The Role of TNRC6 Proteins in Gene Silencing

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All models are wrong, but some are useful.

Statistician George E. P. Box, in 'Science and Statistics',
Journal of the American Statistical Association 71:791-799.

Abstract

Proteins of the GW182 family have recently emerged as key players in miRNA-mediated gene silencing. They have been shown to interact with Argonaute proteins, components of the RISC and are assumed to mediate the repression in metazoa. Three paralogues are encoded in the human genome, TNRC6A (GW182), TNRC6B and TNRC6C and only one in fly. Results in *Drosophila melanogaster* demonstrated that GW182 has the potential to both repress translation and induce mRNA deadenylation and decay.

In this work, we investigate the role of GW182 proteins in miRNA-mediated repression. We demonstrate that the repression mediated by TNRC6C is due to a combination of effects on the mRNA level and mRNA translation. Through deletion analysis, we could identify the C-terminal part of TNRC6C as a key effector domain mediating repression of protein synthesis. Furthermore, we show that two unstructured regions located within the C-terminal part are responsible for the effect. We give evidence for a direct interaction of TNRC6C with PABP and CNOT1. Both interactions are mediated by the C-terminal effector domain, however by different sub-fragments. While repression of protein synthesis is independent of the interaction with PABP, it relies on the interaction with the CCR4–NOT complex. The interaction is mediated by GW-repeats which are located in the two regions flanking the RRM. Finally, we show that the C-terminal effector domain is able to induce repression upon tethering not only of poly(A)$^+$ but also of poly(A)$^-$ reporters.

Our results characterize the role of GW182 proteins in gene silencing and clarify some of the recent contradictions about the diverse proposals for the mode of action of miRNAs. The identified effector motifs function as important mediators of miRNA-induced silencing and reveal the recruitment of the CCR4–NOT machinery to the RNA-induced silencing complex. In addition to inducing mRNA decay, the recruitment of GW182 also results in inhibition of mRNA translation independently of deadenylation.

Contents

List of Figures

Abbreviations

Abbreviations

RNA	ribonucleic acid
DNA	deoxyribonucleic acid
siRNA	small interfering RNA
RNAi	RNA interference
ncRNA	non-coding RNA
miRNA	micro RNA
miR	micro RNA
piRNA	piwi-interacting RNA
PTGS	post-transcriptional gene silencing
TGS	transcriptional gene silencing
ds	double-stranded
dsRBD	double-stranded RNA binding domain
RRM	RNA recognition motif
WT	wild type
Ago	Argonaute
UTR	untranslated region

Units

bp	base pairs
nt	nucleotides
kDa	kiloDalton

1

Introduction

1.1 Genetics and Epigenetics

The beginning of the field that we now call 'classical genetics' was set in the eighteenth century by Gregor Johann Mendel. He monitored one specific trait at a time in a single specimen over several generations [Carlson, 2004]. Mendel's laws, the fundamental outcome of his studies, set the basis for modern genetics. His work was not yet accepted by the scientific community, however it started a search for the material which carries genetic information.

Several important discoveries contributed to the foundation of genetic research: In 1871, Friedrich Miescher published his discovery of a weak acid in the nuclei of white blood cells, which we now know as DNA. He also proposed that it might be the bearer of inheritance [Miescher, 1871]. More than fifty years later, in 1928, Frederic Griffith realized that genetic material from dead bacteria could be functionally integrated in living cells. Oswald Avery proved Friedrich Miescher's hypothesis in 1944 by using specific enzymes to degrade DNA, RNA, or proteins – showing that only DNA carries genetic information [Avery et al., 1944].

Meanwhile, the concept of chromosomes and their role in inheritance had been discovered [Sutton, 1903], and in 1910, Thomas Hunt Morgan showed with his work in *Drosophila melanogaster* that genes are located on chromosomes [Morgan, 1910]. However, a convincing idea for how genes encode proteins and how this information is passed on to daughter cells was still lacking. The first insight into molecular details came from X-ray diffraction images of DNA. The suggested helical structure was finally confirmed by Francis Crick and James Watson [Watson and Crick, 1953]. The complementary structure of DNA also explained how the strands can be copied before cell division since both strands carry the same information.

With the structure of DNA known, the field of molecular genetics developed much faster. Soon, a central dogma was established, which defined the information

flow in a cell: DNA acts as a template for messenger RNA and this information is then translated into proteins. It also stated that this course of action was unidirectional. The discovery of restriction enzymes by Smith and Wilcox [1970] provided an indispensable tool for further investigations, and in 1977, the first genome was sequenced by Frederick Sanger [Sanger et al., 1977]. The logical consequence was the subsequent race for the sequence of the human genome, which was fundamentally completed in 2001 simultaneously by the Human Genome Project and Celera Genomics [Lander et al., 2001; Venter et al., 2001].

Having the whole genome sequenced, people expected an explanation for most questions concerning diseases and inheritance. However, it soon became evident that the DNA sequence alone cannot explain many observations: How can females have two X chromosomes but the same amount of proteins as males? How can stem cells of one organism with the same genotype differentiate into neurons, muscle cells and blood vessels with distinct profiles of gene expression? Why are some genetic elements in heterozygotes – and thereby the phenotype – influenced by the parental generation as discovered in 1958 by Royal Alexander Brink [Brink, 1958]? These questions could not be explained by mendelian genetics and gave birth to the field of epigenetics.

1.1.1 A Short History of Epigenetics

Having understood that mendelian genetics is unable to explain the development of an embryo, Conrad Hal Waddington proposed the word *epigenetics* to deal with the correlation between phenotype and genotype [Waddington, 1942]. The word is a portmanteau of the words 'genetics' and 'epigenesis' and should describe

> „the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being"

[Waddington, 1942]. This definition has not changed since. Nowadays epigenetics is considered as the study of any potentially stable and inheritable change in gene expression or cellular phenotype that occurs without changing the underlying DNA sequence [Goldberg et al., 2007]. Waddington described such phenomenon in his 'epigenetic landscape' as shown in Figure 1.1. The metaphor represents the process of cellular decision-making and how this modulates development. A cell (here represented by a ball) has to take several decisions during development. Each permitted decision corresponds to a trajectory in the metaphor and thereby leads to a different cell fate.

Today, scientists connect the term epigenetics with the combined modifications of a DNA locus that alter its transcription pattern and thereby the phenotype of a cell without changing the underlying genetic information (DNA sequence). Hence the prefix *-epi* (Greek: $\epsilon\pi\iota$ - over, above). Several distinct ways to alter the readout

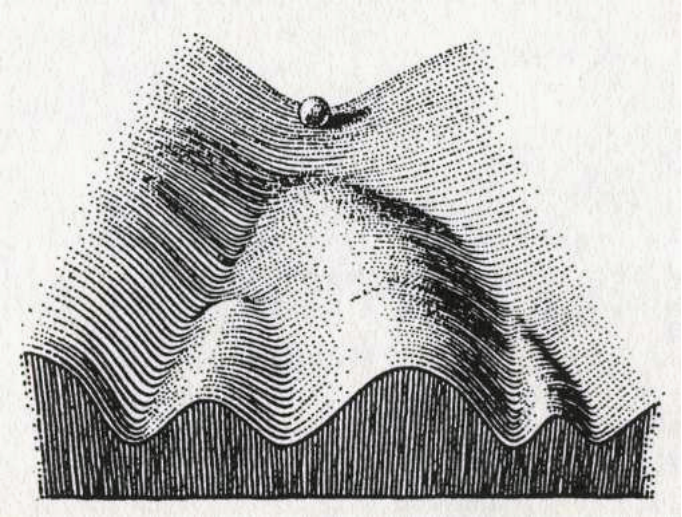

Figure 1.1: Waddington's Epigenetic Landscape. The metaphorical concept of an epigenetic landscape was proposed in 1957 by Conrad Waddington. It illustrates cellular decision-making. The ball (a single cell) can take concrete trajectories which lead to a different end point or cell fate. Figure reprinted from Waddington [1957].

of a given locus have evolved, and much of today's epigenetic research focuses on the study of covalent and noncovalent modifications of DNA and histone proteins [see Goldberg et al., 2007, and other articles in the same issue of Cell].

