhancer	Sc	Neef and Thiele (2009)
Edc3	Decapping activator	Mammals, Dm, Sc	Fenger-Grøn et al. (2005); Kshirsagar and Parker (2004); Tritschler et al. (2007)
eIF3	Translation initiation factor	Sc	Grousl et al. (2009)
eIF4e-T	Translation repressor	Mammals	Andrei et al. (2005)
eIF4e/cdc33	Translation initiation factor	Mammals, Sc	Andrei et al. (2005); Ferraiuolo et al. (2005); Hoyle et al. (2007)
eIF4G	Translation initiation factor	Sc	Hoyle et al. (2007)
eRF1	Translation termination factor	Sc	Buchan et al. (2008)
eRF3	Translation termination factor	Sc	Buchan et al. (2008)
FAST	Fas-activated serine/threonine phosphoprotein	Mammals	Kedersha et al. (2005)
Gbp2	mRNA export	Sc	Buchan et al. (2008)
Gemin5	U snRNP assembly	Mammals	Fierro-Monti et al. (2006)
GW182/AIN-1	miRNA function	Mammals, Dm, Ce	Behm-Ansmant et al. (2006); Ding et al. (2005); Eystathioy et al. (2003)

(continued)

Table 3.1 (continued)

Factor	Function	Organism	Reference
Hedls/Ge-1	Decapping activator	Mammals, Dm	Behm-Ansmant et al. (2006); Fenger-Grøn et al. (2005); Yu et al. (2005)
hMex3A	Translation regulator	Mammals	Buchet-Poyau et al. (2007)
hMex3B	Translation regulator	Mammals	Courchet et al. (2008)
hnRNPA3	Translation regulator, mRNA export	Mammals	Katahira et al. (2007)
hnRNPQ	Translation regulator, mRNA export, splicing, mRNA stability	Mammals	Quaresma et al. (2009)
Hrp1	3′ end processing; export	Sc	Buchan et al. (2008)
Htt	Implicated in Huntington's disease	Mammals	Savas et al. (2008)
Importin-8	Importin	Mammals	Weinmann et al. (2009)
Lin28	Translation regulator	Mammals	Balzer and Moss (2007)
Lsm1-7	Decapping activator	Mammals, Sc, Ce	Gallo et al. (2008); Sheth and Parker (2003); Stoecklin et al. (2006)
Ngr1	Translation regulator	Sc	Buchan et al. (2008)
Nrp1	Putative mRNA binding protein	Sc	Buchan et al. (2008)
NXF7	mRNA transport	Mammals	Katahira et al. (2007)
Pab1	Poly A binding protein	Sc	Hoyle et al. (2007)
Pan2/3	mRNA stability	Mammals	Zheng et al. (2008)
Pat1/PATR-1/ CG5208	Decapping activator and translation repressor	Mammals, Dm, Ce, Sc	Eulalio et al. (2007b); Gallo et al. (2008); Scheller et al. (2007); Sheth and Parker (2003)
Pbp1	mRNA processing	Sc	Buchan et al. (2008)
PCBP2	Translation regulator	Mammals	Fujimura et al. (2008)
PMR1	mRNA decay	Mammals	Yang et al. (2006)
Pub1	Stability of mRNA	Mammals	Buchan et al. (2008)
RAP55/Scd6/ TraI/Dcp5/CAR-1	Translation repressor	Mammals, Sc, Dm, Ce	Barbee et al. (2006); Gallo et al. (2008); Teixeira and Parker (2007); Yang et al. (2006)
RCK/p54/Me31B/ CGH-1/Dhh1	Decapping activator and translation repressor	Mammals, Sc, Ce, Dm	Barbee et al. (2006); Gallo et al. (2008); Sheth and Parker (2003); Wilczynska et al. (2005)
Roquin	mRNA stability	Mammals	Yu et al. (2007)
Rpb4	RNA polymerase IIsubunit	Sc	Lotan et al. (2005)
Rpm2	Rnase P subunit	Sc	Stribinskis and Ramos (2007)
Sbp1	Translation repressor	Sc	Segal et al. (2006)
SMG7	NMD	Mammals	Unterholzner and Izaurralde (2004)

(continued)

Table 3.1 (continued)

Factor	Function	Organism	Reference
Staufen	mRNA localization	Dm	Barbee et al. (2006)
TNRC6B	miRNA repression	Mammals	Meister et al. (2005)
TTP	ARE-mediated mRNA decay	Mammals	Kedersha et al. (2005)
Upf1	NMD	Mammals, Sc	Durand et al. (2007); Sheth and Parker (2006)
Upf2	NMD	Mammals, Sc	Sheth and Parker (2006); Stalder and Muhlemann (2009)
Upf3	NMD	Mammals, Sc	Sheth and Parker (2006); Stalder and Muhlemann (2009)
Vts1	mRNA stability	Sc	Rendl et al. (2008)
Xrn1	5′-3′ Exonuclease	Mammals, Sc	Kedersha et al. (2005)
Ygr250c	Putative RNA binding protein	Sc	Buchan et al. (2008)

within this mRNP interact amongst themselves, possibly forming a "closed-loop" organization of the mRNP. Finally, these mRNPs aggregate through specific protein–protein interaction domains to form a visible cytoplasmic granule.

Three lines of evidence support the conclusion that non-translating mRNA is required for P Body assembly. First, the treatment of semi-purified P Bodies with RNase dissociates their components (Teixeira et al. 2005). Second, decreasing the abundance of available RNA by trapping mRNA in polysomes or inhibiting transcription leads to a reduction in P Bodies (Cougot et al. 2004; Sheth and Parker 2003). And third, augmenting the available pool of non-translating mRNA by the inhibition of mRNA decay, the inhibition of translation initiation, or the overexpression of a decay resistant mRNA fragment in yeast stimulates P Body assembly (Sheth and Parker 2003; Teixeira et al. 2005).

Several observations support, but do not prove, a model where P Body assembly involves the recruitment of preexisting proteins complexes to the mRNA. First, the localization of certain factors to P Bodies is dependent on other P Body factors with localization of Dcp1 and the Lsm1-7p complex being dependent on Dcp2 and Pat1, respectively (Teixeira and Parker 2007). Second, two sub-complexes of P Body components co-purify under a variety of conditions and appear to interact independent of RNA (Bouveret et al. 2000; Fenger-Grøn et al. 2005; Gavin et al. 2006; Teixeira and Parker 2007; Tharun et al. 2000; Tharun and Parker 2001). This includes a putative Dcp1/Dcp2/Dhh1/Edc3 complex, although whether Dcp1/Dcp2 can interact with Edc3 and Dhh1 at the same time has not been established. Similarly, Pat1, Xrn1, and the Lsm1-7p complex co-purify from yeast. Together these observations suggest that the Pat1, Xrn1, Lsm1-7 complex, and a Dcp1/Dcp2/Edc3 complex (possibly including Dhh1) are recruited onto mRNA as two groups, though the exact order of recruitment is unknown. Moreover, since Edc3, Dcp2, and Dhh1 can interact with Pat1, these two complexes are proposed to interact to

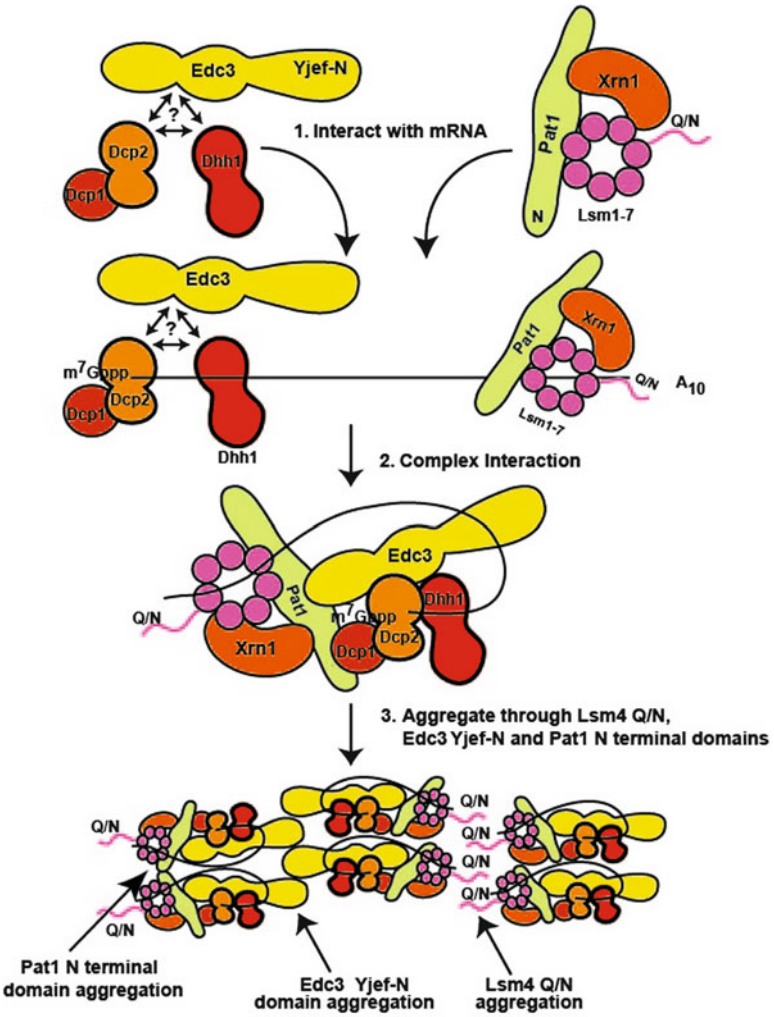

Fig. 3.2 A three step model for P Body assembly in yeast: (1) P Body factors are recruited to the mRNA as complexes. (2) Complex interactions within P Body factors lead to the formation of a "closed-loop" structure. (3) Various such structures aggregate via Lsm4 Q/N domain, Edc3 Yjef-N domain, or Pat1 N Terminal Domain to form visible cytoplasmic granules

form a larger RNA–protein complex (Nissan et al. 2010; Pilkington and Parker 2008). Interestingly, since the Pat1-Lsm1-7p complex has been proposed to bind the 3′ end, and the decapping enzyme has a preference for binding cap structures, this suggests a possible "closed-loop" model for mRNPs that assemble to form P Bodies (Chowdhury and Tharun 2009).

To allow the formation of larger P Bodies, these individual mRNPs must then aggregate into larger structures. In yeast, three proteins have been identified to contribute to this process. First, the Edc3 protein plays a major role in the formation of

P Bodies, both as a scaffold interacting with other P Body components and through a self-interaction domain, the C-terminal YjeF domain (Decker et al. 2007). In the absence of Edc3, P Bodies can still form to some extent and this assembly is driven by a "prion" domain at the Q/N rich C-terminal tail of Lsm4. The deletion of the Q/N domain of Lsm4 (Lsm4ΔC) by itself has no effect on P Bodies, but an Edc3Δ Lsm4ΔC double deletion strain shows a drastic reduction in P Bodies (Decker et al. 2007). The importance of these aggregation domains is underscored by the observation that replacing the C terminal Q/N rich domain of Lsm4 by another prion like domain from Rnq1 is sufficient to restore P Body assembly in an Edc3Δ strain (Decker et al. 2007). Finally, the Pat1 protein can play some role in P Body aggregation as edc3Δ pat1Δ strains are even more reduced than edc3Δ lsm4Δc strains for P Body assembly (Buchan et al. 2008). This severe reduction in the pat1Δ edc3Δ strain is probably due to the Lsm1-7 complex not being present in P Bodies due to its requirement for Pat1 for assembly, and some additional role Pat1 plays in driving aggregation, possibly through its N-terminal domain (Ozgur et al. 2010; Teixeira and Parker 2007). An unresolved and intriguing issue is why these protein components do not aggregate all the time. One possibility is that interaction with mRNA might in some manner promote the aggregation interactions that lead to P Bodies.

The model for P Body aggregation described above is most likely also valid in higher eukaryotes, though subtle differences may exist. For example, self-aggregation domains also seem to play a major role in P Body assembly in mammalian cells, though the proteins they are attached to are different. Lsm4 from higher eukaryotes has an RG-rich domain in place of the C terminal Q/N domain (Brahms et al. 2001). However, with respect to its role in P Body assembly, this aggregation domain is functionally replaced by self-aggregation domains of mammalian P Body factors Pat1, Ge-1/Hedls, and GW182 (Ozgur et al. 2010; Yang et al. 2004; Yu et al. 2005).

3.6 P Bodies, Stress Granules, and the mRNA Cycle

Additional experiments have now suggested that P Body formation and disassembly plays a role in a dynamic cycle of mRNA between different mRNPs and subcellular compartments (Fig. 3.3). This was initially suggested by several lines of evidence that mRNA within P Bodies in both yeast and mammalian cells can return to translation (Bhattacharyya et al. 2006; Brengues et al. 2005). First, trapping mRNA in polysomes by the addition of cycloheximide led to a reduction of P Bodies independent of mRNA decapping, arguing that mRNA within P Bodies are not obligate decapping substrates. Second, during recovery from stress in decay mutants, reemergence of polysomes was still accompanied by a reduction in P Bodies as observed by using both P Body factors and mRNA reporters. Third, yeast cells in stationary phase have large P Bodies containing mRNA that can enter translation when growth resumes (Brengues et al. 2005). Similarly, specific mRNA accumulated in P Bodies under one growth or stress condition can be observed to exit P Bodies with a shift in conditions and enter the polysome

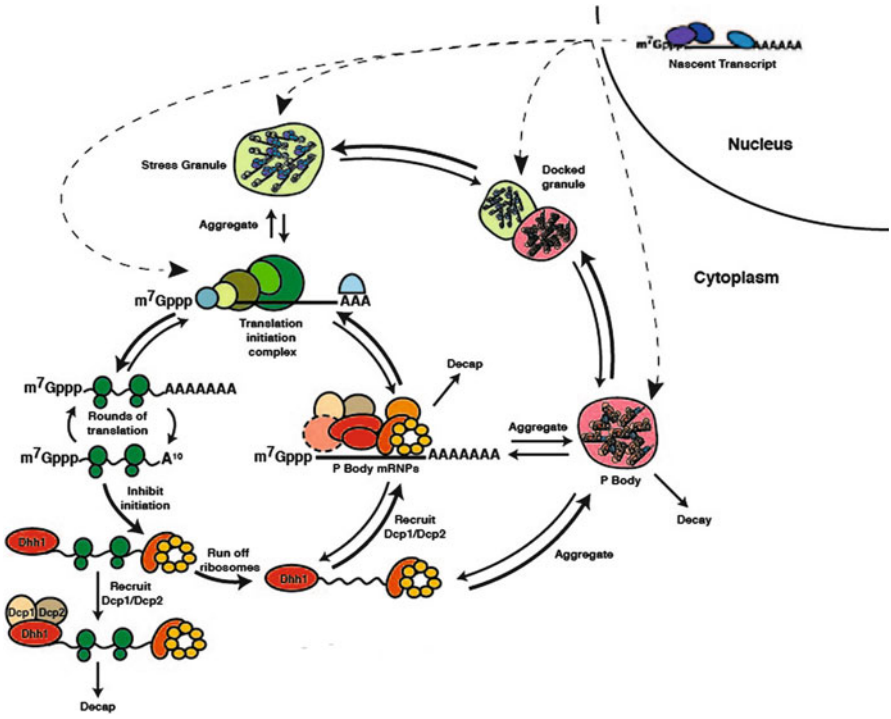

Fig. 3.3 The mRNA cycle showing the dynamic movement of mRNA between polysomes, P Bodies, and Stress Granules

pool (Bhattacharyya et al. 2006; Brengues et al. 2005). These results suggest that mRNA that enter P Bodies can either be degraded or can exit P Bodies to reenter translation, possibly by transitioning from P Bodies to another cytoplasmic mRNP granule, referred to as a Stress Granules.

Stress Granules are a second type of cytoplasmic mRNP granule that can be juxtaposed or overlapping with P Bodies in both yeast and mammalian cells (Brengues and Parker 2007; Buchan et al. 2008; Hoyle et al. 2007; Kedersha et al. 2005). Unlike P Bodies, Stress Granules contain translation initiation factors eIF4G, eIF4A, eIF4B, poly-A binding protein (Pabp), eIF3, eIF2, and the 40S ribosomal subunit (Buchan and Parker 2009). This suggests that Stress Granules are aggregates of mRNPs stalled in the process of translation initiation. Indeed, Stress Granules were first observed under stress conditions, where translation initiation is often inhibited (Kedersha et al. 1999). However, it is now clear that Stress Granule formation is not limited to stress conditions, but can occur in response to a variety of blocks in translation initiation. For example, inhibition of translation initiation using drugs, knock down of translation initiation factors, or over-expression of translation repressors have all been shown to induce Stress Granules (Buchan and Parker 2009). Interestingly, not all blocks on translation initiation induce Stress Granule assembly, suggesting there is a defined window within which translation

needs to be stalled for an mRNP to be targeted to Stress Granules (Mokas et al. 2009; Ohn et al. 2008)

The overlap or juxtaposition of P Bodies and Stress Granules suggests a possible exchange of proteins and mRNA between the two, which has been supported by other observations (Kedersha et al. 2005; Hoyle et al. 2007). First, when induced in yeast and mammalian cells, at least a fraction of Stress Granules form adjacent to, or overlapping with preexisting P Bodies (Brengues and Parker 2007; Buchan et al. 2008; Grousl et al. 2009; Hoyle et al. 2007). Second, mutations that inhibit P Body assembly in yeast reduce Stress Granule formation during glucose deprivation and, to a lesser effect, during Sodium Azide treatment. In contrast, mutations that reduce Stress Granules in yeast do not reduce P Bodies (Buchan et al. 2008; Grousl et al. 2009). Third, over-expression of tristetrapolin (TTP) can promote the juxtaposition of Stress Granules and P Bodies suggesting this mRNA binding protein can trap an intermediate in the exchange process (Kedersha et al. 2005). Taken together, these observations argue that mRNPs exchange between P Bodies and Stress Granules, presumably by remodeling of the mRNP. Unresolved issues in this area include determining whether mRNA can move in both directions between P Bodies and Stress Granules.

Some evidence suggests that Stress Granules might also form independently of P Bodies. First, in mammalian cells and with Sodium Azide stress in yeast, Stress Granules often form independently of visible P Bodies (Buchan et al. 2011; Kedersha et al. 2005; Mollet et al. 2008). It is unresolved whether this is due to individual mRNPs transiting through a P Body like state before forming a Stress Granule mRNP and then aggregating, or because Stress Granule formation can occur independently of P Bodies. Second, knockdowns of some factors in mammalian cells have been described to prevent P Body formation without affecting Stress Granule formation, suggesting the two processes can be uncoupled (Ohn et al. 2008). A key area of future work is determining the mechanisms by which mRNA move between translating pools associated with ribosomes, Stress Granules, and P Bodies and how that process affects the specificity of mRNA translation and the stress response.

The above described dynamic movement of mRNA between various cytoplasmic fates such as translation and storage in P Bodies and Stress Granules has led to the proposal of a cytoplasmic mRNA cycle (Buchan and Parker 2009) (Fig. 3.3). This mRNA cycle suggests that mRNA can exchange their binding partners to move between these different cytoplasmic states. As mentioned in previous sections, translating mRNA can remodel and enter into P Bodies or Stress Granules (Coller and Parker 2004; Parker and Sheth 2007). mRNA can also return to the translating pool from Stress Granules, although for mRNA to exit P Bodies and return to translation, an intermediate mRNP akin to a Stress Granule would be required. Movement between P Bodies and Stress Granules presumably also takes place, with the current evidence suggesting a predominant P Bodies to Stress Granules directionality. Nascent mRNA may also assemble into P Bodies and Stress Granules as they exit the nucleus. This is suggested by the occurrence of various mRNA export and processing enzymes in P Bodies and Stress Granules under various conditions (Table 3.1). Thus as the mRNA is exported out of the

nucleus, it may either directly undergo translation or get stored in P Bodies or Stress Granules as a function of its bound proteins and small RNA.

3.7 P Bodies and mRNA Decay

An important issue is understanding the relationship of P Bodies to the three key processes of mRNA degradation: deadenylation, decapping and 5′ to 3′ degradation, or 3′ to 5′ degradation by the exosome. The exosome and its Ski cofactors have not been observed to concentrate in P Bodies suggesting that P Body formation does not promote 3′ to 5′ degradation of mRNA (Brengues et al. 2005; Sheth and Parker 2003). Indeed, some P Body components such as Pat1 and the Lsm1-7 complex can limit the 3′ to 5′ degradation of mRNA by the exosomes (He and Parker 2001). This raises the possibility that sequestration of mRNA into P Bodies might actually play a role in limiting 3′ to 5′ degradation by the exosome.

Both the Ccr4/Not and Pan2/Pan3 complexes involved in deadenylation have been detected in P Bodies in both yeast and mammalian cells (Cougot et al. 2004; Teixeira and Parker 2007; Zheng et al. 2008). However, since deadenylation in yeast is not affected by preventing P Body formation by trapping mRNA in polysomes (by using Cycloheximide), there is no reason to speculate that deadenylation requires targeting of mRNA to P Bodies (Beelman and Parker 1994; Hilgers et al. 2006).

The relationship between decapping and P Body formation is not yet clear. It is clear that the core components of P Bodies (Pat1, Dhh1, Dcp1/Dcp2, Xrn1, and the Lsm1-7p complex) are required for decapping and 5′ to 3′ degradation of mRNA. Additionally, the yeast strains lacking Edc3 and the Lsm4 prion domain have dramatically reduced P Body assembly yet show normal decay rates for at least a few mRNA, although it has been reported that removal of the Lsm4 prion domain can lead to decreases in the decay rate of at least one mRNA (Decker et al. 2007; Reijns et al. 2008). Thus, a reasonable conclusion is that the aggregation of mRNPs into a larger structure visible in the microscope is not required for the decapping of most mRNA. It remains possible that aggregation into larger structures might affect the decapping of some mRNA, perhaps by increasing the local concentration of mRNA and decapping factors (Yoon et al. 2010). Alternatively, it could be that formation of smaller scale assemblies of mRNPs promote mRNA decapping, even though they are not large enough to be observed in the microscope. Consistent with this latter model, the Dcp1 protein in mammals has a trimerization domain that is required for its assembly into P Bodies and for decapping to occur (Tritschler et al. 2009).

Taking all the observations together, the parsimonious model is that decapping can occur when the translation initiation complex on the cap is replaced by an active decapping complex. Whether decapping occurs while the mRNA is associated with polysomes, as a free individual mRNP, or as a larger P Body; depends on the relative rates of the following: decapping, exiting polysomes, formation of a free P Body

mRNP, and aggregation into a visible P Body. A prediction of this model is that P Body effects might be greatest under conditions where the catalytic steps in decapping are limiting.

3.8 P Bodies and NMD

Eukaryotes have evolved various mRNA quality control mechanisms to inhibit the translation of aberrant mRNA, which could produce potentially deleterious peptides (Fasken and Corbett 2005). One such quality control mechanism is NMD that acts on mRNA that have a premature stop codon (PTC) within the ORF (Baker and Parker 2004). The detection of a PTC on an mRNA leads to the assembly of a complex that includes Upf1-3 in yeast and an additional set of factors namely Smg1, Smg5, Smg6, and Smg7 in higher eukaryotes (Conti and Izaurralde 2005; Amrani et al. 2006). The assembly of this complex leads to deadenylation independent rapid decapping in yeast, while in metazonas the mRNA can be subject to accelerated deadenylation, decapping, and endonuclease cleavage (Cao and Parker 2003; Chen and Shyu 2003; Couttet and Grange 2004; Gatfield and Izaurralde 2004; Lejeune et al. 2003; Mitchell and Tollervey 2003).

Some members of the NMD pathway (SMG7, Upf2, and Upf3) can be found in P Bodies under normal growth conditions in mammals, but not in yeast (Sheth and Parker 2006; Stalder and Muhlemann 2009). However, under conditions that stimulate P Body assembly (such as dcp1Δ, dcp2Δ, and xrn1Δ mutant strains), Upf2, Upf3, and Upf1 colocalize with P Body markers in yeast (Sheth and Parker 2006). Upf1 has RNA-dependent ATPase activity, which has been suggested to be important for mRNP remodeling during NMD (Franks et al. 2010). Interestingly, ATPase mutants of Upf1 have been shown to localize to P Bodies in both yeast and mammalian cells even under normal growth conditions (Sheth and Parker 2006; Stalder and Muhlemann 2009). Together these observations suggest that Upf1 could cycle rapidly through P Bodies, and thus genetic intervention is required to trap it in P Bodies. Additionally, it has also been shown that PTC containing mRNA are targeted to P Bodies in a Upf1-dependent manner (Sheth and Parker 2006). At a minimum, these observations argue that mRNA that are targeted for NMD can assemble into P Bodies when their decay rates are decreased. Whether P Bodies have additional roles in the specificity or rate of NMD under certain conditions remains to be determined.

3.9 P Body and miRNA

MicroRNA are small RNA (length ~22 nt) that are important mediators of posttranscriptional gene expression regulation in higher eukaryotes. Gene silencing by miRNA is dependent on the assembly of the RNA-Induced Silencing Complex

(RISC), of which the Argonaute family of proteins are integral members (Bartel 2004). Argonaute proteins repress translation and promote mRNA degradation by recruiting the GW182 family members, which are the founding members of GW-bodies. As discussed in chapters by Yao, Li, and Chan, Moser and Fritzler, and Izaurralde, GW-bodies can overlap with P Bodies to some extent, which is consistent with many of the components of P Bodies being required for efficient miRNA silencing in a variety of systems (Behm-Ansmant et al. 2006; Chu and Rana 2006; Eulalio et al. 2007c). This suggests a possible relationship between P Body formation and miRNA-mediated silencing.

3.10 P Bodies Associate with Cellular Organelles

An interesting property of P Bodies is their association with various cellular organelles. For example, a subset of P Body-related granules in *Drosophila* oocytes were observed to associate with the endoplasmic reticulum (ER) exit sites (Wilhelm et al. 2005). More recently, P Bodies have been described to associate with the ER by electron microscopy in yeast and components of P Bodies are associated with the ER membrane based on their distribution in density gradients (Kilchert et al. 2010). The interaction with P Bodies appears to be functionally important as alteration of the Scd6/Trailerhitch/Car-1/Rap55 component of P Bodies affects membrane functions. Specifically, over-expression of Scd6 in yeast suppresses a Clathrin mutation, while mutations in the *Drosophila* (Trailerhitch) or nematode (Car-1) orthologs alter ER dynamcis (Nelson and Lemmon 1993; Squirrell et al. 2006; Wilhelm et al. 2005). P Bodies have also been recently described to associate with mitochondria but the significance of this interaction remains to be explored (Huang et al. 2011). One possibility is that P Bodies associated with the ER or mitochondria play an important role in modulating the translation and/or decay of mRNA encoding critical components of these organelles. Alternatively, it could be that these P Body structures play a role in delivering mRNA to these sites, as P Body components can affect mRNA localization as well (Lin et al. 2006).

If P Bodies play a role in intracellular transport, a prediction would be an association of P Bodies with the cytoskeleton. In fact, live imaging of P Bodies has revealed that P Bodies are motile structures and can associate with actin bundles and microtubules (Kedersha et al. 2005). Cytoskeletal motor proteins have been observed in P Bodies, including the yeast Myo2 protein (a class V myosin motor)(Chang et al. 2008). Under certain cellular stress conditions, a microtubule associated molecular motor, Dynein was also observed in P Bodies (Loschi et al. 2009). The association with microtubules has interesting consequences for P Body dynamics. Microtubule disassembly using the drug nocodazole reduces the motility of P Bodies, but also increases P Body number (Aizer and Yaron 2008; Sweet et al. 2007). Taken together, these observations suggest that P Bodies could have an important role in delivering mRNA and/or protein factors to various cellular compartments, and the inability to do so perturbs normal granule assembly dynamics.

3.11 P Body Dynamics and Cellular Processes

A variety of cellular processes are known to alter P Body dynamics. One such cellular process is the cell cycle. In HeLa cells, P Body assembly was stimulated in the late S and G2 phases of the cell cycle and repressed in the early S phase (Yang et al. 2004). Additionally, P Bodies disassembled during mitosis and reappeared in late G1 phase and also proliferating cells had more visible P Bodies as compared to quiescent cells. This was also seen for T cells and mouse splenocytes, wherein P Bodies disappeared on serum starvation and reappeared when feeding was resumed (Yang et al. 2004). A correlation between P Bodies and cellular proliferation has also been observed in germ line cells. However, unlike somatic cells; quiescent, unfertilized eggs assemble P Bodies, which disappear upon fertilization. In mouse oocytes, P Bodies were enriched in meiotic primary arrested oocytes, which rapidly disappeared during in vitro meiotic maturation (Swetloff et al. 2009). Similarly, unfertilized oocytes in *C. elegans* also assemble P Body like aggregates that disappear within 65 min of mating (Jud et al. 2008).

P Body dynamics are also altered in neuronal dendrites as a function of dendritic stimulation. Zeitelhofer et al. reported that in mouse hippocampal neurons, within 2 min of dendritic stimulation with glutamate, BDNF or NMDA, a disassembly of P Bodies at the dendrite could be seen (Zeitelhofer et al. 2008). Thirty minutes poststimulation, the number of dendritic P Bodies recovered to normal levels. An unanswered question is what is the advantage of altering P Body dynamics as a function of the aforementioned cellular processes. One simple possibility is that P Body dynamics are altered under these conditions due to underlying changes in the posttranscriptional control of specific mRNA, which then alters the pool of mRNPs assembled into P Bodies.

3.12 P Bodies Affect Viral Life Cycles

P Bodies, as well as Stress Granules, have been implicated in a variety of different viral life cycles, although in no case is the actual mechanistic role of the different mRNP complexes well understood. One key observation is that many components of P Bodies and Stress Granules function are required for completion of viral life cycles (Beckham and Parker 2008). Alternatively, some antiviral components, such as miRNA (see above) and APOBEC-editing enzymes, can be observed to be concentrated in P Bodies suggesting that these types of mRNPs might also limit viral infection (Gallois-Montbrun et al. 2007; Wichroski et al. 2006). Consistent with that possibility, HIV infection has been proposed to be down-regulated by miRNA and P Body components (Nathans et al. 2009). An important area of future research will be in defining the different types of mRNPs that viral RNAs assemble and how the normal process of cytoplasmic mRNA control affects the translation, degradation, localization, and potentially their packaging into viral particles.

3.13 Future Directions

Recent developments have suggested that P Bodies function as regulators of gene expression, and this function might be more important under stress conditions. But further work is required to establish this role and assess how P Bodies affect various aspects of biology. Three important questions remain unanswered at this time. First, what is the composition and structure of these granules especially with respect to the mRNA species that are targeted to P bodies? Second, how are the assembly and disassembly mechanisms regulated and what impact does that have on mRNA function? In this regard, recent results have indicated that cellular signaling through both the MAP kinase Ste20 cascade and PKA in yeast can control P Body assembly by phosphorylation of Dcp2 and Pat1, although it remains to be determined how these modifications impact the function of specific mRNA (Ramachandran et al. 2011; Yoon et al. 2010). Finally, what is the function of larger scale aggregation on the specificity and efficiency of mRNA degradation, translational control, and possibly mRNA localization. In this regard, the next 10 years look like an exciting time in understanding the assembly, regulation, and functions of P Bodies in eukaryotic cells.

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Chapter 4
Autoantibodies to Argonaute 2 (Su Antigen)

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Abstract Like many other classical autoantibodies in systemic rheumatic diseases, anti-Su antibodies were originally defined by the double immunodiffusion assay in the early 80s. However, despite its high prevalence, only a few reports on anti-Su were published in the following years and the progress in characterizing the target antigens and clinical significance was slow, probably due to its inconsistent or poor reactivity in other standard immunoassays. In 2006 the target antigen was identified as the microRNA (miRNA)-binding protein Argonaute 2 (Ago2). Ago2 is a key component of the RNA-induced silencing complex enriched in cytoplasmic foci called GW bodies. Due to preferential reactivity of human autoantibodies with native antigens, immunoprecipitation is the only method to reliably detect anti-Su/Ago2 antibodies. Anti-Su/Ago2 does not appear to have disease specificity since it is found in 10–20% of patients with various rheumatic diseases, including systemic lupus erythematosus, scleroderma, polymyositis/dermatomyositis, and Sjögren's syndrome, as well as apparently healthy individuals at lower prevalence. The clinical significance and the mechanism of production of anti-Su/Ago2 remains to be clarified.

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4.1 Introduction

Production of autoantibodies to nuclear and cytoplasmic antigens, most commonly identified by immunofluorescence antinuclear antibody (ANA) tests, is a common serological characteristic of systemic autoimmune rheumatic diseases such as systemic lupus erythematosus (SLE), scleroderma (systemic sclerosis, SSc), polymyositis/dermatomyositis (PM/DM), and others. Many different specificities of ANA recognize proteins, nucleic acids, or macromolecular protein and protein–nucleic acid complexes (Tan 1982, 1989; Satoh et al. 2007).

The majority of specific autoantibodies associated with systemic autoimmune rheumatic diseases were originally detected and defined by double immunodiffusion (DID) using calf thymus or rabbit thymus extracts as antigens during the late 60s to early 80s (Tan 1982). Most autoimmune target antigens, which were originally described only by their biochemical characteristics such as molecular weight, sensitivity to RNase, DNase, trypsin, and/or heating, were identified using molecular biology techniques during the 80s and 90s. As originally reported in 1984, anti-Su is probably one of the last specificities defined by DID (Treadwell et al. 1984). However, progress in anti-Su research was slow; only a few clinical reports were published and it took more than 20 years until the target antigen was characterized and identified as the microRNA (miRNA)-binding protein Argonaute 2 (Ago2) (Jakymiw et al. 2005, 2006). Ago2 is a key component of the RNA-induced silencing complex (RISC) present in cytoplasmic foci called GW bodies (GWB). The clinical and immunological characteristics of anti-Su/Ago2 autoantibodies are reviewed in this chapter.

4.2 History of Anti-Su Antibodies

Anti-Su antibodies were first described as a distinctive autoantibody specificity associated with SLE, defined by DID using phosphate buffered saline-extractable fraction of calf thymus as antigen (Treadwell et al. 1984). A study 10 years later identified the target of anti-Su antibodies as a 100/102 kD doublet and 200 kD protein by immunoprecipitation (IP) and reported that anti-Su was commonly found in 10–20% of patients with various systemic rheumatic diseases (Satoh et al. 1994). Anti-Su was also reported in murine models of SLE, MRL/*lpr*, and MRL+/+ mice, at a prevalence of ~25%, which is comparable to that of anti-Sm (Treadwell et al. 1993). In the pristane-induced BALB/c mouse model of lupus, antibodies to Su and small nuclear ribonucleoproteins (snRNPs; Sm, U1RNP) were the two most common specificities identified; each found in ~50% of animals (Satoh and Reeves 1994). Anti-Su was also induced after pristane injection in many other non-autoimmune strains of mice (Satoh et al. 2000). However, as noted above it took more than 20 years to identify the target antigen localized to GWB that are recognized by human anti-GW182 autoimmune sera (Eystathioy et al. 2002).

A few years later, recognition of GWB by anti-Su antibodies was shown and the Su antigen was officially identified as the miRNA-binding protein Ago2 (Jakymiw et al. 2005, 2006). Based on the historical observations and these newer findings, we called the reactive autoantibodies anti-Su/Ago2 antibodies. As a key component of RISC, Ago2 has now become a prime target of research in molecular and cellular biology.

4.3 Su Antigens

An earlier study showed that the target antigen of anti-Su antibodies was a protein based on its sensitivity to trypsin and heat treatment, as contrasted to its resistance to RNase and DNase. The molecular weight was estimated to be approximately 154 kD by Sepharose chromatography using calf thymus extract (Treadwell et al. 1984). A following study reported Su antigen as a 50–55 kD protein by IP and western blot using HeLa (human cervical cancer) cells (Treadwell et al. 1991); however, these results appear to reflect either degradation products or other proteins. Onodera et al. reported that, in indirect immunofluorescence analysis, anti-Tu (same as anti-Su system) autoimmune sera stained cytoplasm with a granular or homogeneous pattern. The Tu antigen was ~70 kD by Sephadex G-200 chromatography and was detected in microsomes and rough surface endoplasmic reticulum fraction (Onodera 1986). It was finally shown clearly that index anti-Su antibodies IP proteins of 100/102 kD doublet and ~200 kD in human HeLa, K562 (erythroleukemia cell line), and murine L929 cells (Satoh et al. 1994) (Fig. 4.1). As for the identity of target antigens, Jakymiw et al. found that anti-Su positive human or murine autoimmune sera, but not control sera, reacted with *in vitro* translated Ago2 protein, which co-migrated with the 100 kD Su protein derived from K562 cell lysates (Jakymiw et al. 2006). Also, the 100 kD protein IP by anti-GW182 autoimmune serum was identified as Ago2 by mass spectrometric analysis of the co-purified protein. In addition, anti-Su positive sera from mice with pristane-induced lupus were strongly reactive with Ago2 recombinant protein in ELISA. Based on these findings, it was concluded that Ago2 is the main target of anti-Su autoantibodies (Jakymiw et al. 2005, 2006). The biology and function of Ago2 is discussed in detail in Chap. 10 and other chapters of this book and will not be discussed here.

Although reactivity with Ago2 appears to be the major component of anti-Su antibodies, Ago proteins are highly conserved and human anti-Su positive sera also reacted with Ago1, 3, and 4 (Jakymiw et al. 2006). The exact contribution of other Ago proteins to the anti-Su antibody system is not known. There was a suggestion that the 200 kD "Su antigen" might be the Dicer protein and some anti-Su positive sera also immunoprecipitated *in vitro* translated Dicer protein (Jakymiw et al. 2006); nevertheless, the nature and identity of the 200 kD Su antigen remains to be determined. The relationship of the 100 and 102 kD component of Su antigen also still needs to be determined; these could be different Ago proteins, posttranslationally modified

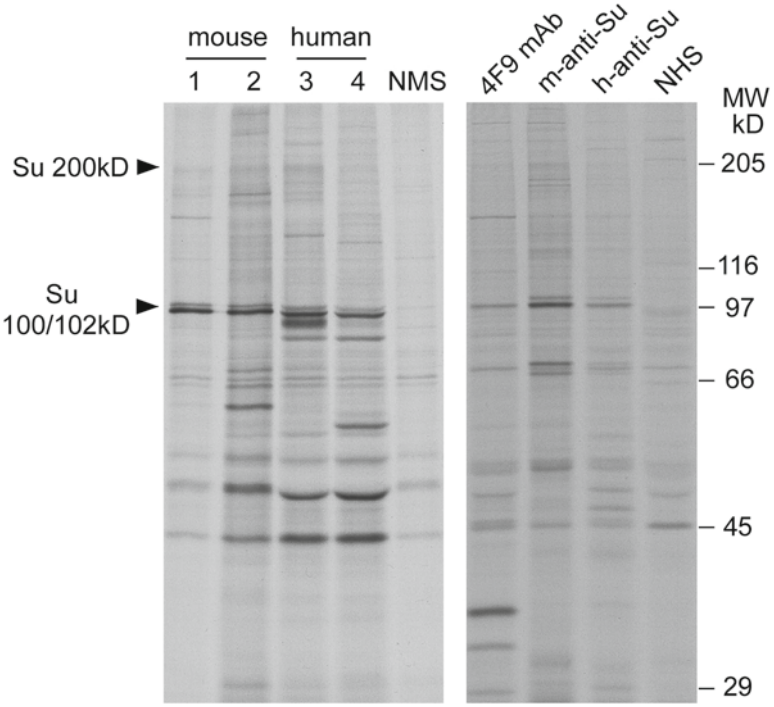

Fig. 4.1 Immunoprecipitation of Su antigens. ^{35}S-methionine-labeled K562 cell extract was immunoprecipitated by human or mouse anti-Su autoimmune sera or mouse monoclonal antibodies to Ago2 4F9. *Left*, immunoprecipitation using 2 sera from pristane-treated BALB/c mice, 2 human autoimmune sera with anti-Su, and normal mouse serum (NMS). *Right*, immunoprecipitation using mouse monoclonal 4F9, mouse or human anti-Su sera, or normal human serum (NHS)

Ago2 proteins, or different proteins. Also, a determination of whether 100 and 102 kD proteins are physically associated, or resulted from cross-reactive autoantibodies, has not been determined. In sucrose gradient experiments, the 100/102 kD co-migrated with the 200 kD proteins (Satoh et al. 1994), although this does not provide conclusive evidence regarding their relationship. In a time course study in pristane-treated mice, some sera IP only the 100 kD protein followed by the appearance of antibodies to the 102 kD protein months later. Mouse monoclonal antibodies to Ago2 4F9 also IP the 100 kD protein with little 102 kD in evidence (Fig. 4.1). This suggests that at least 100 and 102 kD proteins may not be tightly bound all the time but a clear answer on their relationship is not obvious.

One characteristic of human anti-Su antibodies is its preferential recognition of native antigens. It was noted that anti-Su reactivity is represented as a very thin precipitin line compared with most other precipitin lines such as anti-Sm, -U1RNP, or -La (Treadwell et al. 1984; Treadwell et al. 1991). At the time, DID detection of anti-Su antibodies, also known as anti-MoS (Kaburaki et al. 1984), was somewhat

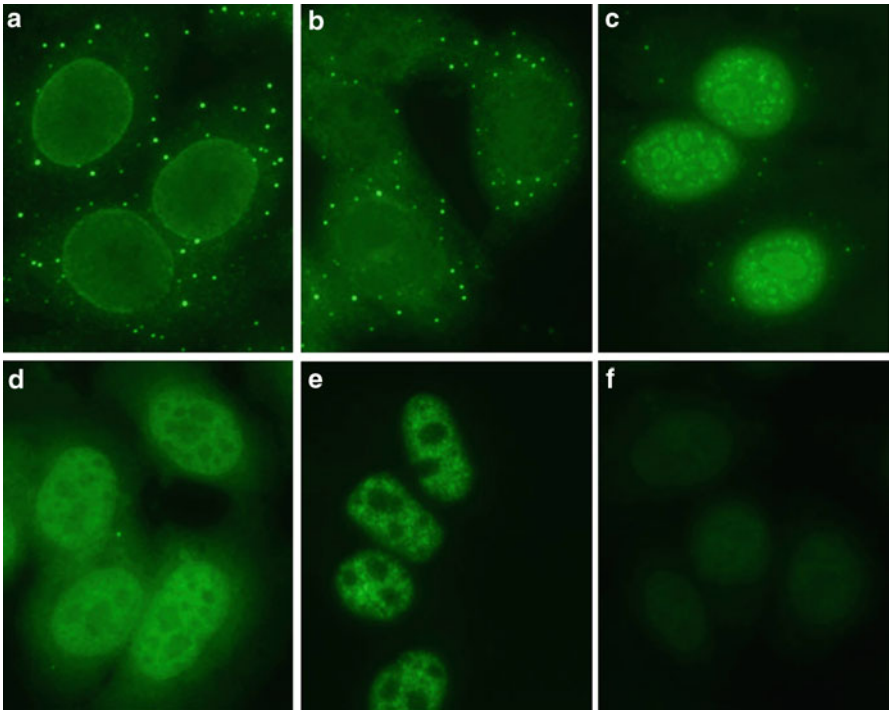

Fig. 4.2 Immunofluorescence using human anti-Su autoimmune sera. (**a**–**e**) Human autoimmune sera anti-Su (+) by immunoprecipitation, (**f**) normal human serum. Some human anti-Su strongly stain GW bodies (**a**, **b**) but others are weak (**c**, **d**), or completely negative (**e**)

inconsistent depending on the preparations of calf thymus extract. IP consistently shows a characteristic pattern of Su antigens though the intensity and the appearance of 200 kD band is somewhat dependent on the batch of cells used. There was nearly a perfect correlation between IP and antigen-capture ELISA, which uses native antigens in cell lysates (Satoh et al. 1995). The majority of human anti-Su positive sera are poorly reactive or unreactive in western immunoblots. Although *in vitro* translated Ago2 was recognized by most anti-Su positive sera (Jakymiw et al. 2006), recombinant Ago2 expressed in E. coli was recognized by only ~30% of human anti-Su positive sera in ELISA (Chan JYF, et al. unpublished). Consistent with denaturation-sensitive anti-Su reactivity, clear GWB staining in immunofluorescence was noted in only ~1/3 of anti-Su positive human sera (Fig. 4.2). Interestingly, some anti-Su positive sera showed GWB staining when cells were fixed using paraformaldehyde followed by saponin treatment (Chan JYF, et al. unpublished) as compared to negative staining after conventional methanol/acetone fixation. All these findings are consistent with the notion that

autoimmune B-cell epitopes of Su antigens are native peptide structures and are likely conformation-dependent.

This delicate and denaturation-sensitive nature of Su antigens might explain why identification of the Su antigen was so delayed despite the high prevalence of anti-Su antibodies in rheumatic disease sera, especially when the majority of other common autoantigens were cloned and identified during 80s and 90s.

In screening for autoantibodies by immunofluorescence, detection of cytoplasmic dot staining by anti-Su antibodies was not appreciated until the description of GWB staining by anti-GW182 antibodies (Eystathioy et al. 2002), followed shortly thereafter by anti-Su antibodies (Jakymiw et al. 2005, 2006). Even though we now appreciate that Su antigens localize to GWB, GWB staining by anti-Su sera is not so obvious nor commonly reported in routine ANA tests. Of relevance to this observation, GWB staining is inconsistent when different fixation methods are used and varies from manufacturer to manufacturer of HEp-2 cell substrates. In fact, immunofluorescence ANA was reported negative in 3 of 37 cases of anti-Su in the original report (Treadwell et al. 1984) and we also reported that anti-Su did not show a consistent immunofluorescence pattern and some sera were negative by indirect immunofluroescence (Satoh et al. 1994). In DID, in addition to the thin precipitin lines (Treadwell et al. 1984), formation of anti-Su precipitin lines was inconsistent between different preparations of calf thymus extract. In IP, not reported until 1994, detection of the characteristic 100 kD doublet requires a good quality IP and SDS-PAGE (Satoh et al. 1994). Finally, in western blot, most human sera are poorly reactive with conventional cell extracts or affinity-purified Su antigens. Taken together, these characteristics in various immunoassays appear to be primary reasons for the delay in characterizing Su antigens.

4.4 Detection of Anti-Su Antibodies

As referenced above, human anti-Su antibodies appear to preferentially recognize native molecules and are poorly reactive with denatured antigens in western blot or ELISA using recombinant Ago2. Although DID positivity is generally reliable, this technique is not used commonly and the inconsistent detection of anti-Su in different preparation of antigens is a problem as discussed above. Thus, IP remains the gold standard and the only reliable method to detect anti-Su, although even it is not a commonly used technique. It is important to verify the characteristic pattern of the 100 kD doublet in combination with the 200 kD antigen (Fig. 4.1) since there are many known and uncharacterized autoantigens in the 90–100 kD molecular weight range, including NOR90 (Chan et al. 1991), golgin-95 (Fritzler et al. 1993), VCP/p97 (Miyachi et al. 2006), Hsp90, Grp94, Sp100, and others (Satoh et al. 1994). Antigen-capture ELISA using mouse monoclonal antibodies to Ago2 and mammalian cell lysates is also a useful approach to developing an immunoassay to detect human anti-Su antibodies but false positives are problematic (Ceribelli et al. 2011). Establishing an ELISA or other immunoassays, such as addressable laser bead

immunoassays, using a stabilized native Ago2 that is recognizable by human autoantibodies is necessary to make the clinical detection of anti-Su widely available.

4.5 Prevalence and Clinical Significance of Anti-Su/Ago2 Antibodies

Considering the long history since the first description of anti-Su in 1984 and the relatively high reported prevalence of 10–20% in SLE and other systemic rheumatic diseases, the clinical information on anti-Su is far from complete. This is probably due to the difficulty in consistent detection of anti-Su as discussed above. Thus, virtually all reports on anti-Su both in human and mouse are either by Treadwell and his colleagues using DID or by our group using IP.

Reports on the prevalence of anti-Su antibodies in human diseases are summarized in Table 4.1. The prevalence of anti-Su in the original DID study in consecutive patients was 3.0% in SLE, 5.6% in probable SLE, 0% in RA and PM, and 3.3% in SSc (Treadwell et al. 1984). Diagnoses among 37 anti-Su positive patients included 13 definite SLE, 4 probable SLE, 10 SLE-overlap, 6 undifferentiated connective tissue diseases (UCTD), 1 probable SSc, and 3 others. By comparison of anti-Su positive SLE patients with other published series of SLE, an increased prevalence of Raynaud's phenomenon and a reduced prevalence of malar rash, alopecia, arthritis, and anemia were noted. Although the title of the report suggested specificity for SLE, it was not as specific for SLE unlike some of the other marker antibodies (Treadwell et al. 1984). Two autoantibody specificities, anti-MoS (Kaburaki et al. 1984) and anti-Tu (Onodera 1986) described in Japanese literature during the similar period were later confirmed to be the same as anti-Su (Satoh M, Kaburaki J, and Miyachi K, unpublished). Anti-MoS was found in ~4% of SSc and other rheumatic diseases (Kaburaki et al. 1984). Autoantibodies to cytoplasmic antigen named Tu as detected by DID were described independently (Onodera 1986) and was found in 20.3% of SLE, 31.6% of SSc, 6.9% of PM/DM, 9.1% of RA, 22.0% of MCTD, 13.1% of Sjögren's syndrome (SS), 20.7% of UCTD with anti-U1RNP, and 27.3% of other CTD. Anti-Tu was detected at a higher prevalence among patients with anti-topo I (42%), anti-Ki/SL (proteasome activator PA-28γ, 38%), and anti-Ku (DNA-binding subunits of DNA-dependent protein kinase, DNA-PK) (30%); however, the prevalence was not compared to each antibody negative group (Table 4.2).

The prevalence of anti-Su in Japanese and American patients with systemic rheumatic diseases was examined by IP (Satoh et al. 1994). Anti-Su was a common specificity found in 10–20% of various rheumatic diseases of both ethnic groups. The prevalence of anti-Su was ~20% in SLE, SSc, and overlap syndrome and 7–8% in PM/DM, SS, and RA, indicating that although anti-Su is associated with systemic rheumatic diseases, it is not specific for any one disease. In this study, prevalence of anti-Su was compared in Japanese patients with various autoantibody positive vs. negative. Anti-Su was more common in anti-Ku positive vs. negative patients (37% vs. 13%, $p < 0.01$) (Table 4.2). This association was found to be due to a coexistence

Table 4.1 Prevalence of anti-Su in systemic rheumatic diseases

	Treadwell et al. (1984)	Kaburaki et al. (1984) (MoS)	Onodera (1986) (Tu)	Satoh et al. (1994)		Vazquez-Del Mercado et al. (2010a, b)	Ceribelli et al. (2011) and unpublished
Country	USA	Japan	Japan	USA	Japan	Mexico	Italy
Method	DID (CTE)	DID (CTE)	DID (RTE)	IP	IP	IP	IP
SLE	3%		20%	17%	21%	24%	(12%)
SSc	3%	4%	32%	17%	20%		(5%)
PM/DM	0%		7%	0% (0/4)	8%	9%	(4%)
RA	0%		9%	7%	7%		
Primary SjS			13%	nd	8%		
Overlap/ MCTD			20%	40% (2/5)	18%		
Others	SLE probable 6%		UCTD 21–27% Liver dis 6% Thyroid dis 0%			HCV 5%	PAPS 13%

CTE calf thymus extract; *DID* double immunodiffusion; *IP* immunoprecipitation; *RTE* rabbit thymus extract; *SLE* systemic lupus erythematosus; *SSc* scleroderma, systemic sclerosis; *PM/DM* polymyositis/dermatomyositis; *RA* rheumatoid arthritis; *MCTD* mixed connective tissue disease; *UCTD* undifferentiated connective tissue disease; *Liver dis* liver diseases; *Thyroid dis* thyroid diseases; *HCV* hepatitis C virus infection; *PAPS* primary anti-phospholipid antibody syndrome; *SjS* Sjögren's syndrome

( ) not random or continuous samples

Table 4.2 Prevalence of anti-Su in patients with other autoantibody specificity

	Onodera (1986)	Satoh et al. (1994)	Vazquez-Del Mercado et al. (2010a); Azucena Palafox-Sanchez et al. (2009) and unpublished
	Prevalence of anti-Su in each specificity +	Prevalence of anti-Su in each specificity + vs. −	Prevalence of anti-Su in each specificity + vs. −
Ethnicity	Japanese	Japanese	Mexican
Subjects	All diagnoses	All diagnoses	SLE
U1RNP	28%	18% vs. 13%	35% vs. 19%
Sm	21%	13% vs. 15%	43% vs. 22%
Ro60	26%	18% vs. 12%	38% vs. 13%[a]
La	27%	12% vs. 15%	17% vs. 25%
Ku	30%	37% vs. 13%[b]	50% vs. 22%
Ki/SL	38%	20% vs. 14%	n.a.
Jo-1	14%	9% vs. 15%	n.a.
Topo I	42%	17% vs. 15%	n.a.
RHA	n.a.	n.a.	29% vs. 23%

n.a. not available; *Topo I* topoisomerase I also referred to as Scl-70; *RHA* RNA helicase A

[a] $p = 0.0282$

[b] $p < 0.01$

of these two specificities in patients with an overlap syndrome, in which prevalence of anti-Su was 46% vs. 0% ($p < 0.05$) in anti-Ku positive vs. negative patients (Satoh et al. 1994). Anti-Su in anti-Ku positive vs. negative SLE was both 20%. It is of interest to note that anti-Ku is found almost exclusively in African Americans but rarely in Caucasian Americans (Wang et al. 2001) and anti-Su also has a much higher prevalence in African Americans vs. Caucasians (Reeves et al. 2005). Thus, it is possible that certain genetic and/or environmental factors associated with anti-Su and anti-Ku are common in a subset of patients. Anti-Su was also detected in 24% of Mexican SLE (Vazquez-Del Mercado et al. 2010a) and was associated with anti-Ro (Table 4.2). Among Italian noncontinuously selected patients, anti-Su was detected in 12% of SLE, 5% of SSc, and 4% of PM/DM (Table 4.1). These results indicate that anti-Su autoantibodies are common regardless of ethnicity.

Detection of anti-Su is not limited to SLE and other typical systemic autoimmune diseases. In a recent study of primary anti-phospholipid antibody syndrome (PAPS), 13% (7/52) of patients were found to have anti-Su, followed by anti-Ro60 in 10% in contrast to the absence of other autoantibodies (Ceribelli et al. 2011). This and previous studies reporting anti-Su in UCTD (Treadwell et al. 1984; Onodera 1986) suggest that anti-Su may be common in atypical cases of rheumatic diseases as well. In other study, anti-Su was found in 5% (4/64) of patients with hepatitis C virus (HCV) infection, 3 cases without interferon (IFN)-α/ribavirin treatment, and one case with treatment (Vazquez-Del Mercado et al. 2010b). No other autoantibodies associated with systemic rheumatic diseases were found in this cohort of HCV infection, suggesting the relatively specific association of anti-Su with HCV infection. Although IFN is associated with autoimmunity, anti-Su production does not appear to be augmented by IFN treatment.

Anti-Su was also found in some cases of healthy donors, undiagnosed patients, and family members of patients with systemic rheumatic diseases (Satoh M, et al., unpublished). In the recent epidemiological study of unselected US population in National Health and Nutrition Examination Survey (NHANES) cohort, prevalence of ANA was estimated to be 13.8% (95% CI, 12.2–15.5%) (Satoh et al. 2012). Among an ANA positive population, anti-Su was the second most common specific autoantibody detected in 2.4%, following anti-Ro60 in 3.9% (Satoh et al. 2012). Anti-Su was predominantly detected in female (3.6% vs. 0.1% in male) and in non-Caucasian (non-Hispanic white 0.8% vs. non-Hispanic black 5.8%, Mexican American 6.7%, other 5.8%). The prevalence of anti-Su in unselected US population was estimated at least 0.33% (female 0.64%, male 0.01%); however, the actual prevalence is likely to be much higher since many anti-Su positive individuals are ANA negative. Thus, it is likely that anti-Su is somewhat similar to anti-Ro60, which can be found in patients under various diagnoses as well as ~0.5–2% of healthy general population such as blood donors (Fritzler et al. 1985).

Another report on anti-Ago2/Su used a different approach (Bhanji et al. 2007). Sera with cytoplasmic discrete speckled pattern were initially selected from samples received at a diagnostic laboratory. Samples were then tested for GWB staining by

immunofluorescence to identify 55 anti-GWB positive sera. Prevalence of anti-GWB positive sera was estimated 0.56% (14/2,500 in a typical 6-month period). Nine of the 55 GWB positive sera (16%) were positive for anti-Ago2 and 3/9 also had antibodies to other components of GWB, Ge-1, and RAP55, by IP using in vitro transcription and translation rabbit reticulocyte lysate kit (TnT-IP). Thus, ~0.09% of the samples of their cohort were anti-Ago2 positive. Clinical information of 6 anti-Ago2/Su positive cases included two cases of SS, one case each of diffuse cutaneous SSc, psoriatic arthritis, motor neuropathy, ataxia (with SS), and sensory neuropathy. Similar to the clinical association of all 55 anti-GWB staining positive patients, neuropathy and SS appear to be common diagnoses for anti-Ago2/Su positive patients. The prevalence of anti-Ago2/Su in this cohort of patients tested for ANA appears to be underestimated, compared with our data of ~6% of sera from unselected rheumatology clinic were positive for anti-Ago2/Su by IP at University of Florida Center for Autoimmune Disease. There are several explanations for discrepancy including methods of screening and differences in the way cohorts were selected for study. Only ~30% of anti-Ago2/Su IP positive sera were clearly anti-GWB positive, thus significant number of anti-Ago2/Su sera will be missed by selection based on GWB immunofluorescence staining alone. Since anti-Ago2/Su reactivity preferentially recognizes native molecule from the cells, all IP positive sera may not be positive in TnT IP. Another factor will be a difference in the various cohorts, in particular race, since the prevalence of anti-Ago2/Su is much lower in Caucasians (Reeves et al. 2005; Satoh et al. 2012). In addition, the sera in the Bhanji et al. study (Bhanji et al. 2007) were selected on the basis of cytoplasmic dot staining pattern that co-localized with GW182 markers.

4.6 Production of Anti-Su/Ago2 in Animal Models

Anti-Su was found in 19% of MRL/*lpr*, 26% of MRL+/+ mice, a prevalence similar to anti-Sm antibodies in these strains but in only 1–2% in NZB and B6/*lpr* and none in BXSB or 110 normal mice by DID (Treadwell et al. 1993). NZB/WF1 mice did not spontaneously produce anti-Su antibodies although anti-Su can be induced in 8% of mice by a single intraperitoneal injection of pristane (Yoshida et al. 2002; Chan et al. 2009). In the pristane-induced model of lupus, anti-Su is one of the two most common autoantibodies, being found 2–6 months after pristane injection in 20–90% of all non-autoimmune prone strains of mice including BALB/c, C57BL/6, CBA, C3H, DBA/1, DBA/2, B10, B10S, A.SW, and others (Satoh et al. 2000). Anti-Su and anti-snRNPs (U1RNP and Sm) are the two most common specificities in several strains such as BALB/c and DBA/1. The prevalence of anti-snRNPs and ribosomal P antibodies varies significantly depending on the murine strains studied. However, anti-Su was produced by all immunocompetent non-autoimmune mouse strains tested regardless of the MHC or other genetic backgrounds.

4.7 Mechanism of Production

Like other autoantibodies associated with systemic rheumatic diseases, the mechanisms of the production of anti-Su antibodies are poorly understood and are likely controlled by the interaction of genetic, epigenetic, and/or environmental factors. To date there are no studies available on HLA and other genetic analysis associated with anti-Su production in humans. Anti-Su is common and found in ~10–20% in African American, Latin, and Japanese patients with various systemic rheumatic diseases, but less common (~5%) in Caucasians. As discussed above, anti-Su was induced in all immunocompetent strains of mice with various background and MHC in the murine model of pristane-induced lupus. These observations in human and mice suggest that the MHC restriction or other genetic restriction of anti-Su production may not be so strong. The prevalence of anti-Su was significantly lower in IFN-γ –/– mice but not in IL-4 –/– (Richards et al. 2001) or IL-6 –/– mice (Richards et al. 1998), a finding similar to that of anti-snRNPs. In IL-12–/– mice, the prevalence of anti-Su was not reduced in contrast to significant reduction of the prevalence of anti-snRNPs antibodies (Calvani et al. 2003). Both anti-Su and anti-snRNPs autoantibodies were diminished in type I-IFN receptor-deficient mice (Nacionales et al. 2007). Regarding the effects of toll-like receptors (TLRs), akin to the production of anti-snRNPs autoantibodies in the pristane-induced mouse model, the production of anti-Su was shown to be dependent on the presence of TLR7 (Lee et al. 2008). In addition, anti-Su was produced by any immunocompetent mouse strain, in contrast to anti-snRNPs antibodies that were produced by few mice in certain strains. This suggests that although both anti-Su and -snRNPs are depending on IFN-γ and I-IFN, there may be some differences in the genetic regulation of these two specificities.

The prevalence of anti-Su in specific pathogen-free (SPF) mice was also reduced as compared to conventionally housed mice (Hamilton et al. 1998) although germ-free mice that lack normal bacterial flora still produced anti-Su (Mizutani et al. 2005). These observations indicate that bacteria or exogenous viruses are not essential in the production of anti-Su in the pristane model. Nevertheless, induction of anti-Su by pristane in NZB/WF1 mice (Yoshida et al. 2002; Chan et al. 2009) that do not spontaneously produce anti-Su, suggest a role for unknown environmental factors in the production of anti-Su.

The production of anti-Su in untreated HCV patients is of particular interest in terms of the mechanisms of the production of this autoantibody (Vazquez-Del Mercado et al. 2010b). Since the E2 HCV virus envelope protein can specifically bind to Ago2 and suppress the RNAi pathway (Ji et al. 2008), a feature that is reminiscent of the mouse model of the induction of autoantibodies to p53. In this model, immunization with p53 alone did not induce autoantibodies to p53; however, immunization of viral SVT protein-self p53 complex induced anti-p53 antibodies in addition to antibodies to viral SVT. This raises the intriguing possibility that the binding of viral proteins to self-proteins can break tolerance by modifying self-antigen processing, intermolecular intrastructural help, or other mechanisms

(Dong et al. 1994). Other potential contributing mechanisms include stimulation of TLR7 by HCV RNA as well as miRNAs that are binding to Ago2 as TLR7 stimulation plays a critical role in anti-Su antibody production in a murine model of pristane-induced lupus (Lee et al. 2008). Type I IFN, which is used as a standard treatment of human HCV infections may also be a contributing factor; however, three of four cases of anti-Su had not been treated with IFN (Vazquez-Del Mercado et al. 2010b). Many viral proteins have interactions with miRNA and miRNA binding proteins and their replication is affected by the regulation of miRNAs. Whether other viral infections are associated with the production of anti-Su or other components of GWB is of interest and requires investigation.

4.8 Conclusions

Anti-Su antibodies that recognize Ago2 proteins in GWB are commonly found in various systemic rheumatic diseases and other conditions. These autoantibodies are not specific for a particular diagnosis, and their clinical association and significance have not been established. Why several components of molecules in the RNAi pathway are common targets of autoimmune response, and why anti-Su is particularly at high prevalence, will need to be investigated in future studies.

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Chapter 5
GW/P-Bodies and Autoimmune Disease

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5.1 Introduction

Some patients with autoimmune disease develop antibodies directed against intracellular structures. Despite more than 50 years of research, the etiology of autoantibody formation in these patients remains unknown. Autoantibody formation may precede the development of clinical disease manifestations by years if not decades (Arbuckle et al. 2003; Prince et al. 2004). It therefore seems likely that autoantibodies are involved in the earliest events that occur in an autoimmune disease. Autoantibodies may provide the only clue to the environmental exposure, such as a viral infection, that initiates autoimmune diseases in susceptible individuals.