1.1.2 Mechanisms of Epigenetic Regulation

Each distinct tissue type has its own set of proteins expressed. This is necessary to gain the crucial differentiation which helps cells to specialize. Typically, cell identities are maintained for a lifetime even though the differentiation signal is experienced only once [Bonasio et al., 2010]. Since all cells in one organism contain the same genetic information, tools to switch genes on and off are crucial. Such epigenetic mechanisms often include the covalent or non-covalent modification of DNA (without altering the sequence) and/or histone modification. Both alterations imply a change in chromatin structure and can thereby regulate the transcriptional potential of the underlying genes. However, not only chromatin provides an attractive way to change a cell's epigenetic landscape. RNA, especially non-coding RNA, acquires an increasingly important role in the field of epigenetics.

It seems legitimate to assume that all mechanisms contribute significantly to the epigenetic reorganization of the genome. It is the joint effort of several pathways that shapes the epigenetic landscape and these entities should be considered collectively.

The individual mechanisms are described briefly in the following paragraphs.

1.1.2.1 DNA Methylation

The addition of a methyl group to DNA is probably the best characterized and most widely studied epigenetic modification. However, since it is not essential to this work, it will be discussed only shortly. The groundwork was accomplished by two independent investigations published in 1975 [Holliday and Pugh, 1975; Riggs, 1975], which offered a role for DNA methylation in epigenetics. The possibility that this modification can be inherited through cell division and that it does not (at least not per se) alter the primary DNA sequence made it especially attractive. Nowadays, at the age of the human methylome being analyzed at single nucleotide (nt) resolution, the chemistry of DNA methylation is uncovered but its function is still not fully understood.

In mammals, DNA methylation occurs almost exclusively in the context of CpG dinucleotides. It involves the covalent attachment of a methyl group to the 5 position of the cytosine pyrimidine ring. This chemical modification does not directly lead to a change in the underlying DNA sequence, however, methylcytosine can spontaneously deaminate to thymine, resulting in the under representation of CpG in the human genome (only 21% of the expected frequency) [Illingworth and Bird, 2009]. DNA methylation is carried out and maintained by DNA methyltransferases [for a comprehensive review see Goll and Bestor, 2005].

Modified CpG pairs are distributed throughout the majority of the genome. In spite of this, there exist areas with a high frequency of CpG dinucleotides, so called CpG islands. These regions consist of an elevated G and C content and the CpGs are not methylated [Illingworth and Bird, 2009]. Furthermore, most of these CpG islands overlap with promoter sequences of 60-70% of all human genes. The methylation of CpG is generally thought to be a strong repressive element in epigenetics.

1.1.2.2 Chromatin Modification

The genetic material in a cell, DNA, is not present as a naked molecule. With the help of DNA binding proteins, it is organized in a highly ordered and structured way inside the nucleus. The combination of DNA and proteins, mainly histones, is called chromatin. This condensed DNA scaffold does not only serve the purpose of compressing the large amount of DNA, it can also regulate which genes are accessible for transcription and which are not. In general, genes which are located on loose chromatin (euchromatin) are actively transcribed, while genes on more tightly packed chromatin (silent chromatin or heterochromatin) are considered to be turned off. A primary component of chromatin that plays a key role in this

regulation is the modification of histones [Bannister and Kouzarides, 2011].

Histone modifications have been investigated intensively [for a review see Kouzarides, 2007, or more recent Bannister and Kouzarides, 2011]. Each of the four histones that build the core nucleosome can be modified at specific amino acid residues, which are generally enriched in the tails. The best known modifications include lysine acetylation, lysine and arginine methylation, and serine and threonine phosphorylation. There is no general rule stating whether a modification alters chromatin structure to a more active way or if its a repressive alteration. Furthermore, mono- or di-methylation at lysine residues can have opposing effects; H3K9 mono-methylation is considered to be active (as is H3K4), while H3K9 di- and tri-methylation are considered to be repressive. It has therefore been proposed that the combinatorial nature of histone modifications reveals a 'histone code', which extends the genetic code and represents a fundamental regulatory mechanism [Jenuwein and Allis, 2001]. Although a lot about histone alterations is already known, new sites and modifications are discovered every year [Tan et al., 2011]. The sheer complexity of covalent histone modification is multiplied by the existence of histone variants that can change the overall composition of the nucleosome and thereby its modifications.

The exact mechanistic details of how histone modifications influence chromatin structure have not yet been determined. Charge-altering modifications of the nucleosome can directly alter the physical properties of the chromatin fiber. Methylation on the other hand has been shown to recruit or stabilize binding partners to chromatin.

Taken together, DNA methylation and histone modification provide stable, heritable, and crucial components of epigenetic regulation. They shape a cells accessible genome, and aberrant placements of these marks or mutations in the machinery are often connected with diseases [Portela and Esteller, 2010]. Both epigenetic tools are tightly connected to each other; Histone deacetylases are recruited by methylation marks on DNA as are nucleosome remodelling factors [Fuks et al., 2000; Wysocka et al., 2006]. The complexity of the interplay between those two machineries has recently been increased by a third player: non-coding RNA.

1.1.2.3 Non-coding RNA

Any RNA molecule which is not translated into protein is considered non-coding RNA (ncRNA). This includes highly abundant and functionally important RNAs such as transfer RNA and ribosomal RNA. More recently, the family of ncRNAs has been expanded by several short regulatory RNAs like microRNAs (miRNAs), small interfering RNAs (siRNAs) and piwi-interacting RNAs (piRNAs).

NcRNAs have become a fundamental element in epigenetics. Although they

are sometimes considered not to be 'epigenetic' in an exact way because they are not directly connected to a specific DNA locus, they do meet the criteria by carrying out a stable and inheritable change in gene expression without changing the DNA sequence. It has become clear that small RNAs play an active role in shaping the state of chromatin [Bernstein and Allis, 2005], and pioneering work in yeast established a direct connection between small RNAs and epigenetic states [reviewed in Moazed, 2009]. Clear examples of RNA involvement in epigenetics range from dosage compensation mechanisms in *D. melanogaster* to the silencing of genes by post-transcriptional and transcriptional gene silencing (PTGS and TGS, respectively).

The discovery of RNA-interference pathways (RNAi) and the expanding world of small RNAs [Grosshans and Filipowicz, 2008] point at the centrality of RNA [Sharp, 2009] in epigenetics. miRNAs especially bring the RNA molecule to the core of gene regulation. It is estimated that miRNAs regulate more than 50% of all genes and are involved in nearly all cellular pathways and diseases. These recent appraisals underline the considerable relevance of miRNAs and emphasize the importance of further research in this field.

1.2 MicroRNA

MicroRNAs (miRNAs) are small, about 22 nt long RNAs that post-transcriptionally regulate gene expression in eukaryotes. After their discovery (see 1.2.1 on page 7), it soon became clear that miRNAs are of immense importance to most biological processes, such as development, proliferation, differentiation, apoptosis, and stress response. These small regulators of gene expression are highly conserved across species and many are tissue or developmental-stage-specific. First estimations made miRNAs responsible for regulating more than 30% of all genes in eukaryotes [Bartel, 2004] while later algorithms suggested a number as high as 60% [Friedman et al., 2009]. Since the number of regulated genes is significantly higher than the numer of known miRNAs, one single miRNA can thereby, by direct or indirect effects, influence protein synthesis from thousands of genes [Selbach et al., 2008]. Although miRNAs are generally thought to make only fine-scale adjustments to protein output [Baek et al., 2008], their misregulation and aberrant expression has been implicated in numerous diseases, including cancer, and miRNA-based therapies are under investigation [Couzin, 2008]. MiRNAs comprise one of the more abundant classes of gene regulatory molecules and are estimated by computational approaches to represent 1% of the predicted genes in most vertebrate genomes [Lim et al., 2003]. As mentioned before in section 1.1.2, they are also embedded in and intertwined with other epigenetic mechanisms like DNA methylation and chromatin formation [Iorio et al., 2010; Amaral et al., 2008].

Overall, the abundance of miRNAs, their wide range of targets and their role as regulators of many cellular processes and diseases (especially in stem cells [for a review see Gangaraju and Lin, 2009]) make them an intriguing and crucial research target.

1.2.1 MiRNA Discovery

The first miRNA was discovered only two decades ago by Ambros, Lee and Feinbaum [Lee et al., 1993]. Their research on the role of the *lin-4* and *lin-14* genes in *Caenorhabditis elegans* revealed that the *lin-4* product is not a protein but instead gives rise to a a pair of small RNAs. The 61 nucleotide long RNA was assumed to fold into a hairpin and to eventually mature to a more abundant 22 nt long transcript. Together with the Ruvkun laboratory [Wightman et al., 1993, in the same issue of Cell], they realized that LIN-14 protein levels are inversely proportional to those of *lin-4* RNA. Further analysis revealed an antisense complementarity between the *lin-4* RNA and multiple sites in the 3' end of the *lin-14* mRNA. They hypothesized that the *lin-4* product can regulate the synthesis of LIN-14 protein and together supported a model which revealed the first miRNA and mRNA target interaction [Lee et al., 1993; Wightman et al., 1993].

This original discovery of a miRNA made no big impression on the field of RNA research. In contrast, at that time it was thought to be a nematode idiosyncrasy and no evidence of similar non-coding RNA was found in nematodes or any other organism. Only seven years later, the discovery of a second miRNA brought the deserved attention, leading to the now so prominent field of miRNA research. In 2000, Reinhart et al. [2000] reported that *let-7*, another gene in the *C. elegans* heterochronic pathway, encoded a 22 nt long RNA. This RNA acts to promote the transition from late-larval state to adult in the same way that *lin-4* acts earlier in development. *Let-7*-RNA has subsequently been reported to repress the genes *lin-14*, *lin-41*, *lin-28* and others. The identification of homologs of the *let-7* gene in human and fly and the detection of the RNA itself [Pasquinelli et al., 2000] stimulated a large cloning effort in the search for new miRNAs. Indeed, only one year later, three labs have reported cloning of hundreds of additional small RNAs from fly, worm and humans [Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001].

Evolutionary conservation of the *let-7* gene indicated the existence of a wider phenomenon. The cloning effort was intensified and a registry has been set up to catalog and name newly identified genes [Griffiths-Jones, 2004]. The first correlation between miRNAs and diseases were drawn soon after their discovery [Calin et al., 2002], and in 2005, genetic evidence for the relevance of miRNAs in mammals came from a study which described the deletion of a gene encoding Dicer, a key miRNA processing molecule (see 1.2.2.2 on page 9) [Yang et al., 2005].

Nowadays, more than 20.000 mature miRNAs in over 150 different species are annotated (www.mirbase.org, [Kozomara and Griffiths-Jones, 2011]) and the number is still growing. All kinds of cellular processes and diseases have been shown to be regulated by miRNAs. First therapeutic trials are on the way and countless algorithms have been developed to search for new miRNAs and targets [Huang et al., 2011; Thomas et al., 2010]. However, despite all efforts and considerable progress, many questions still remain unanswered in the young field of miRNA research.

1.2.2 MiRNA Biogenesis

MiRNAs are genomically encoded. Most miRNA genes are found in intergenic regions and usually contain their own promoter [for comprehensive reviews on miRNA biogenesis see Kim et al., 2009; Carthew and Sontheimer, 2009]. Many miRNA genes are clustered in polycistronic transcripts and share common regulatory elements. MiRNA sequences are also found in introns of protein-coding and non-coding transcription units (in the same orientation) and as such are usually regulated together with their host gene [Rodriguez et al., 2004; Baskerville and Bartel, 2005]. Most of the genes are typically transcribed by RNA polymerase II [Lee et al., 2004a], although some studies report an involvement of RNA polymerase III for certain miRNAs [Borchert et al., 2006]. A typical monocistronic transcript is composed of a ∼33 nt long stem loop with two flanking segments and can be capped, polyadenylated, spliced and edited [Bracht et al., 2004; Cai et al., 2004]. The resulting primary or pri-miRNA transcript is processed in two steps into the mature miRNA (compare Figure 1.2 on page 10).

1.2.2.1 Nuclear processing

The first processing step occurs in the nucleus. The cleavage process excises the ∼33 bp long stem loop from pri-miRNA resulting in formation of a ∼77 nt long precursor or pre-miRNA with a 3'-hydroxyl and a 5'-phosphate group. The core component of the enzymatic complex executing the cleavage is the RNase III enzyme Drosha [Lee et al., 2003; for a review see Kim, 2005]. Drosha is a ∼160 kDa large endoribonuclease and contains two RNase III domains and one double-stranded RNA binding domain (dsRBD) [Han et al., 2004]. Drosha carries out the catalytic cleavage but is dependent on a cofactor for efficient and accurate processing. This cofactor is known as DiGeorge syndrome critical region 8 (DGCR8) protein in mammals and Partner of Drosha (Pasha) in *C. elegans* and *D. melanogaster*. DGCR8 contains two dsRBDs itself and is believed to determine the cleavage site on the pri-miRNA [Landthaler et al., 2004]. Together, Drosha and DGCR8, often in association with additional regulatory proteins, form a complex called the

Microprocessor, which is required for proper miRNA processing [Gregory et al., 2004; Denli et al., 2004].

Not all pri-miRNA transcripts are processed by Drosha. A group of miRNAs are found in very small introns. In those cases, the pre-miRNA sequence matches exactly the size of the intron. These 'mirtrons' are spliced out and can enter the miRNA processing pathway directly, thereby bypassing the Microprocessor step [Okamura et al., 2007; Ruby et al., 2007]. Although being uncommon, mirtrons are found throughout the animal kingdom.

Chronology of splicing and processing is yet unclear. A recent study, using an in vitro system with both splicing and pre-miRNA processing activities, concludes that microprocessor and spliceosome activity are functionally linked [Kataoka et al., 2009]. This may suggest that Drosha processing of pri-miRNAs and mRNA splicing may occur at the same time. Mammalian Drosha contains a serine-arginine rich region, a common protein-protein interaction domain for splicing factors. However, such a domain is not found in the fly homologue. Another study argues for a direct coupling of miRNA processing and transcription [Morlando et al., 2008]. They show that „Drosha cleavage occurs during transcription acting on both independently transcribed and intron-encoded miRNAs". Morlando et al. suggest that exonucleolytic degradation from Drosha cleavage sites may influence the splicing and maturation process of numerous mRNAs.

Following the nuclear processing by the Microprocessor complex, pre-miRNAs are exported to the cytoplasm. Export is mediated by one of the nuclear transport receptors, Exportin-5 [Bohnsack et al., 2004; Yi et al., 2003]. Exportin-5 has been known as a minor transport factor for tRNAs. However, since the affinity of Exportin-5 for pre-miRNAs is much higher, pre-miRNAs seem to be the main cargo. Exportin-5 binds cooperatively to pre-miRNA and GTP-Ran in the nucleus and then releases its cargo in the cytoplasm upon hydrolysis of GTP.

1.2.2.2 Cytoplasmic processing

Once in the cytoplasm, pre-miRNAs undergo a second step of processing, which produces the mature ∼22 nt long miRNA. The main player in this process is Dicer, a cytoplasmic RNAse III protein of ∼200 kDa. Dicer is a highly conserved protein, found in nearly all eukaryotes. Some organisms contain multiple homologues with distinct roles like in *D. melanogaster* [Lee et al., 2004b]. Dicer typically consists of an N-terminal DEXH-box RNA helicase domain, a domain of unknown function (DUF283), a PAZ domain, two catalytic RNase III domains and a dsRBD [see Carmell and Hannon, 2004 and Jaskiewicz and Filipowicz, 2008 for reviews]. The PAZ domain is also found in the Argonaute protein family (see 1.3.1 on page 14), and is in fact named after the three founding proteins, Piwi, Argonaute, and Zwille.