Although the reason patients develop autoantibodies is unknown, it is clear that human autoantibodies have important practical applications. Some autoantibodies are specific for particular autoimmune diseases, and thereby assist physicians in disease diagnosis (reviewed in (Satoh et al. 2007)). Autoantibodies have also proven to be valuable tools for cellular and molecular biologists (reviewed in (Tan 1989, 1993)). Patients often develop autoantibodies that are present in high-titer and have high-affinity for the corresponding antigens. These antibodies have been used as markers of cellular structures, including GW/P-bodies. In addition, in patients with autoimmune diseases, the humoral immune response appears to be antigen-driven, and over time the antibody response spreads from one or a few epitopes on a single antigen within a multi-protein complex, to many epitopes present on proteins throughout

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the complex. Because antibodies develop against many different components of cellular structures, human autoantibodies have been used to define the composition of a wide variety of cellular structures, including nucleosomes, spliceosomes, nucleoli, nuclear pore complexes, centromeres, Golgi apparatus, Cajal bodies, and PML-Sp100 nuclear bodies (Tan 1989; Stinton et al. 2004; Bloch et al. 1996; Sternsdorf et al. 1995).

Human autoantibodies have played important roles in the history of GW/P-bodies. In addition to serving as markers for GW/P-bodies, anti-GW/P-body autoantibodies have been used to identify new components of these structures. This chapter will review the use of human autoantibodies and novel proteomic techniques to identify GW/P-body components Ge-1, RAP55, and YB-1. Although GW/P-body component GW182 was also discovered using human autoantibodies, the identification and characterization of GW182 will be discussed at greater length in other chapters.

5.2 Identification of Ge-1

In 1990, members of the Clinical Immunology Laboratory at the Massachusetts General Hospital identified a serum sample that contained unusual autoantibodies. The antibodies were identified using the traditional screening test for autoantibodies, which involves indirect immunofluorescence and the HEp-2 cell line as substrate. The serum was obtained from a patient ("Ge") who was thought to have the autoimmune disease Sjögren's syndrome, an illness characterized by the gradual destruction of lacrimal and salivary glands and the presence of antinuclear antibodies. Autoantibodies in the patient's serum produced a reticular staining pattern in the cell cytoplasm. The patient's serum also reacted with 5–20 dot-like structures in both the cytoplasm and the nucleus (Fig. 5.1). This staining pattern had not been seen previously. To investigate the structure and function of the novel cellular

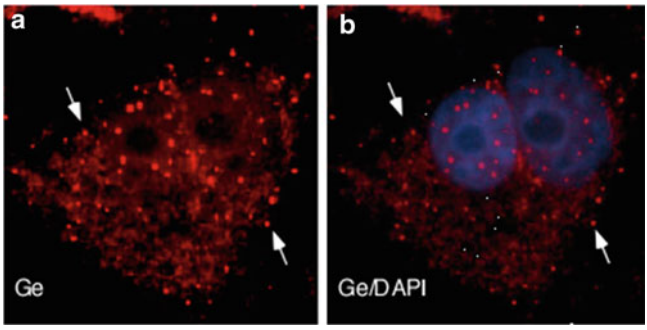

Fig. 5.1 Indirect immunofluorescence using Ge serum and HEp-2 cell substrate. (**a**) Antibodies in Ge serum produced a reticular cytoplasmic-staining pattern. In addition, antibodies reacted with 5–20 dots in both the cytoplasm and nucleus of HEp-2 cells. In (**b**) DAPI staining (*blue*) indicates the location of nuclei. *White arrows* point to representative GW/P-bodies

components recognized by Ge serum, and to further characterize the spectrum of autoantibodies in patients with Sjögren's syndrome, we used Ge serum to identify novel autoantigens.

Ge serum was used to screen a λGT11 bacteriophage expression library prepared from cDNA derived from a human cell line. A cDNA encoding the first immunoreactive protein ("Ge-1") was identified and the full-length cDNA was subsequently cloned from a human umbilical vein cDNA library (Bloch et al. 1994). Using polyacrylamide gel electrophoresis and immunoblot, the protein was found to migrate as a ~160 kDa protein. The predicted amino acid sequence of Ge-1 was relatively unremarkable (as could be assessed in the early 1990s), but was notable for a long stretch of serine residues and a potential bi-partite nuclear localization sequence. The isolated, putative nuclear localization sequence, when fused to the cytoplasmic protein chicken muscle pyruvate kinase (CMPK), induced nuclear localization of CMPK. Partly because of the presence of the functional nuclear localization sequence, Ge-1 was incorrectly identified as a nuclear antigen (Bloch et al. 1994).

In 1997, Garcia-Lorano et al. used serum from a patient with Sjögren's syndrome to identify a cDNA-encoding autoantigen RCD-8 (Garcia-Lozano et al. 1997). The predicted amino acid sequence of RCD-8 was essentially the same as that of Ge-1. Garcia-Lorano and colleagues appreciated that antibodies directed against RCD-8/Ge-1 produced a cytoplasmic-speckled staining pattern (Garcia-Lozano et al. 1997).

With the subsequent discovery of GW182 and the description of GW/P-bodies in the early 2000s (Eystathioy et al. 2002), we reassessed the predicted amino acid sequence and cellular location of autoantigen Ge-1 (Yu et al. 2005). The N-terminus of Ge-1 contains a tryptophan/aspartic acid rich motif, which was originally described by Neer and colleagues and designated "WD40" domain (Neer et al. 1994). The crystal structure of the WD40 motif reveals a seven-blade beta propeller (Renault et al. 1998). This motif is present in a broad range of proteins and is believed to function as a protein–protein interaction domain. The C-terminus of Ge-1 contained a repeating (ψX_{2-3}) motif and was predicted to contain two strong nuclear export sequences (Kutay and Guttinger 2005). To further investigate the cellular location of Ge-1, we expressed a green fluorescent protein (GFP)-Ge-1 fusion protein in HEp-2 cells and showed that the fusion protein co-localized with P-body components DCP1 and DCP2, as well as with autoantigen GW182. The C-terminus of Ge-1 was necessary and sufficient to mediate localization to P-bodies. Depletion of Ge-1, using siRNA, resulted in the loss of staining for DCP1 and DCP2, suggesting that Ge-1 is a central component of P-bodies (Yu et al. 2005).

Fenger-Grön et al. used cell lines over-expressing DCP1 and DCP2 to co-immunoprecipitate Ge-1 and identified the protein as a component of the decapping complex (Fenger-Gron et al. 2005). These investigators showed that Ge-1 functions in vitro as an enhancer of mRNA decapping and renamed the protein "human enhancer of decapping large subunit" (HEDLS, pronounced "headless") (Fenger-Grøn et al. 2005).

A second immunoreactive protein identified in the λGT11 cDNA library using Ge serum proved to be the E2 component of the pyruvate dehydrogenase complex.

Anti-E2 PDC autoantibodies, known clinically as anti-mitochondrial antibodies (AMA), are diagnostic markers of primary biliary cirrhosis (PBC). PBC is an autoimmune disease characterized by slowly progressive destruction of biliary ductules, resulting in hepatic fibrosis and liver failure (reviewed in (Kaplan and Gershwin 2005)). PBC patients may have symptoms that are similar to those of Sjögren's syndrome, including dry eyes and dry mouth. In addition, more than 70% of PBC patients have antinuclear antibodies, making it even more difficult to distinguish between these two autoimmune diseases (Yang et al. 2004). The anti-E2 PDC antibodies in Ge serum explain the reticular cytoplasmic-staining pattern seen in Fig. 5.1. The presence of these antibodies changed the patient's diagnosis from Sjögren's syndrome to PBC.

5.3 Identification of Additional Serum Samples Containing Anti-GW/P-Body Autoantibodies and Use of These Sera to Further Define the Composition of GW/P-Bodies

To permit identification of new GW/P-body components, we first sought to identify additional serum samples containing anti-P-body autoantibodies. Because of the potential relationship between anti-GW/P-body antibodies and PBC, we screened serum from 493 PBC patients for their ability to produce a multiple cytoplasmic dot-staining pattern. Antibodies directed against GW/P-bodies were found in 4% of PBC patients and 15 of the 493 serum samples contained high-titer anti-GW/P-body autoantibodies. Each of the 15 serum samples reacted with several antigens in an immunoblot prepared from a cytoplasmic protein extract derived from HEp-2; five representative immunoblots are shown in Fig. 5.2. The third serum sample (Fig. 5.2), which produced a strong cytoplasmic dot-staining pattern but did not react with Ge-1, was used to screen the λGT11 cDNA library. RAP55 (RNA-associated

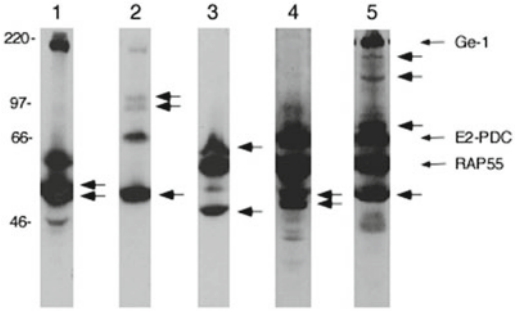

Fig. 5.2 Human sera containing anti-GW/P-body antibodies react with proteins present in an extract prepared from the cytoplasmic fraction of HEp-2 cells. Five representative serum samples were used to probe the nitrocellulose membranes. The location of autoantigens Ge-1 and RAP55, as well as mitochondrial autoantigen E2-PDC, are indicated on the *right*. *Large black arrows* indicate the location of potential, as yet unidentified, GW/P-body autoantigens

protein, 55 kDa) was identified as one of the immunoreactive proteins identified using this serum. This protein had originally been identified in oocytes of *Pleurodeles waltl* and *Xenopus laevis* (Lieb et al. 1998). We demonstrated that a GFP-RAP55 fusion protein localized to cytoplasmic dots in HEp-2 cells and co-localized with GW/P-body components DCP2 and DCP1 (Yang et al. 2006).

5.4 The Use of Human Sera and Protein Arrays to Identify GW/P-Body Components

The screening of λGT11 expression libraries is time-consuming and labor-intensive. In 2000, Bussow and colleagues described the development of a membrane-based macroarray that permits high-throughput screening of human proteins using autoantibodies (Bussow et al. 2000). The technique involves preparation of a cDNA library in a prokaryotic expression vector, transformation of bacteria with these plasmids and growth of individual bacterial clones in microtiter wells. The bacteria are robotically transferred to membranes, and induced to express recombinant protein in situ. We used seven human sera to screen a protein array prepared from phytohemagglutinin-treated human T-lymphocytes Fig. 5.3 (Yang and Bloch 2007).

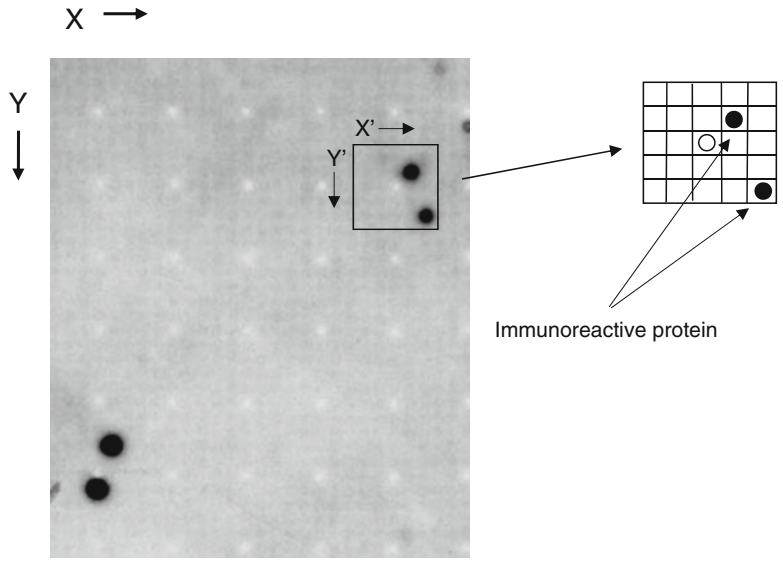

Fig. 5.3 Macroarrays prepared with proteins derived from phytohemaglutinin-treated human T-lymphocytes, were probed with patient sera. A section of the radiograph produced by one array is shown. Twelve proteins are present in duplicate surrounding a central white dot. Detection of two positive dots within a 5×5 block indicates the presence of an immunoreactive protein. The identity of the protein can be determined, based on the *X*- and *Y*-axes of the blocks, and the *x*′ and *y*′ axes of the positive dots within each block

Sixty-seven immunoreactive proteins were identified, including known P-body components DCP1, Ge-1, and RAP55. We further characterized one of the remaining 64 immunoreactive proteins, Y-box protein 1 (YB-1). YB-1 was chosen for further investigation because it was recognized by 4 of the 7 sera and because the protein contains a cold-shock protein domain, which may function as an RNA-binding motif (Evdokimova et al. 2001). GFP-YB-1 localized to cytoplasmic dots in HEp-2 cells and co-localized with Ge-1, DCP1, and other GW/P-body markers. In subsequent studies using 15 serum samples and additional protein arrays, we identified GW/P-body components GW182, EDC3, DCP2, Ago1, Ago2, and Pat1 as additional targets of human autoantibodies. The relationship between GW/P-bodies and more than 300 remaining immunoreactive proteins is the subject of ongoing investigation.

5.5 Reference Sera Contain Antibodies Directed Against Both Ge-1 and GW182

In 2002, Eystathioy and colleagues used serum from a patient with a mixed motor and sensory polyneuropathy to screen a λGT11 cDNA library; an immunoreactive protein fragment was identified (Eystathioy et al. 2002). The nucleotide sequence of the full-length cDNA was determined and the predicted amino acid sequence contained domains that were rich in glycine and tryptophan (G/W) residues. Because of this feature, and the observation that the protein migrated as a ~180 kDa protein in polyacrylamide gel electrophoresis, the protein was designated GW182. GW182 was subsequently shown to co-localize with GW/P-body components DCP1 and LSm4 (Eystathioy et al. 2003). In keeping with the traditional role of human autoantibodies as tools to assist the efforts of molecular and cellular biologists to characterize novel cellular structures, samples of the index serum (18033), and a second reference serum (IC6) were widely distributed to, and used by, investigators in the GW/P-body field.

GW182 and Ge-1 have similar molecular weights and overlapping cellular distribution. Unfortunately, and completely unexpectedly, GW182 reference sera 18033 and IC-6 were found by indirect immunofluorescence and immunoblot to have high-titer antibodies directed against Ge-1 (Bloch et al. 2006). Ge-1 is present in high concentration in all cell lines tested to date and human sera containing anti-Ge-1 antibodies react strongly with the protein in both indirect immunofluorescence and immunoblot (Bloch et al. 2006; Yu et al. 2005). In contrast, GW182 is present in low concentration in cell lines and is difficult to detect by indirect immunofluorescence and immunoblot (Bloch et al. 2006; Moser et al. 2009). It therefore seems likely that 18033 and IC-6 predominantly detect Ge-1, instead of GW182. Studies in the GW/P-body field that relied on reference sera 18033 and IC-6 to detect GW182 require careful reevaluation.

5.6 The Clinical Significance of Anti-P-Body Autoantibodies

Approximately 4% of 493 PBC patients were found to have antibodies directed against GW/P-bodies (Bloch et al. 2005). Although the prevalence of these antibodies is low, it should be noted that indirect immunofluorescence is likely to underestimate the detection of these antibodies, because high-titer anti-E2 PDC autoantibodies, characteristic of PBC patients, may obscure the cytoplasmic dot-staining pattern. Anti-GW/P-body autoantibodies were not detected in any of 248 control patients, suggesting that these antibodies are specific for PBC.

The apparent specificity of anti-GW/P-body antibodies for the diagnosis of PBC has important clinical implications. Because of the presence of these autoantibodies, a 52-year-old woman evaluated at Massachusetts General Hospital with vague musculoskeletal complaints and mildly abnormal liver function tests underwent a liver biopsy. Histological sections of the liver revealed the characteristic findings of advanced (Stage III) PBC (Bloch et al. 2005). The patient has been treated with ursodeoxycholic acid for 8 years and continues to do well, without evidence of disease progression.

In 2007, Bhanji and colleagues reported the clinical manifestations of 42 patients with anti-GW/P-body antibodies identified at the Mitogen Advanced Diagnostics Laboratory at the University of Calgary (Bhanji et al. 2007). In this retrospective, chart-review-based study, 33% of the patients had neurological symptoms, 31% Sjögren's syndrome, and the remainder had other autoimmune diseases including PBC, systemic lupus erythematosus, and rheumatoid arthritis. As patient Ge demonstrates, it is sometimes difficult to distinguish Sjögren's syndrome from PBC, and the latter diagnosis requires an invasive procedure (liver biopsy) for diagnosis. It is therefore possible that additional patients in the University of Calgary patient cohort actually have underlying autoimmune liver disease.

5.7 Other Autoantigens Identified Using Ge Serum: An Unexpected Link Between GW/P Bodies and PML-Sp100 Nuclear Bodies

A third autoantigen identified using Ge serum and the original λGT11 cDNA library was designated Sp140 ("speckled, 140 kDa"), and was shown to be a component of the PML-Sp100 nuclear body (Bloch et al. 1996). Ge serum was subsequently shown to contain autoantibodies directed against several components of this structure, including PML, Sp100, and Sp110 (Bloch et al. 2000). The PML-Sp100 nuclear body is a fascinating cellular structure which has a role in a wide variety of cellular activities including induction of apoptosis and cellular senescence, inhibition of cellular proliferation, maintenance of genomic stability, and the cellular response to viral infections (Bernardi and Pandolfi 2007). Autoantibodies in Ge serum directed against components of the PML-Sp100 nuclear body are responsible for the nuclear dot-staining pattern observed in Fig. 5.1. The observation that Ge serum contained antibodies directed against both

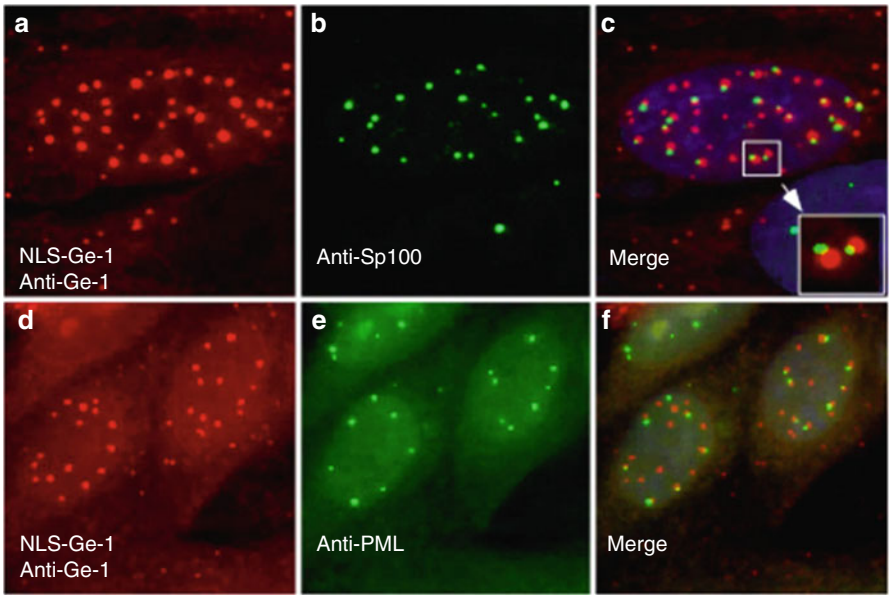

Fig. 5.4 To investigate the potential nuclear location of Ge-1, a nuclear localization sequence (NLS)-Ge-1 fusion protein was expressed in HEp-2 cells. The NLS shifted the location of Ge-1 from the cytoplasm to nucleus. Surprisingly, NLS-Ge-1 was not evenly distributed throughout the nucleus, but was instead localized to nuclear dots (*red* (**a**, **d**)). NLS-Ge-1-containing domains localized adjacent to PML-Sp100 nuclear bodies as determined using anti-Sp100 (*green* (**b**)) or anti-PML (*green* (**e**)) antiserum. (**c**, **f**) Show merge of (**a**, **b**) and (**d**, **e**), respectively. DAPI staining in (**c**, **f**) indicates the location of nuclei. Inset in (**c**) shows the relationship between NLS-Ge-1 nuclear structures (*red*) and PML-Sp100 nuclear bodies (*green*) under higher magnification

PML-Sp100 nuclear bodies and P-bodies raised the possibility that these two structures are functionally related (see below).

In the original report describing the identification of Ge-1, the protein was found to have a functional nuclear localization sequence (Bloch et al. 1994). Subsequent sequence analysis revealed the presence of two putative nuclear export sequences. The presence of both nuclear import and export sequences raised the possibility that Ge-1 shuttles between nucleus and cytoplasm. Because Ge-1 is found exclusively in the cytoplasm by indirect immunofluorescence using fixed tissue culture cells, the normal equilibrium must strongly favor nuclear export. To investigate the potential nuclear location of Ge-1, we used an exogenous nuclear localization sequence, derived from SV40 T antigen (Rihs and Peters 1989), to shift the protein's location from cytoplasm to nucleus. Expression of NLS-Ge-1 in HEp-2 cells resulted in localization of the protein to both the nucleus and the cytoplasm (Fig. 5.4). Nuclear Ge-1 was not distributed diffusely throughout the nucleus, but was instead localized to discrete, dot-like structures resembling PML-Sp100 nuclear bodies. Interestingly, patient Ge had autoantibodies directed against both GW/P-bodies and PML-Sp100 nuclear bodies. To consider the possibility that the NLS-Ge-1-containing nuclear bodies are related to PML-Sp100 nuclear bodies, NLS-Ge-1-transfected cells were stained with anti-Ge-1 antibodies and

anti-Sp100 or anti-PML antibodies (Fig. 5.4a–f). NLS-Ge-1 nuclear bodies localized immediately adjacent to PML-Sp100 nuclear bodies.

Taken together, the observation that patients with PBC develop autoantibodies directed against PML-Sp100 nuclear bodies and GW/P-bodies, and the possibility that a fraction of cellular Ge-1 may localize adjacent to PML-Sp100 nuclear bodies, raise the possibility that these structures are functionally linked. The significance of this relationship, in terms of cellular biology, is unknown.

5.8 Summary

Human autoantibodies have performed admirably in the service of characterizing GW/P-bodies. These antibodies have provided a critical point of reference by which other proteins have been shown to be components of GW/P-bodies. In addition, autoantibodies have been used to identify new GW/P-body components, including Ge-1, GW182, RAP55, and YB-1. Using new, high-throughput screening assays, it is likely that additional, novel GW/P-body components will be identified. Human autoantibodies have also raised the possibility of a functional link between two apparently unrelated cellular structures, PML-Sp100 nuclear bodies and GW/P-bodies.

A key unanswered question remains: What is the role of GW/P-bodies in the pathogenesis of autoimmune disease? Over the next 10 years, as more is learned about the function of GW/P-bodies, it is hoped that molecular and cellular biologists will further consider this question and remember the important contributions of patients with autoimmune disease to the early characterization of these cellular structures.

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Chapter 6
Function of GW182 and GW Bodies in siRNA and miRNA Pathways

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Abstract GW182 is an 182 kDa protein with multiple glycine/tryptophan repeats (GW or WG) playing a central role in siRNA- and miRNA-mediated gene silencing. GW182 interacts with its functional partner Argonaute proteins (AGO) via multiple domains to exert its silencing activity in both pathways. In siRNA-mediated silencing, knockdown either GW182 or Ago2 causes loss of silencing activity correlating with the disassembly of GWBs. In contrast, GW182 and its longer isoform TNGW1 appear to be downstream repressors that function independent of Ago2, whereas the Ago2-GW182 interaction is critical for the localization of Ago2 in the cytoplasmic foci and its repression function. GW182 contains two non-overlapping repression domains that can trigger translational repression with mild effect on mRNA decay. Collectively, GW182 plays a critical role in miRNA-mediated gene silencing.

6.1 Introduction

The protein GW182, discovered in 2002 by using a serum from an autoimmune patient with motor and sensory neuropathy (Eystathioy et al. 2002), is characterized by a large number of glycine/tryptophan (GW and WG) repeats that are distributed in various domains throughout its sequence. GW182 is an 182 kDa phosphoprotein with up to 60 copies of GW/WG motifs, some of which have been

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shown to bind to its functional partner in the Argonaute protein family (Ago1-4) and are critical in microRNA (miRNA)-mediated silencing function. The GW182 protein family has three paralogs in mammals named TNRC6A, B, and C, any of which have been demonstrated to play key roles in small interference RNA (siRNA) and miRNA silencing (Filipowicz et al. 2008; Eulalio et al. 2009c; Krol et al. 2010; Huntzinger and Izaurralde 2011).

The initial discovery of GW182 noted that it was primarily localized to distinct cytoplasmic foci with dynamic morphology and movement (Eystathioy et al. 2002; Yang et al. 2004). Based on their unique GW content and cytoplasmic localization, these foci therefore were provisionally termed GW bodies (GWBs). A set of other proteins involved in RNA degradation were later shown to reside in GWBs linking this structure to the function of RNA turnover (Jakymiw et al. 2007; Meaux et al. 2008; Anderson and Kedersha 2009; Erickson and Lykke-Andersen 2011). These bodies are generally acknowledged to be conserved among species since mammalian GWBs share a similar subset of protein homologs with yeast or *Drosophila* processing bodies (P-bodies). However, Ago2 and GW182 are examples of some key proteins that do not have homologs in yeast P-bodies (Anderson and Kedersha 2006). In addition, the size and number of GWBs increase during cell proliferation in mammalian cells (Yang et al. 2004), whereas as a reflection of their functional differences, the number and size of P-bodies in yeast increase in response to stress (Jakymiw et al. 2007; Erickson and Lykke-Andersen 2011). The heterogeneity of GWBs is denoted by observations that not all foci have the same composition: some foci contain undetectable or very low levels of Ago2, mRNA de-capping activator Dcp1a, and DEAD box RNA helicase rck/p54 (Fig. 6.1).

siRNAs and miRNAs partially share the same pathway in silencing their targets based on their Watson-Crick base pair matching for target recognition (Fig. 6.2). miRNAs are usually derived from endogenous transcripts from their respective gene loci to first form primary miRNAs (pri-mRNAs) with 7-methylguanosine (m^7G) caps and polyadenylated (A_n) 3′-ends flanking hairpin structures as shown in Fig. 6.2 (right panel). Mammalian genes for miRNAs exist as single genes, gene clusters, and within introns of other genes. The RNase III endonuclease Drosha-DGCR8 complex processes pri-miRNAs into about 70-nucleotide hairpin structures referred as precursor-miRNAs (pre-miRNAs). After being transported into cytoplasm, pre-miRNAs further incorporate onto Dicer, another RNase III endonuclease, resulting in a ~22 base pairs miRNA:miRNA* matured duplex (Filipowicz et al. 2008; Jinek and Doudna 2009; Fabian et al. 2010; Huntzinger and Izaurralde 2011). In contrast, siRNAs originate from long double-stranded RNAs that can be derived from viral replication, transposons, or convergent transcripts. The Dicer complex slices these long RNAs into 20 ~ 25 base pairs double-stranded RNA duplex. Synthetic siRNA duplexes of the same size can also knockdown target genes. siRNA or miRNA duplexes undergo a selective loading process to AGO based on the thermodynamic preference of the two strands. Guiding strands directly associate with AGO proteins and the passenger strand will be ejected and eventually degraded (Rana 2007; Hutvagner and Simard 2008; Jinek and Doudna 2009).

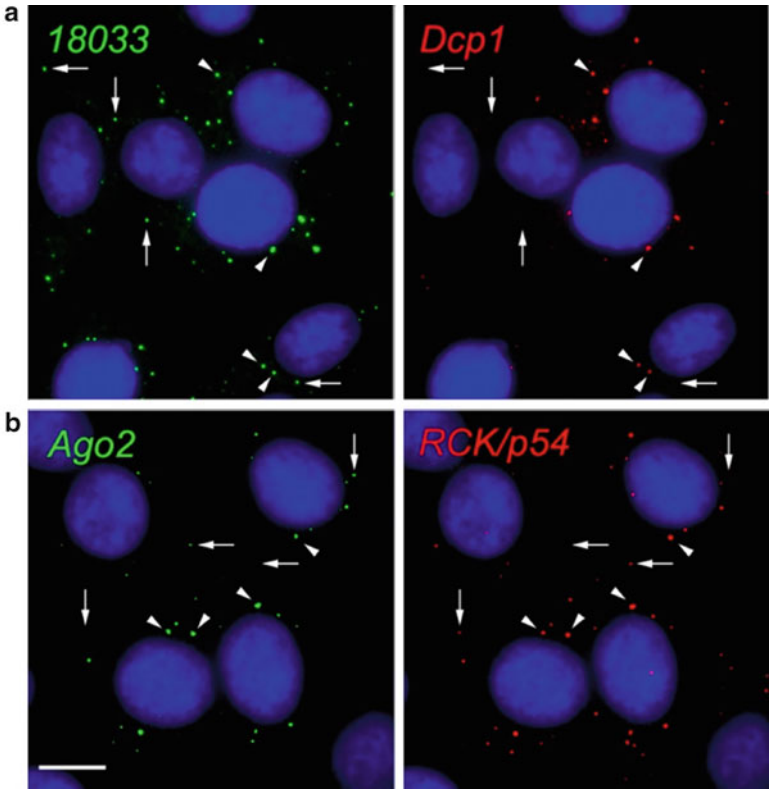

Fig. 6.1 GW bodies (GWBs) are heterogeneous structures with some obvious differences in protein composition. (**a**) HEp-2 cells costaining with prototype human anti-GWB serum 18033 (*green*), known to contain antibodies to GW182, hAgo2, and Ge-1, but not Dcp1, and rabbit anti-Dcp1 antibody (often used as a marker for P-bodies; *red*) demonstrate that although there are many co-stained foci (*arrowheads*), not all GWBs contain detectable Dcp1 (*arrows*). (**b**) HEp-2 cells costaining with mouse anti-hAgo2 monoclonal 4F9 (*green*) and rabbit anti-RCK/p54 (*red*) polyclonal antibodies demonstrate again that most foci have both (*arrowheads*) but not all containing both RCK/p54 and hAgo2 (*arrows*). Nuclei (*blue*) were counterstained with DAPI. Bar, 10 μm

After loaded to Ago2, guiding strands form a perfect complementarity with their target mRNAs, following which, Ago2 cleaves the target mRNA by its endonuclease activity localized in its C-terminal PIWI (RNase H-like P-element induced wimpy testis) domain (Liu et al. 2004). miRNA-loaded AGO complexes are tethered to 3′UTR of target mRNAs and form an imperfect complementarity but are usually strictly matched at miRNA positions 2–8, known as "seed sequence" (Bartel 2009). All 4 AGO proteins (Ago1-4) are involved in miRNA-mediated gene silencing and the GW182 protein family functions downstream as key repressors by inhibiting active translation, as well as trigger bound mRNA deadenylation and eventual degradation (Fabian et al. 2010; Krol et al. 2010; Huntzinger and Izaurralde 2011) (Fig. 6.2).

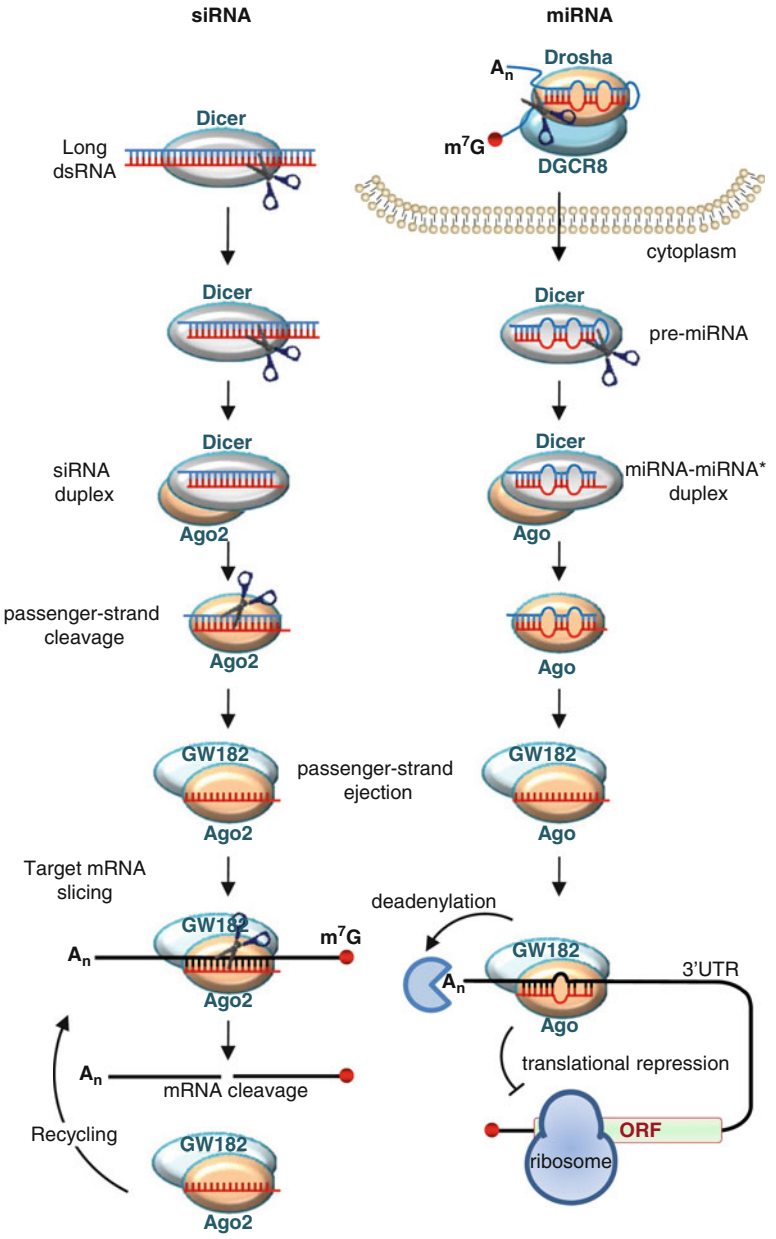

Fig. 6.2 Biogenesis and mechanism of the two main classes of small regulatory RNA. Long double-stranded RNA (dsRNA) derived from viral infection, transposon or convergent transcription can be recognized and processed by Dicer, an RNase III endoribonuclease, to become 20–25 bp small interference RNA (siRNA, *left panel*). The siRNA duplex including both the guiding strand (*red*) and passenger strand (*blue*) is transferred from Dicer onto Ago2, the catalytic component of RNA-induced silencing complex (RISC) complex also possessing endonuclease activity on its C-terminal PIWI domain. Core protein components Ago and GW182 are representing the

Extensive mapping of AGO-interacting and repression domains on GW182 generated consensus that the GW182 N-terminal GW-rich domain primarily binds to AGO on multiple sites, whereas the C-terminal domain possesses intrinsic silencing activity (Baillat and Shiekhattar 2009; Lazzaretti et al. 2009; Lian et al. 2009; Takimoto et al. 2009; Zipprich et al. 2009). However, in addition to that, the *Drosophila* and human N-terminal effective domain, which in fact covers much of the middle region of GW182, also has silencing activity (Chekulaeva et al. 2009; Yao et al. 2011; Zipprich et al. 2009). Although the detailed molecular mechanism underlying translational repression remains elusive, increasing evidence has suggested that the conserved GW182 Pam2 motif (Poly-A binding protein (PABP)-interacting motif 2) functions as a major docking site to interact with PABP (Fabian et al. 2009; Jinek et al. 2010). In addition, GW182 can further recruit CCR4-NOT deadenylase complex to facilitate mRNA decay (Braun et al. 2011; Chekulaeva et al. 2011; Fabian et al. 2011). This dynamic process may occur in GWBs and a general model of how they affect translational repression and mRNA degradation will be discussed towards the end of this chapter.

Although a number of studies have dissected the function of GW182, it needs to be pointed out that there are a few inconsistencies. These discrepancies may arise from differences in the selection of systems (e.g., using Drosophila, yeast, vs. mammals) or methodologies employed (e.g., investigating miRNA and mRNA by qRT-PCR vs. Northern blot). In this chapter we primarily focus on the role of GW182 and GWBs in siRNA and miRNA silencing pathway based on a number of published reports from our laboratory over the past 10 years (Eystathioy et al. 2002, 2003; Yang et al. 2004; Jakymiw et al. 2005, 2007; Lian et al. 2006, 2007, 2009; Pauley et al. 2006, 2010; Li et al. 2008; Yao et al. 2011).

Fig. 6.2 (continued) holo-RISC complex. During the loading process, the passenger strand is cleaved and ejected. Ago2 and GW182 together with the guiding strand identify and hybridize perfectly with the target mRNA. Ago2 then catalyzes the cleavage at the middle region of the siRNA-mRNA duplex to slice the mRNA into two halves. The complex may be recycled for multiple rounds of cleavage. In contrast, miRNA is often derived from primary miRNA (pri-miRNA) which is transcribed from a single miRNA gene locus, multiple gene clusters, or processed from introns. Pri-miRNA has a 5′-end cap, a poly-A tail, and a hairpin stem-loop secondary structure and is processed by Drosha-DGCR8 protein complex in the nucleus to become hairpin precursor miRNA (pre-miRNA). Pre-miRNA is translocated into cytoplasm through a nuclear transporter and then bound by Dicer. Similar to the siRNA pathway, Dicer processes the pre-miRNA into matured miRNA-miRNA* duplex guiding strand and passenger strand (also known as miRNA*) which is then transferred to Ago. The guiding strand loaded complex forms imperfect complementary with its target mRNA preferentially at the 3′UTR. All the Argonaute family proteins can be involved in miRNA-mediated silencing and GW182 plays a critical role in translation repression, deadenylation, and mRNA decay. Modified from Jinek and Doudna (2009).

6.2 Role of GW182 and GWBs in siRNA Silencing Pathway

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

6.3 Disruption of GW Bodies Impaired Mammalian RNA Interference

GW182 and Ago2 co-localized with transfected siRNA in GWBs. Since the discovery of GW182 and GWBs in 2002, substantial efforts have elucidated their functional implications. In 2005, Jakymiw et al. and others simultaneously reported that GW182 bound to Ago2 (Jakymiw et al. 2005; Liu et al. 2005), linking it to the then newly established siRNA and miRNA work (Fire et al. 1998; Lee et al. 1993). By using an Ago2-specific antibody, we demonstrated that Ago2 localized to discrete cytoplasmic foci that were co-stained with an anti-GW182 monoclonal antibody as well as the prototype human anti-GWB serum 18033 (Jakymiw et al. 2005). Co-immunoprecipitation data using polyclonal human serum 18033 or mouse monoclonal anti-GW182 antibody 4B6 pulled down Ago2, demonstrating the strong interaction between Ago2 and GW182. Intriguingly, Cy3-3′-end-labeled lamin A/C-specific antisense siRNA duplex localized to GWB and co-immunoprecipated with anti-GW182 antibody, implying the potential functional connections between GWBs and siRNA pathway.

Disassembly of GWBs using dominant-negative constructs correlates with the loss of RNAi activity. An unexpected finding by Jakymiw et al. (2005) was that overexpression of N-terminal fragment of GW182 termed Δ1 (aa1-498) or the C-terminal PIWI domain of Ago2 causes the disassembly of GWB due to their putative dominant-negative effect. It is now known that Δ1 has at least one Ago2 binding site (Takimoto et al. 2009), while Ago2 PIWI domain binds GW182 (Lian et al. 2009). Thus, transient transfection of GFP-Δ1 or GFP-PIWI into HeLa cells leads to the disassembly of GWBs correlating with the reduction in siRNA efficacy (Jakymiw et al. 2005). This data strongly supported the functional relevance of GWBs in the siRNA pathway.

Directly knocking down of GW182 impairs siRNA function. After demonstrating the role of GWBs in siRNA silencing pathway with overexpression of dominant-nega-

tive constructs, it still remained ambiguous that whether the protein GW182 played a direct role in this process. A direct and specific method was needed to address this concern. Our laboratory had previously demonstrated that knocking down GWB scaffold protein GW182 by specific siRNA could affect the stability of GWBs (Yang et al. 2004). Therefore, a sequential knockdown strategy was performed to first knockdown the endogenous GW182 protein followed by a second transfection conducted with siRNA against lamin A/C 48 h later. The results clearly demonstrated that depletion of GW182 caused the disappearance of the GWB foci, and more importantly, abolished the knockdown of lamin A/C. In contrast, the knockdown of lamin A/C was affected when the cells were firstly transfected with a siRNA against luciferase, which was not a target in these cells. Similar results in reduced lamin A/C siRNA reporter activity were obtained when lamin A/C siRNA was co-transfected with siRNA to GW182 (Lian et al. 2006).

GW182 paralogs TNRC6B and TNRC6C largely share conserved domains with and have been shown to have functional redundancy (Eulalio et al. 2009c; Tritschler et al. 2010). In fact, depletion of individual GW182 family members caused partial de-repression of silenced reporters (Zipprich et al. 2009), and tethering assays mapped similar silencing domains on all GW182 family proteins (Baillat and Shiekhattar 2009; Eulalio et al. 2009a; Lazzaretti et al. 2009; Zipprich et al. 2009). It remained to be determined the distinctive functions of each GW182 paralogs, in particular cellular context.

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

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

After demonstrating the importance of GWBs to siRNA function, as reviewed above, it was still not clear that how siRNA in turn affect the size and number of GWBs. Lian et al. investigated the changes in size and number of GWBs after transfecting with exogenous siRNAs and the major protein players involved in this dynamic process (Lian et al. 2007).

Transfection of siRNAs with endogenous targets increased the size and number of GWBs. To address the correlation between siRNA and GWBs, siRNA against endogenous lamin A/C or siRNA against luciferase, which did not have an endogenous target, was transfected into HeLa cells. The size and number of GWBs were monitored by indirect immunofluorescence using both prototype human serum 18033 and rabbit anti-Dcp1a antibody. Intriguingly, larger and greater numbers of

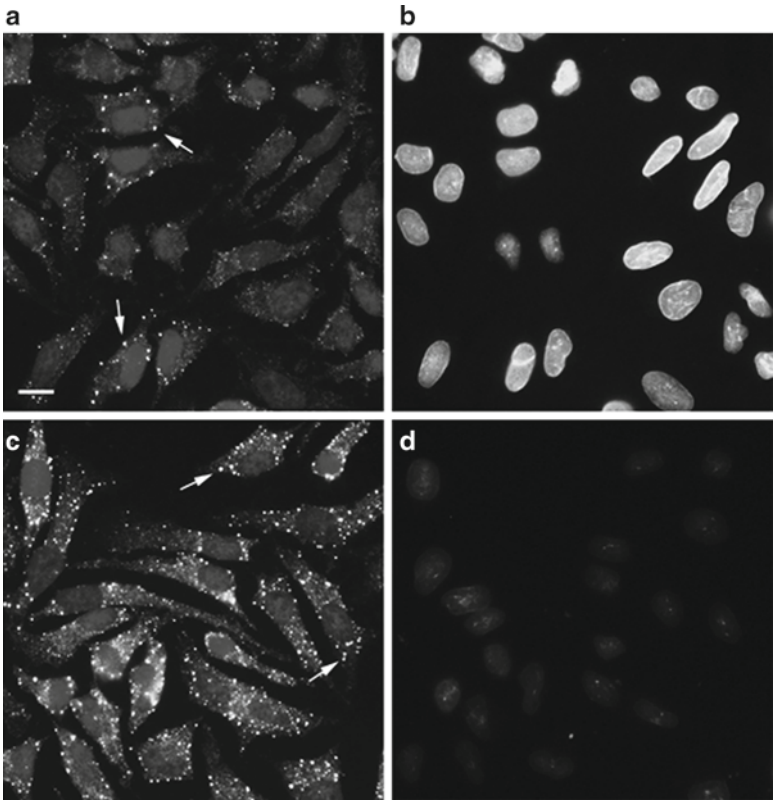

Fig. 6.3 Transfection of siRNA for lamin A/C increased the size and number of GWB in HeLa cells. HeLa cells were mock transfected (**a**, **b**) or transfected with 100 nM siRNA for lamin A/C (**c**, **d**). Transfected cells were fixed 2 d after transfection, and then were co-stained with human anti-GWB serum 18033 (**a**, **c**) and mouse anti-lamin A/C (**b**, **d**) for monitoring the efficient knockdown of lamin A/C. Examples of large GWBs are indicated by *arrows*. Bar, 10 μm

GWBs were observed only in lamin A/C siRNA transfection, but not in mock (Fig. 6.3) or luciferase siRNA that lacked endogenous target (Lian et al. 2007). Another siRNA against endogenous target RAGE showed similar effects as lamin A/C siRNA induced higher numbers and increased size of GWBs, but not by a synthetic "RISC free" chemically modified siRNA, (Lian et al. 2007). siRNAs against endogenous targets do not appear to affect stress granules as detected by marker TIAR, the T-cell intracellular antigen-1(TIA-1)-related protein. The targeting mRNA-dependent effect in these experiments was further examined in 3T3 cells stably transfected with GFP vs. untransfected 3T3 cells using a single siRNA to GFP. The transfection of the GFP siRNA led to a significant increase in both the size and number of GWBs in the stable GFP expressing 3T3 cells, but not in wildtype 3T3 lacking GFP transcripts (Lian et al. 2007). The protein levels of several GWB

components, such as Ago2, Dcp1a, rck/p54, and RNA binding protein Lsm4, did not increase upon lamin A/C siRNA transfection and an observation that is consistent with the concept that these proteins are recruited from free cytoplasmic forms into GWBs.

A detailed 4-day experiment was set up to determine the dynamic change in size and number of GWBs after siRNA transfection (Lian et al. 2007). GWBs were much larger on day 3 and 4 than on day 1 in cells transfected with lamin A/C siRNA, but not in mock transfected, or luciferase siRNA transfected controls in paralleled experiments. Quantitative analysis revealed the average number of GWBs in lamin A/C siRNA transfected cells was higher from day 1 to day 4 with the peak in day 3 compared to mock or luciferase siRNA transfected cells. The highest number of GWBs in day 3 was approximately fivefold higher than controls, suggesting the formation of these foci was correlated with siRNA activity. The increase in number and size of GWB may imply that smaller foci formed at earlier time points correlated with the early silencing of RNA, whereas the larger foci formed in Day 3 might be attributed to the accumulation of mRNA limited by rate of RNA decay.

GW182 and Ago2 were required for siRNA silencing activity. Our previous data suggested that GW182 was a signature protein for GWB assembly and important for siRNA silencing activity (Yang et al. 2004; Jakymiw et al. 2005). It also demonstrated that the number and size of GWBs were closely correlated with siRNA pathway possibly by recruiting essential protein components from cytoplasm and facilitating this process inside the foci.

In related experiments, efficiently knocking down of Ago2 seemed to have very little effects on GWB formation, implying Ago2 was not critical for the formation of these foci (Lian et al. 2007). However, the requirement for Ago2 had not been tested regarding its impact on siRNA induced GWBs. siRNA against Ago2 and lamin A/C or control luciferase siRNA were co-transfected to knockdown Ago2. Remarkably, knockdown of Ago2 generated noticeable effects on siRNA against lamin A/C with 60% silencing activity remained (Lian et al. 2007). Notably, it abrogated the change in size and number of GWBs induced by lamin A/C siRNA, which reduced to a percentage that was comparable to the control cells. Taken together, these data strongly argued that Ago2 was not vital for GWB formation, but was required for newly formed GWBs induced by siRNAs against endogenous targets.

These data suggested Ago2 was not critical for GWB formation as its knockdown had a minor impact on GWB foci. It was important to determine if the function of Ago2 may possibly be compensated by other Ago family proteins and had less impact on GWB foci. Knocking down GW182 showed remarkable effects on GWB formation, emphasizing its importance in forming these foci and in siRNA silencing pathway. Nevertheless, there was insufficient data to elucidate whether GWBs were required or only the consequence for siRNA-mediated silencing function, or depending on the presence of RNAi machinery, the regulation of GWB assembly may vary in different species. As mentioned above, Argonaute and GW182 protein lack their homologs in yeast and this may explain the different observation between yeast P-body and mammalian GWBs (Sheth and Parker 2003; Teixeira et al. 2005). Yeast P-bodies

were considered to process global messages, whereas GWBs might be specifically for small RNA induced silencing.

In summary, transfection of siRNA against endogenous target can induce increase in both the size and number of GWBs. GW182 and Ago2 were required for functional siRNA silencing activity. Based on these data, it can be concluded that RNAi was a key regulatory mechanism for the assembly of GWBs.

6.5 Role of GW182 and GWBs in miRNA Silencing Pathway

The first described miRNA, *lin-4*, can negatively regulate its target gene LIN-14 during different developmental stages in *C.elegans* (Lee et al. 1993). The identification of *let-7* miRNA as the first miRNA conserved across species has opened a door for the remarkable advances in the field of miRNA research dating to 2000 (Pasquinelli et al. 2000). It is currently held that more than 50% of mRNAs are regulated by miRNAs involved in almost all known cellular pathways (Filipowicz et al. 2008; Krol et al. 2010). GW182, discovered 2 years after *let-7* was described in mammals, has become a central player in the miRNA-mediated silencing pathway (Huntzinger and Izaurralde 2011; Krol et al. 2010). Our laboratory has been working on the role of GW182 in miRNA pathway including identification of novel GW182 isoform, elucidation of Ago2 binding domains, and mRNA silencing domains in the past few years (Li et al. 2008; Lian et al. 2009; Yao et al. 2011).

6.6 GW182 Has a Longer Isoform TNGW1 That Both Served as Translational Repressors in Ago2-Mediated Silencing

TNGW1 is a novel isoform of GW182 and distinct in both transcriptional and translational level. Human *TNRC6A* (the gene encoding GW182) is located on chromosome 16p11.2. Since the discovery of GW182 (Eystathioy et al. 2002), homologs have been subsequently characterized in various species including *Drosophila* and *C. elegans* (Ding et al. 2005; Schneider et al. 2006; Eulalio et al. 2007; Miyoshi et al. 2009). However, the isoforms of GW182 were not explored until 2008 (Li et al. 2008). Interestingly, both the NCBI database and University of California Santa Cruz genome browser predicted a novel isoform of GW182, later named as TNGW1 by Li et al. with a nucleic acid sequence identical to GW182, but having an additional 5' extended sequence containing trinucleotide repeats (TNRs). The mRNA for *TNRC6A* longer isoform TNGW1 contained five additional exons upstream of the putative AUG start codon of GW182 and the TNR repeat domain was encoded by the fifth exon. To verify the presence of this isoform, reverse transcriptase PCR using primers flanking the unique N-terminal TNR domain were utilized to examine RNA from a number of different human

cell lines and tissue samples. The amplified PCR products were submitted to direct DNA sequencing, which validated the correct length and an in-frame junction between the novel 5′ exons of TNGW1 and GW182 as found in the various cells examined.

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

We then asked, what is the relationship between these two proteins? They could be independently translated from distinct mRNAs, from the same mRNA with a different AUG start codon, or GW182 could simply be a post-translationally processed form of TNGW1. To distinguish these three possibilities, siRNA specifically targeted TNGW1 mRNA (referred as siTNR, thereafter) was applied to discriminate TNGW1 and GW182 mRNA. Forty-eight hours after transfection, only TNGW1 protein became undetectable, whereas levels of the GW182 protein remained the same compared to the control or mock transfection (Li et al. 2008). These results demonstrated that TNGW1 protein was derived from its own unique mRNA. It remained unclear whether GW182 was the post-translational product from TNGW1 because the GW182 band observed after siTNR treatment could be a stable product processed from TNGW1. However, the Western blot result from cells treated with siRNA targeted common region of TNGW1 and GW182 (referred as siGW182) ruled out this possibility because the disappearance of both proteins suggested both isoforms retained similar turnover rates. We concluded that each protein isoform is reasonably considered as a product translated from its individual mRNA. Collectively, TNGW1 and GW182 appear to be derived from distinct transcription and translation events (Li et al. 2008).

Intracellular localization of TNGW1 and its relationship with other GWB components. It was shown previously that GW182 was a scaffold protein that colocalized with RNA decay factor such as Dcp1a, Ge-1, and Ago2 in GWBs (Jakymiw et al. 2007). Indirect immunofluorescence was used to examine the intracellular location of TNGW1 and its role in GWB formation. Notably, both mouse and rabbit derived anti-TNGW1 only stained a subset (~30%) of GWBs compared to anti-GWB serum 18033. By contrast, a mouse monoclonal anti-GW182 antibody stained the majority, although not all, GWBs recognized by 18033. These data implied that TNGW1 and GW182 only resided in a subset of GWBs that were not necessarily localized in the same GWBs. Double staining

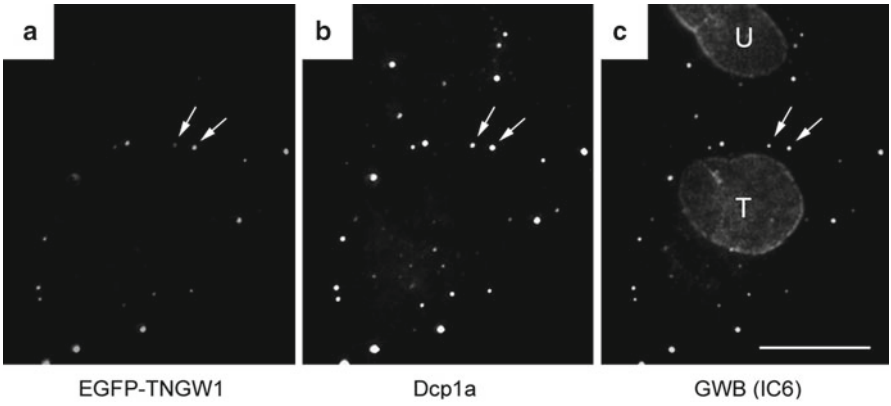

Fig. 6.4 Transfected TNGW1 co-localized with RNA decay factor Dcp1a in GWBs. HeLa cells transfected with EGFP-TNGW1 (**a**) were fixed 24 h after transfection and analyzed by indirect immunofluorescence using rabbit anti-Dcp1a (**b**) and human serum IC6 ((**c**), recognizes Ge-1, RAP55 and unrelated nuclear envelope protein). The transfected EGFP-TNGW1 formed cytoplasmic foci that were co-stained using both anti-Dcp1a and IC6 antibodies. Bar, 10 μm. *T* transfected; *U* untransfected

using both anti-TNGW1 and anti-GW182 antibody confirmed this observation, in that more GWBs were detected by anti-GW182 than by anti-TNGW1-specific antibodies and all GWBs stained by anti-TNGW1-specific antibodies were co-stained by anti-GW182. In summary, in the cells and tissues we have examined to date, TNGW1 is either absent or expressed at much lower levels compared to GW182 in a subset of GWBs.

It was not clear if the extra TNR region on TNGW1 affected the ability of other GWB components to localize to GWB. Co-expression experiments of Ago2 with either TNGW1 or GW182 were performed to determine this possibility. Indirect immunofluorescence showed that either TNGW1 or GW182 was enriched in GWB together with Ago2. Those data supported the theory that the extra N-terminal TNR domain on TNGW1 did not interfere with TNGW1 and its functional partners to locate in GWBs. It was further confirmed by a single transfection with GFP-TNGW1. In this particular experiment, GWBs labeled by GFP-TNGW1 were also co-stained by anti-Dcp1a and another human anti-GWB serum IC6. Intriguingly, transfected cells with either high or low expression of GFP-TNGW1 did not affect the co-localization of endogenous Dcp1a (Fig. 6.4). Taken together, those data suggested GW182 can be substituted by TNGW1 for its putative functions such as formation of the foci that are enriched in RNA decay factors.

Our previous data showed that GW182 is critical for GWB formation as knockdown of GW182 resulted in GWBs becoming undetectable (Yang et al. 2004). However, at the time the existence of TNGW1 was not appreciated and siRNA used in those experiments could knockdown both forms. To examine if TNGW1 was required for GWB formation, siTNR was transfected into HeLa cells to achieve

specific knockdown of TNGW1. In a side-by-side siGW182 transfected experiment, GWBs dissembled 2 days post-transfection as monitored by both anti-Dcp1a and human serum 18033. In comparison, siTNR transfected cells still showed GWB foci 2–3 days after transfection, albeit the protein TNGW1 was non-detectable. These observations demonstrated that TNGW1 was not critical in GWB formation when cells continue to express GW182.

TNGW1 and GW182 exerted a strong repression effects independent of Ago2. To determine the silencing effect induced by TNGW1 and GW182, a reporter tethering assay was adopted from Dr. Filipowicz's work (Pillai et al. 2004). An N-terminal λN-hemagglutinin (NHA) polypeptide tag was fused to TNGW1, GW182, or Ago2. The NHA tag binds strongly to the 5BoxB RNA secondary structure cloned in the 3′UTR of either Firefly luciferase (FL) or Renilla luciferase (RL) reporter (Fig. 6.5a). Co-transfection with NHA-tagged GW182 or Ago2 fragments with the RL-5BoxB reporter allowed discrimination of their repression activity. RL- or FL-no sites in their 3′UTR served as an internal control, respectively. A 46% repression was observed to the reporter when NHA-Ago2 was tethered to the 3′UTR of the reporter, which is consistent with the original report describing this tethering assay (Pillai et al. 2004). In comparison, tethered NHA-TNGW1 or -GW182 induced stronger repression (67.6% and 65.3%, respectively), which was 46.9% or 41.3% stronger than that induced by Ago2, respectively (Li et al. 2008). Quantitative RT-PCR was also performed to analyze the stability of reporter mRNA level. Interestingly, tethered Ago2 induced 50.8% reporter mRNA degradation as compared to tethered TNGW1 and GW182 (24.5% and 23.7%, respectively). These data together indicate that tethered GW182 and TNGW1 possess stronger silencing activity than Ago2. The silencing activity in this experiment is primarily caused by interfering with translation with only mild effects on the reporter mRNA stability. In contrast, tethered Ago2 causes more reporter decay than GW182/TNGW1.

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

The data was not sufficient to distinguish the different functional roles between TNGW1 and GW182. Since TNGW1 was only localized in a subset of GWBs, it was postulated that the N-terminal TNR domain in TNGW1 may be responsible for binding and recruiting additional factors to facilitate some specialized repression activity. The differential co-localization of TNGW1 and GW182 once again emphasized the heterogeneity of GWBs that may reflect their important functional difference in silencing mRNA.

In summary, a novel 210 kDa GW182 isoform, TNGW1, was shown to be transcribed and translated independently from GW182. Both TNGW1 and

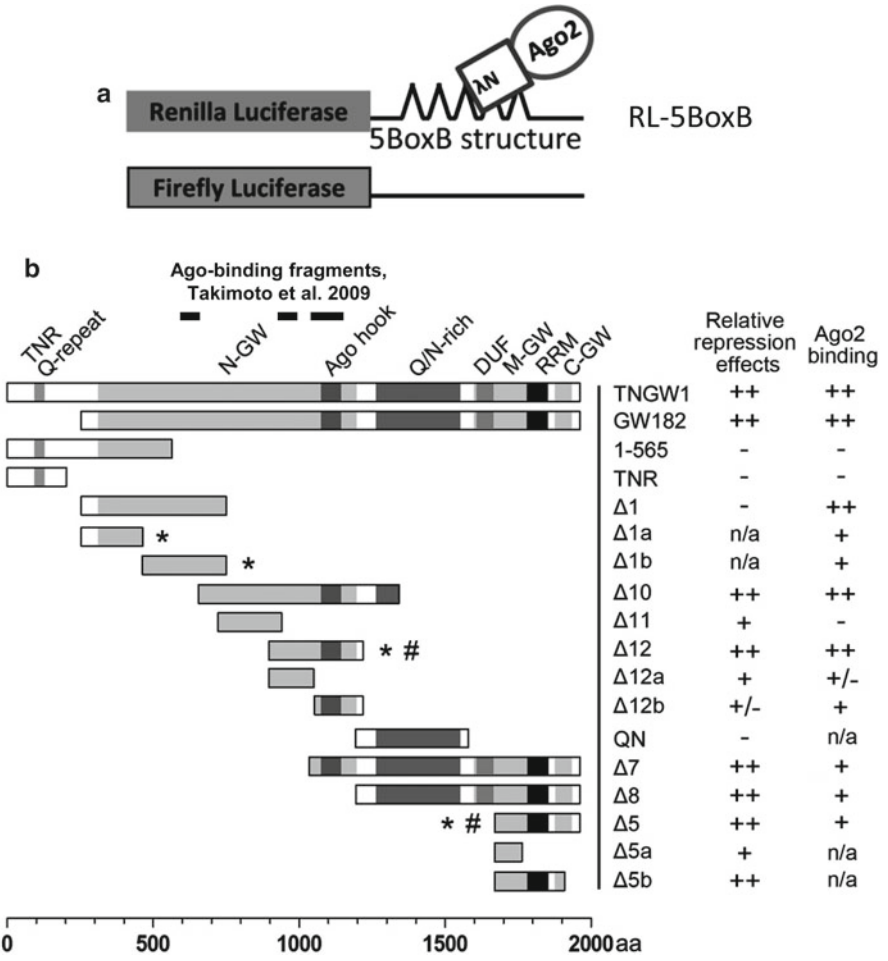

Fig. 6.5 Two non-overlapping GW182 fragments possess repression activity demonstrated in a tethering assay. (**a**) Schematic diagram illustrating the design of the tethering assay using dual luciferase reporters co-transfected to HEK293 cells (Pillai et al. 2004). In this illustration, the Renilla luciferase reporter has a 5boxB RNA element in the 3′UTR. The fusion protein with λN domain binds to the 5boxB element and essentially tethers its fusion partner Ago2 to the 3′UTR. This leads to translational repression as represented by lower Renilla luciferase activity normalized to firefly luciferase activity. This tethering assay thus can determine translation repression activity for any given protein domain fused to λN. (**b**) Summary of the repression activities and Ago2 binding ability for all λN-GW182 deletion constructs generated from full-length TNGW1 and GW182. All repression effects of NHA-tagged constructs in tethering assays are demonstrated. ++, strong repression or binding; +, medium repression or binding; +/−, relatively weak repression or binding; −, no repression or binding. The two non-overlapping repression domains Δ12 and Δ5 are indicated by number signs, while the four non-overlapping Ago2-binding domains are indicated by asterisks. N-GW, M-GW, and C-GW represent N-terminal, middle region, and C-terminal GW-rich domains. DUF, domain of unknown function, recently renamed as Pam2, PABP-interacting motif 2. RRM, RNA recognition motif. (**c**) Fine mapping of identified GW182 Δ12 and Δ5 repression domains in their ability to repress tethered reporter and to interact with Ago2

GW182 were localized to GWBs, but TNGW1 was less abundant than GW182. Knocking down TNGW1 in isolation did not cause noticeable loss of GWBs, whereas knocking down both TNGW1 and GW182 caused significant loss of detectable GWBs. TNGW1 and GW182 induced stronger repression effects in a reporter tethering assay than Ago2, mainly at a translational level. TNGW1 and GW182 were the central repressors and their absence caused the loss of repression ability of Ago2. These data highlighted a critical role of TNGW1/GW182 in miRNA silencing pathway and lead to the next study of mapping their interaction domains with Ago2.

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

The C-terminal PIWI domain of Ago2 bound to 4 non-overlapping regions on GW182. A series of deletion constructs were generated spanning the entire TNGW1 for initial mapping of the Ago2 binding domain(s) (Lian et al. 2009) (Fig. 6.5b). An in vivo GST-pulldown assay was utilized to determine the interactions between different constructs. The binding of Ago2 to each of the GW182 fragments was indicated (Fig. 6.5b). Most of our data was consistent with what Takimoto et al. published almost at the same time: there were three Ago2 binding sites in N-terminal GW182 (Fig. 6.5b, dark lines show the Ago2-binding sites reported by Takimoto et al. (2009)). One was in equivalent to the Δ1 region and other two were in the Δ12 region. An additional binding site localized in the C-terminal Δ5 was observed in our experiments, but not in those of Takimoto et al. Our subsequent experiments confirmed weak Δ5–PIWI interaction compared to that of Δ12–PIWI (Yao et al. 2011). Interestingly, all the Ago2-binding domains were enriched in the GW/WG motif. Therefore, the Δ5–PIWI interaction might result from low affinity of C-terminal Δ5 to Ago2 due to smaller number and density of GW/WG motifs in this region (Eulalio et al. 2009c). Taken together, our laboratory and others identified four Ago2 binding regions on GW182 protein and that the Ago2 PIWI domain interacted with Ago2.

The Ago-GW182 interaction was conserved in human Ago family. There are four Ago proteins bearing high sequence similarity within the human Ago family. It was still not clear if Ago1-4 were functionally distinct in miRNA pathways as they apparently associated with similar sets of miRNA (Filipowicz et al. 2008). When Δ1 and Δ10 GW182 fragments were used to examine the interaction with other Ago proteins in pull down assays, we found that both Δ1 and Δ10 bound to Ago1, 3, and 4. Importantly, Ago3m, a splicing variant of Ago3 missing the C-terminal 66 amino acids (aa757–823) of the PIWI domain, were utilized to address the obligatory presence of the PIWI domain. The data clearly showed that Ago3m did not interact with

either Δ1 or Δ10, indicating the importance of the PIWI domain in interactions with GW182.