Dicer was first discovered to play a role in generating siRNAs, but it was soon

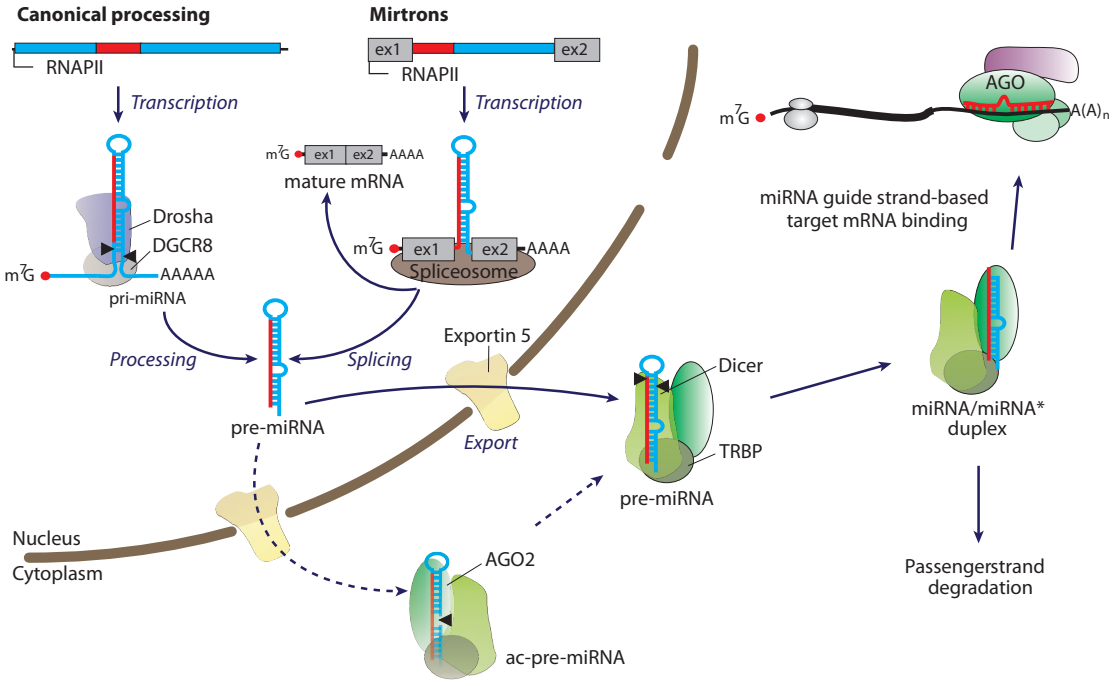

Figure 1.2: MicroRNA biogenesis. MicroRNAs are transcribed by RNA polymerase II. The primary miRNA is processed in the nucleus by the microprocessor, a protein complex with Drosha and DGCR8 being the most important components. This step can be bypassed if the pre-miRNA is produced directly from short introns (mirtrons) as a result of splicing and debranching. The pre-miRNA is exported to the cytoplasm where it is further cleaved by Dicer to yield the mature miRNA duplex. The miRNA guide strand is subsequently loaded into the RNA induced silencing complex (RISC). Figure modified and reprinted from Krol et al. [2010b].

proven that it also participates in processing pre-miRNAs [Knight and Bass, 2001]. The enzyme functions as a monomer, with a single processing center formed by intramolecular dimerization of both RNase III domains. This model resembles the catalytic center of bacterial RNase III [Zhang et al., 2004]. The PAZ domain and the dsRBD stimulate the interaction with pre-miRNAs. The cleavage results in formation of the siRNA-like duplex of two complementary strands, a guide strand and a passenger strand. One of these strands, corresponding to guide or mature miRNA, is then incorporated into the RNA induced silencing complex (RISC, see chapter 1.3 on page 13).

Human Dicer interacts with two closely related proteins, TRBP (TAR RNA binding protein) [Haase et al., 2005] and PACT [Lee et al., 2006]. While these cofactors are seemingly not necessary for cleavage [Zhang et al., 2002], they clearly

facilitate it. Depletion of either TRBP or PACT results in a diminished efficiency of post-transcriptional gene silencing. Although their exact biochemical function remains to be determined, they are proposed to have roles in pre-miRNA cleavage, miRNA stability, and probably contribute to the formation of the RISC (see chapter 1.3).

Latest research revealed a miRNA biogenesis pathway independent of Dicer [Cheloufi et al., 2010; Cifuentes et al., 2010]. The two independent reports identified an alternative biogenesis for pre-miR-451, which uses Argonaute2 (Ago2, see 1.3.1 on page 14) slicer catalytic activity. This miRNA is processed by Drosha and then loaded into Ago where it is cleaved, generating a miRNA intermediate containing an extended 3' end. Ago2 has been previously shown to play a role in miRNA biogenesis: MiRNAs that display a high degree in complementarity along the hairpin can be cleaved by Ago2 to generate Ago2-cleaved pre-miRNAs or ac-pre-miRNAs [Diederichs and Haber, 2007](see Figure 1.2), which are then further processed by Dicer.

1.2.3 Regulation of Biogenesis and Decay of MiRNAs

Due to their strong and general impact on protein synthesis, levels of miRNAs in a cell have to be controlled very tightly. Therefore, regulation of miRNA metabolism including miRNA decay is crucial for a cell. This topic has emerged into a field of its own and will be discussed only briefly in the scope of this work [for extensive reviews on miRNA regulation and decay see Garneau et al., 2007; Winter et al., 2009; Krol et al., 2010b].

MiRNA biogenesis is regulated at all possible levels; transcription, processing in the nucleus, and maturation in the cytoplasm. Furthermore, recent discoveries of maturation steps specific for individual miRNAs have increased regulatory possibilities after transcription. Multiple proteins have been shown to effectively regulate miRNA processing.

Regulation at the level of transcription. MiRNA generation can be controlled and regulated in the same way as the generation of protein-coding mRNAs. This is thought to be a major level of control for tissue-specific or temporal expression of miRNAs. RNA polymerase II, responsible for miRNA transcription, recognizes specific promoters and is regulated by transcription factors. For example, the temporal expression pattern of *let-7* is dependent on a transcriptional enhancer element, known as temporal regulatory element (TRE) [Johnson et al., 2003]. Expression of a specific miRNA also depends on the methylation status of the promoter [Brueckner et al., 2007].

MiRNA transcripts can be edited. Post-transcriptional editing by adenosine deaminases acting on RNA (ADARs) modifies adenosine into inosine, which alters the base-pairing and structural properties of the transcript. Several miRNA transcripts have been reported to be edited this way [Blow et al., 2006; Yang et al., 2006]. Despite multiple examples, the exact function of miRNA editing is not yet known. Modifications might even have different effects on specific miRNAs, since editing has been reported to have both inhibititory and stimulatory effect on miRNA processing.

At the level of Drosha cleavage, processing is regulated mainly by protein-protein interactions. The RNA helicases p68 and p72 are known components of the Microprocessor complex and are thought to positively influence miRNA expression [Fukuda et al., 2007]. Furthermore, transforming growth factor-β (TGFβ) and bone morphogenetic protein (BMP) induce expression of miR-21 by regulating Microprocessor activity. They recruit the ligand-specific signal transducers (SMAD proteins) to pri-miR-21 in a complex with p68. How SMAD proteins exactly modulate miRNA biogenesis is still unclear. Above all, the two main components of the Microprocessor complex, Drosha and DGCR8 mutually regulate each other. DGCR8 stabilizes Drosha through a protein-protein interaction; Drosha cleaves two hairpin structures of the *DGCR8* mRNA, resulting in a negative feedback loop [Han et al., 2009].

Dicer processing in the cytoplasm is regulated similarly to the previous cleavage step by the Microprocessor in the nucleus. The two main proteins interacting with Dicer are TRBP and PACT. These two proteins are not essential for miRNA processing, but clearly facilitate it (see 1.2.2.2 on page 9). Furthermore, a well known negative regulator of *let-7* miRNA, LIN-28, can influence cleavage by Drosha and/or Dicer by interacting with the terminal loop of pri-let-7 or pre-let-7 [Viswanathan and Daley, 2010; Nam et al., 2011]. A recent report revealed the full complexity of this process by showing that LIN28A and LIN28B regulate *let-7* biogenesis by two distinct ways [Piskounova et al., 2011].

MiRNA decay. Research of miRNA turnover has long been neglected despite being a crucial step in regulating miRNA levels. Therefore, not much is known yet about miRNA decay. Experiments using RNA polymerase II inhibitors suggested that miRNAs in general have a long half life [Gatfield et al., 2009]. However, a recent report showed that turnover happens very fast in neurons and is regulated by neuronal activity [Krol et al., 2010a]. Which enzymes degrade miRNAs? In *C. elegans*, Chatterjee and Grosshans [2009] showed that the 5' to 3' exoribonuclease XRN-2 is responsible for miRNA degradation. In plants, a family of 3' to 5'

exoribonucleases encoded by the small RNA degrading nuclease (SDN) genes degrades mature miRNAs to limit their accumulation [Ramachandran and Chen, 2008]. Since the enzymes in plants and *C. elegans* have opposing polarity, not much can be concluded about mechanisms of degradation in other organisms.

1.3 The RNA-induced Silencing Complex

After being processed into its mature form, the miRNA is incorporated into a multiprotein complex called the RNA-induced silencing complex (RISC). From here on, the pathway seems to be biochemically indistinguishable from the central steps of RNA interference (RNAi). RNAi was discovered by Fire and Mello in 1998 [Fire et al., 1998]. Even though miRNAs have been discovered five years earlier, the connection between the two mechanisms was not yet clear at that time. Only later did it become apparent that the mechanisms of most small RNAs (especially siRNAs and miRNAs) share a high similarity. The main difference between siRNA and miRNA function is the degree of complementarity to a target and thus the ensuing resulting way of reducing RNA activity. SiRNAs typically base-pair perfectly and induce endonucleolytic mRNA cleavage only in a single specific site. MiRNA binding to a target usually involves mismatches and one miRNA can have multiple diverse targets (for more details on miRNA mechanism see chapter 1.4 on page 19).

In *D. melanogaster* and *C. elegans*, function of miRNA and siRNA is mediated by distinct Dicer enzymes and Argonaute proteins [Lee et al., 2004b; Okamura et al., 2004]. The RISC for miRNA is also known as a microRNA ribonucleoprotein complex (miRNP), however, for simplicity, it will be referred to as RISC.

The RISC is loaded with a miRNA strand which guides it to its corresponding target RNA. As processing by Dicer produces a double stranded miRNA duplex, the two strands have to be separated and only one of them is eventually incorporated in the RISC. The strand which becomes loaded is called *guide strand*, the other is referred to as *passenger strand*. The guide strand is chosen on the basis of thermodynamic stability of the duplex miRNA ends. In general, the strand with the more unstable base pairs at the 5'-end is selected as miRNA [Schwarz et al., 2003]. The passenger strand is assumed to be degraded [Gregory et al., 2005]. However, in rare cases, both strands of the duplex are functional and may target different mRNAs.

The detailed molecular mechanism of miRISC loading is still under debate. Dicer, together with TRBP and Ago, forms a RISC loading complex (RLC) [Chendrimada et al., 2005; Maniataki and Mourelatos, 2005]. After Dicer releases the miRNA duplex, it is presumed that the more stable duplex end interacts with TRBP while the other is bound by Ago [Tomari et al., 2004]. In *D. melanogaster*, strand

selection is supported by R2D2, which interacts with Dicer2 and binds to the more stable end of the duplex [Liu et al., 2003]. The removal of the passenger strand is facilitated by the slicer activity of Ago2 [Matranga et al., 2005]. However, in case of duplexes with mismatches (common for miRNAs) or in case of Ago proteins with no endonucleolytic activity (Ago 1, 3 and 4 in humans), the unwinding of the duplex is thought to be assisted by a helicase.

Since Ago proteins are essential for a functional RISC and different Ago proteins determine its function, much research has been undertaken to better understand them.

1.3.1 Argonaute Proteins

The most intensely studied proteins and key components of the RISC are members of the Argonaute (Ago) protein family [for comprehensive reviews see Hutvagner and Simard, 2008 and Cenik and Zamore, 2011]. The discovery and the subsequent understanding of these highly conserved proteins is closely linked to the study of RNAi and miRNAs pathways. Ago proteins were first described in *Arabidopsis thaliana* as being important for development [Moussian et al., 1998], and in *D. melanogaster* as important for germ-line stem cell division [Lin and Spradling, 1997]. But only since the association of Ago proteins with RNAi did the research on these central components increase significantly.

The Argonaute protein family members can be divided into two classes, Ago and Piwi (P-element induced wimpy testis) proteins. Sometimes a third class is also discussed, consisting of the worm-specific group 3 of Argonaute proteins [Yigit et al., 2006; Hutvagner and Simard, 2008]. The two main groups can be distinguished by the small RNA they are binding to. Members of the Ago group bind to siRNAs and miRNAs while Piwi family proteins interact with piRNAs (Piwi-interacting RNAs). PiRNAs are 26-31 nt long and are expressed in spermatogenic cells in the germ line of many animal species. They are thought to silence transposons, thereby protecting the integrity of the genome [Kim, 2006; Siomi et al., 2011].

The Ago group includes four ubiquitously expressed proteins in mammals (Ago1-4) and two proteins in *D. melanogaster*, Ago1 and Ago2. In *D. melanogaster*, Ago1 functions primarily in the miRNA pathway while Ago2 is involved in siRNA function [Okamura et al., 2004]. In humans, no preferences of Ago proteins for either miRNAs or siRNAs have been revealed. However, Ago2 is the only one with slicing activity and can therefore act in siRNA mediated cleavage of the target mRNA (see section 1.3.1.2 on page 15).

All eukaryotic Ago proteins that function in gene silencing consist of three distinct domains: PAZ, MID and PIWI. To date, there exists no three-dimensional structure of an entire eukaryotic Ago protein. However, due to research on eubacterial and archaeal Ago proteins and structures of individual domains of eukaryotic

Ago proteins, we have a broad understanding of how these proteins work [Jinek and Doudna, 2009].

1.3.1.1 Functional Domains of Argonautes

The PAZ domain is shared with Dicer and is responsible for binding the 3'-end of the small RNA. It is able to specifically recognize the 3'-overhang of a miRNA duplex which is left after sequential cleavage by Drosha and Dicer. The 2 nt long overhang is inserted into a pocket of conserved aromatic and hydrophobic residues [Ma et al., 2004].

The PIWI domain is the catalytic center of some of the Ago proteins. Surprisingly, crystal structures of prokaryotic Ago-like proteins revealed that the PIWI domain folds similar to the catalytic domain of RNase H [Song et al., 2004; Parker et al., 2004]. RNase H is known to cleave RNA using DNA as a template. It soon became evident that Ago proteins also harbor an endonucleolytic activity which requires Mg^{2+} to cleave a target RNA. Related to the catalytic center of RNase H, the PIWI domain contains three negatively charged and evolutionary conserved amino acids, Asp-Asp-His (DDH), which have all been shown to be essential for cleavage [Liu et al., 2004; Rivas et al., 2005]. Rivas et al. could also prove that Ago contains the catalytic center by reconstituting the RISC from bacterially expressed human Ago2 and single-stranded siRNA.

Interestingly, not all Ago proteins are able to cleave a target RNA. In some Ago proteins (for example human Ago1, 3, and 4), the catalytic center of the PIWI domain has diverged to an extent that its endonucleolytic activity is impaired. However, despite the missing enzymatic activity, all Ago proteins have been shown to function in miRNA-dependent mRNA silencing [Pillai et al., 2004].

The MID domain anchors the 5'-monophosphate of the small RNA. It is proposed that it nucleates the alignment of the small RNA on the surface of Ago [Parker et al., 2004]. Being responsible for binding the 5'-phosphate, the MID domain might also play a role in sorting small RNAs to the various Ago paralogues [Frank et al., 2010].

1.3.1.2 Argonaute function

It is now clear that Argonaute proteins function as the core component of the RISC [for a review see Peters and Meister, 2007]. Similar to RNase H, the catalytic activity requires divalent metal ions and produces a 3'-hydroxyl end and a 5'-phosphate group. The cleavage is very precise and occurs always between the target nucleotides paired to the tenth and eleventh nucleotides of the guide RNA. In analogy to Dicer, Ago proteins which cleave their target are also called „Slicer".

Cleavage of a target RNA requires a perfect match between the guide small RNA

and its target, and therefore usually occurs only for siRNAs. In *D. melanogaster*, the distinction between the two small RNA silencing pathways is made by using different Ago proteins for different mechanisms. While specific small RNA classes are not restricted to associate with Ago1 or Ago2, only Ago1 is able to repress an mRNA with central mismatches in its miRNA binding sites. On the other hand, miRNA-loaded Ago2-RISC only mediates RNA cleavage [Forstemann et al., 2007]. However, more recent reports showed that the separation of both pathways is not that clear and both *Drosophila* Ago proteins can regulate translation without cleavage (although by different mechanisms) [Iwasaki et al., 2009]. In humans, only Ago2 has the capability of cleaving its mRNA target and is therefore the only Ago protein mediating siRNA cleavage [Meister et al., 2004; Liu et al., 2004].