As mentioned above, GW/WG repeats in GW182 are one of its molecular signatures. Studies from two groups showed that short synthetic peptides containing one to two GW/WG interacted with Ago protein (El-Shami et al. 2007; Till et al. 2007). The short peptide sequence studied by Till et al. was termed "Ago hook domain" as originally described in *S. pombe* and conserved in TNRC6B (Till et al. 2007). Sequence alignment of Ago hook with the 4 non-overlapping identified Ago binding domains Δ1a, Δ1b, Δ12, and Δ5 showed some sequence similarity except Δ1a (Lian et al. 2009). It was therefore speculated that the interaction of Δ1a with Ago2 was different from that of the other GW182 truncated constructs (Lian et al. 2009). To address this hypothesis, a series of tryptophan (W) to alanine (A) mutations were generated to test the binding of mutated GW182 protein to the Ago2 PIWI domain. Unexpectedly, any of the five tryptophan's mutations within Δ1a did not abolish their ability to immunoprecipitate PIWI. This observation was inconsistent with other observations that the GW/WG motif played a key role in binding to AGO proteins, although we could not rule out indirect interactions between the Δ1a mutant and Ago2. Our subsequent results proved the functional importance of GW/GW repeats on one of the GW182 silencing domain Δ12 as the GW/GW to AA mutation totally abolished its silencing activity (Yao et al. 2011). Therefore, it should be acknowledged that GW/WG signature motifs on GW182 were certainly important for its binding to AGO or other functional partners such as deadenylase (Braun et al. 2011; Chekulaeva et al. 2011; Fabian et al. 2011), as well as its silencing activity.

The Ago2-GW182 interaction was critical for the localization of Ago2 in GWBs and its repression function. As discussed earlier, Ago2 co-localized with GW182 in cytoplasmic GWB and GW182 was essential for the formation of these foci. However, the dynamic processes by which Ago2 was recruited into GWBs remained elusive. Does the localization of Ago2 to GWBs require its interaction with GW182? To address this possibility, Δ10 that was shown to bind the AGO PIWI but not PAZ domain was transfected into HeLa cells together with either the PIWI or PAZ domain. In the absence of Δ10, neither the PIWI nor PAZ domains formed microscopic foci, but were diffusely expressed in cytoplasm. When the cells were co-transfected together with Δ10, only the PIWI but not PAZ domain drastically changed its localization and formed foci that co-localized with transfected Δ10. These data indicated that it was important that for GWB co-localization it was necessary for Ago2 to interact with GW182.

A Renilla luciferase-5BoxB tethering reporter (Fig. 6.5a) was also used to determine the silencing ability of Ago2 fragments and other Ago family proteins and, more importantly, evaluate their relationship to GW182. When tethered to the 3′UTR of the RL-5BoxB reporter, NHA-PIWI induced almost equally strong repression as the NHA-full-length Ago2 (Lian et al. 2009). In contrast, NHA-PAZ did not repress Ago2. These data suggested that the silencing ability harbored by Ago2 was mainly within its C-terminal PIWI domain.

Other Ago family proteins including Ago1 and Ago4 were tethered to the reporter to assess their repression abilities. Comparable repression effects were observed in Ago1 and 4 similar to that of tethered Ago2 or PIWI. Intriguingly, Ago3m that did not bind to GW182 as described previously did not have repression activity in tethering assays. These observations, together with those of Li et al. (2008), demonstrated that the repression induced by the AGO family is dependent on their interaction with GW182.

Coincident with deriving and publishing these data, there were seven other publications that mapped the interaction of Ago2 with GW182 paralogs, along with the functional significance of these findings in different species (Baillat and Shiekhattar 2009; Chekulaeva et al. 2009; Eulalio et al. 2009a, b; Lazzaretti et al. 2009; Takimoto et al. 2009; Zipprich et al. 2009). The C-terminal region of GW182 was uniformly identified to function as the silencing domain and the N-terminal was shown to interact with AGO proteins: findings consistent with our present data. However, our extended study (Yao et al. 2011) described in next section, together with others (Chekulaeva et al. 2009, 2010), suggested an additional N-terminal domain that covered much of the middle region of GW182 also showed silencing activity (see below).

In summary, Ago2 protein bound to GW182 in multiple non-overlapping regions via its C-terminal PIWI domain and this interaction was critical for its silencing function and co-localization to GWBs. This interaction was conserved along with other AGO family proteins that were also dependent on GW182 to silence their targets. These important conclusions that once again highlighted the key role of GW182 in miRNA-mediated silencing urged us to further dissect the repression domains of GW182.

6.8 Divergent GW182 Functional Domains in the Regulation of Translational Silencing

Non-overlapping GW182 fragments Δ12 and Δ5 harbored comparable repression effects to full-length GW182/TNGW1. It has been demonstrated that GW182 played a central role in miRNA-mediated gene silencing. Yao et al. were interested in further characterizing the region (s) that harbored repression capacity for GW182. To address this question, a series of deletion constructs covering the whole TNGW1/GW182 sequence were fused with NHA-tag and analyzed using a luciferase tethering assay (Fig. 6.5b). Based on the luciferase reading, the constructs can be subgrouped as follows: (1) no repression effect including 1-565, TNR, Δ1, and QN; (2) high repression effect comparable to full-length protein including Δ10, Δ12, Δ8, Δ7, and Δ5; (3) Δ11, which had a low to moderate repression effect. Intriguingly, two non-overlapping regions that were able to induce repression were revealed.

Among these two domains, Δ5 containing the conserved domain RRM and located in the well-defined C-terminal silencing domain was shown in human GW182/TNGW1, TNRC6B, and TNRC6C (Lazzaretti et al. 2009; Zipprich et al.

2009; Baillat and Shiekhattar 2009) and *Drosophila* GW182 (Chekulaeva et al. 2009, 2010; Eulalio et al. 2009a, b) to exert strong translational repression when tethered to the 3′UTR of the reporter mRNA. Our data was consistent with these published studies. However, the middle region Δ12 containing the Ago hook domain was a novel region identified in the described "N-terminal Ago binding domain" to induce comparable repression to full-length GW182. It should be noted that some TNRC6C fragments such as aa1-405 or aa1-1304 have been shown to be partially active with about 50% repression activity in tethering assays (Zipprich et al. 2009). Consistent with our observations, Chekulaeva et al. published a report almost at the same time showing the functional significances of GW/WG repeats in the Drosophila GW182 (dGW182) repression domain (aa205-490) that induced reporter silencing (Chekulaeva et al. 2010). These investigators showed alignment of their repression domain with other GW182 homologs and found that mutation of certain conserved amino acid residues abolished repression induced by these dGW182 fragments. Interestingly, the alignment showed the second dGW182 repression domain corresponded closely to Δ10 (aa655-1343). In agreement with this finding, the Δ10 induced repression in our tethering assay was clearly supported. In addition, the Δ12 repression domain defined in our study represented a new core repression region with somewhat higher repression than in Δ10.

We noted that the two identified GW182 repression domains Δ5 and Δ12 had different defined domains: the Ago hook domain in Δ12 and the RRM domain in Δ5 (Figs. 6.5b and 6.6c). The Ago hook had been shown to bind to Ago2 independently in vitro, but it is not conserved in the TNRC6B isoform 1 or in Drosophila GW182 (Eulalio et al. 2009c). The RRM is linked to RNA binding activity (Eystathioy et al. 2002) and is highly conserved in the GW182 family. Experiments were designed to examine whether the Ago hook or RRM was important for the repression effect of Δ12 or Δ5 in human GW182, respectively, by generating further deletions of Δ12 (Δ12a and Δ12b) or Δ5 (Δ5a and Δ5b) with or without their conserved domains (Fig. 6.5b). Tethering assays revealed that the RRM and its flanking sequences enriched in GW/WG motifs (M-GW and C-GW) were pivotal for the repression, whereas the Ago hook contributed little to the repression induced by Δ12.

GST-pulldown assays were performed to check if the observed repression activity was correlated with the Ago binding function. The results showed that NHA-Δ12, NHA-Δ12a, NHA-Δ12b, and NHA-Δ5 were all pulled down by GST-PIWI albeit at a different efficiency that was independent of RNA and appeared to have no correlation with their repression ability in tethering assays.

Although it was demonstrated earlier that Ago2 protein was not required for tethered GW182-mediated repression in the tethering function assay (Li et al. 2008; Behm-Ansmant et al. 2006; Lian et al. 2009), it was still possible that the repression mediated by Δ12 or Δ5 relied on recruitment of other important factors of the miRNA pathway machinery, including endogenous GW182 paralogs. This possibility was tested in a series of tethering assays in HeLa cells where siRNA knockdown was used as an approach to evaluating the roles of GW182 and/or TNRC6B in the repression mediated by Δ12 or Δ5. These experiments demonstrated that knockdown of endogenous repressors GW182, TNRC6B, and RCK/p54 did not significantly affect the repression activities of Δ12 and Δ5 in the tethering assay. In

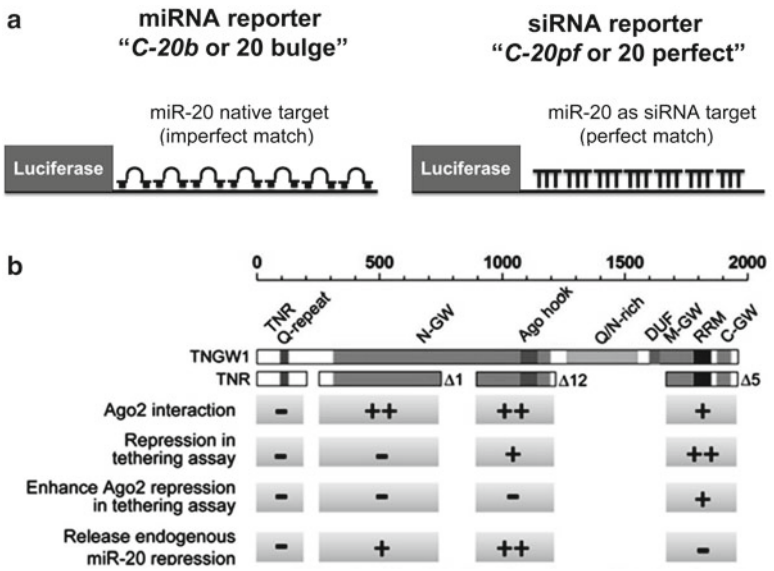

Fig. 6.6 Characteristics of divergent GW182 functional domains. (**a**) Schematic diagram illustrating the 20 bulge and 20 perfect reporters in the quantitation of endogenous miRNA and siRNA function, respectively. (**b**) Summary of GW182 domain functional characteristics. *TNR* Trinucleotide repeats; *Ago hook* a pre-defined Ago2 binding region (Till et al. 2007); *RRM* RNA recognition motif

summary, Δ12 and Δ5 were identified as two "minimum" non-overlapping domains in GW182 to silence their bound targets and independent of endogenous factors.

Δ5 enhances tethered Ago2 repression effects, whereas Δ12 interferes with miRNA silencing activity in a dominant-negative manner. Since both Δ5 and Δ12 have comparable silencing activities when tethered to the luciferase reporter 3′UTR and none of the known endogenous factors tested were required for their repression function, it remained unclear whether their mechanism of inducing repression was similar. Therefore, experiments were designed to determine whether the expression of Δ5 and Δ12 as GST-tag fusion proteins could interfere with NHA-Ago2 tethered assays (Fig. 6.6a). Note that the GST-tagged constructs did not bind to the luciferase reporter 3′UTR for position competition. The typical ~60% repression compared to the NHA control was also observed when NHA-Ago2 was tethered to the RL-5boxB reporter (Li et al. 2008; Pillai et al. 2004). Interestingly, there was significantly (33%) enhanced repression when GST-Δ5 was co-expressed (Yao et al. 2011). In contrast, significant differences were not observed for co-expression of GST-Δ12, -Ago2, or -QN. The Δ5-mediated enhancement in repression by NHA-Ago2 implied that this could be due to binding to other translational machinery or RNA decay factors that remain to be determined. Although Δ12 strongly bind to Ago2, it did not affect repression by tethered Ago2.

Multiple regions on GW182, including Δ1, Δ5, and Δ12, were shown to interact with Ago2 and this interaction is critical for Ago2 localization to GWBs and its

silencing activity (Lian et al. 2009). Do these binding sites on GW182 have an equal role in the miRNA silencing pathway? The RL reporter for miR-20 (RL-20 bulge), which contains seven miR-20 target sites and forms bulge structures with miR-20 (Ebert et al. 2007), was used to monitor the cellular miR-20 functional status (Fig. 6.6b). Meanwhile, another RL reporter with miR-20 target sites forming perfect match with endogenous miR-20, thus acting as reporter for the siRNA pathway, was utilized to determine whether this interference also applies to the siRNA pathway (Fig. 6.6b). Although all these constructs were shown to bind to Ago2, only Δ12 strongly impaired the 20 bulge reporter silencing. Δ12a, which lacked the Ago hook, retained almost the same capability to interfere with the 20 bulge reporter, whereas Δ12b mildly altered the miR20-induced repression. Importantly, this interference was only observed with the 20 bulge but not the 20 perfect match reporter, thus demonstrating overexpression of Δ12 and its deletion constructs impaired reporter silencing in a miRNA-specific manner. These data lead to the hypothesis that the observed interference was not solely due to Ago2 interaction because Δ12a did not contain Ago hook and bound weakly to Ago2. Therefore, other factors on Δ12a such as GW repeats were taken into consideration as alternative functional motifs.

Substitution of GW/WG motifs with alanines in Δ12a hindered its tethering assay activity, as well as its interference with endogenous miRNA repression. Since the results showed that the Ago hook was not critical for function but that Δ12a retained almost the same capability to interfere with RL-20 bulge reporter, it was postulated that the GW/WG motifs in Δ12a might be important for functions besides binding to Ago2. A Δ12a mutant (Δ12am) was then generated to replace all GW/WG residues in Δ12a with alanine-alanine (AA) residues. NHA-Δ12am was devoid of translation silencing when compared to NHA-Δ12a and other relevant controls. Meanwhile, the ability of Δ12a to release miR-20 activity was abolished in Δ12am in the same 20 bulge miRNA functional assay. These data suggested that the GW/WG motifs in Δ12a were important for the silencing in the tethering assay and interference in miR-20 bulge reporter function. It appeared that the GW/WG motifs in different regions of GW182 may have different functional roles. For example, Δ1 also possessed multiple GW/WG motifs and a defined Ago binding site, but it was not efficient in both tethering repression and miR-20 reporter interference assays compared to Δ12. Further, mutation of some of the GW motifs on Δ1 did not abolish the Ago binding (Lian et al. 2009). Collectively, GW/WG motifs in Δ12a region showed significant effects in silencing tethered mRNA and impaired miRNA-induced repression. GW/WG motifs located in different regions of GW182 might have different functional preferences or formed a particular three-dimensional structure that requires further investigation.

Δ12 and Δ5 bound to PABPC1 but only mildly affecting mRNA degradation. In the eukaryotic cap-dependent translation initiation step, mRNAs usually form circularized structures facilitated by the binding between the cap binding complex eIF4E/4G and Poly-A binding protein PABPC1, which favors an association with the 40S ribosome (Kahvejian et al. 2005). GST pull down experiments were used to examine the differential roles of identified GW182 repression domain in mRNA decay.

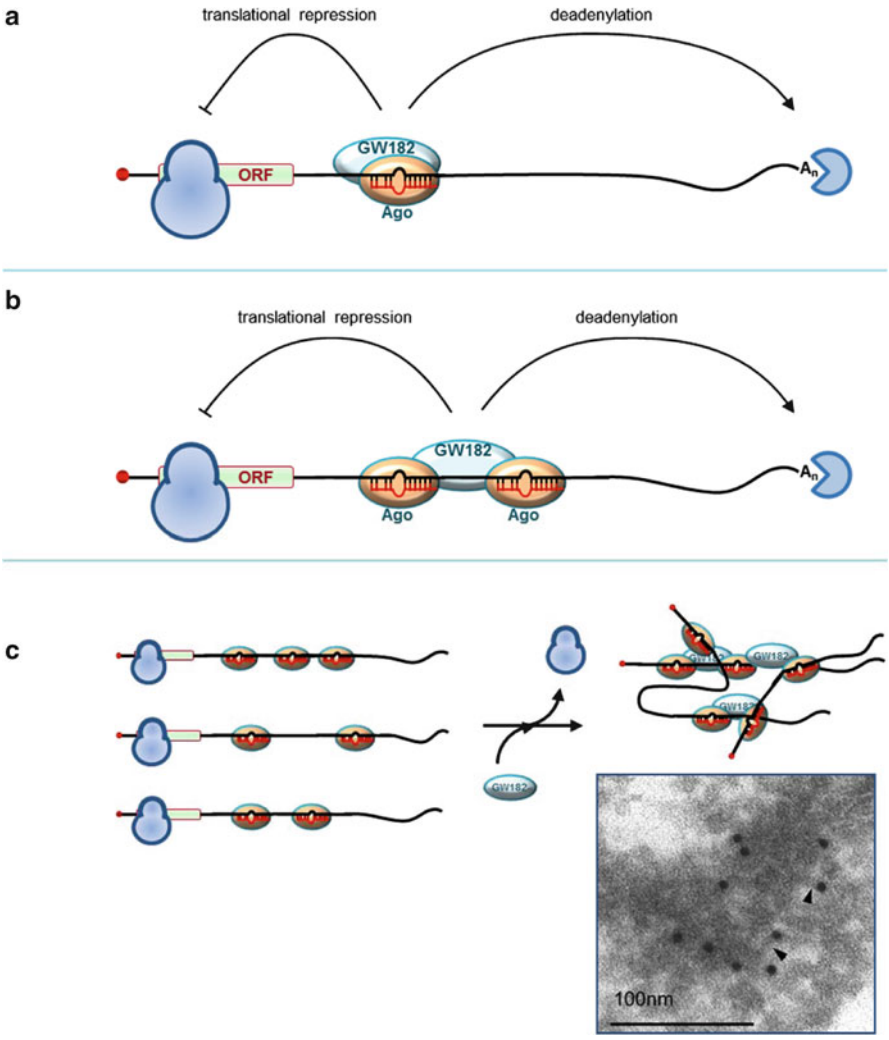

Fig. 6.7 Working models for miRNA-mediated translational silencing

Both Δ12 and Δ5 showed interaction to PABPC1 in an RNA independent manner, although neither of them contain PABPC1 binding sites (Fabian et al. 2009; Jinek et al. 2010). Quantitative RT-PCR was set up to measure the level of degradation of the RL-5Boxb reporter mRNA in tethering assay normalized to the FL mRNA level. The data suggested that both NHA-Δ12 and -Δ5 induced comparable, mild mRNA degradation when tethered to the reporter comparable to the activities of NHA-GW182 and -TNGW1, respectively.

Collectively, two non-overlapping domains Δ12 and Δ5 were shown to silence bound reporter in a functional tethering assay that was independent of examined endogenous repressors. These two domains showed distinct characteristics in that

the C-terminal Δ5 enhanced tethered Ago2 repression, whereas middle region Δ12 interfered with endogenous miRNA activity. GW repeats on Δ12a played a critical role in its repression function as well as miRNA interference activity. Both Δ12 and Δ5 bound to PABPC1, but only mildly induced reporter mRNA degradation (see summary Fig. 6.6c). Observations that GW182 has multiple Ago binding sites with different binding affinities, as well as two distinct repression domains, are highly suggestive of its role in stabilizing multiple "repressed" Ago-miRNA-mRNA complexes or in aggregating Ago-miRNA-mRNA complexes to establish an efficient repressed state. Alternatively, our data also suggest that GW182 may regulate the fate of repressed mRNA and potentially direct the repressed complex to decay or reversal to a translational state (see model in Fig. 6.7).

6.9 Crosstalk Between miRNA and siRNA: Common Roles of GWBs

At the beginning of this chapter, we discussed the evidence that transfected exogenous siRNA was found in GWBs and, the number and size of GWBs correlated with siRNA function. As shown in Fig. 6.2, siRNA and miRNA partially share a functional protein complex for their target recognition and silencing mechanism. Therefore, efforts have been made to examine the correlation between miRNA biogenesis, localization, and functional relevance to GWBs.

6.10 Formation of GW Bodies is the Consequence of microRNA Genesis

miRNA is present in GW bodies. It has been previously demonstrated that key proteins in miRNA silencing pathway, AGO and GW182, were found to co-localize to GWBs. Cy3-3′-labeled miRNA let-7 was transfected into HeLa cells to determine its subcellular localization (Pauley et al. 2006). Good co-localization was observed with miRNA and GWBs stained by GWB-specific serum. The association of miRNA to its functional protein complex was demonstrated by immunoprecipitation and an RNase protection assay (Pauley et al. 2006).

Disassembly of GW body in Drosha-deficient HeLa cells and transfection of synthetic short interference RNAs rescues GW bodies.

As described in Fig. 6.2, endogenous transcribed miRNAs needed to undergo maturation steps to become functionally active. At one step, the Drosha-DGCR8 protein complex processes nascently transcribed pri-miRNA into around 70nt hairpin structured pre-miRNA. Reducing Drosha or DGCR8 by siRNA resulted in the accumulation of pri-miRNA and blocking of miRNA maturation (Krol et al. 2010). Short hairpin RNA (shRNA) targeting Drosha was co-transfected into HeLa cells together with GFP that used to monitor the transfected cells. Seventy-two hours after transfection, most of the cells co-transfected with shRNA-Drosha and GFP

showed a profound and clear reduction of GWB formation, which was strongly correlated with reduction of Drosha protein levels as detected by indirect immunofluorescence and western blot. Note that transfected siRNA that did not target miRNA maturation pathway proteins such as siRNA against lamin A/C described earlier in this chapter showed an increase of GWB, indicating the important role of GWB in miRNA processing and maturation.

6.11 Conclusions and Working Model

It has been 10 years since the discovery of GW182, an amazing protein with many unique features deserved attention. This discovery opened the door for a burst of explorations of miRNA silencing mechanisms, which was poorly understood dating to the identification of the first miRNA Lin-4 in 1993 (Lee et al. 1993). Currently, it has been widely accepted that GW182, together with its paralogs, is a key component of the RISC associated miRNA silencing pathways. The detailed molecular events, however, such as how GW182 and its co-repressors affect gene translation remains elusive. In contrast, it is now increasingly clear that GW182 binds to PABPC1, recruiting two step deadenylase to initiate mRNA decay (Chen et al. 2009; Fabian et al. 2009, 2011; Zekri et al. 2009; Braun et al. 2011; Chekulaeva et al. 2011). Although impressive efforts have been made in the past 10 years to elucidate the function of GW182, there are still fundamental unanswered questions. For example, at which step does GW182 actually interfere with translation? What is the hierarchical order of translational repression vs. mRNA degradation, and what is the role of GW182 in this process? What is the functional relevance of the two non-overlapping silencing domains of GW182 and does the presence of these domains imply a diversified GW182 function? This may highlight the diverse functions of this amazing protein with many aspects yet to be explored. Of course, all these questions need to be addressed by additional research. Nevertheless, we have proposed a working model based on the current available data and knowledge (Fig. 6.7). Three models are illustrated: (A) Simple one-to-one model. A miRNA loaded onto Ago/GW182 complex recognizes a single miRNA-binding site located on the 3′UTR of its target mRNA. GW182 induces translational repression and deadenylation. GW182 simultaneously interferes with translation at many steps, such as initiation, ribosomal joining, elongation, and termination as described by several studies. By interacting with PABPC1, GW182 may also recruit deadenylase to induce deadenylation and followed by de-capping and mRNA decay. (B) Two binding site model. When more than one miRNA binding sites are on the same 3′UTR of a target mRNA, GW182 may "crosslink" using its multiple Ago binding sites to synergistically enhance miRNA silencing activity. (C) GW182 aggregates multiple mRNA targets forming GW bodies. A working model describing the sequence of events whereby miRNA/Ago-mediated mRNA crosslinking via GW182 leads to the formation of GW bodies with structures similar to the immunogold-labeled ultrastructure described for these cytoplasmic foci (Yang et al. 2004). Many mRNAs bound by miRNAs can

form GWB, possibly through the GW182 QN rich domain. The extraction and sequestration of mRNA from the cytoplasm may be a quick and highly efficient method in translation repression. Deadenylation/decay of mRNA are secondary to the need for quick repression of gene translation.

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Chapter 7
Post-transcriptional Stimulation of Gene Expression by MicroRNAs

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Abstract MicroRNAs are small noncoding RNA regulatory molecules that control gene expression by guiding associated effector complexes to other RNAs via sequence-specific recognition of target sites. Misregulation of microRNAs leads to a wide range of diseases including cancers, inflammatory and developmental disorders. MicroRNAs were found to mediate deadenylation-dependent decay and translational repression of messages through partially complementary microRNA target sites in the 3′-UTR (untranslated region). A growing series of studies has demonstrated that microRNAs and their associated complexes (microRNPs) elicit alternate functions that enable stimulation of gene expression in addition to their assigned repressive roles. These reports, discussed in this chapter, indicate that microRNA-mediated effects via natural 3′ and 5′-UTRs can be selective and controlled, dictated by the RNA sequence context, associated complex, and cellular conditions. Similar to the effects of repression, upregulated gene expression by microRNAs varies from small refinements to significant amplifications in expression. An emerging theme from this literature is that microRNAs have a versatile range of abilities to manipulate post-transcriptional control mechanisms leading to controlled gene expression. These studies reveal new potentials for microRNPs in gene expression control that develop as responses to specific cellular conditions.

7.1 MicroRNAs

MicroRNAs are a unique class of small, noncoding, 20- to 24-nt regulatory RNAs that have been demonstrated to modulate both mRNA stability and translation in a highly controlled manner (Valencia-Sanchez et al. 2006). As suggested by their

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strong evolutionary conservation and a large number of bioinformatically predicted target genes, their deregulation or aberrant function leads to immense clinical consequences ranging from immune and developmental disorders to cancers (Sevignani et al. 2006; Stitt-Cavanagh et al. 2009; Sebastiani and Galeazzi 2009; Chico et al. 2010; Taft et al. 2010; Le Quesne et al. 2010). MicroRNAs act as targeting molecules via their sequence-specific patterns of base pairing with other RNAs to guide associated effector RNP complexes and thereby, dictate functional outcomes of target gene expression. A family of critical effector proteins called Argonaute (AGO) or eukaryotic initiation factor 2C (eIF2C) associate specifically with microRNAs to form the functional microRNA-protein complex (microRNP), which is directed by the microRNA to the target RNA/mRNA in a sequence-specific manner. Complete base pairing of the microRNA to its target message leads to mRNA cleavage, degradation, and repression governed by the AGO bound. On the other hand, partial base pairing to target sites leads to mRNA deadenylation (Giraldez et al. 2006; Wu et al. 2006) and to translation regulation (Carmell et al. 2002; Liu et al. 2004; Vasudevan et al. 2007; Chekulaeva and Filipowicz 2009; Fabian et al. 2010; Jackson et al. 2010), either the more frequently observed mRNA translation silencing or as discussed below, translation activation of select messages directed by specific sequences and cellular conditions.

7.2 Translation Regulation

Translation regulation mechanisms promote or suppress gene expression as an adaptive response to intra- and extracellular cues via modulation of general protein synthesis. These mechanisms selectively induce or repress the expression of specific mRNAs under distinct conditions and include relocalization of mRNAs/mRNPs, their modification to alter translation or relocation, recruitment, and alteration of the translation machinery (Gray and Wickens 1998; Jackson et al. 2010). The translation effects of a UTR is measured by normalizing the test UTR reporter activity to a co-transfected second control reporter (Fig. 7.1a, Firefly Luciferase test UTR reporter normalized to Renilla Luciferase co-transfected control) to exclude transfection efficiencies and the overall translation status of the cell. To examine translation effects independent of contributions of mRNA levels, this translation ratio is usually further normalized to the RNA abundance yielding a function called translation efficiency (Fig. 7.1a) or the translation per RNA molecule (Wu et al. 2006). Apart from the normalization to the internal, co-transfected reporter and to RNA levels, a parallel experiment with a comparative control reporter (expressed from the same promoter) mutated at or lacking the key regulatory UTR site, reports the standard reference or general translation efficiency in the absence of the regulatory site to compare against. The expression of the test UTR/target site reporter is assigned basal translation (Fig. 7.1b, (i) white bars with a black dashed line indicates the basal translation level) or no specific translation regulation effect when the translation efficiency of the test UTR/target site reporter equals or is similar to that

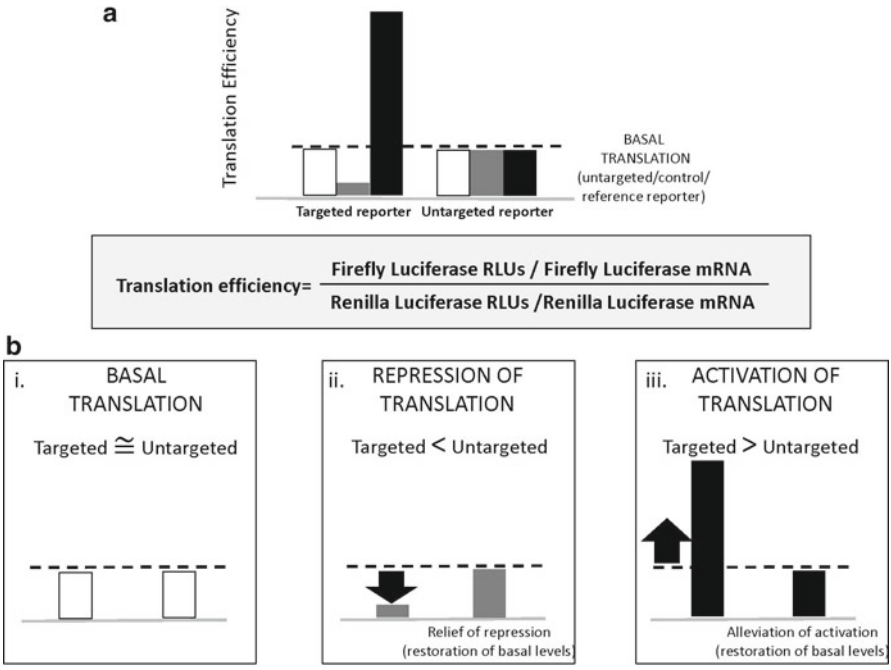

Fig. 7.1 Translation regulation outcomes. (**a**) The activity of an expressed reporter bearing the test target site UTR (Firefly Luciferase with test UTR) is normalized to an internal co-transfected control reporter (Renilla Luciferase) to obtain the test UTR-specific translation ratio. Since the half life of Firefly Luciferase is short (Vasudevan et al. 2007), changes to Firefly Luciferase reporter expression with the test target UTR compared to the control UTR expressed in parallel experiments from the same promoter reflects post-transcriptional (translational and mRNA stability) rather than post-translational effects. The UTR-specific translation ratio can be further normalized to their mRNA levels to yield the translation per RNA molecule or translation efficiency (formula shown below) to compare translation effects. (**b**) In comparison to (i) the basal translation efficiency (*white bars*) as shown by a non-targeted or reference reporter (a reporter mutated at or lacking a target site expressed from the same promoter as the test reporter in a parallel experiment, with normalization for RNA levels), a targeted test reporter can have the following outcomes as shown by the relative change (*wide black arrow*) from basal translation levels marked by a *dashed black line*. It can be (ii) repressed when it is lower than the control reporter ((ii) *gray bar* with the change from basal translation shown by the *wide black arrow*) or (iii) activated ((iii) *black bar* with the change from basal translation shown by the *wide black arrow*) when the translation efficiency is greater than that of the control reporter. Since these compare translation efficiencies (translation ratio after normalization for relative RNA levels as shown in (**a**)), the effects of mRNA levels are normalized allowing analysis of translation effects of the target UTR. When repression is alleviated, translation efficiency is increased from repressed to basal levels ((ii) *gray bar* marked relief of repression). When activation is prevented, the increased translation is lost and brought down to basal levels from activated levels ((iii) *black bar* marked alleviation of activation). RLU=Relative light/luminiscence unit

of the control reporter, indicating that the tested site is not effective in causing a specific translation effect over and beyond general translation, as represented by the control reporter under the tested conditions. There are a few possible translation regulatory outcomes (Fig. 7.1):

1. When the translation of the test UTR/target site reporter is greater than that of the control reporter, then translation is activated or upregulated indicating that the tested site is causing promotion of translation and is increasing translation over and beyond general translation represented by the control reporter (Fig. 7.1b, (iii) black bar with wide black arrow indicating the increase from basal translation).
2. When the translation of the test UTR/target site reporter is lower than that of the control reporter, then translation is repressed or downregulated indicating that the tested site is causing repression of translation and is reducing translation below the level of general translation represented by the control reporter (Fig. 7.1b, (ii) gray bar with wide black arrow indicating the decrease from basal translation).
3. Alleviation or relief of repression is the loss of repression, where the reduction of translation below the basal or control reporter translation is abrogated (Bhattacharyya et al. 2006). In this case, the test UTR/target site reporter demonstrates translation similar to that of the control reporter, behaving like a transcript that lacks microRNA/test UTR repression effects (Fig. 7.1b, (ii) gray bar marked relief of repression). With translation activation, the test reporter translation would be greater than that of the control reporter unlike relief of repression (Fig. 7.1b, compare (ii), gray bar with wide black arrow indicating the decrease from basal translation to the gray bar marked relief of repression with (iii) black bar with wide black arrow indicating the increase from basal translation). Although clearly distinguishable against a control reference reporter, biologically, both activation and relief of repression would yield an increase in the relative levels of the protein produced. The reference state in the absence of microRNA action would clarify whether the microRNA/RNP was relieved of repressive function or whether the microRNA was inducing activation. Alleviation can also be observed for activation where the increased advantage in translation is lost and the test UTR/target site reporter again behaves like a control reporter subject only to general translation effects as observed upon mutation of the microRNA target UTR site (Fig. 7.1b, (iii) black bar marked alleviation of activation).

In eukaryotes, translation repression is common; however, translation activation has also been studied with distinct transcripts and factors as specific phenomena, prominent in natural situations such as developing germ cells, specific cell types, and conditions. These translation activation elements and factors more often function as translation regulatory elements with the ability to repress and activate translation in response to different signals (Gray and Wickens 1998; Wilhelm and Smibert 2005; Kedde and Agami 2008; Radford et al. 2008; Brook et al. 2009). For example, the Cytoplasmic Polyadenylation Element (CPE) represses translation of

specific messages in immature oocytes but activates the same mRNAs upon oocyte maturation (Mendez and Richter 2001; Radford et al. 2008; Richter 2008). Furthermore, studies on specific translation activation have identified not only factors and elements that are indicative of translation activation but also revealed their co-interacting general translation machinery and ribosomal protein factors, thereby providing clues to possible mechanisms of translation regulation.

7.3 Prokaryotic sRNAs-Mediated Post-transcriptional Upregulation of Gene Expression

Bacterial species demonstrate the presence of a large variety of small RNAs called sRNAs that are capable of upregulating or downregulating gene expression. In particular, mRNAs encoding transcription factors such as RpoS that are required under a variety of stress conditions are remodeled from a structurally self-imposed block in the 5′-UTR to a ribosome accessible form by three different sRNAs that are produced in distinct stress conditions. These sRNAs base pair at this 5′-UTR site to open the conformational restraint and recruit an sRNP constituting Hfq to stabilize the RNA-RNA duplex and the consequent open structure (Gottesman et al. 2006; Gottesman 2005). Other bacterial sRNAs employ a diverse variety of mechanisms including associating with the 5′- or 3′-UTR to alter processing and increase mRNA stability, decoying, and preventing the degradation of an activating sRNA or functioning as a pseudotarget mRNA trap to ensnare repressive sRNA and derepress targets (Frohlich and Vogel 2009). Understanding upregulation by bacterial sRNAs will provide mechanistic clues into similar translation upregulation pathways observed in higher eukaryotes.

7.4 Eukaryotic MicroRNA/MicroRNP-Mediated Post-transcriptional Upregulation of Gene Expression

Preceding the discovery of microRNAs and their predominant role in downregulating expression (Roy et al. 1988; Zou et al. 1998; Carmell et al. 2002), the microRNP effector, eIF2C2 or AGO2, was initially identified as a translation stimulatory activity in a rabbit reticulocyte lysate system; the upregulatory activity remains to be understood. The identification of translation activation roles of the related family protein, Aubergine in the Drosophila germ line (Wilson et al. 1996; Harris and Macdonald 2001; Kennerdell et al. 2002) and of the related P-element induced wimpy testis (PIWI) proteins in Drosophila and mice germ lines (Deng and Lin 2002; Grivna et al. 2006b; Unhavaithaya et al. 2009; Wang et al. 2009) further suggested the potential of both the AGO and related family of PIWI proteins in translation regulation, although these studies did not demonstrate

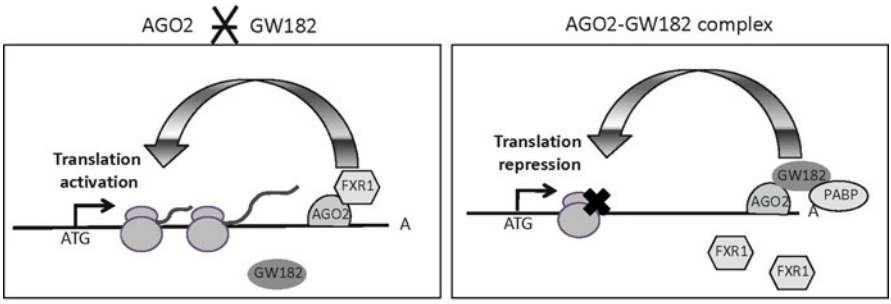

Fig. 7.2 Distinct microRNP complexes mediate translation repression or activation in G0. GW182 is an essential component of the repressive microRNP complex, which is regulated by G0 (Yang et al. 2004). Repression is reduced when GW182 interaction with AGO2 is altered on specific mRNAs (oocytes, G0 cells, dAGO2) (Iwasaki and Tomari 2009; Iwasaki et al. 2009; Vasudevan and Steitz 2007; Flemr et al. 2010; Ma et al. 2010b). In G0, FXR1-iso-a can interact with the microRNP and alter its function to enable activation of specific target mRNAs (Vasudevan and Steitz 2007)

involvement of sRNAs. The first suggestion of microRNAs functioning off the norm in the "wrong" direction was the demonstration of Hepatitis C Virus (HCV) RNA upregulation by miR-122, regulating not the translation but the RNA level (Jopling et al. 2005, 2008). Concurrently, the phenomenon of RNA activation (RNAa) was observed, where transcription at specific promoter regions was upregulated by sRNAs and AGO, suggesting that sRNAs had the potential for additional upregulatory functions (Kuwabara et al. 2004, 2005; Corey 2005; Li et al. 2006). This was followed by translation studies demonstrating alleviation of microRNP repression of specific messages under distinct cellular conditions or in cell type- and tissue-specific patterns (Bhattacharyya et al. 2006; Kedde et al. 2007; Kedde and Agami 2008). Our studies revealed that AGO2/microRNPs can activate translation of specific target reporters in distinct quiescent (G0) cells (Fig. 7.2, Vasudevan and Steitz 2007; Vasudevan et al. 2007, 2008). Subsequent studies with 5′ terminal oligopyrimidine (TOP) tract mRNAs (Orom et al. 2008), specific microRNAs, and targets such as miR-145 with myocardin mRNA (Cordes et al. 2009), miR-346 with RIP140 mRNA (Tsai et al. 2009), miR-223 with Glut4 mRNA (Lu et al. 2010), and miR-122 with the HCV UTR (Henke et al. 2008) as well as of miR-34a on alternatively polyadenylated β-actin mRNA (Ghosh et al. 2008) affirmed that microRNPs have the potential to activate translation (Fig. 7.3). These studies are reminiscent of the wide spectrum of regulation of gene expression influenced by sRNAs in bacteria.

EIF2C2/*AGO2*: The AGO family of proteins across various systems has been associated with common as well as distinct functional outcomes. The fundamental factor in microRNA-mediated regulation in mammalian cells is eIF2C2 or Argonaute 2 (AGO2). AGO2 targets mRNAs that are partially complementary to the associated sRNA for deadenylation and translational regulation while those completely complementary are targeted for cleavage-mediated degradation and repression (Valencia-Sanchez et al. 2006). AGO2 was initially purified as a translation enhancing

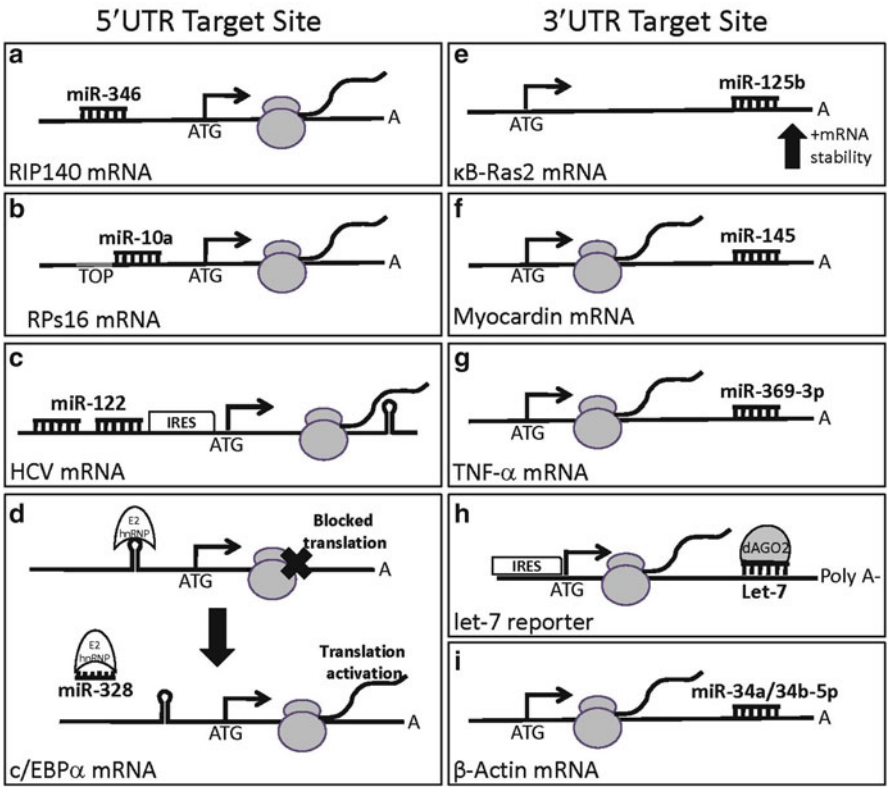

Fig. 7.3 Post-transcriptional upregulated gene expression by microRNAs. microRNAs and specific target mRNAs with target sites in the 5′- or 3′-UTRs demonstrate upregulated expression. (**a**) MiR-346 interacts with the 5′-UTR of RIP140 and upregulates translation in mouse brain tissue and p19 cells (Tsai et al. 2009). (**b**) MiR-10a binds to the 5′-UTR of ribosomal protein mRNAs carrying TOP sites and enhances their translation upon amino acid starvation (Orom et al. 2008). (**c**) miR-122 stimulates translation of HCV mRNA through two target sites in the 5′-UTR (Henke et al. 2008). (**d**) MiR-328 binds to hnRNPE2 and prevents it from repressing c/EBPα mRNA translation (Eiring et al. 2010). (**e**) MiR-125b binding to the 3′-UTR of κB-Ras2 mRNA increases mRNA stability in human macrophages (Murphy et al. 2010). (**f**) Binding of miR-145 to the 3′-UTR of myocardin mRNA increases its translation (Cordes et al. 2009). (**g**) MiR-369-3p binds the 3′-UTR and upregulates translation of TNF-α mRNA in quiescent mammalian cell lines (Vasudevan et al. 2007). (**h**) Drosophila Ago2 (dAGO2) activates translation of 3′-UTR microRNA target reporters lacking poly(A) tails in a Drosophila extract system (Iwasaki and Tomari 2009). (**i**) Mmu-miR-34a/34b-5p binds to the 3′-UTR and upregulates translation of a variant β-actin mRNA in mouse neuronal cells (Ghosh et al. 2008)

activity, co-eIF2a or eIF2C that co-purified with eIF2. EIF2 is a critical translation initiation factor that forms the ternary complex with initiator methionyl tRNA, an obligate intermediate that recruits the tRNA to ribosomes (Roy et al. 1988; Zou et al. 1998; Carmell et al. 2002). The precise functional role of co-eIF2a remains mysterious as these experiments have not been expanded further, although it was

shown to stabilize ternary complex formation in the presence of mRNA at low concentrations by microRNAs.

Aubergine: The PIWI/Aubergine family of proteins is related to the AGO/eIF2C family of proteins. Aubergine, an RNA-associated protein expressed in the Drosophila germ line, was demonstrated to function as a translation upregulatory factor, binding the 3′-UTRs of specific transcripts required for oocyte development (Wilson et al. 1996; Harris and Macdonald 2001; Kennerdell et al. 2002). In Drosophila oogenesis, Aubergine is essential for translation activation of a specific germ cell maternal mRNA, Oskar. Aubergine appears to have two separable functions; the first is limited to early oocytes where it translationally upregulates specific mRNAs while further in development, it is present in polar granules, where it is important for early embryo expression (Wilson et al. 1996; Harris and Macdonald 2001). Additionally, Aubergine is required for the onset of RNAi in embryos, which intriguingly, requires the target mRNAs to be actively translated and coincided with maturation induced activation and maternal mRNA degradation (Wilson et al. 1996; Harris and Macdonald 2001; Kennerdell et al. 2002). Aubergine is associated with piRNAs and involved in their synthesis (Brennecke et al. 2007; Nishida et al. 2007).

PIWI: The AGO-related PIWI family of proteins that are restricted to the germ line of Drosophila and mammals, associate with piRNAs and is predominantly implicated in piRNA-associated transposon and transcriptional silencing (Grivna et al. 2006a; Lau et al. 2006; Brennecke et al. 2007; Nishida et al. 2007). A translation upregulatory role was suggested for a PIWI orthologue in mouse, MIWI, which appeared to be increasingly associated with mRNA cap complexes and polysomes across spermatogenesis stages; its absence causes decreased expression of genes that are required for sperm development (Deng and Lin 2002; Grivna et al. 2006b; Megosh et al. 2006). Another related mouse protein, MILI, was recently demonstrated to be necessary for translation activation of many transcripts during spermatogenesis. It is unclear whether MILI promotes recruitment of specific transcripts to polysomes through direct association or guided by microRNAs. The upregulation involves interactions with eIF3a and the mRNA cap complex, and is required for germ line stem cell self-renewal (Unhavaithaya et al. 2009; Wang et al. 2009).

Oocyte RNAi Regulation: The earliest demonstration that RNAi and microRNA activity may be regulated came from studies on microRNA functions across oocyte development in Drosophila. Comparative proteomic analysis revealed that dicer depletion in oocytes did not cause a loss of repression/downregulation by microRNAs (Kennerdell et al. 2002; Nakahara et al. 2005). These studies suggested that microRNA activity may be regulated at the early stages of oocyte development and that the repression activity of microRNAs may emerge in subsequent stages of maturation. Recent evidences in mouse oocytes demonstrated a similar reduced repressive activity (Flemr et al. 2010; Ma et al. 2010b) despite the presence of microRNPs (Tam et al. 2008), suggesting a conserved regulation of microRNA activity in early oocyte development. The reduced microRNA-mediated repression was attributed to the lack of association between AGO and GW182 observed in mouse oocytes, similar to the alteration of GW182 reported in G0 cells (Yang et al. 2004). Consistently, in *Xenopus*

laevis immature, folliculated oocytes, specific, target reporters injected along with their corresponding microRNAs into the nucleus demonstrated upregulated expression. Additionally, an endogenous transcript, Myt1, is directly targeted and upregulated by xlmiR16, contributing to the maintenance of oocyte immaturity (Mortensen et al. 2011). These results demonstrate the physiological relevance of microRNA-mediated upregulation in the maintenance of oocyte immaturity in *Xenopus laevis*. The early oocyte has been demonstrated to be quiescent-like, mimicking some of the properties and signals such as the cAMP pathway observed in G0 cells (Friedman 1976; Motlik and Kubelka 1990; Pelech et al. 1990; Taieb et al. 1997; Cho-Chung and Nesterova 2005). Interestingly, upregulation by microRNAs in oocytes is regulated by cAMP signaling (Mortensen et al. 2011), which maintains the immature state of the oocyte and the G0 state in some mammalian cells.

Neuronal Small RNAs: In the neuron, specific mRNAs are silenced in mRNPs that are transported to developing synapses and dendrites where neuronal signaling reactivates their translation (Lugli et al. 2005; Ashraf et al. 2006; Banerjee et al. 2009; Khudayberdiev et al. 2009; Schratt 2009). FMRP-Dicer-AGO2-associated complexes (Caudy et al. 2002; Jin et al. 2004) have been observed to aggregate as untranslated microRNP complexes at the synapse until neuronal activation of this Dicer-associated complex (Lugli et al. 2005), upon alleviation of repression in GW-like bodies (Schratt et al. 2006; Schratt 2009) or upon cleavage of associated repressive factors such as Armitage (Ashraf et al. 2006), leading to localized translation at the synapse. While Dicer contributes to the maturation of microRNAs (Chendrimada et al. 2005), Fragile-X Mental Retardation Protein (FMRP) is an important neuronal translation regulator, associated with the microRNP (Jin et al. 2004; Weiler et al. 2004; Park et al. 2008; Bechara et al. 2009; Auerbach and Bear 2010).

Relief of Repression: Interference with and thereby alleviation of microRNA-mediated translation regulation in mammalian cells was demonstrated by Bhattacharyya et al. They reported that a 3′-UTR U rich sequence that binds HuR upon stress interferes with repression upstream mediated by miR-122 on the CAT-1 mRNA (Bhattacharyya et al. 2006). HuR binding was specific and regulated by amino acid starvation and other forms of cellular stress. HuR-mediated alleviation of repression was shown to be mediated by relocalization of the mRNP from repressive GW/P-bodies in the cytoplasm to polysomes, mobilized by HuR association with the mRNA. Similarly, miR-134-mediated repression of Limk1 mRNA in dendritic spines is alleviated in response to extracellular stimuli involving the TOR pathway (Schratt et al. 2006; Khudayberdiev et al. 2009; Schratt 2009). Interestingly, the factors that intervene and restrict microRNA functions may themselves be regulated in expression; thereby, alleviation of repression is further restricted to specific tissues and stages, where these factors may be expressed. Such tissue-specific alleviation of repression was demonstrated by Kedde et al. with another U rich RNA binding protein (RBP), Dead end 1 (DND1). DND1 counteracts the function of several microRNAs by binding to specific mRNA 3′-UTRs via U-rich regions and preventing microRNA association with their target sites, thereby alleviating repression and downregulation (Kedde et al. 2007; Kedde and Agami 2008). DND1 prevents miR-430 association and repression of Nanos1 and TDRD7 mRNAs in

primordial germ cells and is required for germ line development in zebrafish; the same microRNA is essential for the deadenylation and removal of maternal transcripts in the developing embryo where DND1 is not expressed and does not alleviate microRNA functions.

MicroRNP-mediated Upregulation in Quiescent G0 Cells: A key component of the repressive microRNP, GW182, forms GW bodies (Liu et al. 2005) and is a critical mediator of microRNA-controlled deadenylation and repression (Fabian et al. 2010). GW182 was demonstrated to be regulated by the cell cycle with altered interaction in G0 (Yang et al. 2004; Jakymiw et al. 2005). Tethering AGO2 to a reporter activated translation in these G0 arrested cells (Vasudevan and Steitz 2007). Furthermore, microRNAs recruit a modified microRNP comprising AGO2 and a distinct Fragile-X-Mental Retardation-Related Protein 1 (FXR1) isoform, FXR1-iso-a, to mediate translation activation of specific targets and simple reporters in quiescent G0 cells (Figs. 7.2 and 7.3g). The FXR1 isoform also undergoes a switch from a heavy insoluble particle to the soluble polysome-associated fraction in G0. Intriguingly, this mechanism of regulation appears to be restricted to quiescent states where the cells are synchronized in G0 and not in G1 arrest or other arrested states of the cell cycle. Cells transfected with specific reporters bearing the TNFα 3′-UTR or other specific target site reporters and their cognate microRNAs exhibited translation upregulation in G0; in contrast, when the same cells were switched out of their quiescent state into the cell cycle where GW bodies increased, translation repression was restored (Vasudevan and Steitz 2007; Vasudevan et al. 2007, 2008). Consistent with these findings, several, recent studies demonstrated that similar to the upregulation of TNFα and specific target reporters, microRNPs mediate post-transcriptional upregulation of genes involved with the immune system and with cell growth under quiescent conditions. Toll-like receptor 4 (TLR4) is upregulated by miR-511 in quiescent, monocyte-derived dendritic cells and reporters bearing its 3′-UTR or the target site from the TNFα transcript as a control demonstrate upregulated expression under quiescent conditions (Tserel et al. 2011). In immortalized mammary epithelial MCF10A and quiescent RK3E cells, miR-206 activates expression of Kruppel-like factor 4 (KLF4), a transcription factor, via direct base-pairing with a target site in the 3′ UTR. However, the same microRNA, miR-206, in breast cancer as well as a competing microRNA, miR-344 expressed instead of miR-206 in proliferating RK3E cells, repressed KLF4 expression (Lin et al. 2011). These results indicate that specific transcripts and microRNAs are upregulated in quiescent conditions and in immortalized cells.

In quiescent cells, apart from reduced AGO2-GW182 interactions, mRNAs may have shortened poly (A) tails due to increased deadenylation (Seal et al. 2005). Two other studies on microRNA-mediated translation upregulation (Henke et al. 2008; Iwasaki and Tomari 2009) reported similar observations. Additionally, polyadenylated reporters fail to demonstrate activation in oocytes where the endogenous mRNAs have short poly(A) tails in the immature oocyte state (Mortensen et al. 2011). While the overall 3′-UTR interactions dictate the final translation outcome of the message, these studies demonstrated that under quiescent conditions, specific mRNAs recruit a modified RNP that is capable of functioning as a translation activation complex.

True quiescence and activation by microRNAs in mammalian cell lines are less frequently observed than the predominant microRNA-mediated downregulation due to several features. First, G0 is a distinct assortment of states entered selectively as an adaptation to unfavorable conditions. Second, quiescence is controlled by cell-intrinsic factors as well as by cell-to-cell contact, which inhibits entry into quiescence by growth factor deprivation and therefore, prevents translation activation (Friedman 1976; Coller et al. 2006; Schorl and Sedivy 2007). In concurrence, Hwang et al. demonstrated that increasing cell-to-cell contact via increased density of growing cultures enabled increased repression by microRNAs (Hwang et al. 2009). Third, in cells that lack the ability to enter quiescence, a block to cell division by multiple means leads to cell death, G1 arrest or senescence rather than quiescence. Without specific tests and markers to distinguish G0 from G1 arrest and senescence, can misleadingly suggest G0 (Bossis and Stratakis 2004; Cho-Chung and Nesterova 2005). Understanding the essential molecular features of microRNA-mediated activation will provide significant insight into G0 as well as improved ways of control over activation by microRNAs.

5′TOP mRNAs: 5′TOP mRNAs encode ribosomal protein and other protein synthesis-related factor mRNAs. The presence of a 5′TOP tract renders these mRNAs translationally repressed upon cell cycle arrest and upon nutritional deprivation (Meyuhas 2000; Hornstein et al. 2001). When miR-10a is expressed, it binds such target ribosomal protein mRNAs immediately downstream of the TOP sequence at noncanonical microRNA target sites, alleviates TOP-mediated repression and stimulates translation (Fig. 7.3b) during treatments known to activate TOP mRNA translation including: amino acid starvation, following anisomycin treatment or overexpression of a mutant-activated RAS (Orom et al. 2008). Intriguingly, the TOP sequence is required for miR-10a-mediated activation suggesting a synergistic effect of adjacent UTR elements on the microRNA target site. The study also suggested that the TOR pathway was involved since the miR-10a-induced upregulation was alleviated by rapamycin inhibition of mTOR (Orom et al. 2008). Whether an AGO2 microRNP or an alternate RNP is involved remains to be investigated.

HCV RNA Levels: One of the first insights into alternate functions of microRNAs in mammalian cells was demonstrated with HCV and miR-122 by Jopling et al. This study demonstrated that HCV tropism to the liver was dependent on miR-122, a microRNA abundant in the liver. MiR-122 enabled increased levels of the HCV RNA through base pairing at two 5′-UTR miR-122 sites (Jopling et al. 2005, 2008).

HCV RNA Translation: Further investigations revealed that the 5′-UTR miR-122 sites caused translation upregulation dependent on miR-122 base pairing, observed with monocistronic reporters that also contained the HCV 3′-UTR (Fig. 7.3c). The HCV mRNA, upon miR-122 base pairing with the 5′-UTR target site, demonstrated increased association with 40S subunits, increased 48S complex incorporation and, at later stages, enhanced formation of polysomes (Henke et al. 2008; Niepmann 2009). Importantly, this study demonstrated that mutations in the variable region of the HCV 3′-UTR influenced translation efficiency via the 5′-UTR miR-122 target sites, suggesting an important interconnected role between the 5′-UTR and 3′-UTR

to effect translation activation (Henke et al. 2008; Niepmann 2009). The HCV 3′-UTR lacks a poly(A) tail, a feature that appears to be common to some of the studies of translation activation by microRNAs. Intriguingly, placement of the miR-122 sites in the 3′-UTR converted them into translation repression sites in contrast to their upregulatory function in the 5′-UTR. Further investigations are required to determine the differences in the complexes recruited by the microRNA to the 5′-UTR compared to the 3′-UTR and whether the translation effects at the 5′-UTR are AGO2 dependent.

Drosophila Extract/Drosophila AGO2 (*dAGO2*): Studies of a *Drosophila* extract demonstrated that dAGO2 but not dAGO1 caused translation activation of 3′-UTR targets that lacked a poly(A) tail (Fig. 7.3h, Iwasaki and Tomari 2009; Iwasaki et al. 2009). dAGO1 is unable to mediate target cleavage functions but can mediate translation repression requiring GW182 and the cap. Uniquely, human AGO2 as well as dAGO2 do not have this problem and can mediate both target cleavage as well as translation control. dAGO2 causes repression of polyadenylated mRNAs in a cap-dependent manner but independent of GW182, suggesting that other repressive mechanisms mediated by the poly(A) tail must be overcome (Iwasaki et al. 2009). dAGO2 was found in a complex with dFMR1/dFXR1 and an ARE-binding protein, VIG (Caudy et al. 2002), similar to the AGO2-FXR1-iso-a complex identified on an ARE-bearing transcript in G0. G0 mammalian cells (Vasudevan et al. 2007) also exhibit shortened poly(A) tails (Seal et al. 2005) while, similar to the activation observed with dAGO2 on unadenylated target reporters, the HCV RNA is not polyadenylated (Henke et al. 2008; Niepmann 2009). Translation activation in the Drosophila oocyte extract was observed to be stronger for the A capped IRES-driven transcript than for the methylated capped mRNA.

Myocardin mRNA translation by miR-145: A recent study demonstrated that miR-145 was required to regulate the quiescent phenotype of smooth muscle cells. MiR-145 functions as a critical switch to promote smooth muscle differentiation via upregulated expression of Myocardin (Fig. 7.3f), a component of the molecular switch for the vascular smooth muscle development, which bears miR-145 target sites in its 3′-UTR (Cordes et al. 2009). The upregulation by miR-145 was observed specifically with Myocardin 3′-UTR while other targets were repressed.

RIP140 mRNA translation upregulation by miR-346: MiR-346 targets the 5′-UTR of a novel 5′splice variant of RIP140 mRNA and upregulates its protein levels in brain tissue and p19 cells (Fig. 7.3a). This was also observed upon knockdown of AGO2 (Tsai et al. 2009), suggesting mechanistic possibilities such as an alternate complex or RNA structural manipulations that remain to be unraveled.

Glucose Transporter 4 (*Glut4*) *mRNA translation upregulation by miR-223*: In type 2 diabetic patient samples of the insulin-resistant heart, miR-223 is increased. In neonatal rat ventricular myocytes, cardiomyocytes, and other cell lines, the increase of miR-223 leads to Glut4 mRNA translation upregulation without altering mRNA levels in cardiomyocytes (Lu et al. 2010).

Alternative Polyadenylated β-actin mRNA translation by miR-34a/b-5: The neuronal tissue specific, alternatively cleaved and polyadenylated longer form of β-actin mRNA is processed, localized, and translated at neuronal synapses. This longer mRNA harbors an miR-34a/b-5 target site that specifically upregulated translation (Fig. 7.3i) without increasing mRNA levels (Ghosh et al. 2008).

7.5 Mechanisms of Post-transcriptional Upregulation of Gene Expression by microRNAs

MicroRNAs have been demonstrated to repress translation by several mechanisms including cap recognition, translation initiation, ribosome subunit joining, elongation, localization away from polysomes in storage/decay bodies, and nascent peptide turnover (Nottrott et al. 2006; Nilsen 2007; Chekulaeva and Filipowicz 2009; Fabian et al. 2010). MicroRNAs may mediate upregulated gene expression by multiple mechanisms similar to bacterial sRNAs that also show a variety of mechanisms of upregulation (Frohlich and Vogel 2009).

Interconnections between Translation Control and mRNA Stability: sRNA regulation of gene expression in bacteria involves a role for mRNA stability, directly or indirectly (Frohlich and Vogel 2009). Recent studies with mammalian microRNA repression predict that translation regulation by microRNAs is intimately connected with mRNA stability influences: stability RBPs and deadenylation, which contribute to the total gene expression outcome.

mRNA stability and RBPs: The ARE decay pathway often intercalates with microRNA regulation (Jing et al. 2005; Bhattacharyya et al. 2006; Vasudevan and Steitz 2007; Kim et al. 2009), suggesting microRNA-mediated upregulation by stability mechanisms via increased mRNA levels. MicroRNAs have recently been shown to stabilize specific target transcripts: miR-466l upregulates IL10 by preventing TTP binding to the ARE while miR-125b enhances the stability of κB-Ras2 mRNA (Fig. 7.3e), thereby preventing degradation by TTP (Ma et al. 2010a; Murphy et al. 2010).

Deadenylation and the Role of the poly(A) tail: Deadenylation is not required for repression (Wu et al. 2006; Beilharz et al. 2009) but can promote repression (Moretti et al. 2012) and is observed at steady state (Guo et al. 2010) subsequent to initial translation repression of transcripts (Fabian et al. 2010; Bazzini et al. 2012; Djuranovic et al. 2012; Bethune et al. 2012). The poly(A) tail plays an important, regulatory role in activation. In the case of translation regulation by dAGO2, repression ensues with a capped polyadenylated target but activation is elicited by unadenylated target mRNAs, capped or IRES-driven (Iwasaki and Tomari 2009), suggesting that the poly(A) tail is inhibitory to translation activation. This is similar to the required absence of polyadenylation for HCV translation activation by miR-122 (Henke et al. 2008; Niepmann 2009) and the activation in G0 (Vasudevan and Steitz 2007) where increased deadenylation activity results in extensive mRNA

poly(A) shortening (Seal et al. 2005). GW182, an essential repressive component of the microRNP, interacts with PABP and enables microRNA-mediated deadenylation with the recruitment of deadenylases by the microRNP (Fabian et al. 2009; Jinek et al. 2010; Kozlov et al. 2010) as well as promotes translation repression (Moretti et al. 2012), which may be abrogated in these particular cases of activation.

Cofactor influences on the microRNP

While it is clear that the repressive microRNP is distinct from the complexes that mediate translation activation, very few cofactors and mediators have been defined for translation upregulation. Importantly, AGO2 and PIWI proteins are associated with translation effectors including potential activators, which may provide clues to the mechanism of translation activation by the microRNP.