To inhibit mRNA function, the RISC is not obliged to cleave the target mRNA. A cleavage-independent silencing mechanism is especially important when the involved Ago protein has no endonucleolytic activity (as shown for human Ago 1, 3, and 4), or when miRNAs only have restricted complementarity with their targets. In contrast to plants, this is true for most miRNA-mediated gene repression in animals. When there is no cleavage involved, the silencing mechanism is thought to be mediated mainly by Ago interacting with proteins which impair translation and induce mRNA deadenylation. Indeed, comprehensive lists of interacting proteins have been reported for human Ago1 and Ago2 [Hock et al., 2007]. Fore more details on miRNA mechanism see chapter 1.4 on page 19.

1.3.2 mRNA Targeting

Once the RISC is assembled, it is directed to its target mRNA by the sequence of the incorporated guide strand. Target recognition of miRNAs is a widely discussed field of research and most of our comprehension comes from bioinformatic studies. The first hint that miRNAs bind to their regulatory target by partial complementarity came from the early paper on *lin-4* RNAs [Lee et al., 1993, see chapter 1.2.1 on page 7]. Since then, hundreds of miRNA genes have been identified by cloning and computational methods for target identification quickly evolved. In plants, where miRNAs bind their targets with extensive complementarity, many targets have been identified by searching for RNAs with sequences complementary to miRNA binding sites [Rhoades et al., 2002; Sun et al., 2011]. In mammals, perfect binding between miRNA and target occasionally happens but is rather the exception [Yekta et al., 2004]. Therefore, the search for regulatory targets is more complicated and prone to generate false positives.

To date, several independent algorithms have been implemented to identify mammalian miRNA targets, the most prominent being TargetScan (`http://targetscan.org`, [Friedman et al., 2009]) and PicTar (`http://pictar.mdc-berlin.de`, [Lall et al., 2006]). For a more detailed overview of miRNA target recognition

and available prediction tools see Bartel [2009]. Since predicting targets purely based on partial complementarity with a 22 nt long miRNA leads to an immense number of false positives, all computational approaches use some additional features for predicting miRNA binding sites.

First, miRNA sites are generally located in the 3'-UTR (untranslated region) of target mRNAs [Gu et al., 2009]. Considering that the 3'-UTR of mRNAs is a well known site for other types of regulation, this restriction makes sense. If miRNA sites were located in the ORF, they would interfere with the translational machinery [Gu et al., 2009]. By being located in the 3'-UTR, the RISC can bind to the mRNA without completely impairing translation so that miRNAs are able to fine tune gene expression. A further reason why miRNA binding sites are much more common in the 3'-UTR is that they are far more effective than sites in the 5'-UTR or in the ORF [Moretti et al., 2010]. In the case of miRNA binding sites in the 5'-UTR or ORF, Moretti et al. conclude that these sites work by causing a steric hindrance to the scanning or translating ribosome. Taken together, sites in the 3'-UTR are under selective biological pressure and therefore evolutionary conserved. The embedding of this information in search algorithms leads to a better discrimination between true and false miRNA target sites [Lewis et al., 2003].

A further factor which significantly reduced the number of false positives is the so called 'seed' sequence [Lewis et al., 2005]. The initial observation by Lai [2002] concluded that „the 5'-ends of many miRNAs are perfectly complementary to 3'-UTR sequence motifs that mediate negative post-transcriptional regulation". The 6-8 nt long seed region of a miRNA forms a perfect and consecutive stretch of Watson-Crick base-pairing with the mRNA. This perfect complementarity is crucial for miRNA mediated silencing, and mutating the seed sequence has become a standard practice for target site validation.

Despite the diversity of computational approaches and recent experimental progress, there is still room for improvement. Some of the principles are being challenged by biochemical studies like the finding and exploration of functional miRNA target sites located in the ORF [Moretti et al., 2010] or miRNAs with a non-perfect complementarity in the seed region [reviewed in Brodersen and Voinnet, 2009]. More recently, two proteomic studies extended the search for miRNA targets [Baek et al., 2008; Selbach et al., 2008]. Both reports label proteins during translation using a method called SILAC (stable-isotope labeling by amino acids in cultured cells). With subsequent mass spectrometry, whole proteomes of cells grown in the presence of different labels can be compared side by side. Searching whole proteomes for miRNA targets and other advancements as well as fine tuning of computational methods will certainly increase and refine the ranks of miRNA targets. However, biochemical confirmation of the miRNA-mRNA interaction is still necessary.

1.3.3 RISC Localization and P-bodies

It is not yet understood how the active RISC complex finds complementary mRNAs in the cytoplasm. But there is evidence that part of the RISC mediated repression takes place in distinct foci within the cell, so called P-bodies. The first observation of P-bodies occurred in 1997, when Bashkirov et al. reported that XRN1 was „highly enriched in discrete, prominent foci“. Several other names have been suggested, including GW-bodies, referring to the protein GW182 that was discovered early on as a component of P-bodies [Eystathioy et al., 2003], but the name P-bodies was accepted as a more general term.

P-bodies have been demonstrated to play fundamental roles in general mRNA decay, nonsense-mediated mRNA decay, AU-rich element mediated mRNA decay, and microRNA induced mRNA silencing, and the number of proteins detected in P-bodies has increased considerably. This work only emphasizes the miRNA related mechanism but the following reviews provide a detailed overview of other processes and proteins: Kulkarni et al. [2010]; Eulalio et al. [2007a]; Parker and Sheth [2007]; Balagopal and Parker [2009].

In general, P-bodies are sites of mRNA decay. However, it is important to note that not all mRNAs which enter P-bodies are degraded, as it has been demonstrated that some mRNAs can exit P-bodies and re-initiate translation [Brengues et al., 2005; Bhattacharyya et al., 2006]. The connection to miRNA repression was established when Ago was shown to localize to P-bodies [Sen and Blau, 2005; Liu et al., 2005b; Pillai et al., 2005]. Using transfected reporters, researchers could show that miRNAs localize to P-bodies in a complex with their target mRNAs [Liu et al., 2005b; Pillai et al., 2005]. Bhattacharyya et al. showed that the endogenous CAT-1 (cationic amino acid transporter-1) localizes to P-bodies when repressed by miR-122. This reporter is released from P-bodies and reenters translation if cells are subjected to stress.

There is more evidence for P-bodies being important for RNAi processes: The disruption of P-bodies decreases the efficiency of RNAi [Jakymiw et al., 2005] and the human protein RCK/p54, an important component of P-bodies, is required for miRNA mediated silencing [Chu and Rana, 2006]. However, the same report could also show that P-body integrity is not necessary for silencing by siRNAs. Importantly, recent reports gave evidence that P-bodies are rather a consequence of RNA interference than the cause [Pauley et al., 2006; Eulalio et al., 2007b].

Despite the well established link between P-bodies and RNA silencing, the exact role of the distinct foci and the accompanying spatial restriction in a cell is not yet clear. Some reports even challenge the idea of a connection to P-bodies by reporting rather a congregation of GW182 and Ago2 with endosomes and multivesicular bodies (MVBs) [Gibbings et al., 2009]. Indeed, active RISCs have been reported to be physically and functionally coupled to MVBs [Lee et al., 2009] and miRNAs

have been found in exosomes [Valadi et al., 2007]. Further research will clarify the role of spatial distribution and cellular compartments in miRNA and siRNA mediated processes.

1.4 Mechanisms of MiRNA-Mediated Repression

MiRNAs and siRNAs both repress their targets genes. In plants, miRNAs generally have a perfect complementary binding site and their target is cleaved and subsequently degraded [reviewed in Jones-Rhoades et al., 2006]. This mechanism is similar to siRNA mediated cleavage in mammals. The downregulation of gene expression by miRNAs in vertebrates on the other hand, is a highly sophisticated process and probably involves multiple mechanisms and regulation steps. The exact molecular details of how miRNAs repress gene expression are not yet determined and the mechanism of translational repression remains controversial. Many reviews have been written on the complex puzzle of miRNA mediated repression, the most recent being Chekulaeva and Filipowicz [2009], Huntzinger and Izaurralde [2010] and Fabian et al. [2010].

Over the last decade, several different and often contradictory mechanisms have been proposed (compare Figure 1.3 on page 21). Three ideas have been predominantly suggested:

- Deadenylation and subsequent degradation of the mRNA
- Block of translation initiation
- Slowed elongation or ribosome drop-off

To better understand the diverse possibilities of miRNA mediated repression, one needs an insight into the complex mechanism of eukaryotic translation. And although it is now clear that the repressive effect of miRNAs on protein synthesis is due to both mRNA destabilization and translational repression, the order of events and the exact step of translation being regulated remain to be elucidated.

1.4.1 An Insight into Eukaryotic Translation

Eukaryotic translation is divided into three distinct steps: initiation, elongation and termination [refer to Jackson et al., 2010, Van Der Kelen et al., 2009 and Fabian et al., 2010 for reviews]. At the initiation step, the whole complex necessary for translation is assembled at the translation start site of the mRNA. During elongation, the peptide chain is extended with the help of tRNAs. Termination releases the newly synthesized protein and is followed by a disassembly of the ribosomal subunits from the mRNA.

Initiation involves at least 10 different eukaryotic initiation factors (eIFs). It is considered as the rate-limiting step in translation and therefore the most common target for regulation. Most eukaryotic mRNAs contain a 5'-cap structure, a m^7GpppN group and a 3'-poly(A) tail. The cap structure serves as docking point for the eIF4F complex, which contains three main proteins: eIF4A, eIF4E and eIF4G. eIF4A comprises a DEAD-box ATPase and an ATP-dependent RNA helicase and is thought to unwind the mRNA 5' secondary structure. eIF4E is a small protein which specifically interacts with the cap, while eIF4G mainly serves as an assembly scaffold. It is necessary for the coordinated attachment of the translation initiation machinery to the mRNA and facilitates the recruitment of the 40S subunit to the mRNA by binding to eIF3. It also binds to the poly(A)-binding protein (PABP), leading to a potential circularization of the mRNA. Once the 40S subunit of the ribosome is assembled, it scans the mRNA for the start codon AUG [Kapp and Lorsch, 2004]. The subsequent joining of the 60S unit initiates elongation.

Although most mRNAs use the described initiation and scanning mechanism, initiation on some specific RNAs can also be mediated by internal ribosome entry sites (IRESs). This circumvents the necessity of some eIFs and makes the process independent of the 5'-cap [Jackson, 2005].

1.4.2 Repression after Initiation

Several post-initiation mechanisms of repression including inhibition of ribosome elongation or inducing ribosome drop-off have been proposed (see Figure 1.3). Evidence for elongation being blocked by miRNAs comes from studies with polysome sedimentation analysis. However one has to keep in mind that these experiments look at a steady-state which might not reflect the situation *in vivo*. Early studies in *C. elegans* showed that *lin-14* mRNA remains associated with polysomes even while the corresponding protein is severely downregulated [Olsen and Ambros, 1999], suggesting that repression occured after translation initiation. Similar results were obtained in mammalian cells [Petersen et al., 2006; Maroney et al., 2006; Nelson et al., 2004]. Moreover, Petersen et al. found that IRES mediated translation is also sensitive to miRNAs and presented additional data that miRNAs promote premature termination and ribosome drop-off. A similar conclusion, that repression works at a post-initiation step, was drawn by Maroney et al., observing that endogenous miRNAs and their target cosediment with polysomes.

The idea that protein accumulation is inhibited due to the degradation of the nascent peptide chain [Olsen and Ambros, 1999] has been experimentally adressed by Nottrott et al. [2006]. Since they were unable to immunoprecipitate growing polypeptides from repressed mRNA reporters, they concluded that miRNAs recruit proteases to inhibit protein accumulation. In contrast, repression is not prevented

when reporter proteins are targeted to the endoplasmatic reticulum (ER) [Pillai et al., 2005], which makes a degradation in the cytosol unlikely and argues against the 'proteolytic' mechanism.

One has to keep in mind that repression by miRNAs is generally only partial and binding of a single miRNP to mRNA often has no significant effect on translation [Pillai et al., 2005]. It is therefore conceivable that miRNPs can bind to actively translated mRNAs and cosediment with polysomes. This is in agreement with the RISC binding mainly to the 3'-UTR in order not to completely abrogate translation. Also, in contrast to earlier studies in *C. elegans*, shifts in polysome gradients have been observed by Ding and Grosshans [2009]. The debate about this mode of action is still ongoing, but despite the evidence of miRNAs being associated with polysomes, a molecular mechanism for repression at a post-initiation step has not yet been proposed.

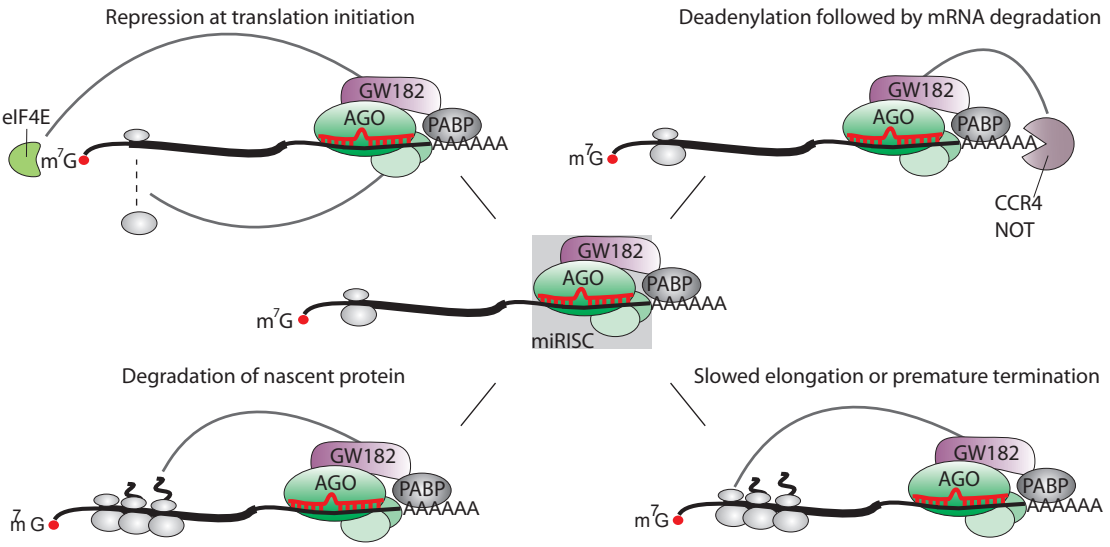

Figure 1.3: Possible mechanisms for miRNA-mediated gene silencing. Four partial contradictory mechanisms have been proposed about how the RNA-induced silencing complex mediates repression. Proposals for a repression at the level of translation initiation (upper left) include the prevention of 60S subunit joining and an interference with cap-recognition via eIF4E. Degradation of nascent protein and ribosome drop-off work at the elongation step. Finally, protein synthesis can be downregulated by deadenylation and subsequent degradation of the mRNA.

1.4.3 Repression of Translation Initiation

In contrast to above mentioned studies, experiments carried out in other laboratories strongly support the idea of repression at translation initiation [Pillai et al., 2005; Ding and Grosshans, 2009; Humphreys et al., 2005; Bhattacharyya et al., 2006]. Data from Humphreys et al. with IRES driven reporters implicate the cap recognition step of eIF4E as a molecular target. Investigations using an IRES by Pillai et al. reinforced the conclusion that the m^7G-cap is essential for translational repression. According to these experiments, cap-dependent cistrons are subject to miRNA mediated repression, while cistrons under the control of an artificially tethered eIF4E or eIF4G are not. Many of the above mentioned studies use artificial reporters with miRNA binding sites in the 3'-UTR. However, a more recent approach by Ding and Grosshans [2009] looks at endogenous targets in *C. elegans*, comparing wild-type worms with worms with mutated miRNA genes. Their data indicate that *lin-14* and *lin-28* repression is accompanied by a decreased association with polysomes and partial mRNA degradation and thus support repression at translation initiation.

Subsequent studies in cell-free extracts of diverse origin have supported the mechanism of repression at translation initiation [Wang et al., 2006; Thermann and Hentze, 2007; Mathonnet et al., 2007; Wakiyama et al., 2007; Zdanowicz et al., 2009]. In all *in vitro* systems, the m^7G-cap was required for efficient repression by miRNAs; mRNAs containing an artificial ApppN-cap structure or an IRES were not inhibited. Zdanowicz et al. demonstrated a crucial role for the m^7GpppN-cap structure and suggested that miRNAs may target cap-dependent translation through a mechanism related to the eIF4E binding proteins. Importantly, supporting the above observations, silencing was suppressed if the extract was supplemented with purified eIF4F (including the cap binding unit eIF4E, see section 1.4.1 on page 19) [Mathonnet et al., 2007].