FXR1/*dFXR1*: Fragile-X Mental Retardation-Related Protein 1 (FXR1) and its Drosophila orthologue, dFXR1/dFMR1, were found to interact with AGO, with MIWI/PIWI and MILI complexes (Caudy et al. 2002; Ishizuka et al. 2002; Megosh et al. 2006) and are essential for germ cell maintenance (Yang et al. 2007; Pepper et al. 2009). Factors such as FXR1-iso-a, which interact with the microRNP, alter the functional output of the microRNP to cause stimulation of gene expression (Vasudevan and Steitz 2007). FXR1 is similar in protein structure to the FMR1 family of KH domain RBPs of translation regulators (Zhang et al. 1995; Siomi et al. 1996, 2002; Khandjian et al. 1998; Kirkpatrick et al. 2001). FMR1 associated with Dicer and microRNPs at the synapse (Caudy et al. 2002; Jin et al. 2004; Lugli et al. 2005; Ashraf et al. 2006). Interestingly, FXR1-iso-a is not required for translation repression by microRNAs and is associated with the microRNP complex in G0, where repressive GW bodies are reduced (Vasudevan and Steitz 2007). Overexpression, tethering, knockdown, and rescue studies demonstrated that this specific isoform of human FXR1 is required for AGO2/microRNA-mediated translation activation of specific transcripts in quiescent cells (Vasudevan and Steitz 2007), suggesting that FXR1-iso-a may contribute to the switch to translation activation in each of these complexes (Fig. 7.2).

Tudor Royal Family of Domains (*TDRD*) *Proteins*: The TDRD family of proteins includes Tudor domain proteins such as TDRD1 and TDRKH as well as Agenet-like domain proteins such as FXR1 (Adinolfi et al. 2003; Maurer-Stroh et al. 2003; Chen et al. 2009; Wang et al. 2009), required for activation of specific transcripts by AGO2 in G0 human cells (Vasudevan and Steitz 2007). The germ cell-specific Tudor proteins are an important set of interacting partners for the PIWI family of proteins and are essential for germ line specification and germplasm formation (Wang et al. 2009). MIWI associates directly with TDRKH, (Chen et al. 2009) where the Tudor/Agenet domain recognizes methylated residues on MIWI (Kirino et al. 2010). Tudor proteins were recently demonstrated to be essential for the local-

ization and function of the PIWI family of proteins (Kirino et al. 2010). Although, their precise roles in translation are not defined, their association with PIWI/MIWI and requirement for MIWI-mediated effects on germ cell expression as well as the requirement for the related family member, FXR1-iso-a, for AGO2/microRNA-mediated activation of specific transcripts in G0 human cells, suggest a potential role in translation regulation.

Vasa: The RBPs, Vasa and Murine Vasa Homologue (MVH), have been implicated as upregulators of specific mRNA translation during oogenesis (Raz 2000; Liu et al. 2009) and are also involved in piRNA biogenesis (Kuramochi-Miyagawa et al. 2010). MVH has been found associated with Aubergine/AGO-related family of proteins and associated factors like Tudor/Agenet-like proteins in germ cell granules (Toyooka et al. 2000; Costa et al. 2006). Vasa has features of a DEAD-box RNA helicase related to eIF4A and could thereby enable translation activation (Liu et al. 2009). Vasa and its associated complexes are thought to bind 3′-UTR U-rich sequences on specific target mRNAs in developing oocytes and cause translation upregulation via interactions with eIF5B, which enables recruitment of the 60S ribosome subunit (Carrera et al. 2000; Liu et al. 2009).

Altered GW182: Several studies suggest that the essential repressive factor, GW182 (Liu et al. 2005; Eulalio et al. 2008) is regulated directly or indirectly to enable activation (Yang et al. 2004; Vasudevan and Steitz 2007; Vasudevan et al. 2007; Iwasaki and Tomari 2009). dAGO2 cannot bind GW182 and mediates translation activation of unadenylated mRNAs in an extract system, suggesting a requirement for a specific microRNP that lacks GW182 in *Drosophila* extracts for translation activation (Iwasaki and Tomari 2009), similar to activation by human AGO2 in G0 conditions where GW182 appears altered (Yang et al. 2004; Vasudevan and Steitz 2007). The fact that disabling the interaction of AGO2 with GW182 usually causes a loss of repression (Till et al. 2007) and that viruses have evolved proteins to decoy GW182 (Azevedo et al. 2010; Jin and Zhu 2010), suggests that manipulating GW182 by the cell cycle/G0 (Yang et al. 2004; Vasudevan and Steitz 2007) and in oocytes, where repression is not observed coincident with altered AGO2-GW182 association (Kennerdell et al. 2002; Nakahara et al. 2005; Ma et al. 2010b; Flemr et al. 2010) or by decoy proteins, might be an inherent method to switch microRNA functions from repression to activation or relief of repression.

Pseudogene RNA Decoys: Pseudogene transcripts can compete with mRNAs for microRNA binding and thereby cause derepression of microRNA-targeted transcripts. PTEN, a tumor suppressor, as well as its pseudogene PTENP1 are targeted by miR-19b and miR-20a. PTENP1 3′-UTR functions as a tumor suppressor by acting as a decoy for these microRNAs to relieve PTEN expression from their repressive activity; thereby, increasing PTEN mRNA and protein levels (Poliseno et al. 2010). Interestingly, the alignment of other genes with their pseudogene sequences such as KRAS and KRAS1P show high conservation of microRNA-binding sites (Poliseno et al. 2010). Overexpression of KRAS1P 3′-UTR lead to increased KRAS levels and accelerated cell growth, suggesting that the action of pseudogenes as

decoy molecules or sponges (Ebert and Sharp 2010; Cazalla et al. 2010) that prevent microRNAs from binding target mRNAs may be a widespread regulatory mechanism of gene expression (Poliseno et al. 2010).

Decoy by the microRNA: MiR-328 was demonstrated to bind and decoy hnRNP E, alleviating repression induced by an RBP of an important target, C/EBPα that was then translated and expressed (Fig. 7.3d). The mechanism and specificity of miR-328 recognition of hnRNP E remains to be investigated (Eiring et al. 2010; Beitzinger and Meister 2010). This example underscores the versatility of microRNAs to alter gene expression by multiple mechanisms, directing a complex to the target or as in this case, away from the target to activate translation.

RNA structure alteration by RBPs in cis: Factors that interact with adjacent sequences, such as HuR, DND1, and Pumilio, modify the functions of the microRNP directly or indirectly and have been demonstrated to cause relief of repression (Bhattacharyya et al. 2006; Kedde et al. 2007, 2010; Kedde and Agami 2008). The cell cycle regulated binding of Pumilio 1 (Pum1, Spassov and Jurecic 2003) to specific mRNAs promotes binding of the microRNA to the microRNA target site on p27 mRNA, which represses its expression exclusively in cycling cells but not in arrested cells (Kedde et al. 2010; Triboulet and Gregory 2010). The mechanism appears to involve an RNA stem loop structure that is opened upon Pum1 binding, revealing the target sites for access by specific microRNAs in cycling cells, where Pum1 is phosphorylated to enable its ability to bind RNA. In arrested cells, Pum1 RNA binding and overall stability is reduced to prevent opening these sites and thereby, p27 translation is enabled.

AGO2-Independent microRNA Role: Translation activation of RIP140 mRNA via miR-346 demonstrated microRNA-mediated activation (Fig. 7.3) despite AGO2 knockdown, suggesting that the microRNA may act independently of AGO2 to cause activation (Tsai et al. 2009). Whether the activation is mediated by other AGOs, a novel microRNP or whether the microRNA is sufficient to alter the mRNP structure and thereby cause activation remains to be investigated.

Translation Machinery: Several reports also suggest that AGO family members can associate with eIF3, including the closely related MILI that associates directly with eIF3a subunit of eIF3 and stimulates translation (Unhavaithaya et al. 2009). EIF3 is an essential translation factor that associates with the 40S subunit of the ribosome to enable its interaction with the ternary complex and the mRNA, thereby stabilizing the ternary complex in the presence of the mRNA (Asano et al. 2001; Hinnebusch 2006; Sonenberg and Hinnebusch 2009). A recent screen in *C. elegans* demonstrated that Let-7 microRNA-mediated functions are affected by eIF3 (Ding et al. 2008). It remains to be investigated whether the association of eIF2C proteins with eIF3 regulates ribosome recruitment for specific microRNP-mediated translation activation or repression. These interactions do not exclude additional mechanistic effects on subsequent steps in protein synthesis as has been found for microRNP-mediated repression (Nottrott et al. 2006; Petersen et al. 2006; Nilsen 2007; Chekulaeva and Filipowicz 2009; Fabian et al. 2010); the precise mechanism(s) influenced by microRNPs to activate translation remains to be elucidated.

7.6 Regulation

G0 and Germ Cells: From the studies discussed above, regulated microRNP-mediated translation activation is also observed with transcriptionally restricted programmed cells such as quiescent cells and germs cells. A quiescent state is a unique adaptive response in a population of dividing cells that provides an advantageous escape from the harsh situations that lead to permanent alternatives; instead the cell is suspended for defined durations in a quiescent state where it can mature, build up levels of regulatory factors, develop, and respond adequately to more favorable conditions (Coller et al. 2006). The suspended cell requires expression of very specific genes to maintain the state, to resist harsh stimuli as well as respond to altered conditions: these are achieved by post-transcriptional mechanisms that may include the translation upregulation by microRNAs (Vasudevan and Steitz 2007; Vasudevan et al. 2008). The germ line follows a quiescence-like program (Motlik and Kubelka 1990; Pelech et al. 1990) with regulated post-transcriptional mechanisms including selective translation for developmental events. Translation activation by RBPs and microRNP-associated factors, has been well observed in the developing germ line, which are non-proliferating like quiescent cells, yet require regulated gene expression for maintenance along with growth and preparations for the next developmental steps (Kobayashi et al. 1991; Smith et al. 1991; Wilhelm and Smibert 2005; Vasudevan et al. 2006; Brook et al. 2009; Unhavaithaya et al. 2009).

TOR pathway: The specific translation enhancement by miR-10a of TOP mRNAs is sensitive to TOR signaling as treatment with rapamycin prevents upregulation mediated by miR-10a. It remains to be investigated how the TOR pathway influences microRNA-mediated translation activation (Orom et al. 2008).

mRNA Localization in the Cell: Regulated localization of the microRNP may determine activation or relief of repression. mRNPs are sequestered away from polysomes in AGO2-GW182-repressive complexes until the cells enter G0 where specific mRNAs are expressed (Yang et al. 2004; Vasudevan and Steitz 2007). mRNPs are also relocalized to polysomes upon relief of repression as a result of release from GW bodies at the synapse upon neuronal activation (Schratt et al. 2006; Banerjee et al. 2009; Schratt 2009) or stress conditions (Bhattacharyya et al. 2006).

microRNA and microRNP Regulation: The levels of microRNAs and unbound AGO dictate the availability and efficiency of microRNA function (Diederichs and Haber 2007; Shi et al. 2007; Diederichs et al. 2008) and may be regulated to control expression. The stability and levels of microRNAs are regulated across different circumstances. Such microRNAs may be the candidate regulators of activation as in the case of G0 with specific enrichment of miR-369-3p (Vasudevan et al. 2007).

Target mRNA Specificity: MicroRNAs are predicted bioinformatically to target over 33% of the genome but they do not all get repressed or activated. Many examples demonstrate that UTRs usually are alternatively polyadenylated to include or preclude essential sites or have additional RBP binding and regulatory sites that

when occupied in a regulated manner can prevent or permit microRNA activity (Bhattacharyya et al. 2006; Kedde et al. 2007; Sandberg et al. 2008). Activation was observed with minimal target UTR reporters in G0 as well as with select natural transcripts, specific for certain 3′-UTRs (Figs. 7.2 and 7.3) that correlate with their biological requirement and corresponding microRNA expression patterns. For example, in G0 mammalian cells, TNFα is upregulated along with its regulatory microRNA, miR-369-3p (Figs. 7.2 and 7.3g, Vasudevan and Steitz 2007). In muscle differentiation, miR-145 upregulated myocardin expression (Fig. 7.3f) while miR-143 and miR-145 downregulated other substrates (Cordes et al. 2009). Specific mRNAs recruit distinct mRNPs induced by G0 that require the absence of GW182 in the RNP, recruitment of the activating AGO2-FXR1-iso-a complex and in several cases, absence of or shortened poly(A) tails (Yang et al. 2004; Vasudevan and Steitz 2007; Iwasaki and Tomari 2009; Mortensen et al. 2011) to demonstrate activation (Table 7.1). These examples suggest that microRNA-mediated activation is dependent not only on microRNA expression but is target specific and dependent on the UTR and the specific history and conditions experienced by distinct target mRNAs.

Sequence Location: MicroRNA-mediated translation activation was identified in the 3′-UTR (Vasudevan and Steitz 2007; Ghosh et al. 2008; Cordes et al. 2009; Iwasaki and Tomari 2009) while other studies demonstrated 5′-UTR-mediated activation (Fig. 7.3) (Henke et al. 2008; Orom et al. 2008). Intriguingly, the HCV miR-122 sites that function to activate translation in the 5′-UTR (Fig. 7.3c) appear to repress translation when relocated to the 3′-UTR (Henke et al. 2008; Niepmann 2009). This suggests the following possibilities: (1) that the miR-122 sites need to be adjacent to or interacting with factors recruited by the HCV IRES; or (2) the overall mRNP structure is conducive to activate expression with the target sites in the 5′-UTR but not in the 3′-UTR where, given the requirement for the 3′end structure, the relocation may inadvertently interfere with activation. Location at the 5′end does not necessarily mediate translation activation effects as tethering AGO2 upstream of an IRES at the 5′end permits repression in mammalian cells (Lytle et al. 2007) and activation is also observed via 3′-UTR sites in quiescent cells, in extracts and with other mRNAs (Fig. 7.3). It remains to be investigated whether specific UTR locations utilize distinct factors or a common translation mechanism elicited on the mRNA via target sites on either UTR.

Additional elements: The presence of additional sequences in a complex UTR that interfere or synergize with the microRNA target site and its normal functions, is expected to dictate the final readout of gene expression (Kedde et al. 2007; Kedde and Agami 2008). The additional UTR complexes recruited are usually themselves subject to modulation, adding another avenue for regulated expression.

3′-UTR Regulatory Sequence Elements and Factors: 3′-UTR regulatory sequences control localization, stability, or translation in a temporal and spatially controlled manner (Gray and Wickens 1998; Ogura et al. 2003; Wilkie et al. 2003; Anderson et al. 2004; de Moor et al. 2005; Wilhelm and Smibert 2005; Schier 2007).

Table 7.1 Distinctive Features of Repression and Activation

		Repression	Activation	References
RNP features	Argonaute	All four human argonautes, dAGO1, dAGO2, and other orthologues	Human AGO2, dAGO2, xlAGO	Wu et al. (2008); Vasudevan and Steitz (2007); Iwasaki and Tomari (2009); Mortensen et al. (2011)
	GW182	Requires AGO-GW182 interaction	Altered or reduced AGO2-GW182 interaction and lack of association of GW182 on specific mRNAs	Yang et al. (2004); Liu et al. (2005); Behm-Ansmant et al. (2006); Vasudevan and Steitz (2007); Li et al. (2008); Ding and Grosshans (2009); Iwasaki and Tomari (2009); Lian et al. (2009); Zekri et al. (2009); Zipprich et al. (2009); Ma et al. (2010b); Flemr et al. (2010)
	FXR1	No	One specific isoform, FXR1-iso-a	Vasudevan and Steitz (2007)
mRNA Features		Targets mRNAs that use general or specialized translation mechanisms, no preference for poly (A) status but poly(A) and PABP can enhance repression	Targets mRNAs with specialized alternative translation mechanisms: IRES, 5′ TOP, absence of or shortened poly (A)	Wu et al. (2006); Vasudevan and Steitz (2007); Henke et al. (2008); Orom et al. (2008); Iwasaki and Tomari (2009); Mortensen et al. (2011); Moretti et al. (2012)
Regulatory features	Regulatory bodies	Associated	No	Yang et al. (2004); Vasudevan and Steitz (2007)
	Regulation	Reduced in oocytes and early development	mTOR, G0, promoted by cAMP-dependent regulation in folliculated, immature, *Xenopus laevis* oocytes	Nakahara et al. (2005); Vasudevan and Steitz (2007); Orom et al. (2008); Flemr et al. (2010); Ma et al. (2010b); Lin et al. (2011); Tserel et al. (2011)

Drosophila Aubergine and MVH (and Vasa) are important 3′-UTR binding proteins that translationally activate specific transcripts in distinct stages of germ line development (Wilson et al. 1996; Harris and Macdonald 2001; Caudy et al. 2002; Kennerdell et al. 2002; Liu et al. 2009). These factors associate with PIWI and AGO family members, linking microRNP-mediated translation regulation with 3′-UTR regulatory sequences.

AU-Rich Elements (*AREs*): AREs and microRNPs affect common functions of deadenylation and translation (Khabar 2005; von and Gallouzi 2008). Historically, the ARE was discovered as a decay element (Chen and Shyu 1995; Wilusz et al. 2001; Brewer 2002) and was found to possess additional control over translation (Kruys et al. 1990; Kontoyiannis et al. 2001; Wilusz and Wilusz 2004; Espel 2005; Garneau et al. 2007); these roles are highly regulated and can increase or decrease gene expression dramatically in response to intracellular and environmental cues. The ARE-binding/U-rich binding protein, HuR and its related family of ELAV proteins pleiotropically influences not only mRNA processing, export and decay but also translation repression and activation in a UTR-specific manner (Atasoy et al. 1998; Peng et al. 1998; Brennan and Steitz 2001; Wang et al. 2002; Mazan-Mamczarz et al. 2003).

Several studies provide substantial evidence for the interaction of the microRNA pathway with the ARE regulatory system. First, BCL2 and other well-known microRNA targets are AU-rich transcripts with known functional AREs (Khabar et al. 2005; Bakheet et al. 2006). Second, several of microRNA targets are predicted to be within AU-rich sequences by multiple programs that align conserved microRNA seed sequences and from experimental microarray data for at least three different microRNAs (Schiavone et al. 2000; Khabar et al. 2005; Robins and Press 2005; Sood et al. 2006). Third, microRNA bound complexes include two ARE-associated proteins, FXR1 and PAI-RBP1, which associate with the TNFα mRNA ARE (FXR1-iso-a, Vasudevan and Steitz 2007) and the PAI mRNA ARE, respectively (Caudy et al. 2002; Jin et al. 2004; Xu et al. 2004). Fourth, a recent study suggested that miR-16-1 regulated the mRNA levels of a TNFα ARE reporter (Jing et al. 2005). Fifth, our data demonstrated that the TNFα mRNA can be translationally activated by microRNAs targeting the AU-rich region (Vasudevan and Steitz 2007). Finally, AREs/associated RNA binding factors and microRNPs compete and cooperate to modulate gene expression. For example, HuR affects microRNP functions, relieving or recruiting microRNPs to their targets while microRNAs compete with TTP to regulate translation and mRNA stability (Fig. 7.3e, Bhattacharyya et al. 2006; Kim et al. 2009; Ma et al. 2010a; Murphy et al. 2010). These evidences suggest that the microRNA and ARE pathways can interact to enforce synergistic post-transcriptional control.

5′ TOP tract: The activation observed by the 5′TOP mRNAs (Fig. 7.3b) depends on miR-10a binding downstream of the TOP sequence and is dependent on the TOP sequence (Orom et al. 2008). It remains to be investigated whether the TOP sequence functions productively with microRNA target sites in a direct manner or contributes indirectly to the overall mRNP structure.

7.7 Similarities and Distinctions Between Repression and Activation

When a microRNA or siRNA is introduced, the predominant feature observed is downregulation; mediated by mRNA cleavage, deadenylation, or translation repression (Wu et al. 2008). Activation is observed naturally with specific transcripts or in distinct conditions. In particular, each case of activation appears synchronized with the biological requirement of the target mRNA, suggesting that the required microRNAs are exclusively expressed in these conditions as in the case of miR-122 and HCV RNA or miR-369-3p and TNFα mRNA (Vasudevan et al. 2007; Henke et al. 2008). There are several apparent features that distinguish the microRNP and its mechanisms for activation from repression and categorize activation as a specialized phenomenon (Table 7.1).

A primary distinction between repression and activation is the limitation of activation to a specialized AGO complex in G0 mammalian systems (Vasudevan and Steitz 2007) and to dAGO2 with unadenylated target mRNAs in Drosophila extracts (Iwasaki and Tomari 2009). While repression can be attributed to all four AGOs in mammalian cells and to all AGOs, dAGO1 and dAGO2, in *Drosophila*, (Pillai et al. 2004; Wu et al. 2008) the ability of introduced (or produced) microRNAs to cause activation has been observed so far only with AGO2, xlAGO (Mortensen et al. (2011)) or dAGO2 (Iwasaki and Tomari 2009), available in distinct conditions in their respective systems. Therefore, activation is limiting and more specialized in comparison to repression, which utilizes all AGOs and can be widespread.

A second feature clearly observed in at least a few examples of activation is that GW182 must be restricted from being recruited to the target mRNA for activation to occur. GW182 fails to interact with dAGO2, and is not recruited to specific transcripts targeted by AGO2 and microRNAs in G0 mammalian cells (Vasudevan and Steitz 2007; Vasudevan et al. 2008; Iwasaki et al. 2009) where GW bodies/GW182 levels are reduced (Yang et al. 2004), suggesting that the interaction with GW182 is restrained on specific transcripts to mediate translation activation. The interaction of AGO2 with GW182 is important for translation repression (Liu et al. 2005; Behm-Ansmant et al. 2006; Li et al. 2008; Lian et al. 2009; Zekri et al. 2009; Ding and Grosshans 2009; Zipprich et al. 2009). This exceptionality limits the cells to activate only in very specific conditions such as in the G0 phase of the cell cycle and in the presence of microRNPs lacking GW182.

A final feature is the observation that many cases of translation activation observed involve mRNAs that likely require alternative mechanisms of translation, either due to cellular conditions or due to specifications of the mRNA. In contrast, repression generally does not exhibit such a preference. Many of the cases of activation occur with transcripts that may have a specialized translation mechanism—IRES with HCV, *Drosophila* extracts with an IRES, 5′TOP, unadenylated mRNAs or conditions with specific translation such as germ cells and G0—adapted to activate selective mRNA translation in response to distinct features of the microRNP/

mRNA (Fig. 7.3 and Table 7.1, Nakahara et al. 2005; Vasudevan and Steitz 2007; Henke et al. 2008; Orom et al. 2008; Iwasaki and Tomari 2009; Ma et al. 2010b). In particular, the absence of a poly(A) tail or the presence of shortened poly(A) tails appears to be a distinctive feature across several examples of activation, indicating that shortened poly(A) tails may be important for the mechanism of translation activation (Seal et al. 2005; Vasudevan et al. 2007; Radford et al. 2008; Henke et al. 2008; Iwasaki and Tomari 2009; Ma et al. 2010b; Mortensen et al. 2011). Additional specifications, conditions, and features of mRNAs that enable alternate functions of microRNAs need to be investigated to fully comprehend the extensive scope of microRNA control over gene expression.

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Chapter 8
Gawky (GW) is *the Drosophila melanogaster* GW182 Homologue

Jing Li, Tom C. Hobman, and Andrew J. Simmonds

8.1 Introduction

While the human GW182 gene was discovered over 10 years Ago, functional characterization of the *Drosophila melanogaster* GW182 othologue—Gawky (GW, previously denoted as CG31992, CG11484, CG9905, or dGW182) has been relatively recent. (Rehwinkel et al. 2005; Schneider et al. 2006) However, the *Drosophila* model has contributed greatly to studying the role(s) of the GW182 family proteins in multiple pathways and in particular their role in RNA interference (RNAi). Of the commonly used metazoan models, *Drosophila* is unique in that there is only one GW protein encoded by the *Drosophila* genome and this homologue retains a high level of sequence and/or organizational identity to vertebrate GW182 proteins (Fig. 8.1). Thus, the potential functional redundancy associated with the multiple GW182 family proteins encoded by the mammalian genome is less of a concern in *Drosophila* studies (Schneider et al. 2006; Eystathioy et al. 2002). The bulk of the currently published literature regarding *Drosophila* GW can be divided into two main categories. Functional studies describing the *Drosophila gw* mutant phenotype and cell-biological/biochemical studies probing the vital role of GW in the mechanics of *Drosophila* miRNA pathway.

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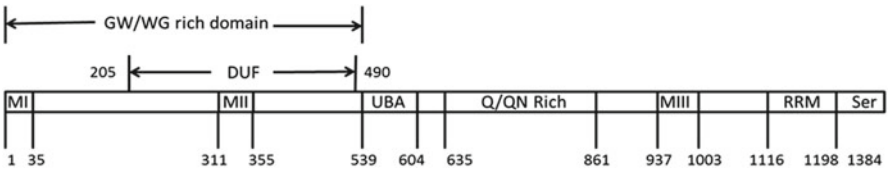

Fig. 8.1 The structural organization of the *Drosophila* GW protein. MI MII MIII—Motif I, highly conserved regions—Motif II and Motif III (Eulalio et al. 2009a). *DUF* conserved domain of unknown function; *UBA* ubiquitin associated domain; *Q/QN Rich* glutamine/glutamine and asparagine rich domain; *RRM* RNA recognition Motif; *Ser* serine rich domain

8.2 Drosophila Cells Make Extensive Use of Cytoplasmic mRNA Regulation

In eukaryotic cells, cytoplasmic mRNA regulation is thought to occur largely within ribonucleoprotein (RNP) complexes. These complexes contain both RNAs and proteins and are often aggregated into larger regulatory structures (Zhang et al. 2003; Muddashetty et al. 2002; Ohashi et al. 2000; Kobayashi et al. 1998). Many investigations of these regulatory mechanisms in *Drosophila* pre-date the discovery of the RNA interference (RNAi) pathway or the functional characterization of *gw*. One of the first RNP structures to be described is the Nuage/Polar granules located within *Drosophila* oocytes and embryos, where regulation of genes critical patterning and development makes extensive use of localized expression of mRNAs at the cellular poles (Hay et al. 1988; Wilsch-Brauninger et al. 1997).

Many other patterning events within the developing *Drosophila* oocyte and fertilized embryos are also extensively regulated by post-translational gene regulation. *Drosophila* screens to identify genes involved in developmental processes have identified several genes encoding multiple components of regulatory RNPs. Examples of these include: Staufen (STAU); Exupurentia (EXU); Ypsilon schachtel (YPS), a Y box binding protein One homologue and Oo18 RNA-binding protein (ORB), the *Drosophila* Cytoplasmic Poly (A) Element Binding protein homologue (St Johnston et al. 1991; Mansfield et al. 2002; Lin et al. 2006). Notably, in *Drosophila*, it seems that multiple mRNA regulatory events can be functionally linked. For example, there is appears to be a coupling of translational suppression and cytoplasmic mRNA localization and/or transport in *Drosophila* embryos. ME31B, a DEAD box helicase translational repressor and decapping activator, transiently localizes with RNP granules during transport, until they reach the posterior of the oocytes (Lin et al. 2006). Notably, many of these previously characterized RNA regulatory proteins have since been associated with GW or mammalian GW182 (Eulalio et al. 2007b; Ikeda et al. 2006; Quaresma et al. 2009; Huntzinger et al. 2010; Tritschler et al. 2010; Yao et al. 2011).

This functional linkage between multiple aspects of mRNA regulation and Recently, Dcp1, a key part of the decapping enzyme complex that is often found associated with GW182 family of proteins in mRNA processing (P-)bodies was also identified as a component of RNP granules that localize to the posterior of

the oocyte (Lin et al. 2006). Degradation of some posteriorly localized transcripts does occur during early embryogenesis (Ephrussi et al. 1991; Kim-Ha et al. 1993). One possibility is that early recruitment of Dcp1 may facilitate the rapid assembly of the degradation machinery at a later time during embryo development (Lin et al. 2006). Much of the early mRNA deposited in the embryo maternally is co-ordinately degraded at approximately 120 min after eggs are deposited at the mid-zygotic (or mid-blastula) transition (reviewed in (Tadros and Lipshitz 2009)). At this stage of development, foci are seen within the embryo that have a typical P-body-like composition including Dcp2 and the 5′-3′ exonuclease Pacman (PCM), a homologue of human Xrn1 (Lin et al. 2008). The fact that the various different regulatory RNPs active in *Drosophila* cells often share many of the same protein components between various regulatory structures supports that they may be linked functionally. This might mean that RNPs containing translationally repressed and localized mRNAs that are initially formed in the oocyte may later acquire additional components to degrade these mRNAs when they are no longer needed.

Extensive regulation of mRNA within cytoplasmic RNPs is not limited to *Drosophila* embryogenesis. *Drosophila* neurons also contain cytoplasmic RNPs that include factors involved in P-body mediated mRNA decay including PCM, Dcp1, Ago2 the RNAi component, and Up-frameshift suppressor (Upf), a component of RNA nonsense mediated decay (NMD) pathway (Metzstein and Krasnow 2006). These neuronal RNPs also have been reported to share components with maternal mRNA regulatory RNPs including STAU, FRMP and Barentz or protein components normally localized to stress granules (G3BP and eIF2) (Barbee et al. 2006). Of particular note is the observation that a number of RNPs contained different subsets of these components. Additionally, the composition of RNPs in neuronal cells appears to be influenced directly by the relative level of particular protein components. Over-expression of STAU or a GFP fusion of dFMR1 resulted in an increase in the degree of co-localization of these two proteins in cytoplasmic RNPs. This concurrent increase in particle size and decrease in particle number suggests that the increase in co-localization may be the result of fusion of different types of RNA granules. Fusion of these granules further supports a model where there is a functional relationship between them. Thus, while many functionally diverse mRNA regulatory bodies have been discovered independently in various *Drosophila* cell types, they share significant similarities, both in composition and function. Thus, there is a distinct possibility that our current differentiation of cytoplasmic RNA regulatory bodies in *Drosophila* could be largely artificial or that there is significant cross-talk between different aspects of mRNA regulation. However, given that most of these proteins were identified in functional screens affecting specific aspects of *Drosophila* development, it is clear these cytoplasmic RNPs have a direct role in regulating many different aspects of cellular function balancing competing cytoplasmic events: mRNA translation and sequestering/degrading mRNAs in RNP complexes. Elucidating the role of *Drosophila* GW in some or all of these various aspects of mRNA regulation during initial cellular differentiation and later homeostasis has only just begun.

8.3 The Drosophila Genome Project Predicted Only one Gene Similar to GW182

The majority of the *Drosophila* genome was first sequenced in 2000 (Adams et al. 2000; Stapleton et al. 2002; Drysdale 2003). *Drosophila* sequences similar to human GW182 were first identified as two different genes on chromosome 4 given the sequential identifiers (Celera Genomics) CG11484, CG9905 (Adams et al. 2000). Later efforts focused on both functional annotation of the genome and conformation of the mRNAs expressed from each identified gene refined this prediction to a single gene id CG31992, which was subsequently named *gawky* (*gw*) (Schneider et al. 2006). Follow up projects that mass sequenced multiple cDNAs indicated that the *gw* locus produces 8 transcripts via alternative splicing: *gw*-RA, *gw*-RB, *gw*-RC, *gw*-RD, *gw*-RE, *gw*-RF, *gw*-RG and *gw*-RH (Fig. 8.2). However, all of these transcripts differ only in their 5′ untranlsated region (UTR) and the open reading frame of each of these alternative splicing forms is identical, encoding a protein with a molecular weight 143 kD. The modENCODE project has confirmed that *Drosophila gw* expression is seen in all development stages. The relative expression levels of *gw* are higher during early embryogenesis and at the beginning stages of pupariation, implying that during these two stages, cells may have elevated requirements for GW (Celniker et al. 2009). Finally, the *Drosophila* genome project has further sequenced *gw* homologues from multiple related species and have found that there is significant conservation of the *gw* locus among the *Drosophilids* (Gilbert 2007).

8.4 Functional Identification of a the Gawky (gw) Mutation

Traditionally, gene discovery in *Drosophila* focuses on the identification of gene mutations affecting specific cellular or developmental activities (St Johnston 2002). Despite an extensive history of screening of the *Drosophila* genome for mutations affecting embryo development, the identification of *gw* as a critical gene required for early embryonic development was quite recent (Schneider et al. 2006). The likely reason for this is that the *gw* gene is located on the right arm of chromosome 4 at sequence location 4:670575..682391, cytological map location 102D2-102D3. Unfortunately, the large scale screens for mutations that are so effective in isolating critical *Drosophila* genes on other chromosomes largely ignore the few genes on chromosome IV. *Drosophila* has two sex chromosomes and 3 autosomes. Chromosome IV has two major regions: the centromeric domain is a-heterochromatic and consists primarily of about ~3–4 Mbp of short, satellite repeats. This region forms part of the highly condensed chromocenter seen in polytene chromosome spreads. The remaining ~1.2 Mbp constitutes cytogenetic regions 101E to 102F (Locke and McDermid 1993).

One aspect of chromosome IV genes that needs to be considered is that this autosome may be regulated by an expression-regulation system similar to some

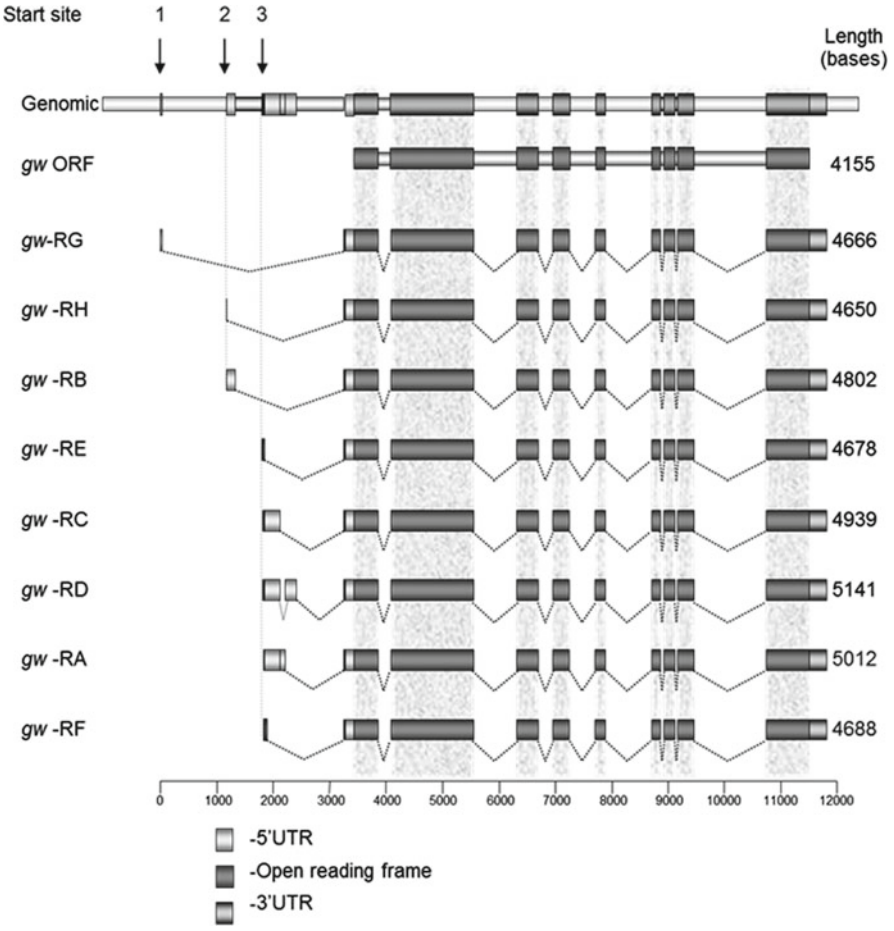

Fig. 8.2 The *gw* gene produces eight mRNA isoforms differing only at the 5′ untranslated region (UTR). The 5′ UTR (*blue*) is composed of several different exons that are selectively spliced and expressed from three different start sites. However, the coding region of the GW protein (*red*) and the 3′ UTR (*green*) are the same in each *gw* mRNA isoform. The *numbered boxes* indicate the positions of RT-PCR primers that can be used to identify mRNAs transcribed from each of the three alternative start-sites and, based upon the length of the resulting product, each of the mRNAs

dosage-compensation systems that regulate sex chromosomes. The Painting-of-Fourth (POF) protein seems to act in concert with Heterochromatic protein 1 (HP1) in a feedback mediated regulatory system to "fine-tune" the expression of genes on chromosome IV (Stenberg et al. 2009; Riddle et al. 2009; Johansson et al. 2007a, b; Tzeng et al. 2007; Larsson et al. 2001, 2004). The POF protein is encoded by a gene that it is itself on chromosome IV. Notably, flies hemizygous for chromosome IV can survive with few ill effects. However, if the *pof* gene is mutated, loss of one copy of chromosome IV is lethal (Stenberg et al. 2009). The DNA encompassing

the *gw* gene locus was found to be bound and potentially regulated by POF (Johansson et al. 2007b). Notably, in *pof* mutant larvae, *gw* mRNA expression is reduced by one half. Such a fine-tuning mechanism which can compensate for the loss of a whole copy of chromosome IV might explain the variability of defects in within homozygous *gw* mutant larvae (Schneider et al. 2006).

The *Drosophila* chromosome IV mapping project has made a concerted effort to expand the relatively small group of mapped and characterized mutations within genes along the fourth chromosome (Sousa-Neves et al. 2005). The *gw* mutation was identified via screening for mutations in the region predicted to encode a potential GW182 homologue by the *Drosophila* genome (Schneider et al. 2006). One particular mutation exhibited a striking phenotype, which caused early embryo lethality due to progressive loss of intact embryonic nuclei due to what appeared to be lack of coordination of the early nuclear divisions. This mutation was termed "*gawky* (*gw*)" based on the uncoordinated nuclear division phenotype and in anticipation that it was a mutation in the *Drosophila* GW182 homologue (Schneider et al. 2006).

Using a novel approach exploiting site-directed terminal deficiencies (Sousa-Neves et al. 2005) the *gawky* recessive zygotic lethal mutation was mapped to a single previously uncharacterized gene, the same locus predicted by the *Drosophila* genome project to be the single *Drosophila GW182* homologue (Adams et al. 2000). Subsequently, this *gw* mutation was confirmed to be the *gw* gene, via PCR-sequencing and western blot analysis (Schneider et al. 2006). A particular quirk regarding *Drosophila* genetic nomenclature, dating back to the original isolation of the *white* mutation by Morgan (Morgan 1910) is that gene names are traditionally derived from the mutant phenotype (Wilkins 2001). Therefore, anticipation that the uncoordinated *gw* mutation identified in the mutation screen would be the GW182 homologue was fortunate as it preserved the nomenclature pattern of "GW" while avoiding the inherent logical lapse of referring to the 143 kDa *Drosophila* GW protein with the name GW182. While some groups still refer to "*Drosophila* GW182," this name is confusing and is not supported by the Flybase consortium which represents the official register of *Drosophila* nomenclature (Ashburner and Drysdale 1994; Gelbart et al. 1997; Misra et al. 2002). The name Gawky (GW) also avoids the situation present in other model organisms where GW182 homologues have been given unrelated names (e.g., *C. elegans* Ain1), while at the same time respecting the long standing tradition of *Drosophila* gene nomenclature.

8.5 The Phenotype of Drosophila *gw*^{1} Mutation

Drosophila embryos (and many other insect eggs) are syncytial during the earliest stages of development. Notably, cellularization of the rapidly dividing cortical nuclei is not complete until after the 14th nuclear division. After fertilization, the zygotic nucleus undergoes several rounds of mitosis within the center of the egg. In *Drosophila*, this continues seven more times until 256 nuclei are present within a

single syncytial embryo. Most of these nuclei then migrate to the periphery of the embryo. During nuclear division cycle nine several nuclei at the posterior pole become surrounded by invaginating apical membrane to generate the pole cells. These pole cells ultimately give rise to the adult gametes. Notably, this process requires extensive post-transcriptional gene regulatory events (Jin and Xie 2006; Mahowald 2001). The majority of the remaining nuclei arrive at the embryo cortex following nuclear cycle 10. These cortical nuclei then undergo four more mitotic division cycles. Also of note is that the earliest nuclear cycles (1–8) are relatively less sensitive to regulation by cyclins (Edgar and Lehner 1996) and seem to move rapidly from S to M phase, with a complete mitotic cycle occurring approximately every 10 min. Later nuclear cycles occur more slowly, and seem to have greater requirements for the cyclin-based mitotic regulatory machinery. During these later nuclear divisions of the cortical nuclei within the syncytial embryo (syncytial blastoderm), mitosis occur less rapidly.

Beginning at nuclear cycle 13, the apical cell membrane surrounding the embryo begins to invaginate between the nuclei, a process that eventually partitions each somatic nucleus into a single cell—commonly referred to as cellular blastoderm (Foe and Alberts 1983; Turner and Mahowald 1977). Thus, after the first 4 h of development, *Drosophila* embryos are composed of a cellular blastoderm of approximately 6,000 cells surrounding a central yolk which then undergoes gastrulation to form the cellular layers of the embryo. Notably, in the developing *Drosophila* embryo, the first 14 nuclear division cycles are precisely synchronized (Edgar and O'Farrell 1989).

The gw^1 mutant lacking the RNA recognition motif (RRM) is the result of a nonsense mutation of the tryptophan codon at position 967 to stop (Schneider et al. 2006). The gw^1 mutant embryos die soon after the nuclear cycle 10 around 2 h after egg deposition (AED) (Schneider et al. 2006). The homozygous mutant shows a disorganized internal structure accompanying abnormal nuclei and cytoskeleton network, consequently failing complete cellularization (Fig. 8.3). High-resolution confocal images of homozygous mutant embryos showed enlarged nuclei accompanied by disposition of centrosomes and severely disorganized microtubule network. These disruptions were confirmed by transmission electron micrography (Schneider et al. 2006).

A difficulty of working with *Drosophila* genes on chromosome IV is the paucity of visible genetic markers that allow unambiguous sorting of wild-type vs. homozygous mutant animals. Genotyping of homozygous gw^1 mutant embryos required a tedious restriction fragment length polymorphism analysis (Schneider et al. 2006). Similarly, the location of the *gw* gene made it impossible to use many of the *Drosophila* methodologies to create embryos that do not have a significant maternal protein contribution (Perrimon 1998). To circumvent these difficulties, a complete loss of GW phenotype was induced by injection of affinity-purified polyclonal anti-GW-antibody into the wildtype developing embryos. Blocking GW function by antibody injection had a rapid effect on embryo nuclear division with the primary phenotypes being mitotic arrest with sister chromatids unable to separate (Schneider et al. 2006). In anti-GW injected embryos the cytoskeleton network was

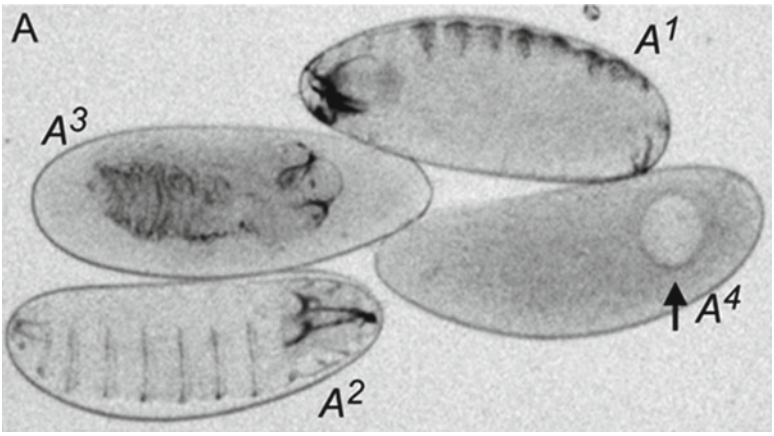

Fig. 8.3 Initial identification of a *gw* mutation causing embryonic lethality. A sample of 22 h old embryos produced by ci^D/gw parents (ci^D is a dominant mutation used to mark the gw^+ chromosome). Embryos A1-A2 have a wild-type cuticle pattern, A3 is characteristic of a homozygous *ci* mutation while A4 is characteristic of a homozygous *gw* mutant. This vacuole was seen consistently in approximately one quarter of the embryos

no longer anchored at the embryo cortex This phenotype shares a great similarity with the gw^1 mutant and the phenotype caused by injection of Ago2 antibody (Schneider et al. 2006).

8.6 Several *Drosophila* Screens Have Implicated *gw* in Multiple Processes

Drosophila, as a genetic model system, is used extensively for unbiased screening to discover genes involved in particular processes (St Johnston 2002). Its development, although analogous to mammals, is less complex, requiring only one or two members of the known gene families with defined roles in embryonic differentiation. Interestingly, there seems to be functional conservation between members of the mammalian *GW182* gene and *Drosophila gw*. This would indicate that the relative simplicity of *Drosophila* compared to mammalian genomes largely represents a lack of redundancy, rather than functional differences in the requirement for a particular gene (Ball and Cherry 2001; Venter et al. 2001). Those working with the *Drosophila* model system have devised multiple methods to screen the *Drosophila* genome for genes involved in specific processes (St Johnston 2002; Mathey-Prevot and Perrimon 2006; Reiter and Bier 2002). Accordingly, several screens for a wide variety of biological processes have identified GW. These include a whole-genome microarray assay of genes involved in the response of females to mating. GW was one of 23 genes that was reduced at least 1.5-fold in virgin females after they were exposed to courtship by males (Lawniczak and Begun 2004).

Drosophila S2 cells are particularly amenable to large-scale dsRNA knockdown screens. Boutros et al. (2004) showed that knocking down *gw* (then named CG9905) caused a significant reduction in S2 growth and viability (Boutros et al. 2004). The *gw* gene was identified as one of 488 genes in a dsRNA based knockout screen for genes involved in cell-cycle progression (Bjorklund et al. 2006). This screen was unique in that it employed flow cytometry to identify specific changes in DNA replication associated with the knockdown phenotypes. Consequently, it identified a large number of loci not found in other screens for cell size and cell cycle progression. One of the most interesting conclusions of this screen was that functional clustering of identified genes tentatively placed *gw* into a category of p38β/MAPK associated regulators of G2 phase. It is particularly interesting that these recent screens have identified a potential role for *gw* in widely divergent functional processes suggesting either that mRNA regulation is also important or that *Drosophila* GW has roles in addition to mRNA regulation.

8.7 The Organization of the *Drosophila* GW Protein is Similar to Mammalian GW182

The *Drosophila* genome project predicted that all splice isoforms of the *gw* gene encoded a 143 kDa protein with a high ratio of glycine and tryptophan as GW/WG repeats throughout its sequences (Adams et al. 2000; Stapleton et al. 2002; Drysdale 2003). This gene encoded a protein with a predicted sequence that is 17.8–20% identical and 24–28.3% similar to the human GW182 protein family (Eystathioy et al. 2002; Schneider et al. 2006). The percentage of glycine (G) and tryptophan (W) in *Drosophila gw* is 12.43% and 2.53 %, respectively with 15 pairs of GW/WG repeats, 12 of which are located within the N-terminal of the protein broadly defined as the GW-rich region (Schneider et al. 2006). This region is followed by a ubiquitin-associated-like domain (UBA) (539–604) and a Q-rich/QN-rich domain (635–861) rich in glutamine (Q) and asparagines (N), whose percentages are 16.81% and 14.61% in this region, respectively. Three additional pairs of GW/WG are interspersed within the following sequences (861–1116) before the RRM (domain—1116–1198). Within the C-terminal region, there is a domain that is rich in serine (S) accounting for 27.62% of the total amino acids (Schneider et al. 2006). Similarly, multiple alignment of *Drosophila* GW with other GW182 family proteins identified an additional three highly conservative regions termed: Motif I (1–35), II (312–355), III/Domain of unknown function (DUF 937–1003) (Fig. 8.1) (Behm-Ansmant et al. 2006; Zekri et al. 2009). Finally, there is functional evidence for the region of Gw encompassing amino acids 205–490 exerts the minimal repressive function in its N-terminal in miRNA pathway. Thus, this region has been termed the N-terminal effector domain (NED) (Chekulaeva et al. 2010).

Many of the notable amino acid motifs found within *Drosophila* GW, including the GW-rich region, Q-rich domain and RRM are also found within all three human GW182 family members (Eystathioy et al. 2002; Schneider et al. 2006). A homologous

region to the UBA domain can only be found between GW and TNRC6C, but iterative PSI-BLAST sequence comparison suggests that all mammalian GW182 family proteins may have this UBA domain (Behm-Ansmant et al. 2006). Therefore, strict comparison to mammalian GW182 would suggest that, TNRC6C is most homologous to GW. However, the basic domain structure of GW is conserved with the entire mammalian GW182 protein family. This is particularly notable as reported GW182 orthologues in another widely used model system *Caenorhabditis elegans,* are considerably more highly divergent in their overall protein organization. For example, neither *C. elegans* AIN-1 nor AIN-2 has a well conserved RRM binding domain (Ding et al. 2005). There is some divergence between the human GW182 family and *Drosophila* GW. A conserved region for binding Ago1 termed as the Ago-hook (Till et al. 2007) reported in human TNRC6B is not present within *Drosophila* GW. Also, human GW182 family members do not have the concentrated Ser-rich domain within the C-terminal domain. Despite these differences, there is evidence for functional conservation. Notably, when human GW182, TNRC6B and TNRC6C are expressed in *Drosophila* Schneider2 cells, they form cytoplasmic foci that also recruit *Drosophila* GW (Schneider et al. 2006). However, a functional conservation for the activities of human GW182 family proteins in S2 cells has not been shown directly.

8.8 Drosophila GW Bodies

Some of the initial biochemical characterizations of the role of GW in the miRNA silencing pathway were reported as early as in 2005 using S2 cells (Rehwinkel et al. 2005). Note that this study referred to GW as *Drosophila* GW182 (dGW182) as the characterization of the mutant phenotype had yet to be published. In cells of most organisms, GW182 family proteins form cytoplasmic foci (Ding et al. 2005; Eystathioy et al. 2002). Fluorescent-tagged GW was seen co localizing with cytoplasmic bodies, Ago2 (Behm-Ansmant et al. 2006), ME31B (Behm-Ansmant et al. 2006). These were subsequently supported by observations showing that *Drosophila* Pacman (PCM), the othologue of human being 5′-3′ exonuclease XRN1 also co-localizes with GW in S2 cells. The best proof that *Drosophila* GW localizes to nonmembrane-bound punctate cytoplasmic bodies shown by transmission electron microscopy and confocal microscopy (Fig. 8.4) (Schneider et al. 2006).

The functional localization of GW182 families appears to be a highly conserved process as all 3 human GW182 family proteins also were targeted to GW containing bodies when these human proteins are expressed in *Drosophila* S2 cells (Schneider et al. 2006). This implies that GW is part of *Drosophila* mRNA processing bodies as it is consistent with the result of others showing that that GW182 co-localizes with XRN1 in human HEp-2 cells (Eystathioy et al. 2003). Similarly, both mammalian and *Drosophila* GW bodies dissociate after RNase A treatment indicating that RNA is a significant component of structures in both cell types (Schneider et al. 2006). Many groups are still expanding the list of known GW-body components using *Drosophila*

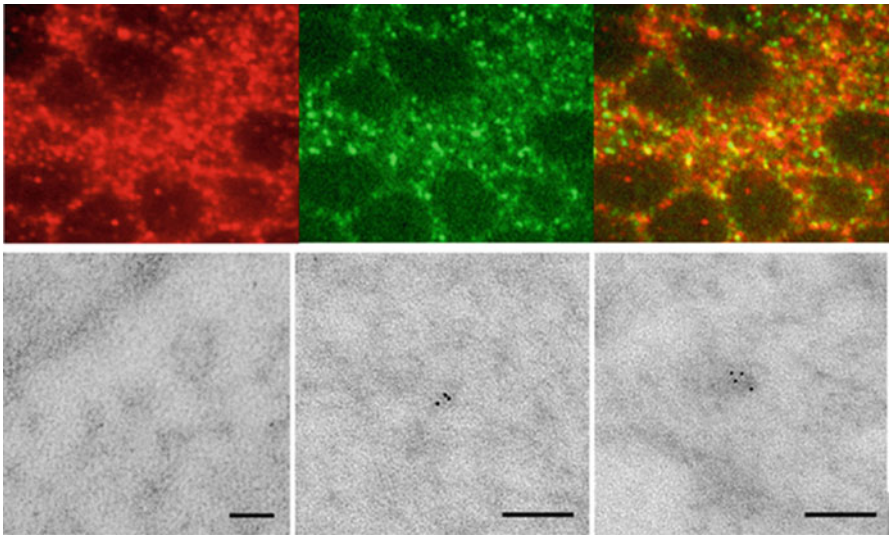

Fig. 8.4 Drosophila GW bodies. (top) Antibody staining against Drosophila GW (*red*) and Ago1 (*green*) in developing embryos. Significant, but not complete, co-localization is seen between thse two proteins. (bottom) Transmission electron microscopy of a section of the cytoplasm of a Drosophila embryo. Immunogold staining using an anti-GW antibody GW bodies detects GW-bodies of various sizes

S2 cells. Recently, an immunoprecipitation assay showed that GW interacts with the decapping activator HPat (Jager and Dorner 2010). Accumulated evidence confirmed that the yeast HPat homologue, Pat1p, is an essential component of P-bodies and required for translational repression and decapping (Eulalio et al. 2007b). Knocking-down HPat in *Drosophila* cells caused the levels of miRNA-targeted mRNAs level to be slightly elevated (Eulalio et al. 2007a).

8.9 The Role of Drosophila GW in Cytoplasmic mRNA Regulation

Much of the recent research on *Drosophila* GW has concentrated on elucidating the specifics of its role in miRNA repression and decay. Depleting Ago1, GW and DCP1:DCP2 does not affect NMD and this observation differentiates Ago1 and GW from NMD pathway components UPF1 and SMG7 (Rehwinkel et al. 2005). Using a specific luciferase reporter that measures activity of specific miRNA silencing, Ago1 and GW were confirmed to be primary effectors of the *Drosophila* miRNA pathway, while Ago2 was revealed to have relatively poor miRNA repression ability (Rehwinkel et al. 2005). This is particularly interesting in light of the fact that some punctate GW co-localized with Ago2 in S2 cells in several studies (Rehwinkel et al. 2005; Schneider et al. 2006). This implies that in *Drosophila,* the

miRNA pathway can function independently of siRNA pathway. Both DCP1 and DCP2assist Ago1-GW miRNA repression activities, as the depletion of these decapping factors increased the release of repression by another twofold (Rehwinkel et al. 2005).

The GW protein itself appears to have silencing function independent of some or all of the other members of the canonical miRNA silencing pathway. This was shown by fusing GW to a phage λN-peptide which binds with high affinity to a phage λ BoxB RNA hairpin. By incorporating repeats of these hairpins into a 3′ UTR (F-Luc-5BoxB) downstream of a luciferase reporter, it was shown that GW could independently promote the target degradation without the presence of either Ago1 or miRNA (Behm-Ansmant et al. 2006). Moreover, artificial targeting of GW to mRNAs increases their degradation rate. However, in these same experiments, co-deletion of deadenylation complex components CAF1, NOT1, or the decapping complex component DCP1:DCP2restored the cellular levels of the reporter mRNA (Behm-Ansmant et al. 2006). This would suggest that GW is able to trigger mRNA degradation by recruiting deadenylation and decapping complexes from the cytoplasmic pool independently of Ago1 (Iwasaki et al. 2009; Eulalio et al. 2007b). This suggests that GW would functions downstream of Ago1 during miRNA repression in *Drosophila* cells. This would agree with studies in human cells where GW182 is co-localized with proteins of the 5′ mRNA decapping and deadenylase complex usually associated with P-bodies (Eystathioy et al. 2002, 2003). However, other studies using different reporters that would interact with a 3′ histone H4 stem-loop structure instead of linked to poly-A tail show that GW also represses mRNA independently of adenylation. Therefore, recruitment of the adenylation complex may be a necessary step ONLY for the degradation of the intact RNAs with poly-A tails. Notably this poly-A tail independent RNA degradation seems to require both GW and Ago1 (Eulalio et al. 2009b).

The mechanism by which GW participates in miRNA-mediated degradation remains unclear. GW is released from the target mRNP only when the deadenylase complex is absent, suggesting GW dissociates from the mRNA target after it is deadenylated (Zekri et al. 2009). The C-terminal region is necessary for the release of GW from the target mRNP. GW without C-terminal is not released from a complex with the Ago1 and miRNA targets. Other functional studies have shown that the middle region conserved sequences MII, together with Motif III and C-terminal region of GW bind to PABP1 (Fig. 8.1). This binding is required for the degradation of and interfere with miRNA target interacting with eIF4G (Zekri et al. 2009). The binding is required for the degradation of target RNA possibly through promoting recruitment of the deadenylase complex. However, what remains to be determined is which subset of the total cellular pool of PABP1 binds to GW. It could be the free PABP1from the cytoplasm pool or as part of a complex that circularizes miRNA-targeted mRNAs.

The biochemical interaction between GW and Ago1 has been probed extensively in *Drosophila*. The Phe594 (F594V) and Phe629 (F629V) amino acids of Ago1 are crucial in miRNA silencing but not important for cap binding (Eulalio et al. 2008). However, mutating both sites may cause a conformational change and

lose the ability to bind either miRNA or GW directly or indirectly. This study also showed that binding of GW to endogenous miRNAs was not impaired after reducing Ago1 function, indicating that GW is not involved in miRNA being loaded onto RNA induced silencing complex (RISC) and acts downstream of the assembly (Eulalio et al. 2008). Notably, overexpression of Ago1 seems to alter the GW-Ago1 complex into an inactive state independently of miRNA binding, resulting in a release of the miRNA repression in S2 cells. Therefore, interaction between Ago1 and GW is necessary for Ago1/miRNA-mediated repression (Eulalio et al. 2008). The GW/Ago1 interaction seems to be a regulated process as Ago1 cannot dissociate from GW as well as the decapping and deadenylase complex when ATP is depleted (Iwasaki et al. 2009). This is particularly notable as Ago1-RISC binding to RNA target requires ATP. Finally, Ago1 seems to require the presence of GW for targeting to cytoplasmic P-bodies (Eulalio et al. 2009a). This would suggest that GW has at least two roles in mRNA repression, one independent of the Ago1/miRNA pathway and the other assisting Ago1 to assist in the miRNA repression function possibly through targeting the GW/Ago2 RNP complex to processing bodies where some or all of the associated mRNAs are degraded. Moreover, GW was also reported not to be related to miRNA repression mediated by Ago2 blocking mRNA's cap structure (Iwasaki et al. 2009). Thus, an unambiguous role for GW in this process is still to be determined.

8.10 Structure/Function Studies of the Role of GW in the *Drosophila* miRNA Pathway

It has been reported that at least three independent domains within GW protein have potential roles during miRNA repression. Fragments of GW containing amino acids 1–605, 605–830 and 940–1215 decrease the rate of mRNA translation similar to full length GW (Chekulaeva et al. 2009). Later studies mapped a minimal region of GW required for miRNA repression more specifically to amino acids 205–490, and the Ago1 binding domain resides within amino acids 1–204. This domain has been proven to be required for miRNA-mediated repression and degradation and this process is independent of poly-A tail (Chekulaeva et al. 2010).

The role of the N-terminal GW-repeat rich region of GW is still not entirely clear. It has been reported that when 12 GW/WG repeats within GW were mutated to AA pairs, the interaction between GW and Ago1 were severely disrupted (Chekulaeva et al. 2010). The GW 1–539 fragment is sufficient to coimmunoprecipitate Ago1 (Behm-Ansmant et al. 2006). However, only GW/WG repeats in Motif I (M I—Fig. 8.1) are required for GW interaction with Ago1. The GW/WG repeats in the middle the GW protein appear not be essential for binding Ago1 and/or miRNA (Eulalio et al. 2009a). This middle region comprising 3 GW/WG repeats as well as the C-terminal regions of GW are thought to be more important for miRNA based gene silencing (Eulalio et al. 2009a). Finally, it has been reported that the Ago1 binding domain and Q-rich domains, but not UBA-like region, are required for the localization of GW in cytoplasmic foci P-bodies (Eulalio et al. 2009a).

Structural studies of the *Drosophila* GW RRM domain indicates that it is an RRM fold, with an additional C-terminal α-helix lying on the β-sheet surface shielding the spot used to bind RNA in canonical RRM domain (Eulalio et al. 2009c). The absence of two aromatic amino acids in RNP1 and RNP2 domains would seem to indicate a low affinity for binding RNA. Rather, this domain was suggested to bind other proteins via its hydrophobic cleft (Eulalio et al. 2009c). This region is not essential for the interaction of GW and Ago1, or for P-body localization and is not required for repression function or poly-A independent deadenylation, but assists in a target-specific manner (Eulalio et al. 2009c; Iwasaki et al. 2009).

8.11 A Link Between *Drosophila* GW-Bodies and Multivesicular Bodies

Many in the field of mRNA regulation have considered GW-bodies and P-bodies as identical structures because GW-containing punctate structures often share many of the same proteins components with P-bodies. This confusion was enhanced by the lack of clear evidence differentiating the biological roles of P-bodies from other GW-containing bodies. However, studies of exosomes (small microvesicles that are released from late endosomal compartments of cells but unrelated to the RNA degradation machinery) in human monocytes first suggested that our concept of a cellular GW-body may need to be considered independently from P-bodies (Gibbings et al. 2009). In these mammalian cells, GW182, Ago2, miRNA and miRNA-repressible mRNA are concentrated with multivesicular bodies(MVB) and endosomes, suggesting that they are the accumulation sites of miRNA-loaded RISC. However while Ago2, which is the core protein in human miRNA-RISC, may be recruited into this subset of GW182 exosome associated structures, these same structures appear to be devoid of the functional P-body marker DCP1. This would suggest that the Endosomal Sorting Complex Required for Transport (ESCRT) may partition GW182 into the exosomes-lysosomes degradation pathway (Gibbings et al. 2009).

Notably, a role in RNAi for ESCRT sorting of GW has also been confirmed in *Drosophila*. In a mutagenesis screen devised to identify genes that increase siRNA-mediated RNA silencing discovered that mutation of the locus *CG4966* can cause stronger RNAi effect. *CG4966* encodes a human Hermansky-Pudlak Syndrome 4 (HPS4) orthologue controlling the turnover of MVBs. Interestingly, RNAi based mRNA silencing is severely impaired when the MVB formation is blocked by mutating *Drosophila* ESCRT genes *hrs* and *vps25* (Lee et al. 2009). Gibbings et al. found that knocking-down the ESCRT genes *vps36*, *hrs* and *alix* in human monocytes mildly compromised miRNA repression but did not change the miRNA accumulation (Gibbings et al. 2009). In human cells, mutations in *HPS4* significantly increase the number of GW-bodies and the quantity of miRNAs being loaded onto the Ago1-RISC, whereas the mutations in MVB formation proteins HRS and TSG101 result in fewer GW-bodies (Gibbings et al. 2009). In *Drosophila* S2 cells, GW and ME31B are found juxtaposed to the cytosolic phase of MVBs and/or

lysosomes. It is also found that mutation in *Drosophila* homologue HPS4 enhances both siRNA and miRNA-mediated silencing, which would seem to support the findings of Gibbings et al. in mammalian cells. Both groups do agree on a hypothesized mechanistic model that recruiting GW into MVBs is a necessary step for miRNA being loaded on to RISC so as to be a rate-limiting step for miRNA silencing. However, the critical details of this process still need to be addressed.

8.12 Summary and Future Directions

The clear conservation of *Drosophila* GW to the mammalian GW182 protein family, in terms of both sequence and function has made it a valuable system to model the requirements for these proteins in both cellular functions like miRNA based repression. However, *Drosophila* studies have also been key to advancing knowledge regarding the function of GW in cellular and developmental processes. An advantage to *Drosophila* studies is that our findings regarding GW can be fit into an extensive knowledge of the role of mRNA regulation in the cell. Further modeling of the developmental role of GW in early embryonic development as well as later tissue formation and cellular homeostasis should be particularly interesting.

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Chapter 9
The Role of GW182 Proteins in miRNA-Mediated Gene Silencing

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Abstract GW182 family proteins are essential for microRNA-mediated gene silencing in animal cells. They are recruited to miRNA targets through direct interactions with Argonaute proteins and promote target silencing. They do so by repressing translation and enhancing mRNA turnover. Although the precise mechanism of action of GW182 proteins is not fully understood, these proteins have been shown to interact with the cytoplasmic poly(A)-binding protein (PABP) and with the PAN2–PAN3 and CCR4–NOT deadenylase complexes. These findings suggest that GW182 proteins function as scaffold proteins for the assembly of the multiprotein complex that silences miRNA targets.

9.1 Introduction

MicroRNAs are key post-transcriptional regulators of gene expression that play critical roles in a wide range of biological processes including cell growth, division and differentiation as well as organism metabolism and development. The number of miRNAs encoded by the genomes of various organisms varies considerably, from a handful of miRNAs up to 500–1,000 of miRNAs in mammals (Bartel 2009). Computational predictions and genome-wide identification of miRNA targets estimate that each animal miRNA can bind hundreds of different mRNAs, suggesting that a remarkably large proportion of the mammalian transcriptome is subject to miRNA regulation (Bartel 2009).

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To exert their regulatory function, miRNAs are loaded into Argonaute family proteins (AGOs) to form the core of miRNA-induced silencing complexes (miRISCs; (Jinek and Doudna 2009)). miRISCs silence the expression of target genes predominantly at the post-transcriptional level. The targets to be silenced are selected through base-pairing interactions between the loaded miRNA and an mRNA containing partially or fully complementary sequences (Bartel 2009; Jinek and Doudna 2009).

In animals, most miRNAs are only partially complementary to their targets (Bartel 2009). In this case, AGO proteins are not sufficient to mediate silencing and must interact with GW182 family proteins (Eulalio et al. 2009a; Huntzinger and Izaurralde 2011). AGO-GW182 complexes silence mRNA targets by repressing translation and expediting mRNA degradation. Target degradation is catalyzed by the enzymes involved in the cellular 5′-to-3′ mRNA decay pathway (Rehwinkel et al. 2005; Behm-Ansmant et al. 2006a, b; Giraldez et al. 2006; Wu et al. 2006; Eulalio et al. 2007a, 2009b; Chen et al. 2009; Piao et al. 2010). In this pathway, mRNAs are first deadenylated by mRNA deadenylases and then decapped by the decapping enzyme DCP2. Decapped mRNAs are ultimately degraded by the major cytoplasmic 5′-to-3′ exonuclease XRN1.

The mechanism by which GW182 proteins contribute to translational repression and enhance miRNA target degradation is not fully understood, although recent studies have provided new insights into their role in silencing by showing that GW182 proteins interact with the cytoplasmic poly(A)-binding protein (PABP) and with the PAN2–PAN3 and CCR4–NOT deadenylase complexes (Fabian et al. 2009, 2011; Zekri et al. 2009; Huntzinger et al. 2010; Jinek et al. 2010; Kozlov et al. 2010; Braun et al. 2011; Chekulaeva et al. 2011). In this chapter, we discuss these recent findings and the emerging picture of the molecular mechanisms underlying miRNA-mediated gene silencing in animals. First, we briefly summarize the evidence for the essential role of GW182 proteins in the effector step of silencing. Next, we describe how the different protein domains contribute to silencing. Finally, we discuss how studies on GW182 proteins are providing new insights into the mechanisms of silencing in animals.

9.2 GW182 Proteins Are Integral miRISC Components in Animal Cells

9.2.1 Overview of the GW182 Protein Family

Although GW182 proteins were originally identified in human cells as an antigen recognized by serum from a patient who had motor and sensory neuropathy (Eystathioy et al. 2002), their link to the miRNA pathway was revealed in subsequent studies in which these proteins were isolated either as interaction partners of AGOs or as proteins required for miRNA-mediated gene silencing in animal cells. These studies included genetic screens in *C. elegans*, RNAi screens in *D. melanogaster* and biochemical purifications of Argonaute-containing complexes in human

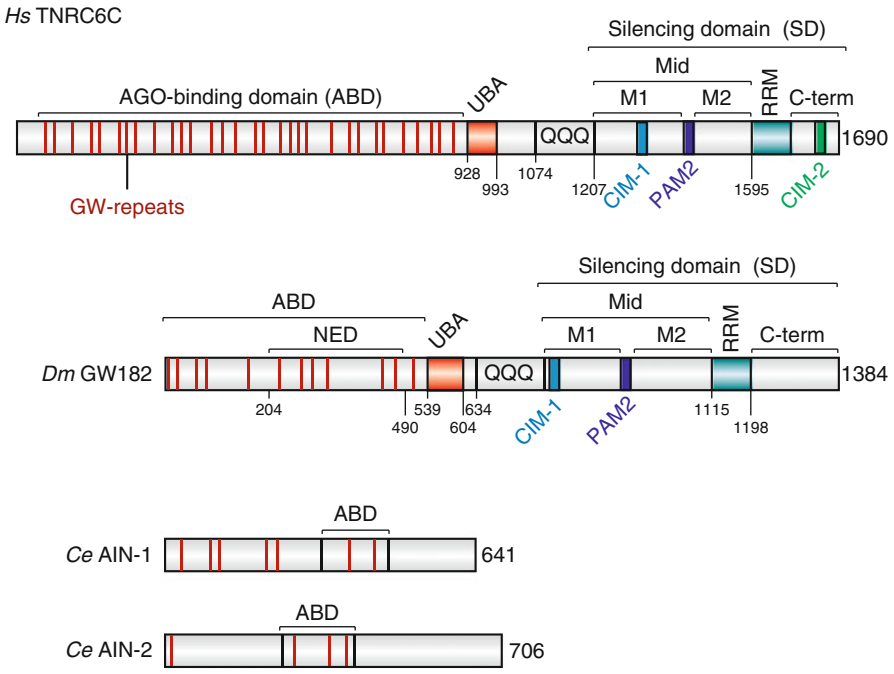

Fig. 9.1 Domain organization of GW182 proteins. *Hs*, *Homo sapiens*; *Dm*, *Drosophila melanogaster*. The AGO-binding (ABD) and silencing domains (SD) are indicated. *C. elegans* (Ce) AIN-1 and AIN-2 proteins are included for comparison. *NED* N-terminal effector domain; *UBA* ubiquitin associated-like domain; *QQQ* region rich in glutamine; *Mid* middle region containing the PAM2 motif, which divides the Mid region into the M1 and M2 regions; *RRM* RNA recognition motif; *C-term* C-terminal region and CIM-1, CIM-2: CCR4–NOT interacting motifs 1 and 2. Amino acid positions at domain boundaries are indicated below the protein outlines

cells (Ding et al. 2005; Jakymiw et al. 2005; Liu et al. 2005; Meister et al. 2005; Rehwinkel et al. 2005; Behm-Ansmant et al. 2006a).

GW182 proteins are found in metazoan organisms. Vertebrates and some insect species possess three GW182 paralogs (TNRC6A/GW182, TNRC6B and TNRC6C), whereas *D. melanogaster* possesses only one (*Dm* GW182). To date, no orthologs have been identified in fungi and plants (Behm-Ansmant et al. 2006a; Eulalio et al. 2009a). The vertebrate and insect proteins are typically characterized by the presence of two annotated structural domains: a central ubiquitin-associated (UBA) domain and a C-terminal RNA recognition motif (RRM). These domains are flanked by regions predicted to be unstructured (Fig. 9.1; Behm-Ansmant et al. 2006a; Eulalio et al. 2009a). The unstructured regions include N-terminal (N-term), middle (Mid) and C-terminal (C-term) sequences containing glycine-tryptophan repeats as well as a glutamine-rich (Q-rich) region located between the UBA-like domain and the RRM (Fig. 9.1; Eystathioy et al. 2002; Behm-Ansmant et al. 2006a; reviewed by Ding and Han 2007; Eulalio et al. 2009a). The number of GW-repeats in the N-, Mid- and C-term regions varies in different GW182 family members; nevertheless,

for all proteins, most GW-repeats lie within the N-term region, whereas the Mid and C-term regions have fewer or no repeats. Remarkably, the unstructured regions are required for GW182 proteins to interact with protein partners and to mediate silencing (reviewed by Eulalio et al. 2009a; Fabian and Sonenberg 2012).

The *C. elegans* genome encodes two proteins, AIN-1 and AIN-2, that interact with Argonaute proteins and are required for miRNA function (Ding et al. 2005; Zhang et al. 2007; Ding and Großhans 2009). AIN-1 and AIN-2 contain a small number of GW-repeats (7 and 4, respectively), and although they are relatively rich in glutamine, these proteins lack a defined Q-rich region (observed in the insect and vertebrate proteins; Fig. 9.1). Furthermore, AIN-1 and AIN-2 lack the UBA-like and RRM and SD domains (Fig. 9.1; Ding et al. 2005; Behm-Ansmant et al. 2006a; Eulalio et al. 2009a). Nevertheless, despite very low sequence homology with vertebrate and insect GW182 proteins, AIN-1 interacts with PABP and deadenylase complexes (Kuzuoglu-Öztürk et al. 2012) and thus carries out a function similar to other GW182 proteins in the miRNA pathway (Ding et al. 2005; Zhang et al. 2007; Ding and Großhans 2009).

9.2.2 GW182 Proteins Are Essential for miRNA-Mediated Gene Silencing

The essential role of GW182 proteins in silencing was first demonstrated in *D. melanogaster* cells, where the depletion of the single GW182 family member (*Dm* GW182) suppresses the silencing of miRNA targets (Rehwinkel et al. 2005; Behm-Ansmant et al. 2006a, b; Eulalio et al. 2008). In other organisms, demonstrating an essential role for GW182 proteins in gene silencing has been complicated by the existence of multiple paralogs with partially redundant functions.

Human TNRC6A/GW182, TNRC6B and TNRC6C are known to be redundant on the basis of the observation that these proteins associate with all four human Argonaute paralogs (AGO1–4) and with a common set of miRNA targets (Jakymiw et al. 2005; Liu et al. 2005; Meister et al. 2005; Landthaler et al. 2008; Lazzaretti et al. 2009; Lian et al. 2009; Takimoto et al. 2009; Zipprich et al. 2009). Despite the redundancy, depleting TNRC6A, TNRC6B or TNRC6C partially relieves the silencing of miRNA targets in human cells (Jakymiw et al. 2005; Liu et al. 2005; Meister et al. 2005; Chu and Rana 2006; Landthaler et al. 2008; Zipprich et al. 2009). Silencing is more efficiently inhibited when at least two of these proteins are co-depleted (Huntzinger et al. 2010), supporting the idea that these proteins have redundant functions. Functional redundancy was also demonstrated for the *C. elegans* proteins AIN-1 and AIN-2 because both proteins interact with *C. elegans* Argonaute proteins 1 and 2 (ALG-1, ALG-2) and their co-depletion suppresses silencing more efficiently than depleting either protein individually (Zhang et al. 2007; Ding and Großhans 2009).

GW182 proteins act during the effector step of silencing, which is downstream of miRNA processing and loading. This conclusion is supported by the following

evidence. First, GW182 proteins are dispensable for miRNA biogenesis (Eulalio et al. 2009c; Miyoshi et al. 2009). Second, GW182 proteins are not necessary to maintain Argonaute protein expression levels (Eulalio et al. 2008, 2009c). Third, GW182 proteins are not required for miRNAs to be loaded onto Argonaute proteins (Eulalio et al. 2009c; Miyoshi et al. 2009). Fourth, GW182 proteins copurify with AGOs in complexes containing miRNAs and mRNA targets, indicating that they are integral miRISC components (Meister et al. 2005; Landthaler et al. 2008). Finally and more importantly, *Dm* GW182 and human TNRC6s induce the translational repression and degradation of the mRNA targets to which they are artificially tethered (Behm-Ansmant et al. 2006a, b; Eulalio et al. 2008; Li et al. 2008; Chekulaeva et al. 2009; Lazzaretti et al. 2009; Lian et al. 2009; Zipprich et al. 2009). This activity is independent of their interaction with AGOs (Behm-Ansmant et al. 2006a; Eulalio et al. 2008; Li et al. 2008; Chekulaeva et al. 2009; Lazzaretti et al. 2009; Zipprich et al. 2009), indicating that GW182 proteins possess silencing activity and are thus involved in the effector step of silencing. Collectively, these and additional observations indicate that target silencing by animal miRNAs is effected by a protein complex consisting of at least one Argonaute and one GW182 protein (Eulalio et al. 2008).

9.3 GW182 Protein Domains

Two domains in GW182 proteins play an essential role in silencing. One domain consists of the N-terminal GW-repeat-containing region, which confers binding to AGOs. The other domain is a bipartite silencing domain, consisting of the Mid and C-term regions, which promotes the translational repression and degradation of miRNA targets (Fig. 9.1). In contrast, and despite conservation, the UBA and RRM domains are not strictly required for GW182 silencing activity, although we cannot rule out that these domains contribute to the silencing of a subset of miRNA targets (Chekulaeva et al. 2009; Eulalio et al. 2009c, d; Lazzaretti et al. 2009; Zipprich et al. 2009).

Most of the published literature concerning the role of the UBA, Q-rich and RRM domains has been reviewed (Eulalio et al. 2009a) and will only be briefly summarized here. Instead, this chapter focuses on the role of the AGO-binding and silencing domains and on how the study of these domains has shed light on the mechanisms of silencing.

9.3.1 The AGO-Binding Domain

Co-immunoprecipitation assays in *D. melanogaster* cells demonstrated that the N-terminal region of *Dm* GW182 is necessary and sufficient for the protein to interact with AGO1 (Behm-Ansmant et al. 2006a). As mentioned above, a striking feature of this region is the presence of multiple GW repeats (GW or WG repeats). Subsequent studies showed that GW repeats mediate the interaction between

GW182 and AGO proteins, with the tryptophan residues playing a critical role (Till et al. 2007; El-Shami et al. 2007; Eulalio et al. 2009c; Lazzaretti et al. 2009; Takimoto et al. 2009). Nevertheless, the context of the repeats also contributes to the strength of the interaction. Two lines of evidence support this conclusion. First, not all GW repeats contribute equally to the interaction (Eulalio et al. 2009c; Lazzaretti et al. 2009). Second, mutational analysis showed that substituting residues adjacent to the repeats affected AGO binding (Till et al. 2007).

Remarkably, GW182 proteins only interact with a subset of Argonaute proteins. For example, *Dm* GW182 interacts with *Dm* AGO1 but not *Dm* AGO2 (Behm-Ansmant et al. 2006a; Iwasaki et al. 2009; Miyoshi et al. 2009). Moreover, members of the PIWI clade of AGOs, which are predominantly expressed in the germline, do not interact with GW182 proteins (Miyoshi et al. 2009). Thus, GW182 proteins appear to bind specifically to AGOs involved in the miRNA pathway. Future structural analysis should clarify how GW-repeats interact with AGOs and should reveal the basis for the specificity of the interaction.

Interestingly, GW-repeats are found in divergent proteins from diverse organisms. In *A. thaliana*, these proteins include NRPE1, a subunit of polymerase IV, and the SPT5-like transcription elongation factor, both proteins interact with AGO4 (El-Shami et al. 2009; Bies-Etheve et al. 2009). In *S. pombe*, the GW-repeat-containing motif of Tas3 confers binding to AGO1 (Till et al. 2007; Partridge et al. 2007). Although these proteins interact with AGOs in a manner similar to GW182 proteins, they are not related to the GW182 protein family. Indeed, in addition to the GW-repeats, these proteins have an unrelated domain organization, and through their binding to AGO proteins, they participate in silencing pathways distinct from the miRNA pathway. Overall, these studies demonstrate that for a variety of proteins, GW repeats act as AGO-binding determinants (Till et al. 2007; El-Shami et al. 2009).

9.3.1.1 Additional Roles for the AGO-Binding Domain

In addition to providing binding sites for AGOs, the AGO-binding domain (ABD) of GW182 proteins is required for their accumulation in P-bodies (Behm-Ansmant et al. 2006a; Eulalio et al. 2009c; Lazzaretti et al. 2009); however, the existence of additional silencing roles for this domain remains unclear. Studies involving human TNRC6A–C showed that the ABD has slight silencing activity in tethering assays (Lazzaretti et al. 2009; Zipprich et al. 2009). In contrast, the ABD of *Dm* GW182 is fully active when tethered to an mRNA reporter and can complement the silencing of a specific miRNA-target pair (Chekulaeva et al. 2009, 2010), suggesting that this domain can induce the formation of silencing complexes in specific 3′ UTR contexts. In agreement with these observations, the N-term region of *Dm* GW182 (termed the N-terminal effector domain (NED); Fig. 9.1) has been shown to bind NOT1 (Chekulaeva et al. 2011).

Consistent with the findings above, it is important to note that several non-overlapping *Dm* GW182 or TNRC6 protein fragments are active in tethering assays (Chekulaeva et al. 2009, 2010; Lazzaretti et al. 2009; Zipprich et al. 2009; Baillat

and Shiekhattar 2009; Yao et al. 2011). However, the contribution of most of these regions to silencing activity remains to be tested in complementation assays. For example, the GW182 N-term domain is not sufficient to silence the majority of miRNA targets tested in complementation assays (E.H. and E.I., unpublished observations), indicating that the silencing activity of *Dm* GW182 resides primarily in the silencing domain.

9.3.2 The UBA-Like Domain

A UBA-like domain was originally only predicted to be present in human TNRC6C; however, using iterative PSI-BLAST searches, we identified UBA-homology domains in all members of the GW182 protein family (Behm-Ansmant et al. 2006a), except AIN-1 and AIN-2. UBA domains are small domains of approximately 40 amino acids and were first identified in proteins involved in ubiquitination (reviewed by Buchberger 2002). These domains bind mono- or poly-ubiquitin chains on ubiquitinated partners. However, distantly related UBA domains (UBA-like domains) that may have evolved different functions have been described in proteins with no role in the ubiquitin-proteasome pathway (reviewed by Buchberger 2002). Whether UBA-like domains in GW182 proteins bind ubiquitin or have different functions remains to be determined. Furthermore, the role of the GW182 UBA domains remains poorly understood, and currently no evidence exists regarding the contribution of these domains to silencing (Chekulaeva et al. 2009; Eulalio et al. 2009c; Lazzaretti et al. 2009; Zipprich et al. 2009). However, these domains may play a role in silencing specific targets during specific cellular conditions.

9.3.3 The Q-Rich Region

The Q-rich region is a common feature of GW182 family proteins; however, aside from the glutamine residues, the composition of this region varies. In *D. melanogaster*, the Q-rich region is also enriched in asparagine residues (14.5 %), but in the human proteins this is not the case. Notably, isoform-1 of TNRC6A contains an additional Q-rich region upstream of the ABD (Li et al. 2008). As mentioned above, AIN-1 and AIN-2 do not contain a defined Q-rich region but are Q-rich overall.

The Q-rich region of *Dm* GW182 plays a role in P-body localization. Indeed, a protein lacking the Q-rich region is dispersed throughout the cytoplasm (Eulalio et al. 2009c); however, the Q-rich region alone is not sufficient for P-body localization because the ABD is also required (Behm-Ansmant et al. 2006a). In agreement with this, GW182 mutants that cannot interact with *Dm* AGO1 also do not accumulate in P-bodies, even thought these mutants have a Q-rich region (Eulalio et al. 2009c). Importantly, the GW182 UBA-like and silencing domains do not contribute to P-body localization (Eulalio et al. 2009c).

For the human proteins, the requirements for P-body localization differ slightly from *Dm* GW182. Nevertheless, in all cases, an interaction with AGOs is required for P-body localization, whereas the Q-rich regions either contribute (human TNRC6A and C) or play an essential role in localization (human TNRC6B-iso1; Lazzaretti et al. 2009).

Despite the role of the *Dm* GW182 Q-rich region in P-body localization, this region is not strictly required for silencing. Indeed, a *Dm* GW182 protein lacking the Q-rich region can complement silencing, even if it does not localize to P-bodies (Eulalio et al. 2009c). These findings are particularly relevant because they show that the silencing activity of *Dm* GW182 is not correlated with its ability to localize to P-bodies. Accordingly, the silencing domains of GW182 proteins that encompass sequences downstream of the Q-rich regions retain full silencing activity in tethering assays, although these domains do not localize to P-bodies (see below, Eulalio et al. 2009c; Lazzaretti et al. 2009; Zipprich et al. 2009). These observations, together with previous studies (Chu and Rana 2006; Eulalio et al. 2007b), indicate that P-bodies that are detectable by conventional microscopy are not required for silencing (Eulalio et al. 2007b).

9.3.4 The RRM Domain

The RRM is highly conserved among GW182 proteins, and its presence was interpreted as indicative of RNA-binding activity (Eystathioy et al. 2002). Whether GW182 proteins bind RNA directly is unknown, but if they do, it is most likely not through the RRM. Indeed, the RRM domain of *Dm* GW182 lacks RNA-binding features (Eulalio et al. 2009d). Although the domain adopts a canonical RRM fold, consisting of a four-stranded anti-parallel β-sheet packed against two α-helices (Eulalio et al. 2009d), an additional C-terminal α-helix (α3) shields the β-sheet surface, which in canonical RRMs is involved in RNA binding. Moreover, the GW182 RRM lacks the conserved aromatic residues that in canonical RRMs interact with RNA through stacking interactions, and it has no positively charged surface patch that is capable of mediating RNA binding. Consistently, *Dm* GW182 RRM exhibits no detectable general RNA-binding affinity in vitro, suggesting that this domain does not bind RNA. Thus, the RRM may instead engage in protein-protein interactions that may occur through an unusual hydrophobic cleft exposed on the opposite face of the β-sheet (Eulalio et al. 2009d). The structural features of the *Dm* GW182 RRM are conserved in the RRM domains of vertebrate TNRC6s, suggesting that vertebrate TNRC6s RRMs are unlikely to bind RNA (Eulalio et al. 2009d).

Despite conservation, the RRM domains are not required for GW182 proteins to localize to P-bodies or to interact with Argonaute proteins (Behm-Ansmant et al. 2006a; Eulalio et al. 2009c, d; Lazzaretti et al. 2009). Furthermore, in complementation assays, a *Dm* GW182 protein lacking the RRM was impaired in silencing but only for a subset of miRNA-target pairs (Eulalio et al. 2009d; Zipprich et al. 2009).

It is important to note that Baillat and Shiekhattar (2009) reported an important role for the RRM in silencing in experiments in which the domain was deleted. However, these authors deleted a region of the protein that extended beyond the structural boundaries of the RRM by including part of the M2 and C-term regions (see Fig. 9.1), which are critical for silencing. Therefore, this deletion mutant was most likely impaired because part of the M2 and C-term regions were deleted. In summary, the RRM is not strictly required for the silencing activity of GW182 proteins, although this domain contributes to silencing of a subset of miRNA targets.

9.3.5 The Bipartite Silencing Domain (SD)

In addition to the N-terminal ABD, the Mid and C-term regions are required for the silencing activity of *Dm* GW182 and human TNRC6A–C (Eulalio et al. 2009c; Lazzaretti et al. 2009; Zipprich et al. 2009; Yao et al. 2011). Indeed, in cells depleted of endogenous *Dm* GW182, silencing of the majority of tested reporters is not rescued by a *Dm* GW182 lacking the Mid and C-term regions, even though this protein interacts with *Dm* AGO1 and is recruited to miRNA targets (Eulalio et al. 2009c; Zekri et al. 2009; Huntzinger et al. 2010). Furthermore, if a protein fragment containing the Mid and C-term regions of *Dm* GW182 or human TNRC6A–C is artificially tethered to a reporter mRNA, then the reporter is silenced (Li et al. 2008; Chekulaeva et al. 2009; Lazzaretti et al. 2009; Zipprich et al. 2009; Yao et al. 2011). As observed in tethering assays for the full-length proteins, these fragments cause bound mRNAs to be translationally repressed and degraded (Behm-Ansmant et al. 2006a; Eulalio et al. 2008; Chekulaeva et al. 2009; Lazzaretti et al. 2009; Zipprich et al. 2009; Yao et al. 2011). The Mid and C-term regions do not interact with AGOs or localize to P-bodies; therefore, these regions possess autonomous silencing activity (Eulalio et al. 2009c; Lazzaretti et al. 2009; Zipprich et al. 2009).

The Mid and C-term regions of GW182 proteins are separated by the RRM domain, which, as mentioned above, contributes but is not strictly required for silencing (Eulalio et al. 2009c, d; Zipprich et al. 2009). On the basis of these observations, we suggested that the Mid and C-term regions define a bipartite SD that is essential in the effector step of silencing (Eulalio et al. 2009a, c; Lazzaretti et al. 2009).

Intriguingly, the Mid and C-term regions of GW182 proteins are not highly conserved, with the exception of short linear motifs. These motifs include the PAM2 motif (PABP-interacting motif 2, previously known as conserved motif III or DUF) that splits the Mid region into the M1 and M2 fragments (i.e., upstream and downstream of the PAM2 motif, respectively, Fig. 9.1, Fabian et al. 2009; Zekri et al. 2009; Huntzinger et al. 2010). Thus, the silencing domains contain four regions: the M1 region, the PAM2 motif, the M2 region and the C-term (Zekri et al. 2009; Huntzinger et al. 2010). Available evidence suggests that the M1, M2 and C-term regions are partially redundant. Indeed, in complementation assays, deleting each region individually has only a minor effect on the silencing activity of GW182 proteins, and the activity is further reduced when at least two of these

regions are deleted in pair-wise combination (particularly when the M2 and C-term regions are deleted). The silencing activity of GW182 proteins is only abolished when the entire silencing domain is deleted, indicating that each region contributes to silencing, although to different degrees (Eulalio et al. 2009c; Huntzinger et al. 2010).

9.4 GW182 Protein Interactions with PABP and Deadenylases

Important clues regarding how GW182 proteins elicit silencing stems from recent studies revealing that their SDs serve as binding platforms for PABP, as well as PAN3 and NOT1, which are subunits of the PAN2–PAN3 and CCR4–NOT deadenylase complexes, respectively (Fabian et al. 2009, 2011; Zekri et al. 2009; Huntzinger et al. 2010; Jinek et al. 2010; Kozlov et al. 2010; Braun et al. 2011; Chekulaeva et al. 2011).

PABP binding is mediated by the highly conserved PAM2 motif (PABP-interacting motif 2) located in the Mid region of the SD (Fig. 9.1). This motif was originally identified in the translational regulators Paip1 and Paip2 (PABP-interacting proteins 1 and 2, (Derry et al. 2006)) and confers direct binding to the C-terminal MLLE domain of PABP (Fabian et al. 2009; Huntzinger et al. 2010; Jinek et al. 2010; Kozlov et al. 2010).

The interactions between human TNRC6 SDs and deadenylases are also mediated by motifs that are predicted to be unstructured. For example, PAN3 binding requires the M2 and C-term regions of the SD (Fig. 9.1; Braun et al. 2011; Chekulaeva et al. 2011). NOT1 binding is mediated by tryptophan-containing motifs in the M1 and C-term regions, termed CCR4–NOT interacting motifs 1 and 2 (CIM-1 and CIM-2), respectively (Fig. 9.1; Fabian et al. 2011). In addition to the CIM-1 and CIM-2 motifs, tryptophan residues in the M2 region also contribute to the interaction with NOT1 (Chekulaeva et al. 2011).

Remarkably, although the interaction between GW182 proteins and PABP and deadenylase complexes are conserved in *D. melanogaster*, the mode of interaction is not similar (Huntzinger et al. 2010; Braun et al. 2011). For example, the CIM-2 motif is absent in *Dm* GW182 (Fabian et al. 2011). Moreover, in contrast to human SDs, which are necessary and sufficient for NOT1 and PAN3 binding, deletion of the *Dm* GW182 SD reduces but does not abolish binding to deadenylases, indicating that sequences upstream of the SD contribute to these interactions (Braun et al. 2011; Chekulaeva et al. 2011). Accordingly, as mentioned above, the N-term region of *Dm* GW182 (the NED; Fig. 9.1) has been shown to bind NOT1 and possess silencing activity (Chekulaeva et al. 2009, 2010, 2011). Finally, in contrast to human proteins, *Dm* GW182 also interacts with PABP indirectly through the M2 and C-term regions in cultured cells (Zekri et al. 2009; Huntzinger et al. 2010). Consequently, the *Dm* GW182 PAM2 motif is dispensable for PABP binding and silencing in *Drosophila* cells (Chekulaeva et al. 2009; Eulalio et al. 2009a; Huntzinger et al. 2010).

9.4.1 Role of the GW182–PABP Interaction in the miRNA Pathway

The interaction of GW182 proteins with PABP is well documented by biochemical and structural studies, and the PAM2 motif is highly conserved among vertebrate and insect GW182 proteins (Fabian et al. 2009; Zekri et al. 2009; Huntzinger et al. 2010; Jinek et al. 2010; Kozlov et al. 2010). Furthermore, several studies have reported that the PABP–GW182 interaction is important for silencing (Fabian et al. 2009, 2011; Huntzinger et al. 2010; Jinek et al. 2010; Walters et al. 2010; Braun et al. 2011; Moretti et al. 2012). This view is supported by three lines of evidence. First, in both *D. melanogaster* and human cells, overexpression of PABP suppresses silencing (Zekri et al. 2009; Walters et al. 2010). Second, depleting PABP from cell-free extracts abolishes miRNA-mediated deadenylation (Fabian et al. 2009). Third, human TNRC6 protein mutants that no longer interact with PABP are impaired in silencing (Huntzinger et al. 2010; Braun et al. 2011).

In contrast to these studies, other studies have indicated that PABP is dispensable for silencing in *Drosophila* cell-free extracts and zebrafish embryos (Fukaya and Tomari 2011; Mishima et al. 2012). For example, the observation that *Dm* GW182 N-term fragments that do not interact with PABP silenced mRNA reporters in tethering assays was interpreted as evidence that the interaction of GW182 proteins with PABP is not required for silencing (Fukaya and Tomari 2011). However, in complementation assays, GW182 N-term fragments fail to complement the silencing of the majority of tested reporters (Eulalio et al. 2009c). Thus, although tethering assays are a powerful tool to study the activity of protein domains in isolation, conclusions from these assays should be validated by complementation assays.

It has also been reported that silencing occurs even when PABP is depleted or removed from mRNA poly(A) tails by an excess of Paip2 (Fukaya and Tomari 2011; Mishima et al. 2012). However, recent studies have indicated that one role of the GW182–PABP interaction is to facilitate PABP dissociation from the poly(A) tail (our unpublished observations; Moretti et al. 2012). This observation may explain why the GW182–PABP interaction becomes dispensable in extracts in which PABP has been depleted or displaced from the poly(A) tail by Paip2. However, it is possible that PABP becomes dispensable for silencing depending on the cellular conditions or the nature of the specific mRNA target.

9.4.2 How Do GW182 Proteins Interfere with PABP Function?

The contribution of the GW182–PABP interaction to silencing is unclear, and several non-mutually exclusive models have been proposed (reviewed by Fabian and Sonenberg 2012). One model proposes that GW182 proteins compete with eIF4G for PABP binding, which prevents mRNA circularization and consequently inhibits translation (Fabian et al. 2009; Zekri et al. 2009). Indeed, actively translated mRNAs

are thought to adopt a closed-loop conformation that is achieved through the interaction of PABP (bound to the mRNA poly(A) tail) with eIF4G (bound to the mRNA 5′ cap structure through eIF4E) (Derry et al. 2006). Preventing the PABP–eIF4G interaction disrupts mRNA circularization and reduces translation efficiency. A second model suggests that by analogy with Paip2, the GW182–PABP interaction may reduce the affinity of PABP for the poly(A) tail, thereby repressing translation (Fabian et al. 2009; Zekri et al. 2009; Huntzinger et al. 2010). A third model suggests that the PABP–GW182 interaction may accelerate miRNA-mediated deadenylation. This model is supported by the observation that PABP depletion prevented miRNA-mediated deadenylation in cell-free extracts from mouse Krebs-2 ascites cells (Fabian et al. 2009). Accordingly, mutations in the PAM2 motif of TNRC6C reduced the rate of deadenylation in tethering assays (Fabian et al. 2009; Jinek et al. 2010). Our current understanding does not allow us to discriminate between these different mechanisms; therefore more work is required to understand how the GW182–PABP interaction contributes to silencing.

9.5 Model for miRNA-Mediated Gene Silencing

The study of GW182 proteins has provided important insight into the mechanism of silencing. The model shown in Fig. 9.2 integrates the data accumulated in the field. Silencing begins with the recognition of the target by a miRNA in a complex with an AGO and a GW182 protein (Fig. 9.2a, b). GW182 proteins interact with PABP bound to the mRNA poly(A) tail and with the PAN3 and NOT1 subunits of the deadenylase complexes. The assembly of this complex on the mRNA represses translation, and this repression most likely occurs at initiation through an unknown mechanism (Fig. 9.2b, Fabian and Sonenberg 2012). The repressed mRNA is then deadenylated. Indeed, translational repression of miRNA targets has been shown to precede complete deadenylation (Bazzini et al. 2012; Béthune et al. 2012; Djuranovic et al. 2012). Depending on the cell type and/or specific target, deadenylated mRNAs may be stored in their deadenylated, translationally repressed state as observed in cell-free extracts (Fig. 9.2d, Wakiyama et al. 2007; Fabian et al. 2009; Iwasaki et al. 2009; Zdanowicz et al. 2009; Wu et al. 2010). However, in animal cell cultures, deadenylated mRNAs are generally decapped and rapidly degraded by the 5′-to-3′ exonuclease XRN1 (Fig. 9.2e, f). Accordingly, the depletion of cytoplasmic deadenylase and decapping complex components suppresses miRNA-mediated mRNA degradation (Rehwinkel et al. 2005; Behm-Ansmant et al. 2006a, b; Eulalio et al. 2007a, 2009b; Chen et al. 2009; Piao et al. 2010).

This model raises several important questions that represent challenges to be addressed in future work. Determining the mechanism of translational repression remains the most urgent of these questions. Moreover, whether translational repression and deadenylation are interconnected or whether these processes represent independent mechanisms used by miRNAs to silence mRNA targets remains unclear. Indeed, although translational repression has been shown to precede deadenylation

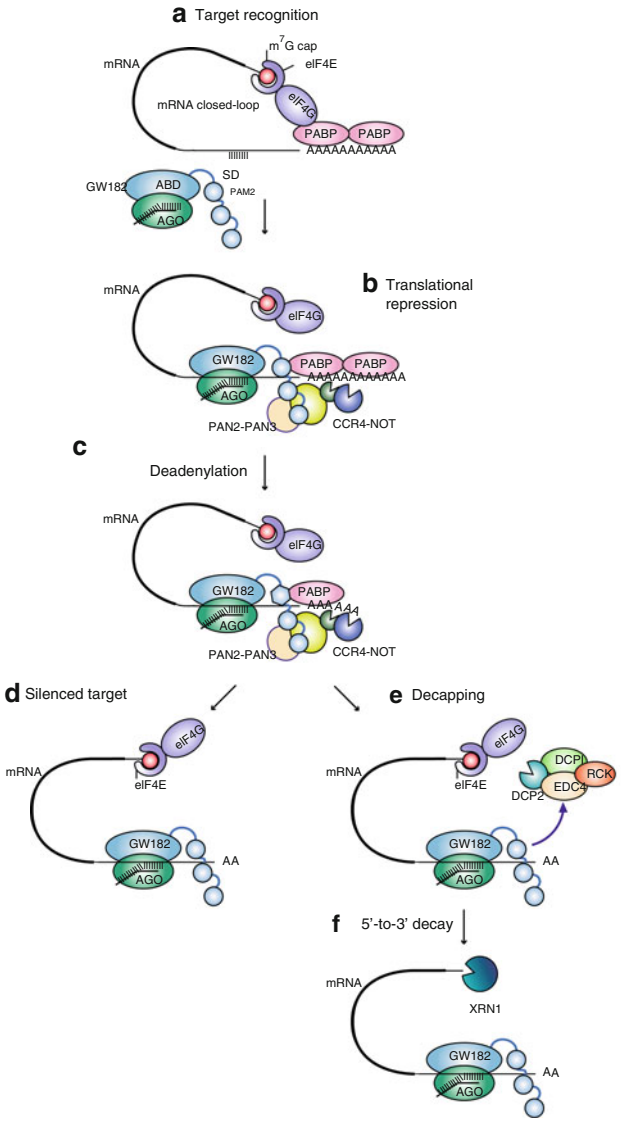

Fig. 9.2 Model of miRNA-mediated gene silencing in animals. miRNA-mediated gene silencing in animals requires at least one Argonaute protein, one GW182 protein, PABP, and the PAN2–PAN3 and CCR4–NOT deadenylase complexes. The CCR4–NOT complex comprises two catalytic subunits and the NOT module containing NOT1. The mRNA target is depicted in a closed-loop conformation (**a**), which is achieved through interactions between PABP bound to the 3′ poly(A) tail and eIF4G (bound to the cap-binding protein eIF4E). (**b**, **c**) Animal miRNAs bound to AGO-GW182 complexes recognize their mRNA targets by base-pairing to partially complementary binding sites, which are predominantly located in the mRNA 3′ UTR. GW182 interacts with PABP bound to the mRNA poly(A) tail via the PAM2 motif. GW182 proteins also interact with the PAN2–PAN3 and CCR4–NOT deadenylase complexes via additional motifs (represented as *circles*). The AGO-GW182 complex represses translation and directs the mRNA to deadenylation (**c**). Translation is inhibited prior to deadenylation. Depending on the cell type and/or specific target, deadenylated mRNAs can be stored in a translationally repressed state (**d**). In animal cell cultures, deadenylated mRNAs are decapped by the decapping enzyme DCP2 and several decapping activators (e.g., DCP1, RCK, EDC4) and rapidly degraded by the major 5′-to-3′ exonuclease XRN1 (**e**, **f**)

(Bazzini et al. 2012; Béthune et al. 2012; Djuranovic et al. 2012), the two processes may still be mechanistically linked; therefore, they may represent two consecutive outcomes of a single molecular mechanism that both interferes with translation and triggers deadenylation.

What might this molecular mechanism be? Although completely speculative at this time, the recruitment of deadenylase complexes to the 3′ UTR of miRNA targets may trigger both translational repression and deadenylation. This possibility is supported by studies demonstrating that the depletion of PAN3 and NOT1 suppresses the silencing of reporters lacking a poly(A) tail (Braun et al. 2011; Chekulaeva et al. 2011). Furthermore, tethering of CCR4–NOT complex subunits to mRNA reporters lacking a poly(A) tail induces translational repression in the absence of deadenylation (Cooke et al. 2010; Chekulaeva et al. 2011). These data suggest that deadenylase complexes not only promote deadenylation, but also contribute to translational repression. If this scenario is correct, it is important to determine the mechanism by which the deadenylase complexes repress translation and the ways in which these complexes interact with the translation and silencing machineries.

9.6 Outlook

Despite their recent identification, GW182 proteins have rapidly become established as key players in the miRNA pathway. Therefore, the study of GW182 proteins is of critical importance for further understanding the silencing mechanisms in animals. The discovery of their interaction with PABP and deadenylase complexes has provided important insights into miRNA silencing mechanisms. However, how these interactions contribute to silencing remains to be established. Additionally, although the role of GW182 proteins in silencing is becoming clear, little is known about how GW182 proteins are regulated. GW182 proteins are phospho-proteins (Eystathioy et al. 2002), but how phosphorylation affects their activity remains unknown. Finally, a major task for future research will be to understand how GW182 proteins orchestrate the translational repression and deadenylation of miRNA targets. We expect that answers to this question will emerge as more studies examine how GW182 proteins interact with their partners to assemble into active effector complexes.

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Chapter 10
Quantifying Argonaute Proteins In and Out of GW/P-Bodies: Implications in microRNA Activities

Anthony K.L. Leung and Phillip A. Sharp

Abstract MicroRNAs (miRNAs) are a class of ~22nt non-coding RNAs that regulate the translational potential and stability of mRNAs. Though constituting only 1–4% of human genes, miRNAs are predicted to regulate more than 60% of all mRNAs. The action of miRNAs is mediated through their associations with Argonaute proteins and mRNA targets. Previous studies indicated that though the majority of Argonaute proteins is diffusely distributed in the cytoplasm, a small fraction is consistently observed to be concentrated in a cytoplasmic compartment called GW/P-bodies. In this chapter, we will provide a quantitative and dynamic view of the subcellular localization of miRNA function, followed by a discussion on the possible roles of PBs in miRNA silencing.

10.1 Introduction

10.1.1 microRNA

microRNAs (miRNAs) are a class of ~22nt short non-coding RNAs that regulate translational potential and stability of mRNAs in the cytoplasm (Bartel 2009; Fabian et al. 2010; Jackson and Standart 2007; Leung and Sharp 2006).

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miRNA action is pervasive: they are predicted to regulate over 60% of all mammalian mRNAs (Friedman et al. 2009) and constitute a sizable class of regulators, e.g., 2237 different miRNAs were identified in humans (miRBase release 19 in August 2012; http://www.mirbase.org/), even outnumbering kinases and phosphatases.

miRNAs are first transcribed as primary transcripts (pri-miRNA), which fold into hairpin structures and are subsequently processed—first by Drosha in the nucleus and then by Dicer in the cytoplasm. These processing steps result in a ~22 nucleotide duplex in which one strand, miR*, is degraded while the mature miRNA is selectively loaded into the RNA-induced silencing complex (RISC) complex where Argonaute is the key protein that binds the mature miRNA (Fig. 10.1).

10.1.2 Argonaute

In animals, most of the miRNAs only require a short "seed" region (second to seventh position of miRNA) of perfect complementarity with the mRNA target to trigger translation inhibition and/or acceleration of mRNA decay (Bartel 2009). On the other hand, if the miRNA is perfectly complementary to its mRNA targets, this results in mRNA cleavage between the corresponding 10th and 11th position of miRNAs. This cleavage event rarely occurs for animal miRNAs, yet this cleavage function is still intact in animals. This is illustrated by the use of chemically synthesized small interfering RNAs (siRNAs) to knock down specific genes in mammals through this mechanism. In fact, miRNA and siRNA silencing pathways share some common activities. For example, endogenous miRNAs can direct cleavage of exogenously expressed targets that contain sites of extensive complementarity, and siRNAs can function as miRNAs in mediating translational repression (Doench et al. 2003; Hutvagner and Zamore 2002). In humans, there are four Argonaute proteins, Ago1–4, which can all mediate translation repression/mRNA decay, but only Ago2 can trigger miRNA/siRNA-directed cleavage (Hock and Meister 2008). These four Argonaute proteins share a common bipartite structure (Parker 2010), where the N-terminal half containing a PAZ domain that binds the 3′ end of the miRNA and a C-terminal half containing a Mid domain that binds the 5′ end of the miRNA as well as a PIWI domain that binds to the "seed" region of the miRNA that targets mRNA (Fig. 10.1). Moreover, the C-terminal half also binds GW182, a downstream effector to mediate miRNA silencing (Eulalio et al. 2009).

To shed light on the potential mechanisms of miRNA functions, we and other groups have used immunostaining and live cell imaging coupled with genetics tools to dissect the localization of Argonaute proteins in different cellular conditions. In most cases examined, but with a few notable exceptions (Gibbings et al. 2009; Lee et al. 2009; Vasudevan and Steitz 2007), all Argonaute members are enriched in a cytoplasmic structure called GW- or P-bodies (PBs). As reviewed elsewhere in this book, PBs, are devoid of any translation machineries, but are enriched with silenced

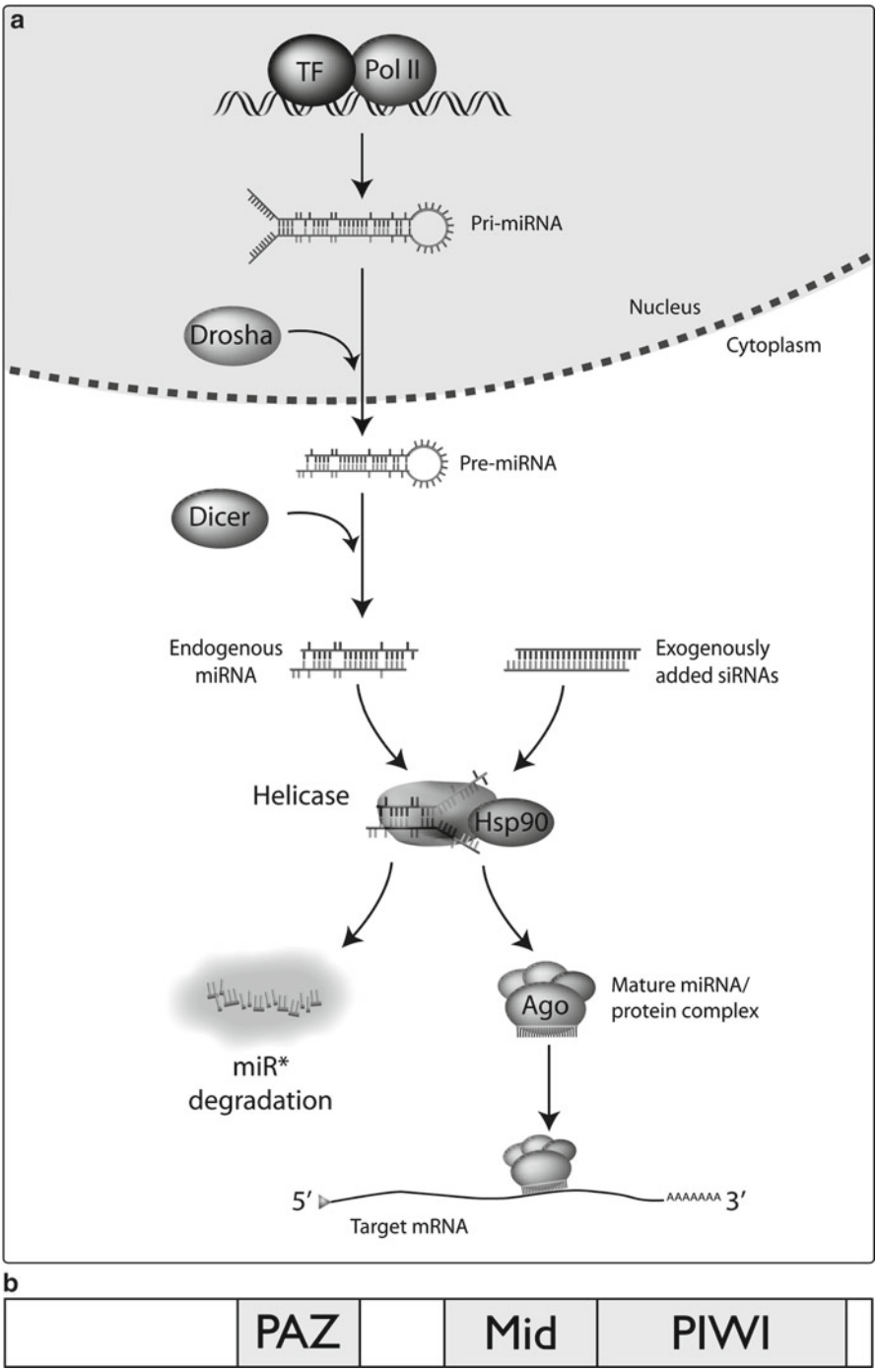

Fig. 10.1 Schematics of (**a**) microRNA biogenesis and (**b**) Argonaute protein domains

mRNAs, translational repressors and RNA decay factors that are involved in nonsense mediated decay, AU-rich element decay or miRNA silencing. In this chapter, we will first review the data on Argonaute localization, specifically focusing on its quantitation and dynamics. This is followed by a discussion on the possible roles of PBs in the context of miRNA silencing.

10.2 Quantitation of Argonaute, miRNA and mRNA Target Localization

10.2.1 Quantitation of Argonaute

To identify where miRNA-mediated silencing occurs in cells, we chose to track the localization of Ago2, the signature component of RISC that can mediate translation repression, acceleration of mRNA target decay as well as miRNA/siRNA-mediated cleavage (Leung et al. 2006). Several HeLa cell lines that stably expressed Green Fluorescent Protein (GFP)-tagged Ago2 were generated and the one with the lowest expression was chosen for quantitation to avoid overexpression artifacts. Consistent with other observations, GFP-Ago2 was enriched at punctate structures that were stained positive for Dcp1a, which is a marker for PBs, and the GFP-Ago2 intensity at PBs is tenfold higher than the cytoplasm (Fig. 10.2). However, when the volume of PBs and the total cytoplasm were taken into account, only ~1% of cytoplasmic GFP-Ago2 was localized at PBs whereas the majority was diffusely distributed elsewhere in the cytoplasm.

To confirm these results, endogenous localization of Argonaute was examined with four different antibodies. Surprisingly, though Dcp1a-positive PBs are present in the parental HeLa cell line, <10% of PBs are co-stained with Argonaute. Several other groups also reported that only a minority of PBs also stain positive for Argonaute. James and colleagues reported that on average only 1 in 7 PBs were stained positively with Ago2 in U2OS cells (James et al. 2010). Vasudevan and Steitz found that Ago2 though co-localized with Dcp1a in HEK293 cells when grown in serum-containing medium, Ago2 dispersed to smaller bodies that no longer co-localized with PBs upon serum starvation (Vasudevan and Steitz 2007). Moreover, homologues of miRNA-binding Argonautes in *Drosophila* S2 cells (dAgo1) and *Caenorhabditis elegans* epidermal/neuronal cells (Alg1/2) were also found to be diffusedly distributed in the cytoplasm (Behm-Ansmant et al. 2006; Ding et al. 2005). However, these Argonaute members localized to punctate PB-like structures in the cytoplasm only when exogenous Ago or GW182 was ectopically expressed. Similarly, GW182 is not always concentrated as punctate structures and such localization depends on the cell cycle and growth conditions (Yang et al. 2004). Together, this data suggest that the majority of miRNA-mediated silencing is not likely at PBs, but rather its localization seems to be sensitive to the endogenous level of Argonaute and its associated proteins.

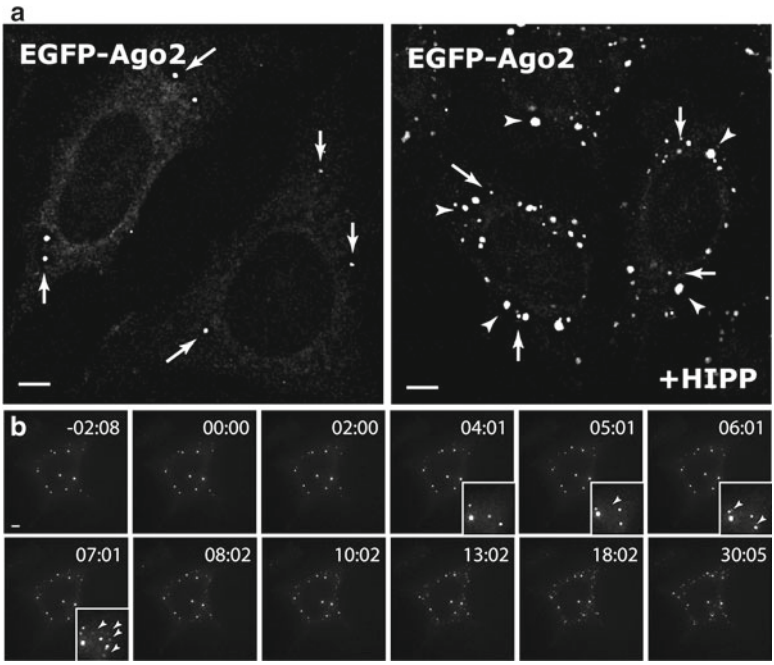

Fig. 10.2 Argonaute protein localization. (**a**) Stably expressed EGFP-Ago2 localized to the cytoplasm and PBs (*arrows*, *left*) and, upon addition of 1 μM hippuristanol (HIPP) for 30 min, also localized to SGs (*arrowheads*, *right*). (**b**) Time-lapse micrographs of stably expressed EGFP-Ago2 in a single live cell. The first appearance of EGFP-Ago2 at SGs (*arrowheads*) occurred between 5 and 6 min after addition of 1 μM hippuristanol (HIPP). Scale bars, 5 μm. (Reprinted, with permission, from Leung et al. (2006); © National Academy of Sciences, U.S.A.)

10.2.2 Quantitation of Mature miRNAs and siRNAs

Several approaches have been used to identify and quantitate the specific localization of mature miRNAs or siRNAs in the cytoplasm. Pillai and colleagues microinjected an in vitro transcribed, fluorescently labeled artificial precursor of *let-7a* into nuclei and found that 26% of the cytoplasmic fluorescent signals localized at (18%) or near (8%) PBs (Pillai et al. 2005). However, both the active miRNA strand and the miR* strand of miRNA were equally labeled and therefore cannot easily be distinguished from each other. The signal observed at PBs could represent the accumulation site for miR* prior to degradation, rather than the active strand. Jakaymiw and colleagues labeled the antisense of a siRNA duplex and found that the labeled strand accumulated in PBs (Jakymiw et al. 2005). In this case, it is similarly difficult to be certain that the labeled antisense strand is the one loaded into the RISC as contrasted to the one targeted for decay. Bhattacharyya and colleagues performed in situ hybridization using locked nucleic acid (LNA) probes against a highly abundant miRNA, miR122, followed by signal amplification and the accumulation of signals was observed at PBs in hepatocytes (Bhattacharyya et al. 2006). Since the

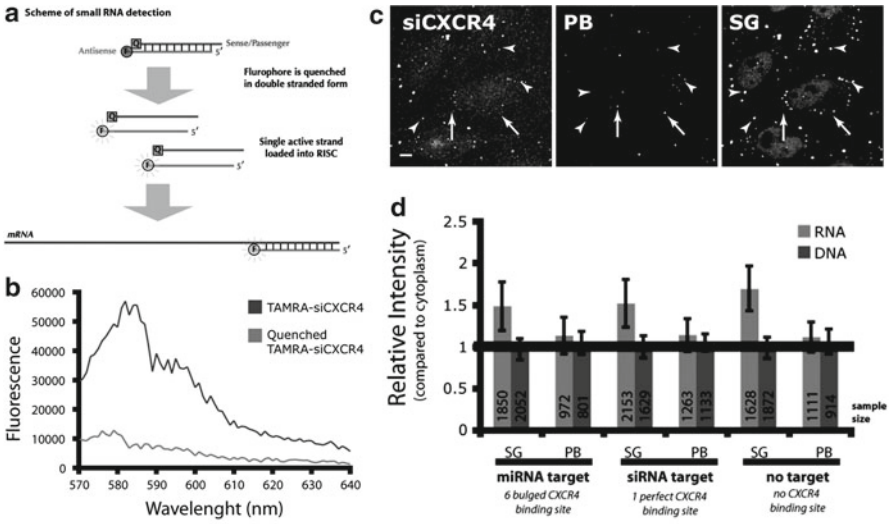

Fig. 10.3 Localization of short RNAs. (**a**) Schematics of active siRNA strand detection. (**b**) Fluorescence emission profiles of unquenched and quenched TAMRA siRNAs against endogenous gene CXCR4 (siCXCR4). (**c**, **d**) The localization of siCXCR4 was compared with PB marker Dcp1a (*arrows*) and SG marker TIA-1 upon addition of 1 μM hippuristanol (HIPP) for 30 min. In this case, siCXCR4 co-localized with SGs (*arrowheads*), but not with PBs, when translation initiation was inhibited (*left*), as shown by the significant enrichment of the intensities at SGs compared with the cytoplasm (two-tailed *t* test, $P < 0.0001$). Scale bars, 5 μm. (Reprinted, with permission, from Leung et al. (2006); © National Academy of Sciences, U.S.A.)

signal has been amplified in a non-linear fashion, it is also difficult to estimate the actual amount of miRNA localized in PBs. Nevertheless, these studies suggest that a fraction of mature miRNAs are present in PBs.

To avoid the ambiguous detection of inactive miRNA/miR* duplexes, or the miR* strand, and to quantitate the signal in the linear range, we have modified a well-characterized siRNA duplex that has been shown to function as a miRNA (Leung et al. 2006). The antisense strand is labeled with tetramethylrhodamine (TAMRA) at its 3′ end and the 5′ phosphate group of the passenger strand is substituted with a black hole quencher 2 (BHQ2) (Fig. 10.3). In this way, the fluorescence is quenched in the siRNA duplex form, thereby enhancing the signal detection from the active single strand. Since the 5′ phosphate group is a prerequisite for RISC loading, the substitution of the phosphate group with the quencher presumably favors the selection of the labeled antisense strand for loading into the RISC complex. The siRNA duplex was electroporated into HeLa cells and their localization was examined after 56 h, a period that allows the decay of endosomally localized siRNAs. Using this modified siRNA, the labeled antisense strand of siRNA was not enriched nor depleted at PBs compared with the cytoplasmic background; instead the signal was diffusively distributed throughout the cytoplasm. The lack of enrichment of siRNA at PBs in HeLa is not surprising given that <10% of PBs are positively stained with anti-Argonaute antibodies (Sect. 10.2.1). However, we recently found

that this modified siRNA can be enriched in PBs in HeLa provided that the cell line overexpresses Ago2. This new data suggest that the level of miRNA/siRNA present in PBs is sensitive to the expression level of Argonaute.

On the other hand, to examine the localization of siRNA duplex, Jagannath and Wood labeled both strands with distinct fluorophores such that they are ~6 nm apart in the native double-stranded configuration (Jagannath and Wood 2009). Such close-proximity configuration resulted in Fluorescent Resonance Energy Transfer (FRET), which requires <10 nm to occur, from the donor dye at the passenger strand to an acceptor dye at the antisense strand. Using this FRET approach, they found that the native siRNA duplex was also localized in PBs. Importantly, a control experiment was performed by transfecting an equal amount of two siRNA duplexes: (1) the same targeting siRNA duplex, but only the antisense strand was labeled with the acceptor fluorophore this time, and (2) a non-targeting siRNA that is labeled with the donor fluorophore at the passenger strand only. In this way, they delineated that the majority of the FRET signal actually came from the siRNA duplex, rather than from two individual, separated siRNA strands that were proximal by chance in PBs, given that this is physically feasible within such a small structure (~100–300 nm in diameter). The FRET signal was highest at 4 h post-transfection and lost at 72 h, yet, at this time point, significant repression was still observed. Consistent with our quantitation data, the miRNA/siRNA in PBs is not the only active fraction. Moreover, this data also raised the possibility that PBs could be the site where miR* and/or the passenger strand of siRNA accumulated.

10.2.3 Quantitation of mRNA Targets

To localize repressed targets, Liu and colleagues used GFP-tagged MS2-binding proteins to track luciferase mRNA that has 24 copies of MS2-binding sites behind two tandemly arranged *let-7* binding sites (Liu et al. 2005b). The target was localized to PBs in a miRNA-dependent manner. Moreover, Pillai and colleagues quantitated the in situ hybridization signal of *let-7* targeted luciferase mRNA, and, similar to *let-7*, ~21% localized at (13%) or near (8%) PBs (Pillai et al. 2005). We also found similar localization of the mRNA targets at or near PBs, while the rest of the signal is diffusely distributed in the cytoplasm (Leung et al. 2006).

10.3 Dynamics of Argonaute and PBs

Fusion of GFP to a protein of interest not only visualizes the protein localization but also enables quantitative measurements of protein dynamics using photobleaching and photoactivation techniques as well as single-particle tracking. In this section, we will examine the kinetics of Argonaute localization in different cellular conditions, and then review data regarding the dynamics of PBs to understand its possible role in miRNA silencing.

10.3.1 Argonaute Localization Is Sensitive to Translation Status

First, we examined the Argonaute localization upon limiting translation initiation by hippuristanol, an inhibitor of initiation factor eIF4A (Leung et al. 2006). Under this condition, while the intensity at PBs remains unchanged, 1.2–10.6% of GFP-Ago2 signal was localized in another cytoplasmic structure called stress granules (SGs; Fig. 10.2). SGs are aggregates of stalled initiation complexes that are enriched with poly(A)+mRNAs and RNA-binding proteins that regulate translation potential and stability of mRNAs (Leung and Sharp 2007; Anderson and Kedersha 2008). SG assembly is commonly induced upon multiple types of stress including oxidative stress, heat shock, viral infection and ischemia. Quantitatively, there was at least threefold more GFP-Ago2 localized to SGs than to PBs. Using our quencher-TAMRA based detection, we detected a 1.5-fold enrichment of miRNAs at SGs compared with the neighboring cytoplasmic signal, in contrast to no enrichment at PBs (Leung et al. 2006). Moreover, in situ hybridization showed that miRNA-repressed targets also accumulated in SGs (Leung et al. 2006). Given that the amount of Argonaute localized at PBs did not change over the course of hippuristanol treatment, those Argonaute relocalized to SGs (along with miRNA and their targets) are likely originated elsewhere from the diffuse cytoplasm.

Biochemical data consistently showed that Argonaute proteins are associated with polyribosomes (Kim et al. 2004; Nelson et al. 2004; Olsen and Ambros 1999; Seggerson et al. 2002), so we then tested whether GFP-Ago2 in SGs could dynamically exchange with these submicroscopic pools of ribosome-associated mRNAs (Leung et al. 2006). If Argonaute proteins at SGs are in dynamic equilibrium with those in polyribosomes, we would expect addition of another translation inhibitor emetine, a drug that stabilizes the association of mRNA and polyribosomes, will shift the exchanging pool toward the polyribosomes. Indeed, these pre-formed, Argonaute-positive SGs dissociate in the presence of emetine, but the signal at PBs remains unchanged under the same conditions. Therefore, Argonaute proteins in SGs possibly originate from, and dynamically exchange with, polyribosomes in the cytoplasm.

10.3.2 Kinetic Behaviors of Ago2 and Other PB Components

Given the distinct behaviors of Argonaute at PBs and SGs in association with the polyribosomes in the diffuse cytoplasm, we further examined their kinetics using photobleaching (Leung et al. 2006). Photobleaching is a photo-induced alteration of a fluorophore that extinguishes its fluorescence irreversibly (Lippincott-Schwartz et al. 2003). GFP is an ideal tool to study protein dynamics because the chromophore has a high fluorescence yield and is resistant to photobleaching at low illumination. On the other hand, when excited by high illumination levels, the GFP fluorophore can be irreversibly photobleached. These properties were exploited to study the movement of non-bleached GFP fusion proteins into the bleached areas in a technique

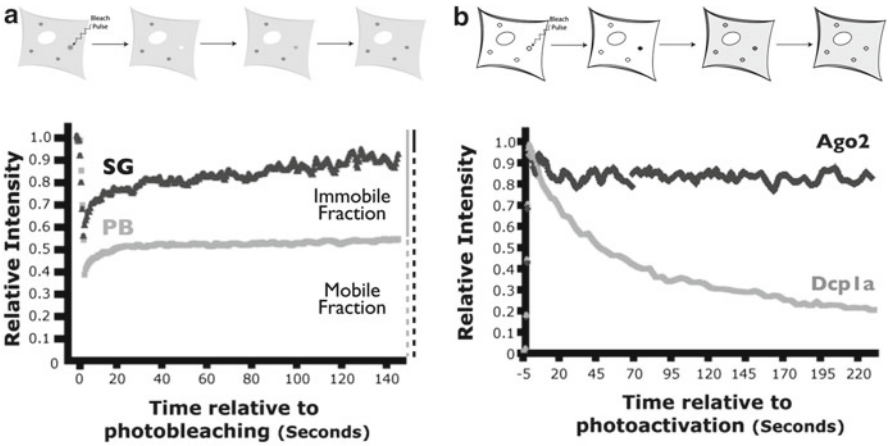

Fig. 10.4 Quantitative dynamics of argonaute protein. (**a**) FRAP analyses of EGFP-Ago2 at single PBs ($n=5$) and SGs ($n=3$) and the intensities at respective structures relative to their initial intensities were compared over time. (**b**) PAGFP-Dcp1a and PAGFP-Ago2 were photoactivated at single PB labeled by mRFP-Dcp1a for 1 s, and the photoactivated cells were imaged over the next 13 min. (Reprinted, with permission, from Leung et al. (2006); © National Academy of Sciences, U.S.A.)

known as Fluorescence Recovery After Photobleaching (FRAP; Fig. 10.4). Though Argonaute proteins at SGs are constantly exchanging with the diffuse cytoplasm, they exhibited much slower kinetics at PBs. The fluorescence at a single photobleached spot at an SG can be fully recovered to its initial intensity level within 6 min, whereas the photobleached spot at a PB never recovered during this period. Indeed, ~50% of Argonaute proteins were deemed "immobile" by FRAP analyses. Consistently, photoactivation experiments suggest that 80% of Ago2 remained at PBs, indicative of a very slow off-rate (Fig. 10.4). The immobility observed could be because (1) Argonaute anchors to fixed molecules or forms aggregates that are restricted in movement and/or (2) Argonaute is confined to a subcellular region that cannot contribute to fluorescence recovery in a separate compartment. The second scenario seems to be unlikely as decapping enzyme co-activators Dcp1a, Dcp1b and Lsm6, all of which co-localize with Ago2 at PBs, exhibit fast recoveries (Aizer et al. 2008; Andrei et al. 2005; Kedersha et al. 2005; Leung et al. 2006). On the other hand, similar slow rates of exchange were observed at PBs for several Argonaute-associated proteins, including GW182, the cap-binding protein eIF4E and the decapping enzyme Dcp2 (Aizer et al. 2008; Andrei et al. 2005; Kedersha et al. 2005). Though one GW182 is able to physically associate with multiple Argonautes through protein-protein interactions (Takimoto et al. 2009), it is unlikely that GW182 anchors Argonaute to PBs. This is because two Argonaute mutants that are devoid of their PB localization were previously shown to be associated with GW182 at the same or greater affinity when compared with the wild-type (Liu et al. 2005a). Instead, Argonaute may anchor to other fixed molecules or form aggregates with other proteins that are restricted in movement at PBs.

10.3.3 Dynamics of PBs

The understanding of Argonaute protein kinetics would not be completed without the dynamic picture of the PB structure itself. Aizer and colleagues followed the movement of PBs in multiple U2OS cell lines that stably expressing GFP-Dcp1a, RFP-Dcp1b or GFP-Dcp2 at a low level using single-particle tracking technique and subsequent mean square displacement (MSD) analysis (Aizer et al. 2008). MSD analyses suggest that only few PBs were moving in a directional manner on microtubules while most were moved by diffusion yet confined to an area. In all three different stable cell lines, ~55% of PBs were confined to an area ranging up to 2 μm^2 and ~30% between 2 and 5 μm^2. The diffusion coefficient, which describes the relative speed at which a particle moves within a defined area, was measured to be in the range of other cytoplasmic organelles (10^{-3} to 10^{-2} $\mu m^2/s$). Such slow diffusion was commonly ascribed to the crowding cytoplasmic environment (Luby-Phelps 2000). In contrast, cytoplasmic mRNPs diffuse at a much faster rate (0.1 $\mu m^2/s$) (Fusco et al. 2003). As cytoplasmic mRNPs diffuse at a rate that is 100 times faster than PBs and are at least 1,000 times more abundant (Femino et al. 1998; Aizer et al., 2008), it is likely that the random movement of both mRNPs and PBs is sufficient to explain how RNAs enters in and out of the relatively confined PBs. Moreover, unlike other cytoplasmic organelles, PBs are not enclosed with a membrane (Yang et al. 2004), thereby allowing constant exchange of mRNPs in and out of PBs and being subjected to regulation upon cellular cues.

10.3.4 Argonaute Protein Kinetics in Neuronal Models

Two recent studies in neurons and in a neurodegenerative disorder model highlighted the importance of Argonaute protein localization and kinetics in miRNA activities (Cougot et al. 2008; Savas et al. 2008). Savas and colleagues reported that Argonaute proteins co-localized at PBs and co-purified with Huntingtin protein (Htt) (Savas et al. 2008). Htt is commonly mutated in Huntington's disease—a dominant autosomal neurodegenerative disorder. Due to an expansion of CAG triplet in its gene, the mutant protein usually has at least additional 36 polyglutamine (polyQ) at the N-terminus. Both the normal and polyQ mutant co-purified with Ago1 and Ago2. Upon siRNA knockdown of Htt in HeLa cells or upon expression of polyQ mutants in striatal progenitor cells from mouse brain, a reduction in the number of PBs as well as silencing activities were observed. Interestingly, FRAP analyses revealed that the mobile fraction of Ago2 in PBs was reduced from 15 to 8% upon expression of the poly(Q) mutant, but the percentage of mobile fraction remained unchanged upon expression of normal Htt. The reduced mobility implies that an even smaller pool of Argonaute at PBs is available to exchange with the diffuse cytoplasm upon expression of mutant Htt, which possibly contributes to the reduction in miRNA silencing in cells with mutant Htt that model the disease.

Cougot and colleagues reported that Ago2 and Dcp1a co-localized in PB-like structures in dendrites of mammalian neurons (Cougot et al. 2008). Consistent with our kinetic observations (Sect. 10.3.2), Ago2 exhibited a similar or slightly slower recovery rate in dendrites. On the contrary, though these authors found that Dcp1a exchanged rapidly with the cytoplasm in HeLa cells similar to our studies, the recovery rate of Dcp1a was very slow in the neurons. However, upon neuronal stimulation, such recovery rate of Dcp1a was dramatically increased to a similar rate as in HeLa cells and half of the Dcp1a-positive foci no longer co-stained with Ago2 within 15 min of stimulation (the shortest time point examined in the study). It is therefore tempting to speculate that such reduction in Ago2 enrichment in PB-like structures is related to the relief of miRNA-mediated repression previously observed upon neuronal stimulation (Schratt et al. 2006).

10.4 What Determines Argonaute to Localize at PBs?

10.4.1 microRNA-Dependency?

To determine whether miRNA is required for Argonaute to localize to PBs, we transfected GFP-Ago2 into three clonal mouse embryonic stem (ES) cell lines that lack mature miRNAs by inactivating *Dicer*, the key cytoplasmic processing enzyme to generate miRNAs (c.f. Fig. 10.1; (Leung et al. 2006)). Similar to the wild-type ES cells, GFP-Ago2 were still localized to PBs that were Dcp1a-positive in *Dicer*$^{-/-}$ ES cells (Fig. 10.5). In contrast, GFP-Ago2 no longer associated with SGs in these cells (Fig. 10.5). However, in the presence of exogenously added *let-7a* miRNA transfected in the form of siRNAs, GFP-Ago2 association with SGs was restored (Fig. 10.5). Thus, while the Argonaute localization to SG is miRNA-dependent, its localization to PBs is independent of miRNAs. This is probably because Argonaute can associate with several PB components, including GW182, cap binding protein eIF4E, translational repressor p54/rck and nonsense mediated decay factor UPF1, through direct protein-protein interactions (Chu and Rana 2006; Hock et al. 2007; Jakymiw et al. 2005; Liu et al. 2005a).

Interestingly, Dcp1a-positive PBs are still present in *Dicer*$^{-/-}$ mouse ES cells, suggesting that neither mature miRNAs nor RNA silencing are required for the formation of PBs. These ES cell data, however, are in apparent contrast with other studies, which showed that the number and size of PBs were reduced in *Drosophila* S2 cells (Eulalio et al. 2007) and HeLa cells upon depletion of key miRNA pathway proteins (Pauley et al. 2006). Eulalio and colleagues reported that the number of PBs cannot be restored by translational inhibitor puromycin in these S2 cells (Eulalio et al. 2007), suggesting that the presence of mRNAs that are not undergoing translation is not sufficient to restore the PB assembly. Instead, these mRNAs must be in a repressed state with perhaps enhanced rates of degradation for the restoration. This is illustrated by the PB assembly upon transient transfection of a functional siRNA/miRNA that repress specific endogenous genes, but not with a non-targeting siRNA/miRNA.

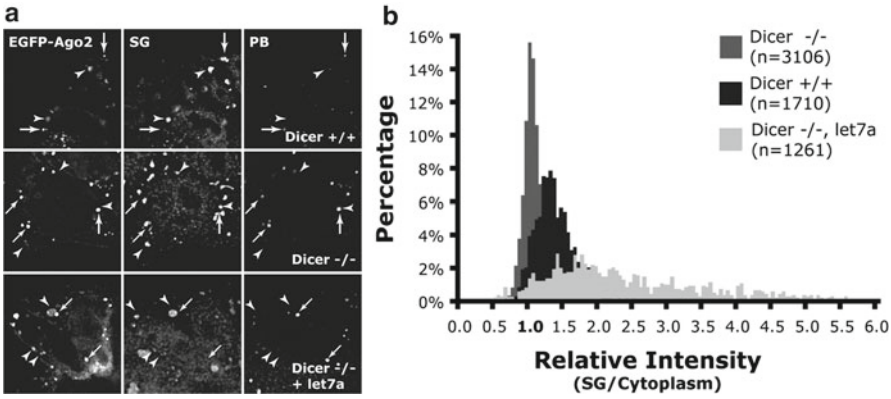

Fig. 10.5 The localization of Ago2 at SGs, but not at PBs, depends on the presence of short RNAs. (**a**) Transiently expressed EGFP-Ago2 still co-localized with PB marker Dcp1a in both *Dicer*$^{+/+}$ (*top*, *arrows*) and *Dicer*$^{-/-}$ (*middle* and *bottom*) cells. However, EGFP-Ago2 no longer co-localized with SG marker TIA-1 (*arrowheads*) in *Dicer*$^{-/-}$ cells. Cotransfection of 100 nM of miRNA *let-7a* in the form of siRNA duplex resulted in the localization of EGFP-Ago2 at SGs in *Dicer*$^{-/-}$ cells (*bottom*). (**b**) The intensities of EGFP-Ago2 at SGs were compared with the cytoplasm in each case, and a histogram was plotted with the percentage of SGs as the *y* axis, for each relative intensity with an interval of 0.05 difference on the *x* axis. Correlated with the image data in (**a**), the intensities of EGFP-Ago2 at SGs relative to the cytoplasm were centered at ≈1.0 in *Dicer*$^{-/-}$ cells, suggesting that there is no enrichment of EGFP-Ago2 at SGs in the absence of short RNAs. Scale bars, 5 μm. (Reprinted, with permission, from Leung et al. (2006); © National Academy of Sciences, U.S.A.)

However, it should be noted that the initial disappearance of PBs in these cells is not due to a lack of repressed targets because the depletion of specific miRNA pathway genes was mediated through RNA silencing in the first place (Eulalio et al. 2007; Pauley et al. 2006). Therefore, the absence of microscopically visible PBs in these cells is likely a kinetic issue and the restoration of PB assembly occurs upon exceeding a critical threshold of repressed mRNP concentration. On the other hand, in the case of mouse *Dicer*$^{-/-}$ ES cells where microscopically visible PBs were observed, perhaps there are already sufficient amount of mRNA degradation/repression mediated through pathways other than RNA silencing in these cells. Consistent with this idea, depletion of translational repressors or RNA decay factors that are not apparently related to RNA silencing is sufficient to trigger PB disassembly (Chu and Rana 2006; Eulalio et al. 2007).

10.4.2 Post-translational Modifications

So far, Argonaute localization to PBs was reported to be controlled by two post-translational modifications: phosphorylation and hydroxylation. Zeng and colleagues reported that Ago2 is phosphorylated at serine-387 in the PAZ domain of Ago2

(Zeng et al. 2008). Mutating the serine to alanine resulted in a reduction of Ago2 localization to PBs. The level of Ago2 phosphorylation at this residue was increased upon arsenite-mediated oxidative stress by a downstream kinase of p38 MAPK pathway, MAPKAPK2. However, the significance of the Ago2 phosphorylation at this residue in miRNA silencing abilities and PB localization remains unclear.

On the other hand, stability of Ago2 is modulated by the prolyl 4-hydroxylation at proline-700 in the PIWI domain (Qi et al. 2008). Mutating the proline to alanine reduced the half-life of Ago2 protein from >10 h to ~6 h. Similarly, siRNA-mediated knockdown or genetic ablation of enzymes required for hydroxylation reduced the stability of Ago2 protein and the destabilization is mediated by proteasome. Interestingly, though these cells had the same number of Dcp1a-positive PBs, Ago2 was no longer associated with these cytoplasmic structures. Consistent with this observation, Ago2 was delocalized from PBs for proline-700 → alanine mutant. Therefore, hydroxylation at this residue can potentially redirect Argonaute to PBs. Alternatively, the observed PB localization could be sensitive to the steady-state level of endogenous Ago2 (c.f. Sect. 10.2.1).

10.4.3 Hsp90 Sensitivity

Even before the discovery of Argonaute function in miRNA silencing, one of the first known protein interaction partners of Ago2 was Hsp90 (Tahbaz et al. 2001). Hsp90 is a stress-induced chaperone that assists a distinct set of ~200 client proteins to adopt their active conformations through ATP binding and hydrolysis (Taipale et al. 2010). Several recent studies in *Drosophila*, human and plant cells have indicated that the HSP90 chaperone machinery facilitates the loading of siRNA duplexes into the RISC complex (Johnston et al. 2010; Landthaler 2010). Addition of Hsp90 inhibitor, such as geldanamycin, resulted in a decrease in the steady state level of endogenous Ago2 through proteasome-mediated degradation, but such decrease can be partially restored by transfecting the cells with siRNA duplex (Johnston et al. 2010). Taken together, these data suggest that Hsp90 stabilizes unloaded Argonaute and facilitates its binding to siRNA duplex. Interestingly, two groups independently observed that Ago2 localization to PBs was abrogated upon treatment of cells with HSP90 inhibitors (Johnston et al. 2010; Pare et al. 2009). This delocalization again, as noted previously, coincides with the low level of steady-state level of endogenous Argonaute (c.f. Sects. 10.2.1 and 10.4.2).

10.5 What Are the Possible Roles of PBs in miRNA Silencing?

Several lines of evidence suggest that PBs are not required for miRNA silencing. First, the majority of Argonaute, miRNAs and mRNA targets are diffusely distributed in the cytoplasm (Leung et al. 2006). Second, cells with or without microscopically

visible PBs are equally competent for miRNA silencing (Behm-Ansmant et al. 2006; Chu and Rana 2006; Eulalio et al. 2007). Third, different aspects of miRNA silencing can be reconstituted in vitro using cell extracts that are likely to be devoid of PBs (reviewed in (Standart and Jackson 2007)). So, what is the significance of PB localization of Argonaute, miRNAs and their mRNA targets?

In the context of miRNA silencing, PB most likely reflects the underlying network of interactions between different components in the pathway. Though equivalent interactions can probably occur elsewhere in the cytoplasm, the local concentration of factors that participate in related steps of miRNA silencing within a physical structure can potentially increase the overall efficiency. For example, PBs are enriched with RNA decay factors, including deadenylase Ccr4-Not complex, decapping enzyme complex and $5' \rightarrow 3'$ exoribonuclease Xrn1. The local concentration of these factors can facilitate the miRNA-mediated mRNA decay process. Yet, in many cases examined in *Drosophila* and human cells, disassembly of these microscopically visible structures does not result in any reduction in siRNA/miRNA-directed cleavage or miRNA-mediated repression both at protein and RNA level (Behm-Ansmant et al. 2006; Chu and Rana 2006; Eulalio et al. 2007). Therefore, the assembly of microscopically visible PBs does not likely confer additional kinetic advantage. Instead, miRNA silencing resulting in mRNA degradation likely occurs in the cytoplasm by submicroscopic complexes that are constantly exchanging with PBs and a majority of these complexes are distributed in the cytoplasm undergoing rapid diffusion.

The existence of such submicroscopic complexes is supported by biochemical fractionation of cell extracts prepared by lysis buffer containing digitonin (Pillai et al. 2005). The resultant pellet from high-speed ($14{,}000 \times g$) centrifugation of these extracts contains the majority of Argonaute, *let-7* miRNA and endogenous *let-7* targets *K-ras* and *N-ras* mRNAs, along with PB components Dcp1 and Xrn1. Yet, the properties of these submicroscopic complexes must be somehow altered upon/following their entry into PBs. As demonstrated by us and others, Ago2 exhibited very slow exchange at PBs, which was not observed in diffuse cytoplasm (Sects. 10.3.2 and 10.3.4). Apart from direct protein-protein interactions with other PB components, the slow-exchanging property of Argonaute proteins at PBs could be due to specific post-translation modifications (Sect. 10.4.2), which increase their binding affinities at PBs for mRNA degradation. Potentially, those Argonaute proteins retained at PBs can be released back to the cytoplasm in a regulated manner upon change in cellular conditions (as observed in neurons upon stimulation in Sect. 10.3.4).

There are precedents for PBs to act as storage repositories for specific transcripts in yeast and possibly in mammalian cells (Bhattacharyya et al. 2006; Brengues et al. 2005). Bhattacharya and colleagues previously reported that the translation of *CAT-1* mRNA is repressed by *miR-122*, but such repression is relieved upon amino acid starvation (Bhattacharyya et al. 2006). Correlated with this, a subpopulation of *CAT-1* mRNAs is localized at PBs when repressed by *miR-122* but de-localized from PBs when de-repressed. It is possible that those *CAT-1* mRNAs formally localized in PBs are relocated to polyribosomes upon amino acid starvation, given that such stress-induced translation can still occur in the presence of transcriptional

inhibitors. However, this conclusion is qualified only if the majority of *CAT-1* mRNA are localized in PBs at steady state. Another dilemma of this model is that neither poly(A)+mRNAs nor the poly(A) binding protein Pabpc1 are detected in PBs by in situ hybridization and immunostaining, respectively (Anderson and Kedersha 2006; Cougot et al. 2004). As Pabpc1 is highly abundant (8×10^6 molecules per cell) and it binds to a minimum tract of 5As (Gorlach et al. 1994), it is difficult to envisage that those miRNA-targeted mRNAs are not deadenylated upon entry to PBs (Sect. 10.2.3). Therefore, these transcripts would have to be protected from degradation following deadenylation in PBs and then undergo poly(A) addition by a cytoplasmic poly(A) polymerase, such as mammalian homologue of Gld-2, prior to translation.

Immunoprecipitation data showed that the associations between Argonaute proteins and other PB components, such as Dcp1a, rck/p54 and GW182, were due to protein-protein interactions, rather than through a common RNA scaffold (Chu and Rana 2006; Jakymiw et al. 2005; Liu et al. 2005a, b). Consistent with this, we observed that the PB association with Argonaute protein does not require miRNAs (Sect. 10.4.1). Since PBs do not contain either ribosomal subunits or initiation factors, one possible model of RNA silencing is that the stable association of Argonaute protein and PBs keeps the bound mRNAs repressed in such a translation-incompetent environment and destined for degradation. On the other hand, the dynamic exchange and distribution of Argonaute in the total cytoplasm suggests an alternative model that silencing at the stage of translation exists elsewhere in the cytoplasm independent of PBs.

10.6 Conclusions and Perspectives

In summary, though most of the miRNA action is likely to be carried out by submicroscopic complexes in the cytoplasm, the distinct kinetics of Argonaute and its associated proteins at PBs indicate roles in translational repression and degradation of mRNAs targeted by miRNAs. To further understand such roles in PBs, it will be important to quantitate the kinetics of protein components required for miRNA silencing in relation with their post-translational modification(s). In addition, efforts should be focused on biochemically purifying PBs such that a quantitative proteomic approach can be used to globally characterize the flux of the full complement in and out of this cytoplasmic organelle under different cellular conditions. Similar effort has previously been applied to the nucleolus, another membrane-less, RNA-rich organelle (Andersen et al. 2005).

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Chapter 11
Deadenylation and P-Bodies

Chyi-Ying A. Chen and Ann-Bin Shyu

Abstract Deadenylation is the major step in triggering mRNA decay and results in mRNA translation inhibition in eukaryotic cells. Therefore, it is plausible that deadenylation also induces the mRNP remodeling required for formation of GW bodies or RNA processing bodies (P-bodies), which harbor translationally silenced mRNPs. In this chapter, we discuss several examples to illustrate the roles of deadenylation in regulating gene expression. We highlight several lines of evidence indicating that even though non-translatable mRNPs may be prepared and/or assembled into P-bodies in different ways, deadenylation is always a necessary, and perhaps the earliest, step in mRNA decay pathways that enable mRNP remodeling required for P-body formation. Thus, deadenylation and the participating deadenylases are not simply required for preparing mRNA substrates; they play an indispensable role both structurally and functionally in P-body formation and regulation.

11.1 Introduction

Regulation of the abundance and translation of messenger RNAs (mRNAs) is important for controlling gene expression because mRNAs carry genetic information from the nucleus to the cytoplasm, where they can be translated into proteins. mRNAs associate with a number of proteins, and the resulting mRNA-protein complexes (mRNPs) undergo a series of remodeling events that impact and/or are influenced by the translation and mRNA decay machineries (Moore 2005; Shyu and Chen 2009). The components of most mRNPs are in dynamic equilibrium with the translational pool, allowing rapid shifts between translation, storage, and degradation (Balagopal and Parker 2009; Kedersha et al. 2005; Wilkinson and Shyu 2001).

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Thus, the metabolism and functions of mRNAs can be regulated through mRNP remodeling to accommodate various cellular processes.

Some protein components of particular mRNPs may promote assembly of microscopically visible, cytoplasmic granules, such as the GW or RNA processing bodies (P-bodies), which harbor translationally silenced mRNPs (Eystathioy et al. 2002, 2003; Sheth and Parker 2003; van Dijk et al. 2002). While many mRNA decay factors are linked to P-bodies, the recent finding that deadenylation (i.e., shortening of the 3′ poly(A) tail of mRNAs) is prerequisite for P-body formation has opened up new aspects of P-body dynamics and regulation (Zheng et al. 2008). Deadenylation is the major step triggering mRNA decay in eukaryotic cells (Chen and Shyu 2011). The poly(A) tail and associated poly(A)-binding protein (PABP) interacts with the 5′ m^7G-cap/cap-binding complex to form a closed loop that enhances translation initiation and protects the mRNA ends from nuclease attack (Jacobson 1996; Sachs 2000). Thus, deadenylation can impact an mRNA not only by inducing its degradation but also by reducing its translatability. Given that deadenylation serves as an important control point for both mRNA degradation and translational silencing, it is not surprising that regulation of deadenylation provides a key means of controlling eukaryotic gene expression. In this chapter, we discuss the importance of deadenylation in regulating gene expression and how deadenylation may impact P-bodies.

11.2 Deadenylation

The 3′ termini of all mRNAs except histone mRNAs contain a poly(A) tail. The length of the poly(A) tail plays a critical role in determining mRNA stability and translation efficiency (Jacobson and Peltz 1996; Wickens et al. 1997; Wilusz et al. 2001). The poly(A) tails of newly synthesized mRNAs entering the cytoplasm are 200–250 nucleotides (nt) long; the poly(A) tails subsequently undergo deadenylation at different rates until their length reaches 10–60 nt (Baker 1993; Brawerman 1981). Recent identification and characterization of at least eight distinct deadenylases in metazoa highlight the diverse biological functions of deadenylases, and thus of deadenylation in gene regulation (Dupressoir et al. 2001; Goldstrohm and Wickens 2008; Zuo and Deutscher 2001).

11.2.1 Deadenylation Is the Major Step Triggering mRNA Decay

RNA destabilizing elements exert their effects mainly by inducing accelerated deadenylation, thereby leading to rapid mRNA decay in mammalian cells (Chen and Shyu 2011). Computational modeling of eukaryotic mRNA turnover also suggests that changes in levels of mRNA are tightly linked to the rate of deadenylation (Cao and Parker 2001). Thus, deadenylation provides a critical point for regulation of gene expression. We propose that the deadenylation rate of any given mRNA in a

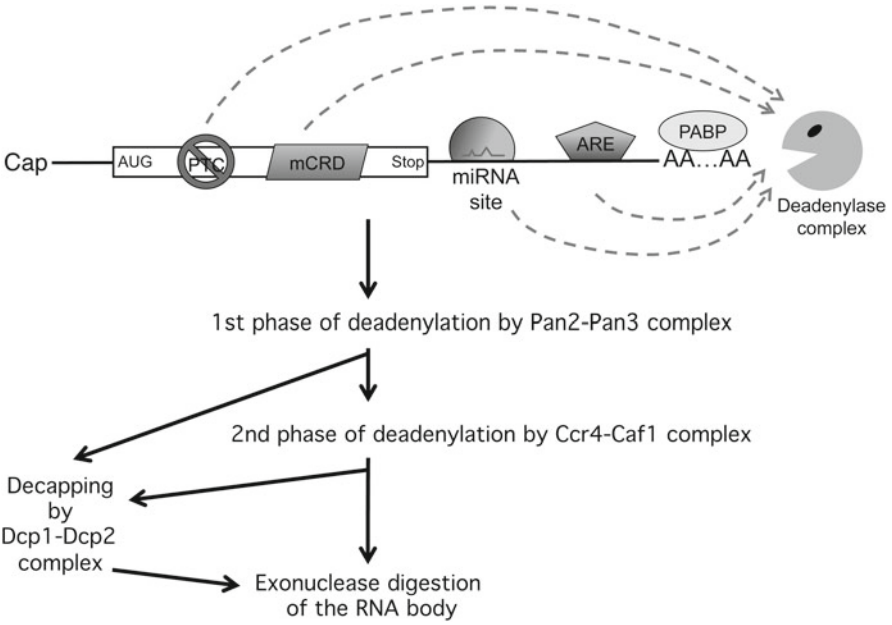

Fig. 11.1 Deadenylation and major mRNA decay pathways in mammalian cells. Sequence elements are depicted for nonsense-mediated decay (NMD) triggered by a premature termination codon (PTC), decay mediated by the c-*fos* coding-region determinant (mCRD), miRNA-mediated decay, and decay mediated by AU-rich elements (ARE). The poly(A) tail, the poly(A)-binding protein (PABP) and the deadenylase complexes are shown at the 3′ end. The four RNA destabilizing elements or mutations and their cognate binding complexes have distinct paths (shown as *dashed lines*) to recruit deadenylation machinery and thus accelerate deadenylation, which occurs in two phases. In mammalian cells, decapping does not occur until the end of the first or second phase of deadenylation. Note that the slow, default decay of stable mRNAs lacking recognized destabilizing elements is also triggered by deadenylation

specific cellular environment reflects interactions among several key components, including deadenylases, the poly(A)-PABP complex, mRNA element-binding complexes, and factors that modulate these interactions.

All major modes of mammalian mRNA decay observed thus far are triggered by deadenylation (Chen and Shyu 2011). These include decay directed by AU-rich elements (AREs) in the 3′ untranslated region (UTR) (e.g., (Chen et al. 1994; Shyu et al. 1991)), decay mediated by destabilizing elements in protein-coding regions (e.g., (Chang et al. 2004; Grosset et al. 2000)), decay of nonsense-containing mRNA (i.e., NMD) (Chen and Shyu 2003), the microRNA (miRNA)-mediated decay (miRMD) (Chen et al. 2009; Wu et al. 2006), and the default decay of stable messages lacking destabilizing elements (Fig. 11.1). Mammalian mRNA deadenylation involves two consecutive phases mediated by the Pan2-Pan3 and the Ccr4-Caf1 complexes, respectively (Fig. 11.1). Decapping takes place after deadenylation and may serve as a backup trigger for mRNA decay if initial deadenylation is compromised (Yamashita et al. 2005; Zheng et al. 2008). Compromising both Ccr4-Caf1

and Dcp2 activities essentially halts various modes of mRNA decay with concomitant accumulation of stable intermediates containing a ~110 nt poly(A) tail (Chen et al. 2009; Yamashita et al. 2005; Zheng et al. 2008). While it remains unknown as to whether deadenylation-independent decapping operates in mammalian cells, it is clear that the major route for mRNA decay in mammalian cells is triggered by deadenylation followed by decapping and 5′ to 3′ exonuleolytic digestion of the RNA body. Thus, even though the mechanisms for mRNA degradation differ in yeast and in mammals, the major mRNA decay pathway is highly conserved (Chen and Shyu 2003; Muhlrad and Parker 1994). As a process that is reversible, deadenylation may serve as an important checkpoint before an mRNA is committed to elimination, which makes deadenylation an important step for regulation of gene expression during a variety of biological processes, such as embryogenesis, cell growth, and cell differentiation.

11.2.2 The Role of Deadenylation in miRNA-Mediated Gene Silencing

Gene silencing is one mechanism to ensure that proteins are expressed at proper levels and miRNAs contribute to gene silencing mainly by accelerating deadenylation to promote rapid decay of their mRNA targets (Chen and Shyu 2011). Here, we discuss some new findings on the mechanism of miRNA-mediated mRNA decay to illustrate the importance of deadenylation in controlling eukaryotic gene expression.

miRNAs induce degradation of mRNA targets in many eukaryotic cells, including those from humans, *C. elegans*, *Drosophila*, and zebrafish (Bagga et al. 2005; Behm-Ansmant et al. 2006; Chen et al. 2009; Giraldez et al. 2006; Wu et al. 2006). Accumulating evidence from kinetic studies supports the idea that miRNAs destabilize target mRNAs through deadenylation and subsequent decapping and 5′ to 3′ exonucleolytic digestion (Chen et al. 2009; Piao et al. 2010). For example, poly(A) length assays indicated that miRNAs mediate deadenylation of a wide array of targets in a variety of systems. In zebrafish, miR-430 mediates the deadenylation of hundreds of maternal transcripts early in embryonic development (Giraldez et al. 2006). Results from a study using mouse P19 embryonic carcinoma cells (Wu and Belasco 2005) demonstrated that *lin-28* mRNA, whose levels decrease during retinoic acid-induced neuronal differentiation, is deadenylated through the action of miR-125, a miRNA whose levels increase during differentiation (Wu et al. 2006). Deadenylation mediated by miRNAs has also been demonstrated in mammalian and *Drosophila* cell-free extracts (Fabian et al. 2009; Iwasaki et al. 2009; Wakiyama et al. 2007).

The precursor-product relationships of mRNAs targeted by let-7 miRNA for degradation were directly demonstrated by using a transcriptional pulsing approach to analyze mRNA decay kinetics combined with a strategy to block specific endogenous decay enzymes (Chen et al. 2009; Wu et al. 2006). These studies were able to

trap mRNA intermediates during decay directed by let-7 via either miRNA-mediated decay or siRNA-mediated decay in mouse fibroblasts. The results showed that let-7 routes target mRNAs to the major cytoplasmic mRNA decay pathway, in which degradation of mRNA is triggered by deadenylation involving Pan2-Pan3 and Ccr4-Caf1 complexes followed by decapping via the Dcp1-Dcp2 complex (Chen et al. 2009). Moreover, tethering AGO proteins or GW182 protein, components of the miRNA-induced silencing complex (miRISC) required for gene silencing (Fabian et al. 2010), promoted decay of target mRNA by triggering rapid deadenylation (Chen et al. 2009). These observations indicate that promoting rapid decay by triggering deadenylation is an intrinsic property of miRISC in mammalian cells. Consistent with this notion is the observation that deadenylation is a widespread feature of miRNA regulation (Eulalio et al. 2009).

11.3 Deadenylation and P-Bodies

P-bodies are dynamic cytoplasmic foci in eukaryotic cells that contain non-translatable mRNAs as well as proteins involved in translational inhibition and mRNA decay (Eulalio et al. 2007a; Franks and Lykke-Andersen 2008; Kedersha and Anderson 2007; Parker and Sheth 2007). As deadenylation triggers mRNA degradation and also leads to inhibition of mRNA translation in eukaryotic cells, it is plausible that deadenylation induces the mRNP remodeling required for P-body formation. Although non-translatable mRNPs can follow different paths leading to assembly into P-bodies, several lines of evidence indicate that deadenylation is always a necessary and perhaps the earliest step that begins the mRNP remodeling required for P-body formation.

11.3.1 Deadenylation Is Required for P-Body Formation

The roles of deadenylation and its participating enzymes and factors in the formation of P-bodies were unclear until recently and thus were largely ignored for a long time after the cytoplasmic foci were discovered. Also contributing to earlier neglect of deadenylation were observations that P-bodies are enriched in factors involved in decapping and 5′ to 3′ decay and that deadenylases are not readily found in yeast P-bodies (Sheth and Parker 2003). Furthermore, an earlier study in yeast reported that deletion of the major deadenylase complex had much less effect on P-bodies than did deletion of factors related to decapping and 5′ to 3′ decay (Teixeira and Parker 2007). This led to the idea that deadenylation is not critical for P-body formation. However, observations that Ccr4 co-localizes with P-bodies and that siRNA-mediated knockdown of Ccr4 blocked P-body formation in HeLa human cells (Andrei et al. 2005; Cougot et al. 2004) hinted at an important role for deadenylation in P-body assembly. Yet, it was unresolved whether Ccr4 deadenylase

activity or just the Ccr4 protein was required for P-body formation. It thus remained unclear, especially in mammalian cells, whether deadenylation is important for P-body formation.

With the recent findings that deadenylation is the major step triggering mRNA decay in yeast and mammalian cells (Cao and Parker 2001; Chen and Shyu 2011), the impact of deadenylation on P-bodies can no longer be overlooked, especially as P-bodies have been found to contain many mRNA decay factors and the corresponding mRNA substrates (Eulalio et al. 2007a; Franks and Lykke-Andersen 2008; Kedersha and Anderson 2007; Parker and Sheth 2007). In 2008, Zheng et al. (2008) reported that P-bodies contain all the major mammalian deadenylases, including the Pan2-Pan3 and Ccr4-Caf1 complexes, thereby linking P-bodies with all major mRNA decay factors except the exosome components. Other results from the same study (Zheng et al. 2008) indicated that deadenylation is required for P-body formation in mammalian cells. First, impairment of deadenylation by knocking down Caf1 led to loss of P-bodies. Second, a dominant negative mutant of Caf1 inhibited both deadenylation and P-body formation, demonstrating that the effect on P-bodies involved loss of deadenylation activity rather than loss of the Caf1 protein per se. Further, co-expression of wild-type Ccr4 with the Caf1 mutant to rescue deadenylation activity restored P-body formation. In contrast, puromycin, which increases the pool of non-translatable mRNAs and thus promotes P-body formation (Cougot et al. 2004; Eulalio et al. 2007b; Yang and Bloch 2007) in control cells, did not induce P-body formation when deadenylation was blocked by expressing the Caf1 mutant or by knocking down Caf1.

Blocking deadenylation impairs P-body formation but the converse is not true. Evidence on this point came from the observation that knockdown of Pan3, a key component of P-bodies, impairs P-body formation but has little effect on deadenylation and decay of ARE-containing transcripts and miRNA targeted mRNAs (Zheng et al. 2008). Thus, deadenylation does not require P-body formation.

With a linkage established between deadenylation and P-body formation, it seems possible that poly(A)-shortened mRNAs are major components of mRNPs in P-bodies. Thus, although non-translatable mRNAs may arrive at P-bodies by different pathways, deadenylation is always required for P-body formation.

11.3.2 Deadenylation Factors Play Different Roles in P-Body Formation

siRNA-mediated gene knockdown experiments show that knocking down Pan3 impairs P-body formation (Zheng et al. 2008), indicating an essential role for Pan3 in P- body formation. Moreover, Pan3 helps enrich Pan2, Ccr4, and Caf1 in P-bodies (Zheng et al. 2008). In contrast, knocking down Pan2 had little effect on P-body formation (Zheng et al. 2008), indicating that Pan2 is dispensable for P-body formation. This is consistent with the observation that expression of a catalytically inactive mutant of Pan2 had no effect on P-bodies in mouse fibroblasts (Zheng et al. 2008).

siRNA-mediated knockdown of Caf1 inhibited P-body formation in NIH 3T3 cells (Zheng et al. 2008) and Ccr4 knockdown blocked P-body formation in HeLa cells (Andrei et al. 2005). Also, as mentioned in Sect. 11.3.1 above, over-expression of Caf1 catalytic mutant inhibited deadenylation and also blocked P-body formation; restoring deadenylation by co-expression of wild-type Ccr4 with the Caf1 mutant led to reappearance of P-bodies (Zheng et al. 2008). Collectively, these observations indicate that P-body formation in mammalian cells requires the deadenylase activity of the Ccr4-Caf1 complex.

It is noteworthy that even though the Ccr4p-Pop2p complex is the major deadenylase in yeast, deletion of either Ccr4p or Pop2p (yeast Caf1) had only a small effect on P-bodies (Teixeira and Parker 2007), suggesting that yeast and mammalian cells have different mechanisms for P-body formation. Consistent with this notion, it was reported that yeast, but not human, P-body components are more likely to contain Q/N-rich aggregation-prone regions (Reijns et al. 2008), which may help accumulate the associated mRNPs into P-bodies.

The observation that Pan3 is required for P-body formation in mammalian cells and its knockdown has little effect on deadenylation (Zheng et al. 2008) argues for an additional role of Pan3 in mammalian P-body formation. This is further substantiated by the finding that Pan3 greatly enhances the localization of other P-body components to P-bodies (Zheng et al. 2008). Thus, deadenylation and the participating deadenylases are not simply required for preparing mRNA substrates; they play an indispensable role both structurally and functionally in P-body formation and regulation.

11.3.3 Deadenylation Triggers mRNP Remodeling for P-Body Formation

Although P-bodies harbor translationally silenced mRNPs, formation of P-bodies is not simply a consequence of increasing the pool of untranslatable mRNAs. Instead, P-body formation requires an active deadenylation process. This requirement was demonstrated by experiments using puromycin, a translation inhibitor that releases ribosomes from mRNAs and enhances P-body formation (Cougot et al. 2004; Eulalio et al. 2007b; Yang and Bloch 2007). When deadenylation was inhibited by either overexpressing a Caf1 dominant-negative mutant or by knocking down Caf1, P-body formation was impaired with or without puromycin treatment (Zheng et al. 2008). This indicates that deadenylation does something more than simply enhancing P-body formation by rendering the mRNAs ribosome-free because the poly(A) tail is known to promote translation initiation (Franks and Lykke-Andersen 2008). Thus, even though an mRNA needs to be ribosome-free to enter or form P-bodies (Cougot et al. 2004; Sheth and Parker 2003), the importance of deadenylation in P-body formation cannot be simply explained as a block on recruitment of ribosomes to the mRNAs. Instead, deadenylation is required for a distinct process, which may involve dissociation of factors that could prevent mRNPs from joining P-bodies. One candidate for such a factor is PABP.

Through its interactions with various decay and translation factors in the eukaryotic cytoplasm, PABP plays a central role in mRNA turnover and in translation (Mangus et al. 2003). Several PABP-interacting proteins are reported to co-localize with P-bodies. For example, the interaction of PABP with Pan3 enhances Pan2 nuclease activity (Boeck et al. 1996; Mangus et al. 2004; Uchida et al. 2004). This suggests that the first phase of deadenylation mediated by the Pan2-Pan3 deadenylase complex is PABP-dependent. However, PABP itself is not a component of P-bodies (Kedersha et al. 2005; Zheng et al. 2008). Thus, it is possible that mRNPs go through the first phase of deadenylation and subsequent dissociation of PABPs before participating in P-body formation. An important implication is that association with PABPs may inhibit mRNPs from joining existing P-bodies or nucleating P-body formation. In this interpretation, deadenylation helps mRNPs become PABP-free for P-body assembly. This is consistent with the observation that mRNAs competent for translation normally carry a poly(A) tail that is long enough to associate with PABPs (Mangus et al. 2003).

Other factors that are important for efficient translation of mRNAs may also dissociate from mRNPs during the remodeling process triggered by deadenylation. For example, eIF4G, a translation activator, can simultaneously interact with both the cap-binding protein eIF4E and PABP to circularize poly(A)$^+$ mRNAs (Jacobson 1996; Sachs 2000). The resulting closed-loop conformation enhances translation initiation and provides an effective way to prevent mRNAs from degradation and P-body localization. Deadenylation would disrupt the binding sites for factors that stabilize the closed-loop structure, which increases the untranslatable pool of mRNPs and promotes P-body formation.

The mRNP remodeling triggered by deadenylation may also promote recruitment to the mRNP of translation repressors or other P-body components, such as decapping complexes (Coller and Parker 2004; Franks and Lykke-Andersen 2008). This notion is supported by the observation that deadenylation promotes the interaction between the poly(A)-shortened mRNAs and the Lsm1-7 complex, a decapping activator that contains some P-body components and prefers to bind the 3′ end of deadenylated mRNAs (Chowdhury and Tharun 2008). Based on these current observations, it is plausible that deadenylation triggers mRNP remodeling that is critical for P-body assembly.

11.3.4 Deadenylation May Occur in P-Bodies

Some observations raise a question as to whether deadenylation occurs in P-bodies. For example, both the Pan2-Pan3 and the Ccr4-Caf1 deadenylase complexes, the two main deadenylase complexes responsible for cytoplasmic poly(A)-shortening in eukaryotes, can co-localize with P-bodies of mammalian cells (Andrei et al. 2005; Cougot et al. 2004; Zheng et al. 2008). In yeast, both Ccr4p and Pop2p (yeast Caf1) co-localized with P-bodies when decapping or 5′ to 3′ mRNA decay was inhibited (Teixeira and Parker 2007). One interpretation of these observations is that

the Ccr4-Caf1 complex transits through P-bodies quickly and localizes to P-bodies under restricted conditions. Interestingly, the detection of poly(A)$^+$ mRNAs in yeast P-bodies during glucose deprivation and in stationary phase (Brengues and Parker 2007) suggests that some mRNAs in P-bodies have poly(A) tails that are longer than oligo(A). This is consistent with the observation that yeast mRNAs can be released from P-bodies and recruited to polysomes for translation in response to stress (Sheth and Parker 2006). One possibility is that some translationally inhibited poly(A)$^+$ mRNAs are temporarily stored in P-bodies; these mRNAs can subsequently be deadenylated, decapped, and degraded in P-bodies, or be released for translation before complete deadenylation.

11.4 A Model Linking Deadenylation and P-Body Formation

Based on the current information, we have devised a model for the linkage between deadenylation and P-body formation in mammalian cells (Fig. 11.2). The 3′ poly(A) tail-PABP complex stimulates poly(A) shortening by the Pan2-Pan3 complex but inhibits the activity of the Ccr4-Caf1 complex (Chen et al. 2002; Tucker et al. 2002). Thus, the first phase of deadenylation is initiated when PABPs on the mRNA poly(A) tail interact with Pan3 to recruit the Pan2 deadenylase. After the poly(A) tail is significantly shortened by Pan2, the remaining bound PABPs are less effective in inhibiting the deadenylase activity of the Ccr4-Caf1 complex, allowing the second phase of deadenylation to occur. During the first phase and/or early second phase of deadenylation, factors for efficient translation (such as PABP, eIF4G, ribosomes, etc.) may dissociate from the mRNPs, which would allow mRNPs to reversibly associate with P-bodies. At this stage, the mRNPs could either be released from P-bodies for translation or remain in the P-bodies to be further deadenylated by Ccr4-Caf1, resulting in recruitment of the Lsm1-7 complex and other decapping factors. The resultant mRNPs would constitute the core of P-bodies and their mRNA components would be decapped and degraded. Alternatively, the second phase of deadenylation mediated by Ccr4-Caf1 and the subsequent recruitment of decapping complex and related factors could occur outside P-bodies. In this case, the remodeled mRNPs would aggregate into P-bodies. Thus, it is possible that the mRNPs could not form the cores of P-bodies or aggregate into P-bodies when the deadenylase activity of Ccr4-Caf1 is inhibited. The proposed model helps explain why the deadenylation complexes co-localize with P-bodies, why deadenylation is required for P-body formation, why PABP, eIF4G, and ribosomes are not enriched in P-bodies, and why some mRNAs released from P-bodies can still be translated.

Future research addressing the key changes in mRNP composition at each stage of the life of an mRNA after it arrives in the cytoplasm will be crucial for understanding what targets an mRNP to P-bodies. Along this line, determining when and how PABPs dissociate from an mRNP, a particularly critical unresolved issue, may further elucidate the importance of mRNP remodeling and the link between deadenylation and P-bodies.

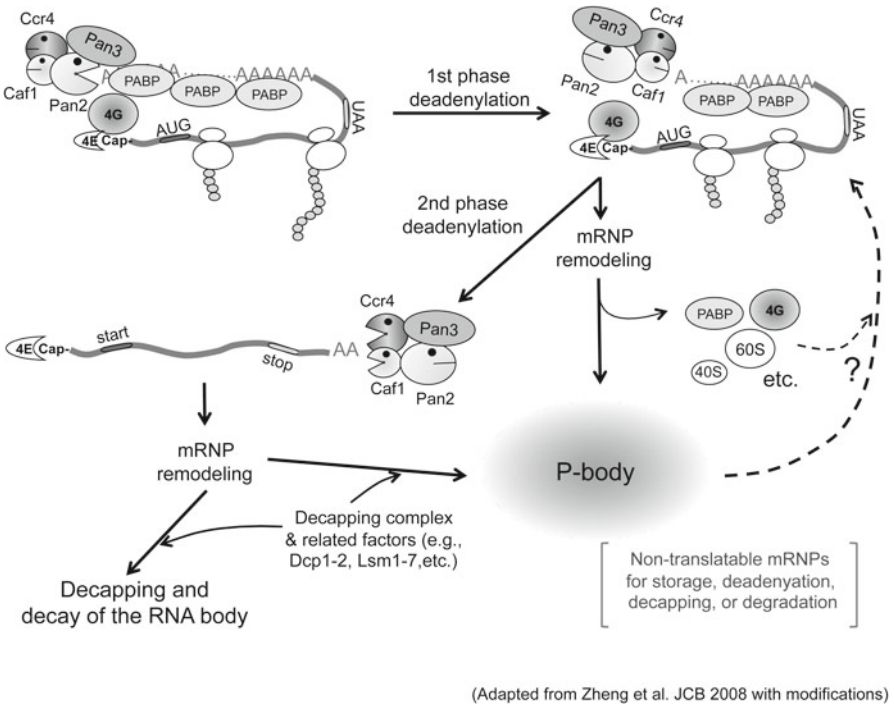

Fig. 11.2 Working model linking deadenylation and P-body formation

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Chapter 12
Relationship of GW/P-Bodies with Stress Granules

Georg Stoecklin and Nancy Kedersha

Abstract Whereas P-bodies are intimately linked to the cytoplasmic RNA decay machinery, stress granules harbor stalled translation initiation complexes that accumulate upon stress-induced translation arrest. In this Chapter, we reflect on the relationship between P-bodies and stress granules. In mammalian cells, the two structures can be clearly distinguished from each other using specific protein or RNA markers, but they also share many proteins and mRNAs. While the formation of P-bodies and stress granules is coordinately triggered by stress, their assembly appears to be regulated independently by different pathways. Under certain types of stress, P-bodies frequently dock with stress granules, and overexpressing certain proteins that localize to both structures can cause P-body/stress granule fusion. Currently available data suggest that these self-assembling compartments are controlled by flux of mRNAs within the cytoplasm, and that their assembly mirrors the translation and degradation rates of their component mRNAs.

12.1 Stress Granules Assemble When Translation Initiation Is Stalled

While P-bodies (PBs) assemble around the key enzymes of cytoplasmic RNA degradation, stress granules (SGs) assemble around essential components of the translation machinery. Heat shock or heat stress granules, characterized as reversible aggregates

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of ribonucleoprotein complexes containing untranslated mRNA, were initially described in 1989 in tomato cell cultures (Nover et al. 1989). In the late 1990s, reversible aggregates of mRNPs were "re-discovered" in mammalian cells (Kedersha et al. 1999) and dubbed mammalian stress granules to acknowledge the presumed connection to the plant studies. Ironically, it was recently reported that the original tomato heat stress granules do not contain mRNA after all (Weber et al. 2008) although plants can also assemble both SGs and PBs. Thus, in hindsight, the first descriptions of "modern" SGs are relatively recent (Kedersha et al. 1999, 2000, 2002).

Mammalian SGs were originally defined as large cytoplasmic mRNA aggregates that become microscopically visible when global protein synthesis is inhibited in response to different types of stress. The original definition was updated upon discovering that SGs are aggregates of stalled or abortive preinitiation complexes and associated RNA-binding proteins (RNA-BPs). Heat shock, oxidative stress, viral infection, UV irradiation, or energy depletion all cause polysomes to disassemble, owing to the inhibition of translation initiation while elongation and termination rates remain normal (Fig. 12.1a, b). Blocked initiation is most commonly driven by the phosphorylation of the translation initiation factor eIF2, a trimeric GTP-binding protein that delivers initiator $tRNA_i^{Met}$ to the small 40S ribosomal subunit (Holcik and Sonenberg 2005). eIF2 thereby allows the initiating 40S subunit within the 48S pre-initiation complex to scan the beginning of the mRNA for the AUG start codon. When phosphorylated by one of four stress-responsive kinases on its α-subunit, eIF2 no longer dissociates from its GDP exchange factor eIF2B, and thus cannot be recharged with $tRNA_i^{Met}$.

The arrest of translation initiation causes ribosomes to run off their mRNAs and 48S pre-initiation complexes to accumulate (Fig. 12.1b). In a subsequent step, stalled pre-initiation complexes can then form large aggregates that become microscopically visible as SGs (Fig. 12.1c). Accordingly, SGs contain poly(A)-mRNA, 40S, but not 60S ribosomal subunits, as well as most translation initiation factors such as eIF3, eIF4A, eIF4E, eIF4G, and the cytoplasmic poly(A)-binding protein (PABP) (Kedersha et al. 2002; Kimball et al. 2003). The use of different translation inhibitors that either freeze or disassemble polysomes suggested that mRNAs in SGs are not static, but rather remain in a dynamic equilibrium with polysomal mRNA (Kedersha et al. 2000). Photobleaching studies have directly confirmed that the mRNPs within SGs are indeed in a highly dynamic flux (Kedersha et al. 2000, 2005; Mollet et al. 2008). In addition to components of the translation initiation apparatus, numerous RNA-BPs accumulate in SGs including PABP, TIA1, TIAR, FMRP, FXR1, and G3BP (Kedersha et al. 1999, 2002; Tourriere et al. 2003; Mazroui et al. 2002). The TIA proteins and G3BP contain aggregation-prone domains, which participate in the aggregation process that underlies SG assembly (Gilks et al. 2004; Tourriere et al. 2003). Ataxin-2, a protein that interacts with PABP, is also involved in SG formation (Nonhoff et al. 2007). Moreover, posttranslational modifications such as the dephosphorylation of G3BP (Tourriere et al. 2003) and the conjugation of O-linked N-acetylglucosamine to ribosomal proteins (Ohn et al. 2008) are important for SG assembly. Nevertheless, the molecular details of the actual aggregation process during SG formation are not well understood.

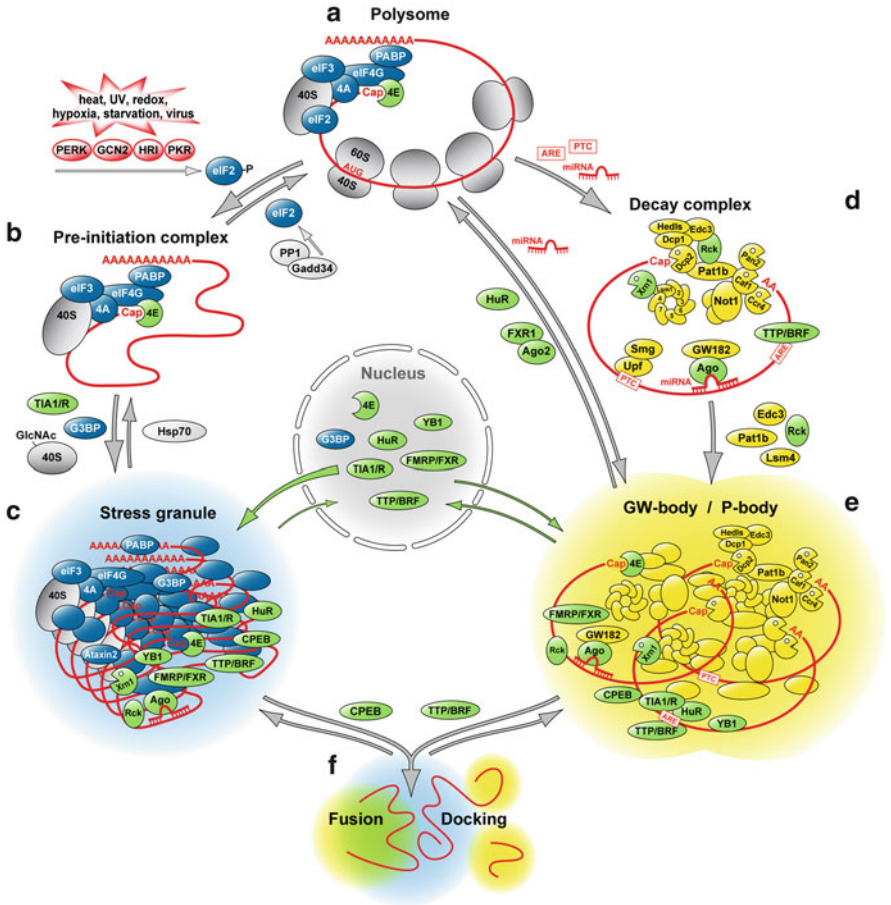

Fig. 12.1 Scheme of mRNP complexes forming stress granules and P-bodies. (**a**) Actively translating mRNAs are capped, polyadenylated and form polysomes. (**b**) Under conditions of severe stress, global mRNA translation is inhibited by phosphorylation of eIF2 through activation of stress-responsive kinase PERK, GCN2, HRI or PKR. As a consequence, polysomes disassemble, and translation pre-initiation complexes accumulate. The protein phosphatase PP1 together with its regulatory subunit Gadd34 dephosphorylates eIF2 and thereby re-activates translation. (**c**) RNA-binding proteins such as TIA1 and G3BP contain aggregation-prone domains that drive the assembly of stalled pre-initiation complexes into cytoplasmic stress granules. The conjugation of N-acetylglucosamine (GlcNAc) to ribosomal proteins is also important for stress granule assembly. When stressful conditions are overcome, the chaperone Hsp70 facilitates disassembly of stress granules. (**d**) mRNAs are specifically degraded if they have acquired a premature termination codon (PTC), associate with miRNAs or contain AU-rich elements (AREs). Such mRNAs are either cleaved by an endonuclease or subject to rapid deadenylation, which induces decapping and degradation through the 5′-3′ exoribonuclease Xrn1. (**e**) mRNAs targeted for decay together with key enzymes of cytoplasmic RNA degradation assemble in processing (P)-bodies. The RNA helicase Rck and aggregation-prone proteins such as Pat1, Edc3 and Lsm4 are important for P-body formation. miRNAs can also suppress the translation of target mRNAs by recruiting them to P-bodies, from where such mRNAs can also exit and re-engage in translation. (**f**) P-bodies are frequently observed in close physical contact to stress granules as if they were docking. The overexpression of certain RNA-binding proteins further enhances the association between P-bodies and stress granules, and causes fusion of the two structures. Such activity is observed for the cytoplasmic polyadenylation element binding protein (CPEB1) as well as for tristetraprolin (TTP) and butyrate response factor-1 (BRF1), two zinc finger proteins that accelerate the degradation of ARE-containing mRNAs. Stress granule proteins are shown in *blue*, P-body proteins in *yellow*, and proteins that localize to both structures are shown in *green*

12.2 Stress Granules and P-Bodies Are Distinct Structures

In mammalian cells, SGs can be clearly distinguished from PBs, although both contain non-polysomal mRNPs. PBs are formed from mRNAs targeted for degradation (Sheth and Parker 2003; Cougot et al. 2004; Franks and Lykke-Andersen 2007) (Fig. 12.1d), and PB assembly is driven by a distinct set of aggregation-prone proteins that include Lsm4, Edc3 and Pat1b (Ozgur et al. 2010; Teixeira and Parker 2007; Decker et al. 2007) (Fig. 12.1e). By light microscopy, small numbers of PBs are detected in most somatic cells under normal conditions (Bashkirov et al. 1997; van Dijk et al. 2002; Ingelfinger et al. 2002; Eystathioy et al. 2002), whereas SGs only emerge in response to severe translation arrest induced by stress or energy starvation (Kedersha et al. 1999, 2000). Electron microscopy of SGs and PBs (Souquere et al. 2009; Yang et al. 2004; Gilks et al. 2004) confirms the general observations made at the light level: PBs exhibit a compact, dense substructure that may contain a fibrillar component, while SGs are larger, more irregular, looser and rather granular structures that often contain small regions of cytoplasm. Importantly, both structures lack any limiting membrane.

PBs can be distinguished from SGs because numerous proteins are specific for either of the two structures (Kedersha and Anderson 2009). On the SG side, translation factors (such as eIF3b, eIF4A, eIF4G) and RNA-BPs such as PABP and G3BP can be used as specific markers (Kedersha et al. 1999, 2002, 2005; Tourriere et al. 2003). On the PB side, components of the cytoplasmic RNA degradation machinery such as Dcp2, Dcp1 or Hedls serve as reliable marker proteins (van Dijk et al. 2002; Kedersha et al. 2005; Ozgur et al. 2010). GW-bodies share many proteins with PBs, yet seem to preferentially form around miRNA effector proteins such as GW182 and Argonaute (Eystathioy et al. 2002). Since PBs and GW-bodies are morphologically not distinct in many cases (Liu et al. 2005a, b), we will treat them as one entity in this Chapter.

Although PBs and SGs can contain the same species of mRNA (Kedersha et al. 2005), the two compartments differ with regard to the state of the mRNA they contain: In SGs, mRNAs are polyadenylated and can be easily visualized by in situ hybridization with oligo-dT probes (Kedersha et al. 1999, 2000). Moreover, the presence of eIF4G and PABP suggests that SG-associated mRNAs might be circularized. In contrast, mRNAs in PBs lack a poly(A) tail, and deadenylation is thought to be an early step in the assembly of PBs, or in the recruitment of mRNAs to pre-existing PBs (Zheng et al. 2008). This difference indicates that mRNAs in SGs are translationally stalled, but not subject to immediate degradation. In PBs, however, most mRNAs seem to be specifically primed for decay (Sheth and Parker 2003, 2006; Franks and Lykke-Andersen 2007; Cougot et al. 2004).

12.3 Parallels Between P-Bodies and Stress Granules

Despite important differences, PBs and SGs also have several features in common: (1) They are both cytoplasmic, seemingly amorphous RNA-protein aggregates that are not surrounded by any membrane as determined by electron microscopy

(Gilks et al. 2004; Eystathioy et al. 2002; Souquere et al. 2009), (2) both are induced by stress conditions (Kedersha et al. 1999; Teixeira et al. 2005; Raaben et al. 2007; Wilczynska et al. 2005), (3) growth in size of both SGs and PBs depends on retrograde transport along microtubules (Loschi et al. 2009), (4) both SGs and PBs are in exchange with polysomes and contain translationally stalled mRNAs that can re-engage in translation (Bhattacharyya et al. 2006; Kedersha et al. 2000; Cougot et al. 2004), and (5) there is a large number of proteins, listed in Table 12.1, which localize to both PBs and SGs.

Proteins commonly observed in both compartments include the cytoplasmic cap-binding protein eIF4E (Kedersha et al. 2005; Andrei et al. 2005), the RNA helicase Rck (Wilczynska et al. 2005), which is involved in suppressing translation, and the argonaute proteins Ago1 and Ago2 (Sen and Blau 2005; Leung et al. 2006; Pare et al. 2009; Gallois-Montbrun et al. 2007) that play a central role in miRNA- and siRNA-induced silencing of mRNA expression. Several other RNA-BPs that localize to both PBs and SGs are known to control either the stability or translation rate of their target mRNAs: the cold shock domain containing nucleic acid-binding protein YB1 (Yang and Bloch 2007), the poly(rC)-binding protein PCBP2 (Fujimura et al. 2008), Roquin—an RNA-BP important for suppressing autoimmunity (Athanasopoulos et al. 2010; Glasmacher et al. 2010), Smaug—an RNA-BP essential in embryonic development (Eulalio et al. 2007; Baez and Boccaccio 2005), and CPEB1—an RNA-BP that controls cytoplasmic mRNA polyadenylation during oocyte maturation (Wilczynska et al. 2005). Dual localization was also reported for several RNA-BPs with affinity to AU-rich elements (AREs), regulatory elements that typically destabilize mRNAs or repress their translation. This includes the zinc finger proteins TTP and BRF1—both enhancers of mRNA degradation (Kedersha et al. 2005), the translation repressors TIA1 and TIAR (Kedersha et al. 1999, Fig. 12.2 and N.K., unpublished observations), FMRP and FXR1 (Vasudevan and Steitz 2007; Mazroui et al. 2002; Didiot et al. 2009; Kim et al. 2006), as well as HuR, typically a stabilizer and activator of mRNA translation (Gallouzi et al. 2000 and N.K., unpublished observations). Surprisingly, the 5′–3′ exoribonuclease Xrn1 not only localizes to PBs, where most mRNA decay enzymes are concentrated, but small amounts of Xrn1 can also be detected in SGs (Bashkirov et al. 1997; Kedersha et al. 2005). Does the localization of Xrn1 suggest that mRNA decay may also occur in SGs? For the bulk of mRNAs, this is unlikely. First, many mRNAs are stabilized under stress conditions in both yeast and mammalian cells (Fan et al. 2002; Hilgers et al. 2006; Bollig et al. 2002). Second, mRNAs in SGs have retained their poly(A) tails (Kedersha et al. 1999), suggesting that they are protected from deadenylation, which is generally the first step of mRNA degradation. Third, Xrn1 cannot degrade capped mRNA, and the decapping enzyme Dcp2 does not localize to SGs. Hence, SGs appear to serve as sites where mRNAs are protected from degradation. Two RNA-BPs, HuR and ZBP1, were in fact proposed to play an active role in stabilizing mRNAs in SGs (Gallouzi et al. 2000; Stohr et al. 2006).

So what might be the function of Xrn1 in SGs? Interestingly, the miRNA- and siRNA associated endonoclease Ago2 was found to move from PBs to SGs in response to stress (Leung et al. 2006), and it requires miRNA in order to do so. Thus, it is conceivable that the 3′ fragment generated by siRNA-induced cleavage of an mRNA is degraded by Xrn1 at SGs.

Table 12.1 Proteins common to P-bodies and stress granules

Protein	Function	References
Ago1	Argonaute-1, component of the RNA-induced silencing complex	Sen and Blau (2005); Leung et al. (2006)
Ago2	Argonaute-2, component of the RNA-induced silencing complex, endonuclease activity associated with siRNAs	Sen and Blau (2005); Leung et al. (2006); Pare et al. (2009); Gallois-Montbrun et al. (2007)
APOBEC3G	Cytidine deaminase involved in RNA editing, antiviral function	Wichroski et al. (2006); Kozak et al. (2006); Gallois-Montbrun et al. (2007)
CPEB1	Cytoplasmic polyadenylation element-binding protein, inhibitor of translation, overexpression causes fusion of PBs and SGs	Wilczynska et al. (2005)
eIF4E	Translation initiation factor, cap-binding protein	Kedersha et al. (2005); Andrei et al. (2005)
FAST	TIA1-interacting protein, splicing regulator, antiapoptotic and pro-inflammatory, mostly in PBs, some in SGs	Kedersha et al. (2005)
FMRP/FXR	RNA-BPs involved in translational control of specific mRNAs	Vasudevan and Steitz (2007); Mazroui et al. (2002); Didiot et al. (2009); Kim et al. (2006)
HuR	RNA-BP, enhances mRNA stability and regulates translation	Gallouzi et al. (2000) and N.K., unpublished observations
Importin 8	Required for import of Ago2 into nucleus	Weinmann et al. (2009)
Lin28	RNA-BP, blocks let7 miRNA processing	Balzer and Moss (2007)
Lsm14	Also Rap55, SCD6 (*S. cerevisiae*), Tral (*D. melanogaster*), CAR-1 (*C. elegans*), involved in cytokinesis and endoplasmic reticulum organization	Yang et al. (2006)
MEX3B	RNA-BP, regulator of mRNA translation and germline development in *C. elegans*	Courchet et al. (2008)
Musashi1	RNA-BP, neuronal stem cell maintenance	Kawahara et al. (2008)
PCBP2	Also hnRNP-E2, αCP2, poly(rC)-binding protein, involved in control of mRNA stability and translation	Fujimura et al. (2008)
Rck	Also DDX6, Dhh1 (*S. cerevisiae*), Me31B (*D. melanogaster*), SGH-1 (*C. elegans*), RNA helicase involved in mRNA translation	Wilczynska et al. (2005)
Roquin	RNA-BP and suppressor of autoimmunity, enhances mRNA decay	Athanasopoulos et al. (2010); Glasmacher et al. (2010)
Smaug	RNA-BP, control of mRNA translation and decay	Eulalio et al. (2007); Baez and Boccaccio (2005)

(continued)

Table 12.1 (continued)

Protein	Function	References
TIA1/TIAR	RNA-BPs, alternative splicing and inhibition of mRNA translation	Kedersha et al. (1999) Fig. 12.2 and N.K., unpublished observations
TTP/BRF1	RNA-BPs, enhance mRNA decay, overexpression causes fusion of PBs and SGs	Kedersha et al. (2005)
Xrn1	Exoribonuclease 1, cytoplasmic 5′-3′ exoribonuclease, mostly in PBs, some in SGs	Bashkirov et al. (1997); Kedersha et al. (2005)
YB1	RNA and DNA-BP, involved in transcription, translation and mRNA stability	Yang and Bloch (2007)

12.4 Docking and Fusion of P-Bodies with Stress Granules

With certain types of stress, one can frequently observe PBs grouped around SGs. For instance, treatment of various human cell lines with sodium arsenite, an inhibitor of the citric acid cycle and inducer of oxidative stress, causes many PBs to cluster around SGs (Wilczynska et al. 2005; Kedersha et al. 2005; Souquere et al. 2009). Similarly, the mitochondrial poison FCCP causes PBs to group around SGs in certain cell types (Fig. 12.2). In contrast, heat shock or clotrimazol, which induces energy starvation by displacing hexokinase from the mitochondrial outer membrane, induce SGs but do not cause SG-PB association. SGs induced by overexpression of SG components display transient contacts with PBs, as shown by live imaging. PBs appear to dock with SGs as they touch them for short periods of time (min) before they separate again (Kedersha et al. 2005). When examined by electron microscopy, PBs in close proximity to arsenite-induced SGs remained distinct: PBs retain their dense structure as opposed to the more granular appearance of SGs (Souquere et al. 2009).

Contacts between PBs and SGs can be dramatically stabilized by increasing the expression levels of specific proteins (Fig. 12.1f). Overexpression of TTP or BRF1, two RNA-BPs that can both target ARE-containing mRNAs to PBs for degradation (Franks and Lykke-Andersen 2007), causes tight clustering of PBs around and within SGs (Kedersha et al. 2005). Interestingly, this phenomenon is linked to the activity of these proteins: When TTP is phosphorylated at two specific serine residues that cause binding of 14-3-3 adaptor proteins, its mRNA destabilizing activity is reduced, and TTP no longer localizes to SGs (Stoecklin et al. 2004). Likewise, clustering of PBs around SGs is disrupted when TTP is phosphorylated (N.K., unpublished data). One way to interpret these data is that active TTP/BRF1 recruits stalled mRNPs in SGs for translocation into PBs. If this happens at a high rate upon overexpression of TTP/BRF1, the two compartments may literally fuse. Indeed,

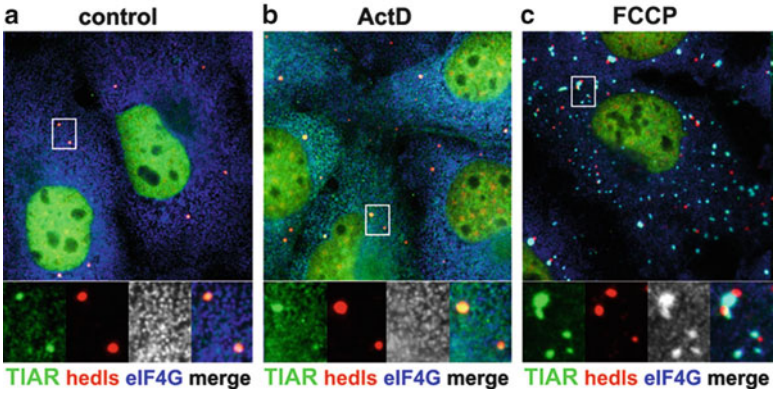

Fig. 12.2 Immunofluorescence micrograph of P-bodies docking to stress granules. African green monkey COS7 kidney cells were (**a**) grown under normal conditions, (**b**) exposed to 5 μg/mL actinomycin D (ActD) for 1 h, or (**c**) treated with carbonyl cyanide p-trifluoromethoxyphenylhydra-zone (FCCP) in glucose-free media for 1 h. Cells were then fixed and stained for TIAR (*green*), the stress-granule specific marker protein eIF4G (*blue*), and the P-body-specific protein Hedls/GE-1 (*red*). Actinomycin D treatment does not induce stress granules but causes TIAR accumulation at P-bodies, whereas FCCP treatment triggers stress granule formation and promotes docking of P-bodies with stress granules

live microscopy reveals that PBs no longer dissociate from SGs in cells overexpressing TTP or BRF1 (Kedersha et al. 2005).

Another dual SG/PB protein, CPEB1, can also increase contacts between PBs and SGs. When CPEB1 is overexpressed, PBs are found to tightly cluster around SGs (Wilczynska et al. 2005). In some cells, PB components were observed to completely redistribute into SGs, effectively abolishing the distinction between the two compartments (Wilczynska et al. 2005). A similar observation was made after inhibiting the expression of the RNA helicase Rck. Knock down of Rck leads to the loss of PBs and, in stressed cells, causes the PB protein Dcp1 to re-localize in SGs (Serman et al. 2007). These examples of tight PB/SG clustering induced by manipulating the expression of a defined group of proteins argue that contacts between PBs and SGs do not simply result from random collisions of moving entities. Rather, it is tempting to speculate that specific proteins and/or RNAs are exchanged between PBs and SGs during such contacts.

What could be the molecular basis of PB-SG docking and fusion? One possibility is that an increased flux of mRNPs from PBs to SGs, or vice versa, may cause docking or fusion of the two compartments. The TTP/BRF1 proteins might act in this way by promoting the transfer of mRNPs from SGs to PBs for rapid deadenylation and degradation. Since TTP associates with polysomes (Brooks et al. 2002), but also binds to proteins of the decapping complex (Fenger-Gron et al. 2005), it could form transient bridges between stalled 48S-complexed mRNAs in SGs and core proteins in PBs. This model is consistent with photobleaching studies indicating that the interactions of TTP with SGs are very fleeting (Kedersha et al. 2005).

CPEB1 might also cause SG-PB fusion by increasing the flux of mRNPs between the two compartments. CPEB proteins recognize cytoplasmic polyadenylation elements within the 3′UTR of mRNAs, and by interacting with a poly(A) polymerase, mediate cytoplasmic polyadenylation of mRNAs both in germline and somatic cells (Richter 2007). Given that CPEB1 localizes to PBs in unstressed somatic cells, one may speculate that CPEB1 re-adenylates mRNAs in PBs. CPEB1 might thereby rescue mRNAs from degradation in PBs and promote their transfer to SGs, which could cause PB-SG fusion.

A second mechanism to explain PB-SG docking or fusion is that scaffold proteins within PBs and SGs may co-aggregate. Such co-aggregation could occur when chaperones that normally assist in dissolving aggregates, e.g., chaperones of the Hsp70 family, become limiting. Elevated expression of chaperones during heat shock could explain why SG-PB docking or fusion is typically not seen under these conditions (Kedersha et al. 2005).

A third possibility is that the cytoskeleton might connect PBs with SGs. Indeed, both SGs and PBs can be associated with the microtubule network, and motor proteins were found to influence both the assembly and movement of SGs and PBs (Loschi et al. 2009; Aizer et al. 2008). Extensive SG-PB docking induced by some mitochondrial poisons such as FCCP (Fig. 12.2) could indicate that an energy-dependent, motor-driven step is needed for their separation.

12.5 Mammalian Stress Granules and P-Bodies Form Independently

The ability of PBs and SGs to dock and fuse under certain conditions raises the question whether the two compartments are related. Since PBs exist in unstressed cells lacking SGs, it is clear that “basal” PBs do not depend on SGs for their assembly. Additional genetic data provided clear evidence that stress-induced PBs form independently of SGs. Mouse embryonic fibroblast (MEFs) expressing a non-phosphorylatable mutant of eIF2α do not form SGs under conditions of arsenite-induced oxidative stress, whereas PBs are induced several fold (Kedersha et al. 2005).

The reverse question is whether SGs are formed out of PBs under conditions of severe stress. Using video microscopy of mammalian cells, we do not observe SGs emerging at or growing out of PBs (N.K., unpublished observations), whereas other labs report that some but not all SGs appear to grow out of preexisting PBs (Mollet et al. 2008). This apparent contradiction may be due to the different marker proteins used: the Mollet study used stably expressed GFP-CPEB in HeLa cells as their SG marker, whereas our studies used stably expressed GFP-G3BP in U2OS cells. Since CPEB is present in both SGs and PBs (Wilczynska et al. 2005), its overexpression possibly enhances the interaction between nascent SGs and PBs, which G3BP does not do. Regardless, both studies agree that some SGs can and do arise independently of PBs.

Another way to address this question is to test whether SGs would still form in the absence of PBs. This experiment, however, turns out to be challenging. Knock down of Lsm4 abolishes PBs under normal growth conditions, but does not prevent the formation of either PBs or SGs under stress conditions (Kedersha et al. 2005). A surprising effect is observed with Rck, an RNA helicase generally viewed as an inhibitor of translation. Reducing expression levels of Rck very efficiently prevents the assembly of PBs under normal conditions, whereas in stressed cells, Rck knock down causes the PB-specific protein Dcp1 to re-localize in SGs (Serman et al. 2007). This would suggest that SGs do form in the absence of PBs, but that PB proteins have a tendency to co-aggregate with SG proteins when they lack factors required for canonical PB assembly. Again, the distinction between PBs and SGs becomes blurred once the experimenter starts to manipulate important regulators of translation and mRNA decay.

12.6 Con-Fusion with P-Bodies, EGP-Bodies and Stress Granules in Yeast

S. cerevisiae also forms SG-like aggregates in response to glucose deprivation (Buchan et al. 2008; Hoyle et al. 2007) or heat shock (Grousl et al. 2009). When induced by glucose deprivation, these aggregates are more accurately termed EGP-bodies because they contain eIF4E, eIF4G and Pab1, the PABP ortholog in yeast (Hoyle et al. 2007), but lack eIF3 and small ribosomal subunits, both of which are present in stalled 48S pre-initation complexes, which define metazoan SGs. Yeast EGP-bodies share similarities with mammalian SGs in that they contain polyadenylated mRNA, eIF4E, eIF4G1, eIF4G2 and Pab1, as well as Pub1, Ngr1 and Pbp1, the yeast orthologs of mammalian TIA1, TIAR and ataxin-2, respectively. The assembly of yeast EGP-bodies upon glucose removal depends on Pub1 and Pbp1 (Buchan et al. 2008), similar to the importance of the two respective orthologs, TIA1 and ataxin-2, for mammalian SG formation (Gilks et al. 2004; Nonhoff et al. 2007). There are, however, important differences between yeast EGP-bodies and mammalian SGs. eIF3 subunits are defining components of mammalian SGs (Kedersha et al. 2005), whereas eIF3 is absent from yeast EGP-bodies induced by glucose deprivation (Buchan et al. 2008; Hoyle et al. 2007). Moreover, formation of yeast EGP-bodies does not require eIF2α phosphorylation. Interestingly, severe heat shock was reported to cause eIF3 to localize to "yeast SGs" (Grousl et al. 2009), but the relationship between glucose starvation-induced EGP-bodies and heat shock-induced "yeast SGs" remains to be elucidated. Based on the localization of ribosomal protein Rps30A (Grousl et al. 2009), 40S ribosomal subunits were also proposed to localize to heat shock-induced "yeast SGs." However, this remains to be verified, and in stark contrast to mammalian SGs, there is no clear evidence that "yeast SGs" contain either ribosomal subunits or stalled pre-initiation complexes.

What adds to the confusion is that yeast EGP-bodies show a much closer spatial connection to PBs. After glucose deprivation, about half of all EGP-bodies in yeast

overlap with PBs, whereas the remaining ones do not contain PB markers (Buchan et al. 2008; Hoyle et al. 2007). Moreover, the formation of EGP-bodies is clearly stimulated by factors that are important for PB formation such as Edc3, Lsm4, Pat1 and Dhh1, the Rck ortholog in yeast (Buchan et al. 2008). The authors of this study also observed that both PBs and EGP-bodies become larger and more numerous in yeast strains lacking Dcp1 or Xrn1. These data indicate that EGP-bodies are closely related to PBs in budding yeast. Based on live imaging, one study came to the conclusion that EGP-bodies form independently of PBs (Hoyle et al. 2007), whereas another study observed that EGP bodies form next to and require pre-existing PBs (Buchan et al. 2008). The difficulties of distinguishing between "yeast SGs," EGP-bodies and PBs in *S. cerevisiae* suggests that mRNP aggregates may in fact have a variable composition. According to this model, each granule is positioned within a continuum that ranges from a typical PB containing mRNA decay factors to a canonical SG comprised of stalled translation pre-initiation complexes.

12.7 Conclusions

Stress-induced assembly of SGs and PBs represents a profound reorganization of the cytoplasm. Both structures illustrate the cell's ability to form compartments by means of transient protein aggregation. While SGs form as a result of stress-induced translation arrest and polysome disassembly, SG formation itself is not required for the inhibition of translation. Likewise, PBs harbor almost all enzymes of the basic RNA degradation machinery in the cytoplasm, yet the assembly of microscopically visible PBs is not required for mRNA degradation. Rather, SGs and PBs create cytoplasmic domains at which mRNPs remain partially assembled, possibly allowing their mRNAs to re-engage in translation more efficiently once stressful conditions are overcome. Contacts between PBs and SGs are not random collisions, but result from the specific activity of RNA-BPs exemplified by TTP, CPEB and Rck. Docking and fusion of PBs with SGs could be the result of enhanced trafficking of mRNPs between the two compartments. An important task for future research will be to visualize trafficking at the level of single mRNPs.

In addition to being hubs of mRNP trafficking, PBs and SGs may serve as platforms that allow integration of signaling events within the cytoplasm. The sequestration of signaling molecules such as RACK1 and TRAF2 in SGs indicates a role of SGs in signal transduction (Arimoto et al. 2008; Kim et al. 2005). Given the tight link between SGs, PBs and stress, we envision that protein aggregation at PBs and SGs helps in coordinating the various processes that together shape an integrated stress response: the adjustment of mRNA and protein expression to a survival mode, the re-direction of energy resources and metabolites to damage control, the activation of catabolic programs to cope with periods of starvation, and the timing as to when cell death should be induced in the event that all other strategies fail. Exploring the broader role of PBs and SGs as part of integrated stress responses will require careful dissection of the localization, activity, interactoins and functions of individual proteins that are associated with PBs and SGs.

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Chapter 13
Relationship of Other Cytoplasmic Ribonucleoprotein Bodies (cRNPB) to GW/P Bodies

Joanna J. Moser and Marvin J. Fritzler

Abstract GW/P body components are involved in the post-transcriptional processing of messenger RNA (mRNA) through the RNA interference and $5' \rightarrow 3'$ mRNA degradation pathways, as well as functioning in mRNA transport and stabilization. It is currently thought that the relevant mRNA silencing and degrading factors are partitioned to these cytoplasmic microdomains thus effecting post-transcriptional regulation and the prevention of accidental degradation of functional mRNA. Although much attention has focused on GW/P bodies, a variety of other cytoplasmic RNP bodies (cRNPB) also have highly specialized functions and have been shown to interact or co-localize with components of GW/P bodies. These cRNPB include neuronal transport RNP granules, stress granules, RNP-rich cytoplasmic germline granules or chromatoid bodies, sponge bodies, cytoplasmic prion protein-induced RNP granules, U bodies and TAM bodies. Of clinical relevance, autoantibodies directed against protein and miRNA components of GW/P bodies have been associated with autoimmune diseases, neurological diseases and cancer. Understanding the molecular function of GW/P bodies and their interactions with other cRNPB may provide clues to the etiology or pathogenesis of diseases associated with autoantibodies directed to these structures. This chapter will focus on the similarities and differences of the various cRNPB as an approach to understanding their functional relationships to GW/P bodies.

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13.1 Introduction

Historically, human autoantibodies directed to nuclear and cytoplasmic antigens have aided clinicians in the diagnosis of many autoimmune diseases and, for cell and molecular biologists, have been remarkably powerful tools to discover and understand the structure, composition and function of novel macromolecules and cellular compartments (Tan 1991; Fritzler 1996). These include small nuclear ribonucleoproteins (snRNPs) and components of the spliceosome, kinetochore and components of the mitotic spindle apparatus, nucleoli, the Golgi complex and endosomes, to name a few (reviewed in Stinton et al. 2004). Some of the target autoantigens such as Sm, U1-RNP, SS-A/Ro, SS-B/La, HuD, and Nova-1 bind specific RNAs, which then associate with other proteins to form macromolecular complexes that perform a variety of functions (Musunuru and Darnell 2001; Mansfield and Keene 2009). At the clinical interface, autoantibodies directed to Sm, U1-RNP, SS-A/Ro and SS-B/La are important biomarkers that facilitate an earlier and more accurate diagnosis while elucidating the immune aberrations and pathogenesis of autoimmune diseases such as systemic lupus erythematosus (SLE), systemic sclerosis and Sjögren's syndrome (SjS) (Perl 2009; Rosen and Casciola-Rosen 2009).

GW bodies [GWB, glycine (G)- and tryptophan (W)-rich cytoplasmic RNP bodies (cRNPB); also known as mammalian processing (P) bodies or Dcp containing bodies (hereafter referred to as GW/P bodies)], were initially identified through immunoscreening of cDNA expression with human autoantibodies that produced a unique cytoplasmic discrete speckled staining pattern on human tissue culture cell lines (Eystathioy et al. 2002a). Since then, many investigators have focused their attention on elucidating the protein components of GW/P bodies as an approach to understanding their structure and function. Of these GW/P body protein components, autoantibodies to GW182/TNRC6A, GW2/TNRC6B, GW3/TNRC6C, Ge-1/Hedls/RCD8, LSm1-7, Ago2/EIF2C2, RAP55/LSm14A, and diacyl-phosphatidylethanolamine have been clinically correlated with autoimmune diseases that include idiopathic ataxia, motor and sensory neuropathy, SjS, SLE, rheumatoid arthritis and primary biliary cirrhosis (Table 13.1) (Bhanji et al. 2007; Yamochi et al. 2008; Eystathioy et al. 2003a; Bloch et al. 2005; Yu et al. 2005; Marnef et al. 2009; Yang et al. 2006; Jakymiw et al. 2006; Laurino et al. 2006). Of relevance to cell and molecular biologists, GW/P body components are involved in the post-transcriptional processing of messenger RNA (mRNA) through RNA interference (RNAi) or RNA silencing pathways (Jakymiw et al. 2005; Liu et al. 2005a, b; Pillai et al. 2005; Rehwinkel et al. 2005; Sen and Blau 2005), $5' \rightarrow 3'$ mRNA degradation (Andrei et al. 2005; Bashkirov et al. 1997; Cougot et al. 2004; Eystathioy et al. 2003b; Fenger-Gron et al. 2005; Ingelfinger et al. 2002; Rehwinkel et al. 2005; Sheth and Parker 2003; van Dijk et al. 2002), and mRNA transport and stabilization (Barbee et al. 2006; Moser et al. 2007).

RNA interference (RNAi) is a key pathway involved in the post-transcriptional silencing of >50% of all mRNAs in a variety of organisms (Friedman et al. 2009). The RNAi pathway begins in the nucleus where miRNA genes are transcribed by RNA polymerase II into primary transcripts (pri-miRNA) that are then cleaved by the

Table 13.1 Summary of GW/P body autoantigens as recognized by human sera with autoimmune diseases

GW/P body autoantigens	Autoimmune disease	Reference
Ge-1/Hedls/RCD8	PBC	Bloch et al. (2005);Yu et al. (2005)
hLSm1-7 complex	SLE	Eystathioy et al. (2002b)
GW182/TNRC6A	SLE/SjS/neurological[a]	Eystathioy et al. (2002b, 2003a); Bhanji et al. (2007)
Ago2/EIF2C2	SLE/SjS/neurological[a]	Bhanji et al. (2007)
GW2/TNRC6B	SjS, ataxia, SLE, sensory neuropathy, limited cutaneous SSc, PBC, hypothyroidism	Bhanji et al. (2007)
GW3/TNRC6C	SjS, arthritis, celiac disease, PBC, limited cutaneous SSc, hypothyroidism, neuropathy	Bhanji et al. (2007)
Ro52/TRIM21	SjS	Yamochi et al. (2008); Bhanji et al. (2007)
RAP55/LSm14A	PBC	Marnef et al. (2009); Yang et al. (2006)
Su	SLE	Jakymiw et al. (2006)
Diacyl-phosphatidylethanolamine[b]	SLE, other autoimmune disease (no clinical antiphospholipid syndrome)	Laurino et al. (2006)

[a]Neurological features include ataxia, motor, and sensory neuropathy
[b]Partial colocalization with GW/P bodies

Drosha-DGCR8 complex into precursor miRNAs (pre-miRNA) (Rana 2007). Pre-miRNA are then rapidly processed into miRNA duplexes of 18–22 nucleotides in length by Dicer, an RNase III-specific endonuclease (Meister and Tuschl 2004). RNAi can also be mediated by exogenous dsRNA that is processed by Dicer into small interfering (siRNA) duplexes of similar length (Meister and Tuschl 2004). These small RNA duplexes (miRNA and siRNA) are then incorporated into the RNA-induced silencing complex (RISC) where the passenger RNA strand is dissociated by cleavage, degradation or a bypass mechanism (Matranga et al. 2005) by interacting with Argonaute 2 (Ago2), one of four Ago proteins (Liu et al. 2004; Ares and Proudfoot 2005). The RISC then recruits one or more heteromeric protein complexes (e.g., GW182 and Rck/p54) to associate with the mRNA leading to the formation of the ribonucleoprotein (RNP) structure known as GW/P bodies. Depending on the degree of complementarity between the guide-strand miRNA or siRNA and its target mRNA, this augmented RISC then initiates post-transcriptional inhibition of gene expression through cleavage or translational repression (Jakymiw et al. 2007; Eulalio et al. 2009d). Importantly, each miRNA is predicted to regulate hundreds of different target mRNAs while a single mRNA has the potential to be regulated by dozens of different miRNAs.

The $5' \rightarrow 3'$ degradation of targeted mRNA by XRN1 exonuclease is initiated after the poly(A) tail is shortened by the deadenylase CCR4:NOT complex and the $5'$ cap is removed by decapping factors that include the LSm1-7 ring and the Dcp 1/2 complex (Eystathioy et al. 2003b; Rehwinkel et al. 2005; Behm-Ansmant et al.

2006). While some evidence suggests a central role for GW/P bodies in RNAi, other studies have shown that the process of active RNAi can occur in the absence of microscopically visible GW/P bodies (Chu and Rana 2006; Rehwinkel et al. 2005; Lazzaretti et al. 2009). Additionally, in Dicer knockout cells it has been observed that GW/P bodies exist in the absence of active RISC (Leung et al. 2006).

In some cells, sequestration of mRNAs targeted for repression or degradation may need to be stabilized while being transported to other cellular regions. For example, neurons contain specialized regions such as axons and dendrites that can extend great distances from the cell body and, in such cells, certain mRNAs are transported to these regions and stabilized until the appropriate signal can either remove the repressor or degrade the transcript (reviewed in Bolognani and Perrone-Bizzozero 2008). In neurons and astrocytes, stabilization and transport proteins [i.e., Hu antigen R (HuR), fragile X mental retardation protein (FMRP), heterogeneous nuclear ribonucleoproteins (hnRNPs), and Staufen] have been shown to have variable association with GW/P bodies (Barbee et al. 2006; Moser et al. 2007) while possibly awaiting a signal to initiate mRNA degradation, repression or release of translational inhibition.

It has been suggested that silencing and degrading factors are partitioned to these specialized cRNPB to increase the efficiency of post-transcriptional regulation and to prevent the inadvertent degradation of functional mRNA. The discovery and characterization of GW/P bodies in *S. cerevisiae* (Sheth and Parker 2003, 2006; Teixeira et al. 2005; Nissan and Parker 2008), *C. elegans* (Ding et al. 2005; Zhang et al. 2007; Gallo et al. 2008), *D. melanogaster* (Schneider et al. 2006; Lin et al. 2006, 2008; Miyoshi et al. 2009), *H. sapiens* (Eystathioy et al. 2002a, 2003b; Jakymiw et al. 2005, 2007; Liu et al. 2005a; Pauley et al. 2006; Moser et al. 2007, 2009; Zee et al. 2008; Li et al. 2008) and somatic cells from a variety of species has led to speculation that other unique cytoplasmic RNP structures may be related to GW/P bodies in cells that have specialized functions (i.e., neurons, germline cells) or in cells induced by certain stimuli (i.e., stress, prion protein). This chapter will discuss the key features of GW/P bodies and compare and contrast these structures with other cytoplasmic RNP-rich microdomains that include neuronal transport RNP granules, stress granules, germline granules/chromatoid bodies, sponge bodies, cytoplasmic prion protein-induced RNP granules, U bodies and TAM bodies (Table 13.2).

13.2 GW/P Bodies

13.2.1 Discovery

Distinct cytoplasmic foci similar to GW/P bodies were initially reported in 1997 when Bashkirov and colleagues examined the cellular localization of mXRN1p in mouse E10 cells by indirect immunofluorescence (IIF) (Bashkirov et al. 1997). Related cytoplasmic foci were rediscovered 5 years later when a human autoimmune serum from a patient with ataxia and polyneuropathy was used to immunoscreen a

Table 13.2 Structural characteristics of RNP-rich cytoplasmic bodies

Cytoplasmic body	Cytoplasmic foci	Size (nm)	Quantity (foci/cell)	Membrane-bound	Associated with ribosomes	Movement	Microtubule-dependent	Contain mRNA	miRNA
GW/P body (P body in yeast)	+	100–300	3–4[§] 3–8[¤] >30[*]	–	–	+	+	+	+
Neuronal transport RNP granule	+	150–1,000	n/a[§] 10–15[¤] >20[*]	–	+ (small 40S and large 60S)	+	+	+	+
Stress granule	+	100–2,000	1–5[§] ND[¤] >20[*]	–	+ (small 40S only)	– (change shape)	+ (formation)	+	+
Chromatoid body	+	200–900	n/a[§] ND[¤] 2–10[*]	–	–	+	+	+	+
Sponge body	+	500–3,000	ND[§] 10–20[¤] ND[*]	–	–	+	ND	+	ND
cyPrP-induced RNP granule	+	4,000–5,000	ND[§] ND[¤] 1[*]	– (vimentin cage)	ND	ND	+	+	+
U body	+	200–400	ND[§] 5–15[¤] ND[*]	–	ND	ND	ND	+	ND

+ = positive/yes; − = negative/no; ND = no data; n/a = not applicable
The approximate number of foci/cell is indicated by [§] in *S. cerevisiae*, [¤] in *D. melanogaster* cells and [*] in mammalian cells
GW/P body glycine and tryptophan-rich cytoplasmic processing body; *cyPrP* cytoplasmic prion protein; *miRNA* microRNA; *mRNA* messenger RNA; *nm* nanometer; *RNP* ribonucleoprotein; *U* uridine-rich

HeLa expression cDNA library that led to the discovery of the novel phosphoprotein named GW182, which localized to distinct cytoplasmic foci containing hDcp and LSm4 (Eystathioy et al. 2002a, 2003b).

13.2.2 Cellular Location and Structure

These foci, initially named GW bodies (GWB), were distinct from other cytoplasmic organelles such as endosomes, lysosomes, Golgi complex vesicles or peroxisomes (Eystathioy et al. 2002a). When examined by immunogold electron microscopy, it was noted that these electron-dense structures had a diameter of 100–300 nm and did not have a limiting membrane (Table 13.2) (Eystathioy et al. 2002a; Yang et al. 2004). An ultrastructural study has further elucidated the fine structure of GW/P bodies in unstressed and arsenite-stressed conditions as marked by GWB component Rck/p54 (Souquere et al. 2009). Under unstressed conditions, GW/P bodies were confirmed to be round, electron dense bodies with a diameter of up to 300 nm where anti-Rck/p54 labeled gold particles clustered on 10–15 nm fibril strands (Souquere et al. 2009). When stressed, the fundamental ultrastructure of GW/P bodies appeared unchanged, but an increased number of gold particles marking Rck/p54 were detected on the fibril strands (Souquere et al. 2009).

GW/P body components were subsequently identified in other eukaryotic cells such as those in *S. cerevisiae*, *D. melanogaster*, *C. elegans* and mammals (reviewed in Parker and Sheth 2007; Jakymiw et al. 2007; Eulalio et al. 2009d). Since a homolog of the GW182 protein marker protein has yet to be identified in yeast, it is considered inappropriate to refer to yeast P bodies as GW/P bodies. Nevertheless, GW/P bodies have been studied in numerous human cells including but not limited to HeLa cells (Eystathioy et al. 2002a; Jakymiw et al. 2005; Moser et al. 2009), normal and malignant breast cells (Luft 2005), astrocytes and astrocytoma cells (Moser et al. 2007), and skin cells (Zee et al. 2008). Whereas GW/P bodies are thought to be ubiquitous, they are variably expressed in cells and tissues of different origins; vary in size and number in proliferating and malignant cells (Luft 2005; Moser et al. 2007) and in different phases of the cell cycle (Yang et al. 2004); in stress responses (Anderson and Kedersha 2009a); mRNA decay inhibition (Andrei et al. 2005; Cougot et al. 2004; Sheth and Parker 2003); stalled translational initiation (Brengues et al. 2005; Sheth and Parker 2003; Teixeira et al. 2005) and during transcription inhibition and deadenylation or translational elongation of mRNA (Cougot et al. 2004; Sheth and Parker 2003).

13.2.3 Protein Components

There is compelling evidence that GW182 is a requisite component of GW/P bodies. Of note, after knockdown of GW182 by a cognate siRNA, GW/P bodies became inconspicuous by conventional microscopy (Yang et al. 2004) (reviewed in Jakymiw et al. 2007; Eulalio et al. 2009d). Furthermore, GW182 is a key co-factor of Ago2 which

plays an essential role in siRNA and miRNA-mediated gene silencing (Jakymiw et al. 2005; Liu et al. 2005a; Meister et al. 2005; Rehwinkel et al. 2005; Behm-Ansmant et al. 2006; Chu and Rana 2006; Eulalio et al. 2008, 2009a, b, c, d). Serman et al. also noted that GW/P bodies assembled in the absence of GW182 under arsenite-induced stress conditions (Serman et al. 2007) where it was suggested that Rck/p54 helicase rather than GW182 was the requisite component of GW/P bodies. In addition, RNAi depletion of other proteins such as eIF4ET, LSm1, Rck/p54, CCR4 (Andrei et al. 2005), LSm4 (Kedersha et al. 2005), Ge-1 (Yu et al. 2005), RAP55 (Yang et al. 2006), CPEB1 (Serman et al. 2007) and PatL1 (Scheller et al. 2007) induced GW/P body disappearance suggesting that these proteins are also critical components of GW/P bodies.

Recently, heat-shock protein (Hsp) 90 was found to be a key modulator of Argonaute function as evidenced by the observation that Hsp90 activity was required for efficient targeting of human Ago2 (hAgo2) to GW/P bodies (Pare et al. 2009). This study also showed that the miRNA-dependent translational repression and siRNA-directed cleavage functions of hAgo2 are dependent upon the activity of Hsp90 (Pare et al. 2009). Taken together, these findings suggested that GW/P body assembly and stability are governed by the activity of the Hsp90 chaperone, which may be dependent upon adenosine triphosphate (ATP), previously shown to stimulate RISC activity (Gregory et al. 2005).

Although there are likely additional protein components of GW/P bodies yet to be identified, components of GW/P bodies determined by methods such as IIF and Western blot analysis include mRNA (Liu et al. 2005b; Brengues et al. 2005; Eystathioy et al. 2002a), miRNA (Lian et al. 2006; Pauley et al. 2006), $5' \rightarrow 3'$ mRNA degradation pathway proteins (Andrei et al. 2005; Bashkirov et al. 1997; Cougot et al. 2004; Eystathioy et al. 2003b; Fenger-Gron et al. 2005; Ingelfinger et al. 2002; Rehwinkel et al. 2005; Sheth and Parker 2003; van Dijk et al. 2002), proteins involved in the RNA silencing pathway (Jakymiw et al. 2005; Liu et al. 2005a, b; Pillai et al. 2005; Rehwinkel et al. 2005; Sen and Blau 2005; Baillat and Shiekhattar 2009; Lazzaretti et al. 2009; Zipprich et al. 2009), and proteins involved in mRNA stabilization, transport, and processing (Moser et al. 2007) (Table 13.3) (for reviews that list GW/P body components in multiple species see Eulalio et al. 2007; Jakymiw et al. 2007; Parker and Sheth 2007; Eulalio et al. 2009d). Some of these proteins only partially colocalize to GW/P bodies in human U-87 astrocytoma cells (Moser et al. 2007) (Table 13.3). IIF analysis showed that Dicer, HuR and Staufen colocalized to less than 15% of GW/P bodies, suggesting that some proteins transiently associate with GW/P body components (Moser et al. 2007). Arguably, the dynamic interactions between GW/P bodies and their constituent proteins over time would best be determined from real-time fluorescent imaging experiments.

13.2.4 GW/P Body Intracellular and Intercellular Dynamics

The intracellular movement of GW/P bodies is evidenced by a number of vectoral dynamics: passively within a confined cytoplasmic region where interactions with

Table 13.3 Common GW/P body proteins found in other RNP-rich cytoplasmic bodies

		RNP-rich cytoplasmic bodies						
	Protein	GW/P body	Neuronal transport RNP granule	Stress granule	Chromatoid body	Sponge body	cyPrP-RNP granule	U body
RNA interference	Dicer	±	+	ND	+	ND	+	ND
	Ago2/EIF2C2	+	+	+	+	ND	ND	ND
	GW182/TNRC6A/AIN-1	+	+	−	+	ND	ND	ND
mRNA degradation	CCR4	+	ND	−	ND	ND	ND	ND
	Dcp1	+	+	−	+	+	+	±
	Dcp2	+	ND	−	+	+	ND	ND
	Sm proteins (LSm1-7)	+	ND	−	+	+	ND	+
	RAP55/LSm14A	+	ND	+	ND	ND	ND	ND
	XRN1	+	±	+	+	ND	ND	ND
	Ge-1/Hedls/RCD8	+	ND	−	ND	ND	ND	ND
	Rck/p54/Dhh1/DDX6/Me31B	+	+	+	+	ND	+	±
RNA stabilization, processing, transport	HuR	±	ND	+	+	ND	ND	ND
	FMRP	+	+	+	ND	ND	ND	ND
	SYNCRIP/hnRNPQ/NSAP1	+	+	+	ND	ND	ND	ND
	Staufen	±	+	+	ND	ND	ND	ND
	hnRNPs	+	+	+	+	ND	ND	ND
	CPEB	+	+	+	+	ND	ND	ND

+ = localize to foci, ± = partially localize to foci and other places in cell, − = no localization to foci, ND = no data

Ago2 Argonaute 2; *CPEB* cytoplasmic polyadenylation element-binding protein; *EIF* eukaryotic initiation factor; *FMRP* fragile X mental retardation protein; *GW/P body* glycine and tryptophan rich cytoplasmic processing body; *hnRNP* heterogeneous nuclear ribonucleoprotein; *HuR* Hu antigen R; *PrP* prion protein; *RNP* ribonucleoprotein; *SYNCRIP* synaptotagmin-binding cytoplasmic RNA-interacting protein; *TNR* trinucleotide repeat; *U* uridine-rich

mRNA transcripts may be based on random encounters rather than on an active or directed mechanism; bidirectionally along microtubules to facilitate interactions with mRNA transcripts; actively along the periphery of the nuclear envelope where interactions with the nuclear pore complex and exported mRNAs may occur (Aizer et al. 2008). Aizer et al. also reported that GW/P bodies move in channel-like regions between mitochondria where, after disrupting the microtubule network with nocodazole, the areas of movement and diffusion were reduced (Aizer et al. 2008).

Besides intracellular cytoplasmic movement, there is recent evidence that GW/P body components, GW182 and a nascent fraction of miRNA-loaded Ago2, are found in 50–100 nm extracellular microvesicles (also referred to as extracellular exosomes) that are derived from intracellular endosome–lysosome–multivesicular bodies (Gibbings et al. 2009; Lee et al. 2009b). These microvesicles are particularly enriched in GW182 and contain inactive forms of mRNA and miRNA that are thought to be transferred between cells and exert a functional role in the new cellular environment (Gibbings et al. 2009). These and other recent observations have highlighted the potential importance of extracellular exosomal miRNA and GW/P body components as biomarkers for a number of diseases (Simpson et al. 2009; Rabinowits et al. 2009; McLellan 2009).

13.2.5 GW/P Bodies: Unanswered Questions and Future Directions

Although there has been remarkable progress over the past 10 years, an understanding of the mechanism of GW/P body formation is still in complete (Jakymiw et al. 2007). A question that still remains is whether GW/P bodies form around specific mRNAs and associated proteins or are they targeted to preexisting mRNP structures that contain similar mRNAs and protein components shared between all cytoplasmic RNPs (Table 13.3). In addition, since GW182 was shown to be a phosphoprotein (Eystathioy et al. 2002a) the functional role of phosphorylation of the GW182/TNRC6A, GW2/TNRC6B and GW3/TNRC6C paralogs remains to be clarified. A considered hypothesis is that phosphorylated GW182 protein may be the active hAgo2 ligand. Interestingly, hAgo2 has been shown to be post-translationally modified by phosphorylation of serine-387 by the p38 MAPK (mitogen-activated protein kinase) signaling pathway, which when mutated to alanine, led to a reduction in localization of hAgo2 to GW/P bodies (Zeng et al. 2008). This observation suggested that phosphorylation of hAgo2 is essential for localization to GW/P bodies, but specific investigation of the phosphorylation state of hAgo2 and its influence on GW182 binding was not reported. In addition, this study suggested that hAgo2-mediated gene silencing may be linked to distinct signaling pathways and highlights the importance of defining whether the same or different signaling pathways phosphorylate GW182.

From protein colocalization and related studies, it has become apparent that at any given time GW/P bodies are quite heterogeneous in protein composition. The complete proteome of GW/P bodies as analyzed by mass spectrometry analysis has

not yet been fully elucidated and, therefore, other functions of these important structures likely remains to be discovered.

13.3 Neuronal Transport Ribonucleoprotein Granules

Neuronal transport RNPs are also referred to as neuronal RNA granules, RNA granules, RNA particles, dendritic P-body like structures (dlP-bodies), FMRP granules and Staufen granules; herein they will be referred to as neuronal transport RNP granules. Neuronal transport RNPs are broadly identified by the presence of two highly conserved proteins: Staufen, a double-stranded RNA-binding protein (Barbee et al. 2006; Kiebler and Bassell 2006; Thomas et al. 2005) and FMRP (Barbee et al. 2006; Eddy 1975; Mazroui et al. 2002), both of which have been identified by immunofluorescence (often including fluorescence-labeled cDNA constructs) and Western blot.

13.3.1 Cellular Location and Structure

Similar to GW/P bodies, neuronal transport RNP granules are cytoplasmic, non-membrane bound 150–1,000 nm foci that store translationally repressed mRNAs (reviewed in Krichevsky and Kosik 2001). In contrast to GW/P bodies, neuronal transport RNP granules contain ribosomal RNA (40S and 60S) and to date they have been primarily studied in mammalian and *D. melanogaster* neurons (reviewed in Sossin and DesGroseillers 2006; Hillebrand et al. 2007) (Table 13.2).

13.3.2 Protein Components

Neuronal transport RNPs have been shown to be structurally and functionally similar to GW/P bodies with respect to protein components such as GW182/TNRC6A, Ago2/EIF2C2, Rck/p54/Dhh1/DDX6/Me31B, FMRP, Staufen, SYNCRIP/hnRNPQ/NSAP1, hnRNPs, CPEB, mRNA and miRNA (Anderson and Kedersha 2006; Zeitelhofer et al. 2008; Cougot et al. 2008) (Table 13.3). In astrocytes and astrocytoma cells, these neuronal transport RNP components were only present in subsets of GW/P bodies (Moser et al. 2007). Further, miRNA-mediated regulation of mRNA presumably in the GW/P body microdomain is important in the controlled regulation of neuronal development and plasticity (Ashraf and Kunes 2006; Schratt et al. 2006).

13.3.3 Neuronal Transport RNP Granule Intracellular Dynamics

Transport of mRNA to subcellular domains is essential for establishing cellular polarity (St Johnston 2005). In highly polarized cells, such as neurons, the dendritic

localization of mRNAs and their subsequent translation at synapses is thought to contribute to remodeling of synapses and the subsequent establishment of long-term memory (St Johnston 2005; Sutton and Schuman 2006). In mammalian cells, there are two distinct phases of neuroplasticity: an early phase (~ 1–3 h) that is independent of new protein synthesis and a late phase lasting longer than 8 h that is dependent on new protein synthesis in dendrites (Sutton and Schuman 2006). Recently, the molecular mechanisms underlying new protein synthesis have focused on local dendritic regulation of mRNA translation. In neurons, local mRNA translation is important for the regulation of synaptic plasticity as well as development and growth of axons and dendrites (Hillebrand et al. 2007). To ensure that the appropriate mRNA will be available for translation upon a specific stimulus, neurons have developed elaborate systems for delivering RNPs into distant cytoplasm domains, which include the movement of neuronal transport RNPs along the microtubule cytoskeleton by the kinesin protein KIF5 (Hirokawa 2006; Kiebler and Bassell 2006). It is likely that the composition of neuronal transport RNPs at a given time determines whether specific mRNAs are transported to a certain location (i.e., synapse), translated locally [i.e., at the postsynaptic density (PSD)], or targeted for storage and/or degradation to GW/P bodies. Indeed, GW/P bodies marked by antibodies to GW182 localize to postsynaptic densities in rat hippocampal neurons marked by antibodies to PSD-95 (Moser and Fritzler 2010). These observations suggest that mRNA or miRNA targeted to dendrites may be stored and/or silenced in GW/P bodies until an appropriate signal directs the mRNA for local translation or degradation.

13.3.4 Neuronal Transport RNP Granules: Unanswered Questions and Future Directions

MiRNAs and protein components of GW/P bodies have been observed in neurons and astrocytes, however, it remains to be determined which physiologically relevant signals activate Dicer to process precursor-miRNAs (pre-miRNA) into miRNAs in these excitable cells. In part, this has been elucidated by two studies that focused attention on the somatodendritic compartment of neurons in the adult mouse brain (Lugli et al. 2005; Fierro-Monti et al. 2006) where Dicer and Ago2 were localized to dendritic spines and enriched in PSDs. In a separate study, GW182 was found to localize in close proximity to PSDs (Cougot et al. 2008) whereas we found that GW182 colocalized directly to PSDs (Moser and Fritzler 2010). In addition, Dicer and Ago2 were biochemically associated with FMRP macromolecular complexes in PSDs (Lugli et al. 2005). This cellular localization placed PSDs in contact with intracellular ionic fluxes, such as calcium (Ca^{2+}) influx, and second messenger cascades generated by neurotransmitters binding to ligand-gated Ca^{2+} channels and, accordingly, provide a suitable microenvironment for neurotransmitter-mediated Ca^{2+} influx and subsequent activation of signaling proteins. Lugli et al. showed that Dicer was inactive when localized to PSDs (Lugli et al. 2005), but upon Ca^{2+} influx, NMDA (*N*-methyl-D-aspartic acid) stimulation or exogenous treatment with the protease calpain, it together

with Ago2 was released into the cytosol and became enzymatically activated (Lugli et al. 2005). The calpain-specific protease inhibitor, calpeptin, was observed to reverse the effects of calpain even in the presence of Ca^{2+} indicating that Dicer-mediated RNAse III activation was calpain-dependent (Lugli et al. 2005). Lugli et al. (2008) went on to examine the expression of miRNAs and pre-miRNA in synaptic fractions of the adult mouse forebrain and found that pre-miRNAs were predominantly associated with PSDs whereas miRNAs were enriched in soluble synaptic fractions (Lugli et al. 2008). The observation that Dicer only interacted with pre-miRNA (Lugli et al. 2008) suggested that Dicer dissociated from mature miRNA.

Taken together, these observations suggested that synaptic stimulation mediated by Ca^{2+}-induced calpain activation released pre-miRNA, Dicer, FMRP and Ago2 from PSDs into the cytosol where activated Dicer cleaved pre-miRNA into mature miRNA. These data also suggested that mature miRNA associated with Ago2 and GW182 are capable of regulating target mRNA perhaps within GW/P body components that may be present in the soluble components of synaptic fractions. Future studies into the physiological cues that regulate GW/P bodies and their interactions with neuronal transport RNP granules will be useful to understand their role in cell signaling pathways.

13.3.5 Relationship to GW/P Bodies

A number of studies have shown that neuronal transport RNPs colocalize with GW/P bodies. Zeitelhofer et al. observed that they are distinct compartments because only 3–4% of the structures were colocalized and were not transported in the same particles in the dendrites of mammalian neurons (Zeitelhofer et al. 2008). Using time-lapse video microscopy, 50% of GW/P bodies and transport RNPs transiently interacted, however, the authors emphasized that the nature and purpose of this interaction was unknown (Zeitelhofer et al. 2008). After synaptic stimulation with glutamate, approximately 60% of GW/P bodies disassembled, which implied that the mRNAs stored in GW/P bodies were translated in concert with this event (Zeitelhofer et al. 2008). These data support the possibility that mRNAs localized to dendrites might be stored in GW/P bodies and are then released and translated when synapses are activated. Clearly, further studies that focus on the relationship between neuronal transport RNPs and GW/P bodies (and their corresponding mRNAs and miRNAs) in neurons under physiologically relevant stimuli, such as long-term potentiation (LTP) or long-term depression (LTD), are still required.

13.4 Stress Granules

13.4.1 Cellular Location and Structure

Stress granules are 100–2,000 nm nonmembranous cytoplasmic foci that are formed as a cellular response to environmental stress, including heat shock, hypoxia (oxygen

deprivation), osmotic shock, stress responses that include treatment with sodium arsenite, glucose deprivation, UV irradiation or viral infection (Table 13.2) (reviewed in Anderson and Kedersha 2009b). Ultrastructural analysis showed that stress granules are loosely organized fibrillo-granular aggregates of moderate electron density (Souquere et al. 2009). They have been studied in mammalian cells and tissues, and more recently in *S. cerevisiae* (Hoyle et al. 2007; Teixeira et al. 2005; Buchan et al. 2008), *S. pombe*, protozoa (*Trypanosoma brucei*, *C. elegans*) and plant chloroplasts (Anderson and Kedersha 2009a).

13.4.2 Assembly and Protein Components

Upon induction of stress, a series of molecular changes silence ongoing translation of certain housekeeping mRNAs presumably to conserve energy for the repair of stress-induced molecular damage by enhancing the translation of other mRNAs (including heat shock proteins) (Kedersha and Anderson 2007). Specifically, the assembly of stress granules is dependent on the phosphorylation of translation initiation factor eIF2α by stress-activated kinases, which reduces the availability of the eIF2-GTP-$tRNA_i^{Met}$ ternary complex and subsequently blocks translation initiation thus promoting polysome disassembly (Anderson and Kedersha 2006). Assembly of stress granules is also promoted by aggregation of specific proteins that act downstream of the phosphor-eIF2α pathway including TIA (T-cell intracellular antigen) or G3BP (Ras-GAP SH3 binding protein) proteins, to name a few (Gilks et al. 2004; Tourriere et al. 2001). Stress granule components that are unique to these cytoplasmic RNPs include stalled 48S preinitiation complexes containing small ribosomal subunits (40S only, not large ribosome subunit 60S) and early translation initiation factors eIF2, eIF3, eIF4E, eIF4A, eIF4B and eIF4G (Kedersha and Anderson 2002; Tourrière et al. 2003); PABP (poly(A)-binding protein), G3BP, TIA-1 (Kedersha et al. 1999; Tourrière et al. 2003); translationally arrested mRNA from disassembling polysomes. These proteins, with the exception of eIF2, eIF4E, TIA-1 and G3BP, are considered to be core stress granule components that serve as universal markers of stress granules (Anderson and Kedersha 2008; Kedersha and Anderson 2007).

13.4.3 Stress Granule Intracellular Dynamics

As observed by real-time fluorescent imaging, stress granules dynamically interacted with GW/P bodies, suggesting that under these stress conditions there is transfer of mRNA targeted for degradation (Wilczynska et al. 2005; Kedersha et al. 2005; Anderson and Kedersha 2008). While there is a possibility that stress granules exchange protein components due to their physical proximity, a study by Mollet

et al. showed that proteins present in mammalian stress granules originated in the cytosol and not from adjacent GW/P bodies (Mollet et al. 2008). Nevertheless, suggestions that these two RNP granules exchange not only mRNA but also protein components is supported by observations showing that stress granules contain some GW/P body components such as Ago2/EIF2C2 (Leung and Sharp 2007), APOBEC3G (Kozak et al. 2006; Gallois-Montbrun et al. 2007), CPEB (Wilczynska et al. 2005), FAST (Kedersha et al. 2005), Rck/p54/DDX6/Dhh1/Me31B helicase (Wilczynska et al. 2005), RAP55/LSm14A (Yang et al. 2006), 5′→3′ exonuclease XRN1 (Kedersha et al. 2005), eIF4E (Kedersha et al. 2005), HuR (Gallouzi et al. 2000), Staufen (Thomas et al. 2005), FMRP (Antar et al. 2005) SYNCRIP/hnRNPQ/NSAP1 (Quaresma et al. 2009) and TIA-1/TIAR (Kedersha et al. 2005) (Table 13.2). As previously discussed, a recent study indicates Hsp90 is a critical modulator in the targeting of hAgo2 to stress granules (and GW/P bodies) (Pare et al. 2009).

13.4.4 Relationship to GW/P Bodies

Although stress granules and GW/P bodies share protein components and interact intimately with each other, they are structurally, compositionally, and functionally distinct cytoplasmic foci (Souquere et al. 2009). This is supported by observations that two key markers of GW bodies, GW182 and Dcp1/2, are not found in mammalian stress granules (Kedersha et al. 2005). On the other hand, as discussed above, GW/P bodies and stress granules do have some protein constituents in common and it has been shown that stress granule assembly is dependent on GW/P body formation (Buchan et al. 2008). Taken together, these studies suggested that stress granules are formed from mRNPs in preexisting GW/P bodies. Recently, this hypothesis has been challenged by an ultrastructural study of stress granules and GW/P bodies showing that they are distinct entities with unique structural integrity (Souquere et al. 2009). Unlike some GW/P bodies that exhibit rapid intracellular movement, stress granules remain relatively fixed within the cytoplasm and constantly change shape by fusing and dividing (Kedersha et al. 2005), although their formation but not movement was shown to be microtubule-dependent (Table 13.2). Accordingly, microtubule disruption with nocodazole or vinblastine prevented stress granule formation, suggesting that a functional and intact microtubule array is necessary for their formation (Ivanov et al. 2003).

13.5 Germinal Granules/Chromatoid Bodies

13.5.1 Discovery

Germinal granules (also termed nuage) are cytoplasmic RNA and protein-rich amorphous nonmembrane bound structures found in germline cells of over 80 divergent

species that have been localized adjacent to the nuclear envelope by light and electron microscopy (Table 13.2) (Eddy 1975). Most studies of germinal granules used *D. melanogaster*, *C. elegans*, *X. laevis* (Kloc et al. 2002) and, more recently, murine cells (Chuma et al. 2006; Hosokawa et al. 2007).

13.5.2 Cellular Location and Structure

In *D. melanogaster*, *C. elegans* and *X. laevis*, germinal granules are asymmetrically partitioned to prospective germ cells during early embryogenesis where they direct the timing of maternal mRNA translation to facilitate early embryogenesis and establish the germ line of the progeny (Schisa et al. 2001; Leatherman and Jongens 2003). Mammalian germinal granules are observed in the later stages of germ cell differentiation (i.e., spermatogenesis and oogenesis) but are not observed in early embryogenesis (reviewed in Chuma et al. 2009).

In mammals, there are two types of germinal granules: the chromatoid body that is apparently restricted to differentiated male germ cells, and inter-mitochondrial cement which is found in differentiated male and female germ cells (Chuma et al. 2009). The differences between species suggest that germinal granules have a role in development within early embryos of *D. melanogaster*, *C. elegans* and *X. laevis* but not in mammals (Chuma et al. 2009), highlighting evidence that mammals have a more complex partitioning of germinal granules during germ cell differentiation. *D. melanogaster* germinal granules are sometimes referred to as polar granules and *C. elegans* germinal granules as P granules; however, some studies have used chromatoid bodies as a collective nomenclature for *D. melanogaster*, *C. elegans* and *X. laevis* germinal granules. Although there is a paucity of published information about inter-mitochondrial cement, the term chromatoid body will be used herein to describe all germinal granules and will explore their striking similarities to GW/P bodies.

Murine chromatoid bodies are cytoplasmic fibrous, granular structures that are localized amongst mitochondrial clusters in meiotic pachytene spermatocytes (Fujiwara et al. 1994). After meiosis, the chromatoid body condenses to form a single filamentous perinuclear granule in rounded spermatids (Parvinen 2005; Nagamori and Sassone-Corsi 2008). During early spermiogenesis, the chromatoid body is localized adjacent to the nuclear envelope where it is juxtaposed to nuclear pore complexes (Parvinen 2005; Nagamori and Sassone-Corsi 2008).

13.5.3 Protein Components

Chromatoid bodies contain RNAs, mitochondria, histone and ubiquitin-related proteins (for a complete list of proteins in chromatoid bodies see Table 1 in Chuma et al. 2009) (Hess et al. 1993; Haraguchi et al. 2005; Toyooka et al. 2000; Tsai-Morris

et al. 2004; Werner and Werner 1995). Given the location of chromatoid bodies in the germline, as well as their protein composition which include mitochondrial proteins, histone and ubiquitin-related protein components, chromatoid bodies are likely to have a specialized role in processing, degradation and/or repression of mRNAs that are important in germline development or differentiation of germline cells. Therefore, they may not contain some of the somatic GW/P body components and it is expected that their complement of mRNA and miRNAs, and to a certain extent protein, would be different from that found in somatic GW/P bodies and may remain in a quiescent state until receiving appropriate cues for embryonic developmental progression. To fully elucidate the function of chromatoid bodies, future studies will likely include a thorough examination of their mRNA, miRNA and/or protein components.

13.5.4 Relationship to GW/P Bodies

As determined by IIF, chromatoid bodies contain key GW/P body proteins involved in mRNA degradation, RNA silencing/translational repression and mRNA stabilization, processing and transport (Table 13.3) (Anderson and Kedersha 2006; Chuma et al. 2009; Gallo et al. 2008). For example, Ago2/EIF2C2, Dicer, GW182/TNRC6A, Dcp1/2, XRN1, LSm proteins, Rck/p54/DDX6/Dhh1/Me31B, HuR, hnRNP and CPEB proteins are found in both chromatoid bodies and GW/P bodies. Although there are other GW/P body protein components that have not been mapped to chromatoid bodies (i.e., CCR4, RAP55/LSm14A, Ge-1/Hedls/RCD8, FMRP, SYNCRIP/hnRNPQ/NSAP1, and Staufen), the structural and protein mapping evidence thus far suggests that germline chromatoid bodies may be analogous to GW/P bodies in somatic cells.

13.6 Sponge Bodies

13.6.1 Discovery

Sponge bodies were originally characterized by their enrichment with the Exuperantia (Exu) protein that functions to localize the bicoid (bcd) mRNA and other mRNAs to specific sites in oocytes (Berleth et al. 1988; St et al. 1989; Wilhelm et al. 2000).

13.6.2 Cellular Location and Structure

Sponge bodies are 500–3,000 nm structures identified and characterized in *D. melanogaster* nurse cells and oocytes (Wilsch-Brauninger et al. 1997; Snee

and Macdonald 2009). They lack a surrounding membrane, are characterized by endoplasmic reticulum-like cisternae embedded in an electron dense matrix that is free of ribosomes (Wilsch-Brauninger et al. 1997) (Table 13.1). Under optimal experimental conditions, sponge bodies were diffusely distributed throughout the *D. melanogaster* ovary as small punctate structures particularly in nurse cells and oocytes (Snee and Macdonald 2009). However, under less optimal conditions, they formed large, extensively networked reticulated bodies (Snee and Macdonald 2009). Sponge bodies were reported to exhibit rapid intracellular movement and lie in proximity to, but do not directly associate with, microtubules (Wilsch-Brauninger et al. 1997).

13.6.3 Protein Components

To understand the function of sponge bodies, various proteins (Exu, Btz, Tral, Cup, eIF4E, Me31B, Yps, Gus, Dcp1/2, Sqd, BicC, Hrb27C, Bru) were co-localized with Exu-GFP indicating that these structures have a role in post-transcriptional gene regulation (discussed in Snee and Macdonald 2009).

13.6.4 Sponge Body Intracellular Dynamics

The rapid movement of sponge bodies in *D. melanogaster* oocytes and their protein composition suggested that these structures have a dynamic role in cell physiology (Snee and Macdonald 2009). It was also reported that reticulated sponge bodies contained the Staufen protein, which was not enriched in the dispersed sponge body foci (Snee and Macdonald 2009). At this writing, the mode of mRNA regulation in the reticulated sponge bodies has not been reported.

13.6.5 Relationship to GW/P Bodies

Some sponge body proteins are *D. melanogaster* homologs of GW/P body protein components (i.e., Me31B/Dhh1/DDX6/Rck/p54, Dcp1/2, eIF4E, and Tral/Car1) suggesting that either sponge bodies may be closely related to GW/P bodies or that they interact and exchange components with GW/P bodies (Snee and Macdonald 2009) (Table 13.3). In addition, both mammalian GW/P bodies and sponge bodies are currently considered to be devoid of ribosomal components (Table 13.2). Further investigation into the function of sponge bodies and their interactions with GW/P bodies would benefit from systematic protein mapping of GW/P body-specific proteins such as GW182, Ago2, LSm4, and XRN1.

13.7 Cytoplasmic Prion Protein-Induced Ribonucleoprotein Granules

Prion-related diseases are typically progressive neurodegenerative disorders that include variant Creutzfeldt-Jakob disease in humans, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease in deer, elk and moose, and scrapie in sheep (Prusiner 1998). A feature of prion disorders is the post-translational conversion of the endogenous cellular α-helix prion protein (PrP^{C}) to a β-sheet isoform of the scrapie prion protein (PrP^{Sc}), believed to be the main component of infectious prions (Collinge 2001; Aguzzi and Polymenidou 2004). PrP^{C} is mostly localized to lipid rafts (Vey et al. 1996) or associated with membranes due to hydrophobic transmembrane residues (Hegde et al. 1998). However, a number of reports indicated that PrP^{C} was localized to the cytoplasm in neurons, pancreatic cells, glioblastoma cells, and sperm (Mironov et al. 2003; Strom et al. 2007; Kikuchi et al. 2008; Ecroyd et al. 2004) and is denoted as cyPrP (Beaudoin et al. 2008). The presence of cyPrP is thought to inhibit a normal cellular stress response by preventing the assembly of protective stress granules and the synthesis of Hsp70 following environmental stress (Roucou 2009). It has been shown that cyPrP-induced interference with the stress response correlates with the aggregation of poly (A) mRNAs into large cytoplasmic RNP granules (Roucou 2009).

13.7.1 Discovery, Cellular Location and Structure

CyPrP-RNP granules, which are approximately 4,000–5,000 nm in diameter, have been characterized in mouse neuroblastoma cells and observed in murine and human neuronal and non-neuronal cells (Beaudoin et al. 2008). CyPrP-RNP granules share characteristics of PrP aggresomes (large juxtanuclear misfolded protein aggregates) including a vimentin protein cage that is related to the collapse of intermediate filaments that surround this RNP granule (Beaudoin et al. 2008). The characteristic vimentin cage is a key difference between this cytoplasmic RNP granule and all other nonmembrane bound cRNPB reviewed here (Table 13.2). Mitochondria were clustered adjacent to cyPrP-RNPs that localized to the centrosome (Beaudoin et al. 2008). This phenomenon of mitochondrial clustering seems to be unique to cyPrP-RNPs, however, localization to the centrosome is a feature also observed with some GW/P bodies (Aizer et al. 2008; Moser et al. 2011). In addition, cyPrP-RNP formation was shown to be completely dependent on microtubule integrity where disruption with nocodazole inhibited cyPrP-RNP formation (Beaudoin et al. 2008). In contrast, GW/P body movement was stalled upon microtubule disruption and, interestingly, GW/P bodies increase in number after nocodazole and vinblastine treatments (Aizer et al. 2008). This difference in microtubule dependence and cytoplasmic localization suggests that cyPrP-RNPs

are not entirely analogous to GW/P bodies but may have a role in sequestering mRNAs for centrosomal functions.

13.7.2 Protein Components and Relationship to GW/P Bodies

Although the literature suggests that cyPrP-RNPs are distinct from GW/P bodies, Beaudoin et al. provided the first evidence for the similarities between cyPrP-RNPs and chromatoid bodies (which share features of GW/P bodies) (Beaudoin et al. 2008) by demonstrating that cyPrP-RNPs contain mRNA, 5S rRNA and U1 snRNPs (Figueroa and Burzio 1998; Moussa et al. 1994), miRNA (miR-122a, miR-21 and let-7a) (Kotaja et al. 2006) and GW/P body proteins Dcp1a, DDX6/Rck/p54/Me31B/Dhh1, and Dicer (observed from in situ hybridization and IIF analysis) (Table 13.3) (Kotaja et al. 2006; Nakamura et al. 2001; Biggiogera et al. 1990; Moussa et al. 1994). Although cyPrP-RNPs have components that are similar to those in chromatoid bodies, it would be premature to suggest that cyPrP-RNPs are analogous to chromatoid bodies or GW/P bodies without evidence for the presence of GW182 and/or Ago2, the two key GW/P body/chromatoid body components. It is more likely that cyPrP-RNPs have their own distinct role in the post-transcriptional processing of cell cycle, proliferative or centrosome-related mRNAs in the cell cytoplasm in the nondiseased state. However, it might be speculated that upon development of a prion-related disease, PrP^{Sc} and PrP^{Sc}-RNPs will have a role in cell death leading to the spongioform pathology that is the hallmark of these neurodegenerative diseases. The composition and function of cyPrP-RNPs would benefit from a thorough proteomic and ribonomic (miRNA and mRNA) analysis to determine their specific role and relationship, if any, to other cytoplasmic RNP granules. In addition, live cell imaging with a fluorescent cyPrP construct could provide clues as to the movement of these granules and their potential interactions with GW/P bodies.

13.8 U Bodies

13.8.1 Discovery, Cellular Location and Structure

U bodies have recently been described in *D. melanogaster* ovary, testis, brain and gut and in *X. laevis* and human cells as discrete 200–400 nm nonmembranous cytoplasmic microdomains containing uridine-rich small nuclear ribonucleoproteins U1, U2, U4/U6 and U5 (U snRNPs) (Liu and Gall 2007). These components of the spliceosome have a number of functions including the removal of introns from pre-mRNAs (Tarn and Steitz 1997) (Table 13.2). SnRNPs are involved in pre-mRNA processing in the nucleus of eukaryotic cells and are enriched in discrete nuclear

domains that include nuclear speckles (Huang and Spector 1992; Misteli et al. 1997), Cajal bodies (Carmo-Fonseca et al. 1991; Gall 2000), and histone locus bodies (Liu et al. 2006; Liu and Gall 2007). U snRNPs are assembled in the cytoplasm into U bodies, which also contain essential snRNP (splicesome) assembly factors, suggesting that U bodies are sites for assembly or storage of snRNPs before being imported into the nucleus (Liu and Gall 2007).

In addition to snRNPs, U bodies contain LSm1-7, and the survival of motor neurons (SMN) protein (Liu and Gall 2007). Each snRNP contains a small RNA associated with the ring of LSm1-7 proteins (Schumperli and Pillai 2004; Will and Luhrmann 2001; Bertrand and Bordonne 2004) where properly assembled Sm cores, which must include the SMN protein, are required for snRNP import into the nucleus (Narayanan et al. 2004; Liu and Gall 2007; Lee et al. 2009a). Although SMN protein was diffusely localized in the cytoplasm, it was also localized to cytoplasmic, nonmembranous U bodies (Liu and Gall 2007; Lee et al. 2009a) (Table 13.2). SMN complex proteins, Gemin2, Gemin3 and Gemin5, have recently been shown to colocalize with SMN proteins in U bodes (Cauchi et al. 2010).

13.8.2 Relationship to GW/P Bodies

U bodies, as marked by antibodies to either LSm11 or SMN, do not colocalize with the Golgi complex, mitochondria or centrosomes, but partly overlap with the endoplasmic reticulum and GW/P bodies as marked by antibodies to Dcp1, eIF4E, Me31B/Rck/p54/Dhh1/DDX6 (Liu and Gall 2007) (Table 13.3). Partial overlap of U bodies with GW/P bodies was most abundant in mitochondria-rich regions of the cytoplasm (Liu and Gall 2007). Liu and Gall also showed that every U body is attached to one or more GW/P bodies, but not every GW/P body is associated with a U body and on occasion, U and GW/P bodies form a string of alternating bodies (Liu and Gall 2007). An immunohistochemical study by Cauchi et al. in *D. melanogaster* egg chambers revealed that U body components Gemin2, Gemin3 and Gemin5 invariably associate with GW/P bodies that contain Me31B but do not colocalize to GW/P bodies (Cauchi et al. 2010).

Other studies of interest demonstrated that genetic mutations of GW/P body components (Trailer Hitch (Tral) and Ago2) disrupted the organization of U bodies suggesting that the formation and/or organization of U bodies depended on proper functioning of GW/P bodies (Liu and Gall 2007). More recently, Lee et al. confirmed this finding by showing that GW/P body mutations disrupt SMN protein distribution and the organization of U bodies (Lee et al. 2009a). They also showed that mutations in the *smn* gene disrupted the function and organization of U bodies and GW/P bodies, suggesting that the SMN protein is an essential component for the functional relationship between U bodies and GW/P bodies (Lee et al. 2009a). Although U bodies and GW/P bodies partially colocalize with and share LSm1-7 protein components, their expression patterns differ between *D. melanogaster* nurse cells and oocytes where U bodies are evenly distributed between nurse cells and oocytes whereas GW/P bodies are expressed predominantly in oocytes. Further research into the relationship between U bodies and

GW/P bodies and their role in snRNP assembly, storage, processing, degradation and exchange to the nucleus is required to elucidate their functional relationship and their possible role in snRNP biogenesis and nuclear organization.

13.9 TAM Bodies

Specialized structures in *S. cerevisiae* referred to as TAM bodies (temporal asymmetric MRP bodies) are observed exclusively during mitosis (Gill et al. 2006). Like GW/P bodies, these structures contain Xrn1p but also RNase mitochondrial RNA processing (MRP), which is an essential and highly conserved RNP complex found in eukaryotes. RNase MRP is involved in ribosome biogenesis by processing ribosomal RNA transcripts to produce primers for mitochondrial DNA replication and it cleaves *S. cerevisiae* B-type cyclin (*CLB2*) mRNA (Martin and Li 2007). Colocalization of Xrn1p with RNase MRP to GW/P body-like structures suggests these ribonucleases regulate mitosis by degrading mRNAs encoding cell cycle regulators. Human MRP/RNase P, originally named Th/To (Gold et al. 1989), is a target autoantigen of systemic sclerosis and other related disease sera (Karwan 1998; Kuwana et al. 2002; Yamane et al. 2001), where the immunodominant targets were the Rpp25 and hPop1 subunits (van Eenennaam et al. 2002). Of note, Pop1 was a key component that localized to TAM bodies by IIF (Gill et al. 2006). Considering the features of TAM bodies described to date, it is likely that other RNA processing components will link them even more closely to GW/P bodies in the future.

13.10 Conclusion

The cytoplasm of eukaryotic cells is a complex, dynamic and highly partitioned composite of organelles and structural components. In addition to a variety of membrane bound organelles, the cytoplasm contains a number of nonmembranous RNP bodies that house specific mRNAs at various stages of post-transcriptional processing. GW/P bodies and their roles in RNA stabilization, transport, translational repression and/or degradation have emerged from this list as key players in the modulation of mRNA expression. Evidence to date also indicates that GW/P bodies transiently interact with other RNP bodies in a molecular “dance” moving protein, mRNA and miRNA from one body to another. It is obvious from protein colocalization studies that GW/P bodies are heterogeneous in protein composition and at any given time a subset of GW/P bodies may contain protein cargo that is different from another subset of GW/P bodies. The complete proteome of these similar, yet diverse, RNP bodies has not yet been elucidated and, therefore, other functions of these important bodies remain to be discovered.

Given the evidence that these RNP bodies share similar components to GW/P bodies (Table 13.3), it is entirely possible that they interact with GW/P bodies to

allow their cargo mRNA to become associated with the RNAi or decapping machinery. Regulation of molecular signals or physiologically relevant cues that direct interactions of RNP bodies with GW/P bodies has yet to be elucidated. Furthermore, it will be important to determine if these cues alter or affect GW/P body protein composition and thereby their function. These questions are essential to understanding GW/P body assembly, function, their interactions with other RNP bodies, disassembly and ultimately are key to understanding the role of these structures in cellular processes such as the regulation of mRNA expression and diseases such as in autoimmunity, neurological disease and cancer.

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Chapter 14
An SNP in the Trinucleotide Repeat Region of the *TNRC6A* Gene Maps to a Major TNGW1 Autoepitope in Patients with Autoantibodies to GW182

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Abstract GW/P bodies contain two *TNRC6A* protein isoforms (GW182 and TNGW1) that function as translational repressors of mRNA through Ago2-mediated RNA silencing. Autoantibodies to GW/P body components GW182, Ge-1 and Ago2 have previously been correlated with clinical autoimmune diseases including neurological disease, Sjögren's syndrome, systemic lupus erythematosus, rheumatoid arthritis and primary biliary cirrhosis. No studies were published to date examining if patients with autoantibodies directed against GW/P bodies contain autoantibodies to the trinucleotide repeat (TNR) region of TNGW1, which differs from GW182 only by the addition of an N-terminal QP-rich 253 amino acid sequence. Our data show that 85.7% of GW/P body positive plasma contain autoantibodies to various epitopes in the TNR region of TNGW1. Given the association of neurological diseases with autoantibodies directed to the TNR region on exon 5 of *TNRC6A*, this study examined whether there were TNR expansions as described in other neurological diseases and/or mutations in the nucleotide sequence of the CAG/CCA/G-rich region in seven anti-GW/P body positive patients, six control and eight breast cancer patients. Although a TNR expansion was not identified, 28.6% of patients containing autoantibodies to the TNR of TNGW1 were shown to have a single nucleotide polymorphism (SNP) at c.344C>A in the CAG/CCA/G-rich region of *TNRC6A*, which when translated, would produce a protein variant of p.Pro115Gln. The amino acid change may alter the structure of TNGW1 and/or perturb its miRNA regulatory function although this has not been examined experimentally. A putative change in protein structure may lead to a loss of tolerance to the TNGW1 protein or

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result in a "neo-antigen" in patients containing the specific *TNRC6A* SNPs. Further studies of a larger cohort of GW/P body positive patients and structure–function relationships of the variant *TNRC6A* are required to fully understand the role that such SNPs play in GW/P body autoantibody production and/or pathogenesis of related autoimmune diseases.

14.1 Introduction

The mechanisms that underlie human autoimmune diseases are notably complex because multiple domains, which include environment, genetics, and tissue injury, are involved (Rosen and Casciola-Rosen 2009). While this highly complex system has presented challenges in developing an overarching paradigm of etiology and pathogenesis, the immune response in individual autoimmune diseases is remarkably specific as evidenced by distinct autoantibodies associated with various disease phenotypes (reviewed in Stinton et al. 2004). The aberration of B- and T-cell responses in these conditions has provided important insights into disease mechanism (reviewed in de Souza et al. 2010). Some data have suggested that the selection of target autoantigens in autoimmunity is based on molecular mimicry of unrelated viral and other microbiological vectors (Shoenfeld 2009; Naugler and Conrad 2010); the release of altered antigens during apoptosis, necrosis or exocytosis (Rosen and Casciola-Rosen 2009); the impact of environmental factors (Gutierrez-Roelens and Lauwerys 2008); aberrations in miRNAs that play a vital role in the regulation of immunological functions and the prevention of autoimmunity (Pauley et al. 2009); or the appearance of neo-antigens due to mutations of target macromolecules. In addition, environmentally induced epigenetic effects such as DNA hyper- or hypo-methylation are known to contribute to multiple systemic autoimmune disease pathogenesis in genetically predisposed patients (reviewed in Strickland and Richardson 2008). Taken together, distinct autoantibody patterns in autoimmune conditions may be attributed to an integrated and amplified output of any of these interacting systems. Since B-cell antigen expression and structure may occur independently under these conditions, it is important to determine if an autoimmune reaction is generated as a result of an epigenetic change in expression of an allele carrying a single nucleotide polymorphism (SNP).

One of the more recently described targets of the human autoimmune response is GW bodies [GWB, also known as mammalian processing (P) bodies or Dcp containing bodies (hereafter referred to as GW/P bodies)]. These cytoplasmic domains, characterized by glycine- and tryptophan-rich protein GW182, were initially identified through the use of autoantibodies from a patient with a neuropathy and ataxia that produced a unique cytoplasmic discrete speckled staining pattern on human tissue culture cell lines (Eystathioy et al. 2002). Since then, many investigators have focused their attention on elucidating the protein components of GW/P bodies as an approach to understanding their structure and function. Primary functions of GW/P body components include the post-transcriptional processing of messenger RNA (mRNA) through RNA interference (RNAi) or RNA silencing pathways (Jakymiw et al. 2005;

Liu et al. 2005a, b; Pillai et al. 2005; Rehwinkel et al. 2005; Sen and Blau 2005; Moser and Fritzler 2010b), 5′→3′ mRNA degradation (Bashkirov et al. 1997; Ingelfinger et al. 2002; van Dijk et al. 2002; Eystathioy et al. 2003c; Sheth and Parker 2003; Cougot et al. 2004; Andrei et al. 2005; Fenger-Gron et al. 2005; Rehwinkel et al. 2005), and mRNA transport and stabilization (Barbee et al. 2006; Moser et al. 2007) (for review see Moser and Fritzler 2010a).

As the list of GW/P body-protein components expands, there is continued effort to identify the autoimmune diseases associated with GW/P bodies in anticipation that such studies will provide clues to the genesis of the cognate autoantibodies and also their pathogenic role in the associated diseases. Common clinical presentations of patients that had antibodies directed to GW/P bodies included neurological symptoms of ataxia, and motor and sensory neuropathy (33%), Sjögren's syndrome (SjS) (31%) and the remainder had a variety of other diagnoses such as systemic lupus erythematosus (SLE) (12%), rheumatoid arthritis (RA) (7%) and primary biliary cirrhosis (10%) (Eystathioy et al. 2003b; Bhanji et al. 2007). The relevance of circulating GW/P body autoantibodies to the etiology or pathogenesis of disease, the loss of tolerance to autoantigens such as GW182/TNRC6A and/or prognosis is currently unknown. Recent microRNA (miRNA) expression studies in RA (Nakasa et al. 2008; Pauley et al. 2008; Stanczyk et al. 2008) and SLE (Dai et al. 2007, 2009) suggest that these autoimmune conditions are characterized by unique RNAi/miRNA profiles (reviewed in Pauley et al. 2009; de Franca et al. 2010) implicating GW/P body proteins, GW182, TNGW1 and Ago2, as key players in the induction and/or production of autoantibodies or related autoimmune phenomena.

Independently expressed isoforms GW182 and TNGW1 have been previously shown to localize to GW/P bodies, function as translational repressors in Ago-2 mediated RNA silencing (Li et al. 2008; Lian et al. 2009; Takimoto et al. 2009; Eulalio et al. 2009) and differ only by the addition of an N-terminal QP-rich 253 amino acid (aa) sequence, termed the trinucleotide repeat (TNR) region, to the TNGW1 protein (Fig. 14.1a, c). At the genetic level, the TNR region of the trinucleotide repeat containing 6A (TNRC6A) gene is encoded on exon 5 of chromosome 16p11.2 (Fig. 14.1b) and is rich in CAG/CCA/G codons (Fig. 14.1c).

Potential relevance for our studies were linked to observations that genetic aberrations of TNR regions have been associated with a number of neurological diseases including Huntington's disease and spinocerebellar ataxia type 2 (SCA2). Huntington's disease is an autosomal dominant neurodegenerative disorder caused by an expansion of polyglutamines (CAGs) in the Huntington (Htt) protein. Of relevance to the RNAi pathway, the Htt protein has been shown to be associated with Ago2 in GW/P bodies where the depletion of Htt resulted in compromised RNA-mediated gene silencing (Savas et al. 2008). Unfortunately this study did not elucidate the exact molecular role of Htt in the RNAi process, but did provide compelling evidence that Htt may function as an Argonaute accessory factor involved in RNAi-mediated mechanisms and/or other translation repression/mRNA decay pathways associated with GW/P bodies (Savas et al. 2008). Ataxin-2 protein is subject to polyglutamine (CAG) expansion which is implicated in the pathogenesis of SCA2. Ataxin-2 is reported to localize to GW/P bodies where it affects the assembly of GW/P bodies and stress granules through

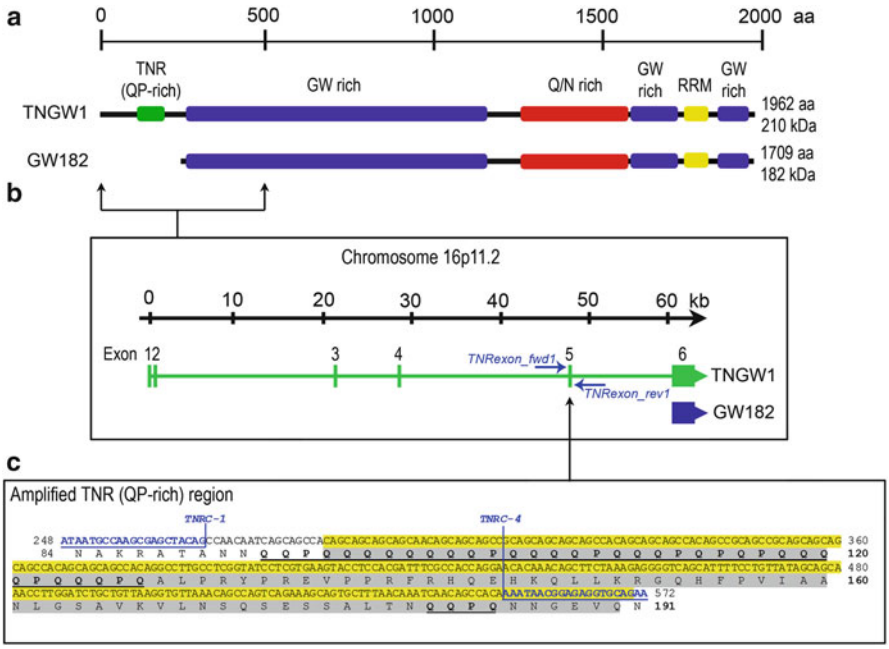

Fig. 14.1 Schematic diagram of the human TNRC6A gene products GW182 and TNGW1 protein and the respective location of the TNR region of interest. (**a**) GW182 is a 182 kDa protein containing three GW-rich regions, a QN-rich region and a RNA recognition motif (RRM). TNGW1 is a 210 kDa isoform of the GW182 protein that contains an extra N-terminal 253 amino acid polypeptide which contains a stretch of glutamine-proline repeats (QP-rich) translated from multiple CAG and CCA/G repeats. (**b**) The TNRC6A gene resides in human chromosome 16p11.2 where the 5′ end corresponding to the mRNA of these two isoforms is separated by 60 kb. The TNR QP-rich domain is encoded from exon 5 and was amplified using PCR between desalted forward and reverse primers TNRexon_fwd1 (5′-GTCTTGGCCGAAGGTCCCTGG-3′) and TNRexon_rev1 (5′-TGGGCAGAACCTGAAGGGAGAA-3′) (*blue*). (**c**) The CAG/CCA/G repeat region within the TNR region was amplified using PCR between desalted forward and reverse nested primers TNRC-1 (5′-ATAATGCCAAGCGAGCTACAG-3′) and TNRC-4 (5′-TTCTGCACCTCTCCGT TATTT-3′) respectively (*blue*). All DNA primers were synthesized by University Core DNA Services (University of Calgary, Calgary, AB). The region highlighted in yellow indicates the specific nucleotide sequence analyzed within this study and the respective translated amino acids of this TNR QP-rich region (highlighted in *grey*)

interaction with GW/P body component DDX6/Rck/p54 (Nonhoff et al. 2007). Taken together, these studies suggest that expansions and/or mutations in CAG-rich TNR-containing genes may alter post-transcriptional regulation of their respective proteins and perturb the integrity or miRNA regulatory function of GW/P bodies. To date, it is unclear whether these genetic changes may affect tolerance and the production of autoantibodies to GW/P bodies.

The foregoing served as the basis for our study to examine whether plasma from patients with GW/P body autoantibodies also contained autoantibodies to the TNR region of TNGW1 and if the TNR region of *TNRC6A* was mutated in seven GW/P body patients, six control patients and eight breast cancer patients. The primary

analysis was based on the polymerase chain reaction (PCR)-amplified CAG/CCA/G-rich TNR region of the TNRC6A gene, which specifically encodes the TNGW1 protein (Fig. 14.1) (Li et al. 2008).

14.2 Background Clinical Information

Seven patients with anti-GW/P body antibodies were selected based on the availability of cells for DNA extraction and on serum autoantibodies that produced a cytoplasmic speckled pattern of staining by indirect immunofluorescence (IIF) on HEp-2 cells and specific reactivity to GW182 as determined by addressable laser bead immunoassay (ALBIA) in the Mitogen Advanced Diagnostics Laboratory (University of Calgary, Calgary, AB) (Table 14.1). The clinical features of the patients obtained by a retrospective chart review and consultation with the attending physicians revealed a common theme of neurological disease in addition to features of SLE, SjS and RA (Table 14.1). Control patients were selected based on the absence of autoantibodies directed to GW/P bodies as determined by IIF on HEp-2 cells (Table 14.1). The six control subjects included a normal control, and the others had various autoimmune conditions that included hypothyroidism, multiple sclerosis, SjS, idiopathic neuropathy, and diffuse cutaneous systemic sclerosis. Eight breast cancer patients were chosen as a disease-specific control group because GW/P bodies are overexpressed in breast cancer cells and tissues compared to normal breast cells and tissues (Luft 2005). The mean age of GW/P body subjects was 62 years (range = 33–84) and of control subjects was 49 years (range = 27–77) (Table 14.1). Ethics review and approval (ID# 20173) was provided by the Conjoint Health Research Ethics Board (University of Calgary, Calgary, AB) and blood samples for this study were collected after informed written consent was obtained. Patient anonymity was maintained throughout the study.

For DNA and related genetic studies, whole blood was collected from these patients by venipuncture and then centrifuged at $2{,}000 \times g$ at 4°C for 10 min to separate whole blood into plasma, buffy coat and red blood cell fractions. Genomic DNA was extracted from the buffy coat cells with the QIAamp DNA Mini Kit (Qiagen, Mississauga, ON) using the protocol provided by the manufacturers. DNA concentration was determined using the NanoVue spectrophotometer (GE Healthcare Life Sciences, Baie d'Urfe, QC).

14.3 Autoantibodies to GW/P Bodies are Validated by Co-Staining with GW182 Monoclonal Antibody

To validate that the cytoplasmic discrete dot staining pattern was consistent with anti-GWB reactivity, co-localization studies were first performed (Fig. 14.2). Commercial prefixed HEp-2 cells (ImmunoConcepts) were incubated for 30 min in a blocking buffer containing 10% normal goat serum (Antibodies Incorporated,

Table 14.1 Demographic and clinical characteristics of seven patients with GW/P body autoantibodies and six patients containing no autoantibodies or autoantibodies unrelated to GW/P bodies

Patient No.	Age	Sex	Clinical profile	ANA/ENA profile
T01	50	F	Generalized muscle weakness, neuropathic pain	GW/P bodies
T02	78	F	RA, neuropathy	GW/P bodies
T03	55	F	SLE, multiple personality disorder	GW/P bodies, cytoplasmic speckled
T04	84	F	SjS, ataxia	GW/P bodies
T05	63	M	Sensory neuropathy	GW/P bodies
T06	73	F	SLE with central nervous system involvement, SjS	GW/P bodies, cytoplasmic speckled, centromere, diffuse cytoplasmic
T07	33	F	Intermittent migratory joint pain, psoriasis and onycholysis	GW/P bodies, cytoplasmic speckled
N01	40	F	Hypothyroid	Nucleolar speckles, few cytoplasmic speckles
N02	27	F	Normal control	Negative
N03	53	M	Multiple sclerosis	Unknown
N04	36	F	Anti-Ku and anti-PL7 antibodies	Diffuse cytoplasmic speckled
N05	63	F	Neuropathy, SjS, Raynaud's, diffuse Scleroderma	Cytoplasmic speckled, cytoplasmic foci
N06	77	F	Lichen sclerosis et atrophicus, peripheral neuropathy	Cytoplasmic speckled, cytoplasmic foci, nuclear

Mean age of test subjects = 62; mean age of control subjects = 49
ANA antinuclear antibody; *ENA* extractable nuclear antigens; *RA* rheumatoid arthritis; *SLE* systemic lupus erythematosus; *SjS* Sjögren's syndrome

Davis, CA) and 2% bovine serum albumin (Sigma-Aldrich) to minimize nonspecific antibody binding. The cells were then incubated for 1 h with mouse monoclonal 4B6 antibodies to recombinant GW182 (1:10) (Eystathioy et al. 2003a) and with patient serum containing anti-GW/P bodies diluted between 1:500 and 1:5,000. After washing with phosphate buffered saline (PBS), cells were incubated in a dark chamber with the corresponding secondary goat fluorochrome-conjugated antibodies diluted 1:200. Alexa Fluor (AF) 488 (green) and 568 (red) secondary antibodies were purchased from Invitrogen (Burlington, ON, Canada). After washing with PBS, the cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI), mounted in Vectashield (Vector Laboratories, Burlingame, CA) and examined using a 100× objective on a Leica DMRE microscope equipped with epifluorescence and an Optronics camera. Appropriate IIF controls with one primary antibody or both secondary antibodies alone or in combination revealed no observable nonspecific staining and no detectable bleed-through between microscope filter sets.

In agreement with the clinical antinuclear antibody (ANA) testing results (Table 14.1), N01 plasma was confirmed to have predominately dense fine nuclear

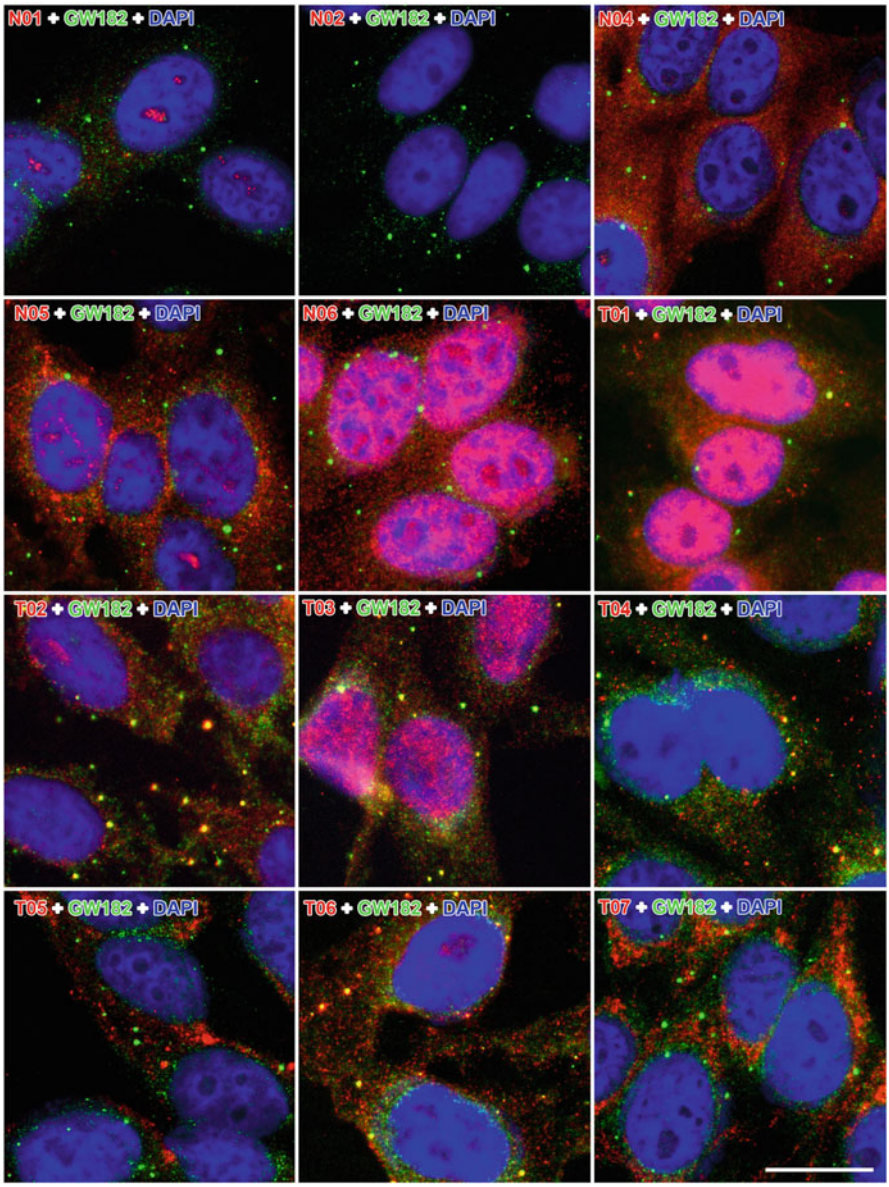

Fig. 14.2 Indirect immunofluorescence (IIF) colocalization analysis of autoantibodies present in patient plasma with mouse monoclonal antibody to GW182 on HEp-2 cell substrates. Patient plasma (*red*; AF568) were double-stained with mouse monoclonal anti-GW182 antibodies (*green*; AF488) to mark GW/P bodies. Colocalization was observed for all seven anti-GW/P body samples whereas the control plasma showed no staining of GW/P bodies

speckled staining with a few cytoplasmic speckles, N02 plasma had a negative ANA, N04 had a diffuse cytoplasmic speckled staining pattern, N05 plasma had cytoplasmic speckled and foci staining and N06 plasma had cytoplasmic speckles and foci staining in addition to nuclear staining (Fig. 14.2, red). None of the control patient autoantibodies (red) colocalized with the GW182 monoclonal antibody (green) indicating that these control samples did not contain detectable autoantibodies directed to GW/P bodies. Similarly, the breast cancer patients did not contain autoantibodies to GW/P bodies (data not shown). In contrast, patient samples T01 through T07 (red) all showed colocalization with GW182 foci (green) indicating that all seven of the test patients contained autoantibodies to GW/P body components as marked by the monoclonal anti-GW182 (Fig. 14.2).

14.4 Patients with Autoantibodies to GW/P Bodies Recognize the TNR Region of TNGW1

A set of addressable beads bearing laser reactive dyes (Luminex, Austin, TX) were coupled to purified recombinant full-length early endosome antigen 1 (EEA1), the C-terminal domain of Ge-1/Hedls/EDC4, full-length GW182, the TNR-specific region of TNGW1 (aa 1–204), partial GW2/TNRC6B (aa 1218–1306) and full-length GW3/TNRC6C polypeptides and analyzed for antibody reactivity as previously described (Eystathioy et al. 2003a). Human 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 min at room temperature. This was followed by the addition of 40 μL of phycoerythrin-conjugated antihuman IgG (Jackson ImmunoResearch, West Grove, PA; diluted 1:50) to each well and incubated on an orbital shaker for an additional 30 min. The binding of human antibodies to the analytes bound to specific beads was determined on a Luminex 100 dual-laser flow cytometry (Luminex, Austin, TX). Each assay included negative and positive controls, and results were expressed as median fluorescent units (MFUs).

The reactivity of the antibodies present in the plasma samples as detected by multiplexed ALBIA showed that the anti-GW/P body samples (T01–T07) contained varying titers of antibodies to various GW/P body components including Ge-1/Hedls/EDC4, GW182 and TNGW1 (a peptide representing the TNR region) (Fig. 14.3). T04 and T05 had the highest titer of antibodies to GW182 compared to the other anti-GW/P body samples (Fig. 14.3). In contrast, the control patient plasma (i.e., N01–N06) had only very low/background MFU values (Fig. 14.3).

Having established reactivity to GW182, the autoantibody reactivity to the 253 aa TNR domains of the TNGW1 protein was accomplished by an epitope mapping protocol wherein membranes containing *in situ* synthesized sequential 15-mer peptides that were offset by five amino acids were prepared by Eve Technologies (Calgary, AB, Canada) using protocols as previously described (Li et al. 2008). All anti-GW/P body positive plasma, with the exception of T07, contained antibodies

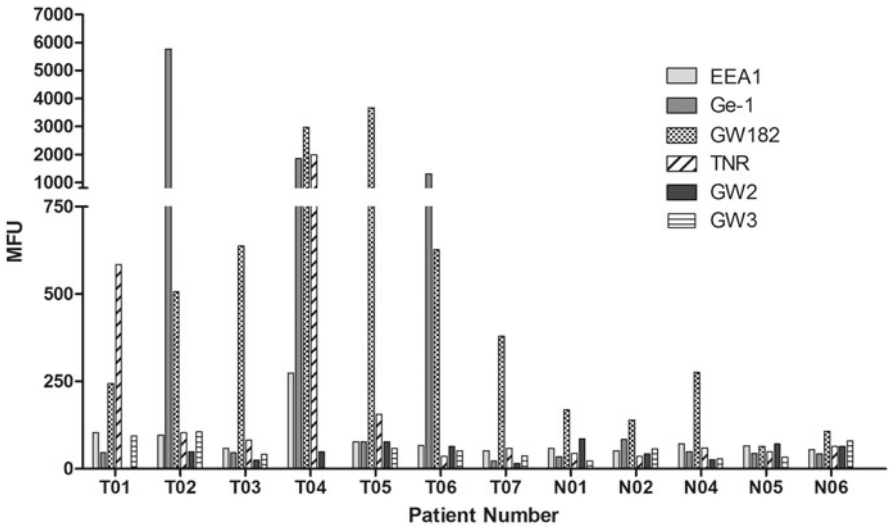

Fig. 14.3 Addressable laser bead immunoassay (ALBIA) autoantibody profile of anti-GW/P body positive plasma compared with control plasma. Reactivity of autoantibodies in plasma samples is expressed as a mean fluorescent unit (MFU) value

that recognized one or more epitopes of the TNR peptide (Table 14.2) including the LSRDLVQEEEQLMEEKKKKKDDKKKKEAAQKKATEQKIKV "K-rich" peptide sequence (with variable peptide binding affinities for each sample). Samples T01 through to T06 also contained autoantibodies which bound with variable affinity to the QP-rich region of the TNR peptide array (Table 14.2, bold sequence). T07 did not contain autoantibodies to the QP-rich region and along with T01, T02, T03, T05 and T06 showed only minimal affinity to the small peptide region (LSRDLVQEEE). T01 and T02 contained autoantibodies which specifically bound to epitopes within the QP-rich region of the TNR (STNNNAKRATANNQQPQQ QQQQQQPQQQQPQQQPQP). T03, T04, T05, and T06 contained autoantibodies which mapped with high affinity to the entire QP-rich region (Table 14.2, bold sequence). All samples (with the exception of T07) showed some apparent epitope spreading downstream of the QP-rich region. Samples N01–N06 and BCA1–BCA8 showed no reactivity to the TNR peptide array (data not shown).

14.5 Some Patients with Anti-GW/P Bodies Have a Common SNP in the TNR Region of the GW182/TNRC6A Gene

Having established that some patients with anti-GWB/P body antibodies reacted with specific domains of the TNR region of GW182, we then set out to determine if these reactive TNR domains corresponded to DNA sequence abnormalities that could conceivably render their cognate proteins immunogenic. The purified DNA

Table 14.2 TNR peptide epitope recognition by antibodies in seven GW/P body patient plasma samples [AU1]

	Sample						
	T01	T02	T03	T04	T05	T06	T07
Peptide							
MRELEAKATKDVERN				1			
AKATKDVERNLSRDL		2	2	2	2	3	
DVERNLSRDLVQEEE	1	3	3	3	3	3	1
LSRDLVQEEEQLMEE	2	3	1		2	1	1
VQEEEQLMEEKKKKK					1	4	
QLMEEKKKKKDDKKK					1	4	
KKKKKDDKKKKEAAQ				1	2	4	
DDKKKKEAAQKKATE				1	3	4	
KEAAQKKATEQKIKV				1	2	3	
KKATEQKIKVPEQIK		2		2	4	3	
QKIKVPEQIKPSVSQ		1	1	3	4	2	
PEQIKPSVSQPQPAN							
PSVSQPQPANSNNGT							
PQPANSNNGTSTATS							
SNNGTSTATSTNNNA	1				1		
STATSTNNNAKRATA	2	3	4	1	4	3	
TNNNAKRATANNQQP		3	4	3	4	4	
KRATANNQQP**QQQQQ**	1	2	4	3	4	4	
NNQQP**QQQQQQQQPQ**	3	1	1	3	1		
QQQQQQQQPQQQQPQ			2	4	1	1	
QQQPQQQQPQQQPQP	1	1	3	4	1	2	
QQQPQQQPQPQPQQQ	1	1	1	4	1	2	
QQPQPQPQQQQPQQQ	2		3	4	1	3	
QPQQQQPQQQPQALP			3	4	2	3	
QPQQQPQALPRYPRE			2	4	2	1	
PQALPRYPREVPPRF				3			
RYPREVPPRFRHQEH			3	4	3	3	
VPPRFRHQEHKQLLK			1	2	4	4	
RHQEHKQLLKRGQHF				1	4	4	
KQLLKRGQHFPVIAA			1	1	4	4	
RGQHFPVIAANLGSA							
PVIAANLGSAVKVLN				1		2	
NLGSAVKVLNSQSES			1	1	1	2	
VKVLNSQSESSALTN					2		
SQSESSALTNQQPQN							
SALTNQQPQNNGEVQ	2			1			
QQPQNNGEVQNSKNQ							
NGEVQNSKNQSDINH							
NSKNQSDINHSTSGS	3	3	3	2	2	2	
SDINHSTSGSHYENS		1	2	1	2	2	
STSGSHYENSQRGPV							
HYENSQRGPVSSTSD					2		

(continued)

Table 14.2 (continued)

	Sample						
	T01	T02	T03	T04	T05	T06	T07
QRGPVSSTSDSSTNC		3					
SSTSDSSTNCKNAVV		4					
SSTNCKNAVVSDLSE							
KNAVVSDLSEKEAWP	2						
SDLSEKEAWPSAPGS							
KEAWPSAPGSDPELA							
SAPGSDPELASECMD							
DPELASECMDADSAS							
SECMDADSASSSESE							
ADSASSSESERNITI		3			2		
SSESERNITIMASGN							
RNITIMASGNTGGEK				1		1	
MASGNTGGEKDGLRN							
TGGEKDGLRNSTGLG							
DGLRNSTGLGSQNKF		2	4	3	3	3	
STGLGSQNKFVVGSS		1	3	3	1	2	

was subjected to PCR, which was performed in 50 μL reactions containing 5 μL Surestart Taq 10× reaction buffer with $MgCl_2$ (Stratagene, La Jolla, CA), 1 μL 10 mM dNTP mix (dATP, dCTP, dGTP, dTTP, Amersham Biosciences, Piscataway, NJ), 1.5 μL of TNRexon_fwd1 and TNRexon_rev1 primers (10 pmol/μL), 1 μL of Surestart Taq DNA polymerase (1.25 U/μL) and 100 ng of genomic DNA. The reactions were thoroughly mixed and transferred to a thermocycler (Perkin Elmer Gene Amp PCR System 2400) for amplification using the following steps: initial denaturation step at 95°C for 10 min, 35 cycles of 95°C denaturation for 1 min, 55°C annealing for 1 min and 72°C extension for 1 min, followed by a 72°C extension step for 10 min and indefinite hold at 4°C. PCR-amplified DNA samples were frozen at −20°C until use.

Using this protocol, the TNR region of *TNRC6A* was amplified using intronic primers that flanked exon 5 in genomic DNA isolated from the buffy coat layer of whole blood (Fig. 14.1b). The intronic PCR-amplified 893 bp product DNA was examined on a 1.2% agarose gel (Fig. 14.4), gel-purified and sequenced by automated DNA sequencing using the same intronic primers (Fig. 14.1b) and nested TNRC-1 and TNRC-4 primers flanking the QP-rich TNR region (Fig. 14.1c). Sequence chromatograms were analyzed using ChromasPro software. We observed that the DNA from N01–N06 and BCA1–BCA8 contained the same genetic sequence for the TNR region of the TNRC6A gene, which was 100% identical (data not shown) to the *H. sapiens* TNRC6A mRNA reference sequence NM_014494.2 in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/nuccore/116805347). When the anti-GW/P body positive samples T01–T07 DNA sequence chromatograms were compared to the DNA sequence from the controls, breast cancer patients and the mRNA reference sequence NM_014494.2, it was remarkable that two out

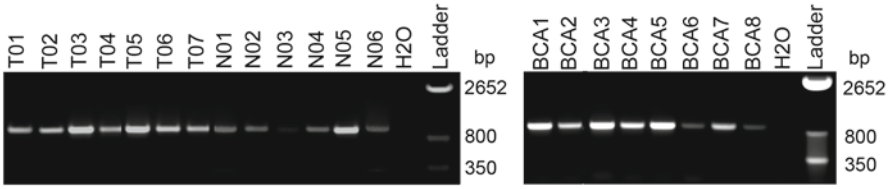

Fig. 14.4 PCR amplification products on an electrophoretic 1.2% agarose gel shows the 893 bp exon 5 for healthy controls (N01–06), breast cancer (BCA1–8) and anti-GW/P body antibody positive patients (T01–07). The 893 bp band was excised from the gel and purified using the QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions and sequenced as described in Fig. 14.5

of seven patients (T04 and T05) contained the same SNP in the CAG/CCA/G-rich TNR region of the TNRC6A gene specifically at c.344C>A (Fig. 14.5a, b, red arrows, NM_014494.2:c.344C>A). A subsequent search of the NCBI Reference SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP) revealed that this specific SNP was predicted as refSNP ID# rs11644562. When translated, the SNP was predicted to yield a single amino acid substitution of p.Pro115Gln (Fig. 14.5c, NP_055309.2:p.Pro115Gln).

14.6 Discussion

GW182 is the predominant gene product of *TNRC6A*, however the longer isoform TNGW1 is expressed independently of GW182 and localizes to a subset of mammalian GW/P bodies (Li et al. 2008). Although the exact function of TNGW1 has yet to be fully elucidated, TNGW1 has been shown to induce translational repression and mRNA degradation in Ago2-mediated RNA silencing to the same extent as the GW182 protein (Li et al. 2008). It has been suggested that the unique TNR region at the N-terminus of TNGW1 may play an important role in protein folding and localization with Argonaute proteins (Li et al. 2008). In general, TNR mutations and/or expansions are known to play a key role in many neurological diseases (reviewed in Di Prospero and Fischbeck 2005; Batra et al. 2010), leading us to question whether patients with autoantibodies to GW/P bodies who have clinical neurological signs and symptoms contain mutations or expansions in the CAG/CCA/G-rich TNR region of *TNRC6A*.

Six out of seven (~86%) patients containing autoantibodies to multiple GW/P body components also contained autoantibodies to the N-terminal TNR region of the TNGW1 protein, which mapped to 15mers in the K-rich and QP-rich regions. Out of these seven patients, T04 and T05 contained the same SNP in the CAG/CCA/G-rich TNR region of the TNRC6A gene specifically at c.344C>A which upon translation would lead to a protein mutation of p.Pro115Gln. This SNP has been predicted in the NCBI Reference SNP database (refSNP ID# rs11644562), however our study is the first to report a clinical association with the NM_014494.2:c.344C>A SNP.

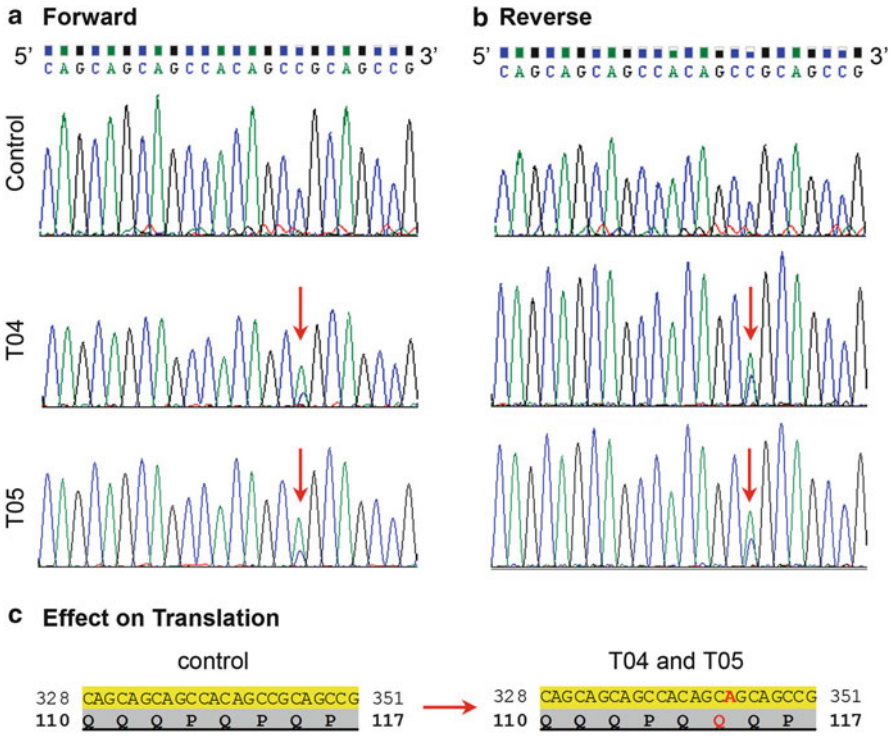

Fig. 14.5 Forward (**a**) and reverse (**b**) DNA sequence chromatograms of anti-GW/P body positive patient T04 and T05 showing the C to A single nucleotide polymorphism (*red arrow*) at position 344 from the start of the TNRC6A gene (NM_014494.2:c.344C>A). (**c**) The C to A mutation changes the codon from CCG to CCA and when translated leads to an amino acid change from Pro to Gln. DNA samples were sequenced using both forward- and reverse-nested primers (TNRC-1 and TNRC-4, Fig. 14.1c) by the University of Calgary DNA Sequencing Lab (Calgary, AB) using an Applied Biosystems 3730xl (96 capillary) genetic analyzer (Applied Biosystems, Foster City, CA). DNA sequences and chromatograms were subsequently analyzed using the ClustalW2 bioinformatics tool (version 2.0.12, European Bioinformatics Institute, http://www.ebi.ac.uk/Tools/clustalw2/) and ChromasPro software ver 1.5 (Technelysium Pty Ltd, Australia)

The sequence chromatograms for T04 and T05 clearly showed that two heterozygous alleles carrying the normal C-allele and the SNP A-allele are present at position 344 in exon 5 of *TNRC6A*. We hypothesize that in these two patients, the C-allele is normally active during transcription while the A-allele is normally suppressed by an epigenetic hypermethylated promoter region. During the activation of an autoimmune state, the A-allele may become hypomethylated if peripheral blood mononuclear cells change their epigenetic methylation status. This may lead to the translation of the A-allele thereby generating a second aberrant form of the native TNGW1 protein normally encoded by the C-allele. The antigenic load of accumulated aberrant TNGW1 protein (a neo-antigen) may be an important trigger of the autoantibody response specifically to GW/P bodies. This proposed mechanism would require further study.

Glutamines (Gln, Q) are polar and are generally found on the surface of proteins exposed to an aqueous environment and therefore thought to be frequently involved in protein–protein binding interactions. Consequently, a mutation from a nonpolar hydrophobic proline (Pro, P) to a polar hydrophilic Gln residue could potentially alter the protein–protein interactions of the TNGW1 protein. Hypothetically, it is possible that if the TNGW1 protein becomes mis-folded or otherwise recognized as a foreign protein in these patients, it could lead to a loss of B-cell tolerance and also to an increase in GW182 production to compensate for underproduction of TNGW1. Alternatively, the function of "mutated" TNGW1 may be altered by changing its protein-binding partners. Lian et al. showed that Ago2 binding domains are distributed in multiple domains distinct from the TNR region of TNGW1 suggesting that a mutation in a non-Ago2 binding domain would not affect its Ago2-miRNA-mediated translational repression ability (Lian et al. 2009). The TNR region of TNGW1 does not function as a translational repressor (EKLC, unpublished observations); however, the results of this current study underscore the importance of determining the exact function of the TNR region in relation to the key role that miRNA regulation plays in the prevention of autoimmunity. Albeit a small cohort, a SNP does exist in the TNR region of the *TNRC6A* gene in GW/P body positive patients but not in control subjects or in a cohort of breast cancer patients. Whether this SNP alters TNGW1 function in regards to miRNA regulation remains to be studied. Future studies would benefit from examining the miRNA profile of these GW/P body patients to examine which miRNAs are significantly upregulated or downregulated compared to normal and other unrelated autoimmune disease phenotypes.

The observation that some patients with anti-TNR antibodies possess SNPs in the cognate region are interesting in the context of studies by Winter et al. who examined p53 autoantibodies in 46 small cell lung cancer patient sera (Winter et al. 1992). They showed that 13% (6/46) of lung cancer patient serum contain autoantibodies reactive to the p53 protein, but also that 21% (5/24) produced antibodies to a missense mutation in the p53 gene (Winter et al. 1992). In addition, they observed that five available tumors from these patients all had a mutated p53 gene but no p53 autoantibodies were present in the patients whose tumors had a p53 stop, splice/stop or frameshift mutation ($n=10$) ((Winter et al. 1992) and reviewed in Tan 2001). Mutations in *p53* lead to an increased half-life of p53 compared to the wild-type p53 and were correlated with the presence of anti-p53, however the anti-p53 polyclonal response was not directed specifically to the p53 mutations. It follows that in future studies it will be interesting to determine whether the A-allele-derived TNGW1 will have a longer half-life than the native C-allele TNGW1. Potentially, the overexpression of TNGW1 may contribute to autoantibody production.

Although 28.6% (2/7) GW/P body positive patients have the SNP in the TNR region of the TNRC6A gene, it is still to be determined why the other 71.4% (5/7) patients do not contain the SNP. The answer to this apparent dilemma may be related to the clinical diagnosis, where T04 was the only patient to exhibit ataxia and T05 the only patient to exhibit sensory neuropathy (Table 14.1). Interestingly, T04 and T05 had the highest GW182 autoantibody titers of all GW/P body patients tested

suggesting the SNP in the TNR region may enhance the antigenicity of the GW182 protein. In future studies, a cohort of ataxia and sensory neurological disease patients without GW/P body autoantibodies could be examined for the presence of an SNP in the TNR region of *TNRC6A* to examine if this SNP is common to these specific neurological conditions. It is possible that patients containing autoantibodies to GW/P body components may contain underlying genetic mutations to these GW/P body antigens. In this cohort of GW/P body patients, two out of seven contained *TNRC6A* TNR region mutations, however, it is possible that some of this patient cohort may have mutations to other GW/P body antigens such as GW182, Ge-1/Hedls/EDC4 or Ago2 and would be an interesting avenue for further investigation.

14.7 Summary

Approximately 86% of patients containing autoantibodies to multiple GW/P body components also contained autoantibodies to the N-terminal K- and QP-rich TNR region of the TNGW1 protein. Of these patients, ~29% contained the same SNP (NCBI refSNP ID# rs11644562) in the CAG/CCA/G-rich TNR region of *TNRC6A* specifically at c.344C>A, which would lead to a protein mutation of p.Pro115Gln. This is the first study to report a clinical association with the NM_014494.2:c.344C>A SNP and to show that there may be a genetic basis for GW/P body autoantibody production; however, the exact molecular implications related to this particular SNP is unknown. Further studies of a larger cohort of GW/P body positive patients targeting the TNGW1 protein and other GW/P body autoantigens is required to fully understand the role that genetic polymorphisms play in autoantibody production. Future investigations in the GW/P body field would benefit from a greater knowledge of the structure of the TNR region of the TNGW1 protein and its protein binding partners.

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Chapter 15
Reflections on Ten Years of History of, and Future Prospects for, GW182 and GW/P Body Research

Edward K.L. Chan, Bing Yao, and Marvin J. Fritzler

15.1 GW/P Bodies as a Target of Human Autoantibodies

The literature on human autoantibodies in systemic rheumatic diseases has clearly elucidated major autoimmune targets, many of which are nucleic acid-protein macromolecular complexes or subcellular particles (Tan et al. 1988). Well-documented examples of these include the ribonucleoprotein Sm/RNP complex comprised of key components of U-rich small nuclear ribonucleoproteins (UsnRNPs) that are critical for processes in mRNA splicing, and chromatin subunits composed of DNA, histones and high mobility group proteins. Although there are still unanswered questions about why these are the targets of the B-cell response, the current thinking is that these nucleic acid-protein complexes are preferred target autoantigens in systemic autoimmune diseases because of their interactions with toll-like receptors (TLR). TLR3, 7, and 9 which are primarily located in the endosomes and are responsible for sensing of endogenous RNA and DNA ligands (Kawai and Akira 2009). Thus, endogenous nucleic acids-protein complexes have a higher tendency to stimulate a B-cell autoimmune response.

It is of special interest that GW/P bodies are enriched in several autoantigens such as AGO, GW182, and Ge-1 that have direct or indirect interactions with RNA. As discussed in Chap. 4, autoantibodies to AGO proteins are common in systemic lupus erythematosus (SLE) and other systemic autoimmune rheumatic diseases. Anti-GW182 autoantibodies are detected in Sjögren's syndrome, neurological

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diseases, SLE and other conditions (Bhanji et al. 2007; Stinton et al. 2011) while anti-Ge-1 and RAP-55 more commonly found in primary biliary cirrhosis (Chap. 5) (Stinton et al. 2011).

It is reasonable to ask why autoantibodies to different components of GW/P bodies are seemingly specific or predominant in different diseases. This is counterintuitive because conventional thinking suggests that GW/P bodies are presented as a whole structure to the immune system and the ensuing autoimmune response to different components is expected to be dominant in the same disease. However, this is not surprising in autoantibody studies as there are well-documented precedents in the literature. The best example is the autoimmune response to the UsnRNP complex, which is composed of U-RNA bound to the Sm core proteins B/B′, D, E, F, and G. In U1snRNP, there are additional proteins U1-70kD, A, and C that are in complex with the Sm core. The observation that anti-Sm autoantibodies recognize primarily B/B′ and D proteins and are specific marker antibodies for SLE has been puzzling. Yet anti-U1RNP autoantibodies recognize primarily the U1-70kD, A, and C proteins and are specific marker antibodies for mixed connective tissue disease (MCTD). Thus, the finding of multiple autoantibodies that bind to different components of GW/P bodies and their apparent link to different diseases has been previously documented in other autoantibody systems. One conclusion from these observations is that GW/P bodies are likely to be presented as autoantigens in many diseases and it will take additional studies to examine the underlying causes.

Although a number of GW/P proteins have been identified (see Tables 3.1, 12.1, and 13.1), the entire GW/P body proteome has yet to be reported and it is anticipated that the specific reactivity of autoantibodies to these structures will grow in number and complexity. More work is needed to determine whether there are novel autoantibodies with strong disease-specific associations. In this regard, it becomes important to determine whether a given protein found to be enriched in these structures is an integral or passenger component. It is not surprising that many mRNA binding proteins are detectable in GW/P bodies. One explanation is that more than 60 % of all mRNA are estimated to be regulated by miRNA (Friedman et al. 2009) and thus many mRNA binding proteins, such as the hnRNP proteins may be detectable in GW bodies as passengers on the target mRNA (Katahira et al. 2008). The question of whether such specific proteins have unique functions in GW/P bodies will need careful analysis and distinction from others described to date. Since Argonaute (AGO) and GW182 are currently considered the core proteins of the RNA-induced silencing complex (RISC) (Fabian and Sonenberg 2012), it seems appropriate to suggest that the RISC is the main target of human autoantibodies.

15.2 Limitations in Using Prototype Human Anti-GW Body Sera

Following publication of our initial studies on GW bodies (Eystathioy et al. 2002, 2003; Yang et al. 2004), there have been many requests for index human antibodies from colleagues around the globe for their own research studies. It is important to

appreciate that a drawback in using human sera is that the supply is usually limited. In the case of anti-GW182, patients with "monospecific" reactivity as defined by indirect immunofluorescence (IIF) are in the minority. The first prototype serum, anonymously identified as Amy, was used in the initial screening of cDNA expression libraries (Eystathioy et al. 2002) and all of the immuno-electron microscopy work (Yang et al. 2004). Initially, Amy was thought to be monospecific for anti-GW182 but due to high demand the supplies rapidly dwindled. When the Amy serum was no longer available, another serum IC6 was used as an anti-GWB serum (Yang et al. 2004). It was clear to us that IC6 had other antibodies because IIF clearly showed nuclear envelope staining (Ou et al. 2004). Another anti-GW182 serum 18033 from a patient with virtually identical clinical features to Amy became available in 2004. It was later pointed out that IC6 had predominant anti-Ge-1 and little or no anti-GW182, and 18033 also had strong reactivity to Ge-1 by Western blot and weaker reactivity to GW182 (Bloch et al. 2006). The experience in our laboratory agrees in part with the report that IC6 recognized predominantly Ge-1 and RAP55; however, in our hands serum 18033 also recognized GW182 and Ago2 (Li et al. 2008). Our policy in providing anti-GWB sera to colleagues has been revised to acknowledge limitations in the specificity of antibody and the interpretation of data derived from these sera must take their polyspecificity into consideration.

15.3 GW182 and Paralogs: A Proposal for Renaming

GW182 was provisionally named because of the apparently novel distribution of the glycine-tryptophan (GW/WG) motifs throughout the 182 kDa protein (Chap. 2). The largely irregular distribution of the GW motifs was most characteristic for this protein and no defined domain was recognized at the time except for a single RNA-recognition motif (RRM) in the C-terminal region (Eystathioy et al. 2002). The significance of the GW motifs is turning out to be very interesting as some GW motifs are involved in binding the PIWI domain of AGO proteins (Lian et al. 2009; Takimoto et al. 2009), exerting translational silencing activity (Chekulaeva et al. 2010; Yao et al. 2011), or binding to the CCR4-NOT complex leading to deadenylation and mRNA decay (Chekulaeva et al. 2011; Fabian et al. 2011). It is unfortunate that the Gene Nomenclature Committee decided to name the GW182 gene TNRC6A and that the two paralogs we had designated GW2 and GW3 were named TNRC6B and TNRC6C. The three paralogs appear to have equal silencing potentials (Li et al. 2008; Baillat and Shiekhattar 2009; Eulalio et al. 2009; Lazzaretti et al. 2009; Zipprich et al. 2009). Knocking down one of the three paralogs partially de-repressed silenced targets indicating their functional redundancy (Zipprich et al. 2009). Furthermore, translational silencing assay-based tethering constructs showed comparable results among all three paralogs (Baillat and Shiekhattar 2009; Eulalio et al. 2009; Lazzaretti et al. 2009; Lian et al. 2009; Zipprich et al. 2009). Their functional differences, if any, have yet to be demonstrated. Intriguingly, since HeLa cells expressed tenfold less TNRC6C compared to HEK293 cells, TNRC6C seems to be dispensable (Yao et al. 2011).

The rationale for using TNRC6A as the gene name was probably based on the fact that ~60 kB upstream of GW182, there was a gene already identified as TNRC6A (CAGH26) based on screening of trinucleotide-repeat (TNR) CAG containing genes in the human brain (Margolis et al. 1997). Expansion of TNR is known to be related to a set of diseases, most notably those with neurologic features, such as muscular dystrophy and Huntington disease (La Spada and Taylor 2010). TNRC6A is one of the TNR-containing genes in the human genome, but to date it has not been related to trinucleotide expansion diseases; although, point mutations in the TNR region have been identified in a few patients with anti-GW182 antibodies (Chap. 14). Thus, the longer isoform TNGW1 was identified as the 210-kDa GW182 isoform with an extra N-terminal 253-amino-acid polypeptide containing a stretch of glutamine-repeats (Q-repeat), which is translated from the CAG TNR (Li et al. 2008). TNRC6A resides on human chromosome 16p11.2 and the mRNA of TNGW1 and it contains five additional exons upstream of the putative AUG start codon of GW182.

The problem in naming TNRC6A as the gene name for GW182 is related to the fact that GW182 is the major isoform and it does not have the Q-repeat or the TNR. TNGW1 is a minor isoform and it has the TNR-derived Q-repeat. Most of the studies to date are based on GW182 and the functional role of the TNR-derived Q-repeat has yet to be published. Another major consideration is that TNRC6B and TNRC6C do not have TNR regions. Thus, the current nomenclature of these genes is misleading as their gene structure, except for a minor form of TNRC6A, is not linked to TNR at all. An effort should be directed to changing the names of these genes so that their names will be more consistent with their expressed protein structure and their clearly established function in miRNA-mediated gene regulation.

15.4 GW182, GW/P Bodies, and miRNA Function

Our earlier publications showed that siRNA (Jakymiw et al. 2005) and miRNA (Pauley et al. 2006) are highly enriched in GW bodies and, by extension, we suggested that they may be critical for RNA interference. Other investigators have shown that siRNA are localized to GW/P bodies and they showed that it is Ago2-dependent and transfection of siRNA results in upregulation of Ago2 and GW182 (Jagannath and Wood 2009). Functional siRNA activity correlates with increases in both number and size of GW bodies (Lian et al. 2007). In the classical lipopolysaccharide (LPS)-stimulation of human monocytes, there is an increase in both the number and size of GW bodies: the number of GWBs is highest at 8 h after LPS stimulation compared to unstimulated controls. These data are consistent with increases in miRNA-mediated activity during LPS stimulation (Pauley et al. 2010; Nahid et al. 2011).

It is now well accepted that AGO and GW182 are core components of the RISC (Fabian et al. 2010). AGO proteins play important roles in miRNA maturation, unwinding, and loading, whereas GW182 is the apparent silencer, and in cooperation with other co-factors, it triggered translational repression and mRNA degradation. In fact, depletion of either Ago2 or GW182 is associated with severe consequences.

Ago2 knockout mice showed embryonic lethality and displayed several developmental abnormalities (Liu et al. 2004). Disruption of GW182 in mouse yolk sac led to growth arrest and apoptosis (Jiang et al. 2012). These data indicate their critical role in maintaining normal cellular activities.

It seems clear that tethering of Ago2 or GW182 leads to poly(A) tail deadenylation of reporter mRNA and that is the first step toward mRNA decay (Chap. 11). The speed at which this process occurs is of importance and it is possible that the time frame for this event may differ depending on other factors, such as the metabolic status of the host cells. Utilizing in vitro translation extracts from mouse Krebs-2 ascites cells, miRNA-mediated inhibition of translation initiation proceeds as early as 15–40 min after addition of mRNA to the cell extract and deadenylation occurred 1–2 h later (Fabian et al. 2009). Although the data are limited to a specific cell and a single combination of miRNA:mRNA, generalizing from this, there appears to be a 1–2 h "window of opportunity" during which the miRNA-mediated event can be reversed and the de-repressed mRNA sends back to translation. In practice, the time may be affected depending on whether there is a backup in the mRNA decay pathway. If there is a limitation on the decay machinery, there may be accumulation of GW/P bodies (Erickson and Lykke-Andersen 2011). Several studies have shown that it takes 1.5–2 h for decay of the reporter to be detected (Fabian et al. 2009; Bazzini et al. 2012; Bethune et al. 2012; Djuranovic et al. 2012). FRAP experiments in live cells show that many factors rapidly cycle in and out of GW/P bodies, suggesting that the release of mRNPs from GW/P bodies can take place with the mRNA sent back into active translation again (Kedersha et al. 2005; Leung et al. 2006). Our data using GFP-GW182 transfected in HeLa cells also show rapid recovery within minutes in similar FRAP experiments (unpublished data). There is also an example of a specific miRNA-repressed mRNA in human cells observed to leave the GW/P bodies once repression is lifted (Bhattacharyya et al. 2006).

Another important unanswered question is the temporal hierarchy and dynamics between translational repression vs. mRNA deadenylation and decay. In some instances, pure translation repression is observed with little effect on mRNA stability (Pillai et al. 2005; Yao et al. 2011). On the other hand, global miRNA-induced mRNA decay is proposed to be predominant for miRNA silencing effects (Guo et al. 2010). Recent progress has been made to dissect these events in zebrafish (Guo et al. 2010; Bazzini et al. 2012) and Drosophila S2 cells (Djuranovic et al. 2012) where it was agreed that translational repression and mRNA decay are independent events. Translational repression usually occurs in the early stage of repression before complete deadenylation while mRNA decay takes place in a later stage. Bethune et al. performed kinetic analysis and revealed successive steps in mammalian cells, further supporting the observations described in zebrafish and Drosophila (Bethune et al. 2012). These observations are consistent with previous in vitro experiments that translation repression occurs in the early stages before complete deadenylation in mouse Krebs-2 ascites cells (Fabian et al. 2009).

One model argues that the newly identified interaction of GW182 with poly A binding protein (PABP) could competitively disrupt PABP-eIF4G interaction, loosen the circularized mRNA structure and impair translation. GW182-PABP interaction may facilitate the dissociation of PABP from poly-A tail to directly expose unpro-

tected poly-A tail for the deadenylase (Tritschler et al. 2010; Huntzinger and Izaurralde 2011). Three recent studies simultaneously reported that GW182 can directly interact with and recruit the CCR4-NOT deadenylase complex through its C-terminal domain containing conserved tryptophan in adjacent to glycine/serine/threonine-rich region; they also showed that the formation of the GW182-deadenylast complex is highly dependent on PABP (Braun et al. 2011; Chekulaeva et al. 2011; Fabian et al. 2011). This model, however, has been subsequently challenged because in the in vitro Drosophila embryo system it was demonstrated that neither PABP nor GW182-PABP interaction is a prerequisite for shortening of poly-A tail and pure translational repression (Fukaya and Tomari 2011). Nonetheless, Moretti et al. have proposed a revised model supporting the importance of PABP in miRNA silencing; their observations were that PABP and poly-A tail may stimulate the association of miRISC with target mRNA, and PABP can be subsequently dissociated from mRNA in order to facilitate mRNA deadenylation (Moretti et al. 2012). The absolute requirement of PABP in the miRNA-mediated deadenylation process is still to be determined and may vary in different species or different target mRNAs.

Although there is still much to be learned about the structure and function of GW182, disassembly of GWBs by depleting GWB components LSm1, LSm3, HPat, or Ge-1 did not reduce GW182 activity in silencing its tethered reporters (Eulalio et al. 2007). However, the possibility remains that there might still be "submicroscopic" GW/P bodies that effectively silence translation. Our observations that the number and size of GWBs increase upon the introduction of siRNA or miRNA (Lian et al. 2007) suggests a functional relevance of GW/P bodies in siRNA/miRNA-induced gene silencing. Many mRNAs bound by miRNA can form GW/P bodies, possibly through the GW motifs of GW182 that play a role in aggregating miRNA bound mRNA complexes (Fig. 6.7). The extraction and sequestration of mRNA from the cytoplasm may be a quick and highly efficient method in translation repression (Chap. 6). Deadenylation/decay of mRNA is secondary to the biological need for quick repression of mRNA translation. It is also possible that GWBs help to stabilize miRNA by limiting their accessibility to exoribonucleases.

15.5 Some Directions for Future Studies

Phosphorylation in the regulation of GW182 function. It was clear from our first report that GW182 is a phosphoprotein (Eystathioy et al. 2002). Within this 182 kDa protein there are 15.8 % serine residues and many potential phosphorylation sites. To date, there have been no published studies on the role(s) of phosphorylation (or other post-translational modifications) on the function of GW182. There are many interesting questions to address. Does phosphorylation affect the assembly of GW182 into GW bodies and interfere with miRNA-mediate silencing? What are the dominant kinases and phosphatases controlling GW182 phosphorylation status?

Transcription control and function of isoforms. There have also been no published studies that examined the transcriptional control of GW182, TNRC6B, or

TNRC6C. It is generally known that GW182 and TNRC6B are expressed in most tissues whereas TNRC6C expression is more restricted to testis. It is clear that there are multiple isoforms for each of the family members but their roles are not defined with a focus primarily on the longest isoform of each paralog. This, in part, is related to the limited availability of isoform specific reagents. What are the differences in function of these isoforms?

How does miRNA find targets? The current paradigm is that Ago-bound miRNA recognizes its intended mRNA target somewhere in the cytoplasm. This "cat-and-mouse game" is clearly not an effective mechanism for miRNA to find its target. Is it possible that GW/P bodies, devoid of a bilayer membrane, are the highly dynamic structures that serve as the foci that facilitate effective interaction of miRNA:mRNA?

15.6 Thanks for the Past 10 Years

The discovery of GW182 and GW bodies is attended by much excitement and continues to enrich our research lives. One benefit is that we have opportunities to travel to new places for meetings and presentations and meet new colleagues and investigators, some of whom have contributed chapters in this book. We are grateful for the collaborations and the positive reception of our first discovery of GW182 and the many independent and collaborative studies. Another benefit from this discovery is the recruitment of many students and postdoctoral fellows, all of whom are highly dedicated and work zealously on various interesting projects. This volume is, in part, dedicated to their efforts in driving the research forward. Dr. Theo Eystathioy was the first student who was responsible for driving the first paper to publication (Chap. 2). Since then, there were many PhD students and postdocs who have contributed to the efforts. EKLC laboratory: Ph.D. students, Shangli (Jesse) Lian, Kaleb Marie Pauley, Songqing Li, and Bing Yao; postdoctoral fellows, Zheng Yang, Andrew Jakymiw, Keigo Ikeda; summer students and undergraduates, Frank Han, Rebeca Alvarez, Grant Abadal, Lan La, Anna Pyatigorskaya; technical support of John Hamel. MJF laboratory: Kevin Griffith, LeeAnne Luft, Joanna Moser, and Rahima Bhanji.

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