One has to consider that current *in vitro* models are not very robust and show only a repression of about three to four times in contrast to other experimental setups like tethering where a repression up to ten times can be observed. Although this minor repression could quite possibly reflect physiological conditions, further requirements like preannealing of the synthetic miRNA [Wang et al., 2006] or overexpression of RISC components [Wakiyama et al., 2007] make these results difficult to interpret.

The discrepancies about the mechanism of repression at translation initiation are clearly reflected in the amount of different possibilities suggested. Wang et al. showed that in a rabbit reticulocyte extract, mRNAs being repressed by an artificial miRNA are enriched in 40S but not 60S ribosomal subunits. They proposed that joining of the 60S ribosomal subunit is impaired by miRNAs. A similar conclusion is drawn by Chendrimada et al. [2007]. The authors reported that eIF6 – together

with the 60S subunit – co-immunoprecipitates with the RISC. eIF6 is required for 60S biogenesis in *Saccharomyces cerevisiae* and is known to regulate translation by preventing 60S joining [Ceci et al., 2003]. Depletion of eIF6 in either human or *C. elegans* cells partially alleviated the repression by miRNAs. They concluded that the RISC associates with eIF6 and disrupts polysome formation.

A completely different step of translation initiation being regulated by miRNAs was suggested by Kiriakidou et al. [2007]. They identified a limited sequence homology of the Ago2 MID domain to the cap-binding factor eIF4E. They observed that Ago2 binds directly to the cap structure; an interaction which is lost upon mutating two conserved aromatic amino acids to valines. However, the appealing conclusion that Ago2 prevents the cap-eIF4E interaction and thereby inhibits translation initiation was soon challenged by later reports. A recent bioinformatic study could show that the two aromatic residues are too far apart to form a sandwich like structure with the cap [Kinch and Grishin, 2009]. Moreover, data from Elisa Izaurraldes lab [Eulalio et al., 2008a] demonstrated that the mutation of the two aromatic amino acids interferes with Ago2 binding to GW182, a crucial element in gene silencing downstream of Ago2 (see chapter 1.5 on page 26). In the same report, they also knock down eIF6 without seeing an effect on miRNA-mediated repression, questioning the work of Chendrimada et al., see above.

Several possibilities could explain the above mentioned contradictions. The most obvious are differences in the experimental settings used by various laboratories. Effects on repression might vary depending on transfection reagents, if endogenous or artificial miRNAs were analyzed, the number of binding sites in the reporter, if measurements are carried out *in vitro* or *in vivo*, and, in general, which step of translation is investigated. Distinct modifications, like using cap-analogues can change the rate-limiting step in translation initiation which in turn can influence the result of repression [Nissan and Parker, 2008]. In addition, conditions and especially confluency of tissue culture can influence miRNA levels as shown by Hwang et al. [2009].

Another possibility is that miRNAs inhibit protein synthesis via multiple mechanisms. Depending on the experimental setup, one might observe only one of the mechanisms. One example is that different promoters of reporter genes might lead to distinct mechanisms of miRNA-mediated repression [Kong et al., 2008]. Another more controversial example is the observation of upregulation of protein synthesis by miRNAs [Vasudevan et al., 2007; Orom et al., 2008]. Here too, the results strongly depend on the conditions of the experiment. In particular, Vasudevan et al. could observe the activation of translation only in quiescent cells arrested in G0/G1. They further found that Ago2 associates with fragile X mental retardation syndrome-related protein 1 (FXR1) in 'activating' RISCs. A special case is the observation by Orom et al. [2008], who reported an upregulation of mRNAs

bearing a 5'-TOP motif. Such motifs are common in mRNAs encoding proteins involved in translation and generally serve as regulatory elements. Surprisingly, binding of miR-10a to the 5'-UTR downstream of the 5'-TOP motif was observed and it led to an upregulation of translation of the target mRNAs.

The diversity of approaches makes it difficult to compare the proposed mechanisms. To date, there is no irrefutable proof for any of the above listed models. In addition to the disputes about initiation versus post-initiation, leading laboratories still do not agree about the contribution of mRNA destabilization to miRNA-mediated repression.

1.4.4 Deadenylation and Degradation

Degradation of mRNA is a fundamental process in a cell and serves three main functions. First, it is a part of RNA surveillance which ensures the quality of mRNA molecules. Non-functional transcripts are recognized and targeted to degradation, mainly by either nonsense-mediated decay (NMD) [Fasken and Corbett, 2005; Amrani et al., 2006] or non-stop-mediated mRNA decay (NSD). NMD is responsible for degrading mRNA molecules with a premature termination codon, while NSD degrades any that lack translation termination codons. The second important aspect of RNA degradation is antiviral defense [Ding, 2010]. In plants, it includes components of the basic RNA decay machinery as well as elements from more specialized systems like RNAi. Finally, mRNA turnover plays a key role in eukaryotic cells by setting the basal level of gene expression and as a regulatory response [Parker and Song, 2004]. We know now that this regulatory response is mainly triggered by RNA binding proteins and miRNAs.

Despite the diversity of control and stability-regulation mechanisms, all mRNA transcripts eventually get degraded. The pathway of mRNA turnover is best studied in yeast and includes three main steps. First, the poly(A) tail at the end of the 3'-UTR is shortened, a mechanism referred to as deadenylation. Following deadenylation, the 5'-cap structure is removed by a decapping complex consisting of Dcp1 and Dcp2. This step fully exposes the mRNA to 5'→3' digestion by the exonuclease Xrn1. Alternatively, transcripts can be degraded in a 3'→5' direction by the cytoplasmic exosome complex [Wang and Kiledjian, 2001]. The final step of degradation is thought to occur in P-bodies (see chapter 1.3.3 on page 18).

Multiple eukaryotic mRNA deadenylases are known, including the CCR4–NOT complex and the PAN2–PAN3 complex [see Garneau et al., 2007, for a review]. While most of the research has been accomplished in *S. cerevisiae*, these complexes are highly conserves across eukaryotic organisms including yeast, fly and vertebrates. The PAN2–PAN3 complex is a PABP-dependent poly(A) nuclease and consists of PAN2 and PAN3 with PAN3 containing the 3'→5' exonuclease domain. This complex is mainly involved in the first phase of trimming the poly(A) tail to

∼80 nt in mammals [Yamashita et al., 2005]. The CCR4–NOT complex is a much bigger machinery, consisting of several core subunits and is involved in various cellular functions [see Collart and Panasenko, 2011 for the *S. cerevisiae* complex and Lau et al., 2009 for CCR4–NOT composition in human]. It is the main deadenylase in *S. cerevisiae*, with the CCR4 subunit being the 3'→5' exonuclease. In higher eukaryotes, CCR4 and CAF1 provide the exonucleolytic activity. The complex furthermore contains a E3 ligase, CNOT4, which provides ubiquitination activity. The CNOT1 subunit, consisting of 2108 amino acids in *S. cerevisiae*, is the largest component of the complex and servers mainly as a scaffold for the other subunits. In *S. cerevisiae*, it is the only subunit essential for viability, and most interactions seem to be mediated via the C-terminal region [Collart and Panasenko, 2011]. Despite the abundant knowledge about the single components, there is little structural information available about the whole complex due to its heterogeneous composition. In contrast to PAN2–PAN3 activity, PABP blocks the nuclease activity of the CCR4–NOT complex [Tucker et al., 2002].

It has been debated for a long time if mRNA decay plays a role in miRNA-mediated silencing. It is now widely accepted that miRNAs have an effect on both translation and mRNA stability, but many factors still remain in the dark: Which comes first, translational repression or mRNA destabilization? Which mechanism predominates? Are all targets regulated by the same mechanisms to the same extent?

Although first studies on miRNA did not show degradation of target mRNAs, recent reports in different organisms could clearly show a correlation between repression of protein synthesis and mRNA levels [Bagga et al., 2005; Wu and Belasco, 2005; Krutzfeldt et al., 2005; Giraldez et al., 2006]. Most of the studies investigated one miRNA-target pair and observed a frequent correlation between repression and destabilization of the target. Microarray studies of whole transcriptomes also provide evidence for a role of miRNAs in mRNA destabilization [Lim et al., 2005; Rehwinkel et al., 2006].

Looking at single miRNA-mRNA pairs or even whole transcriptomes, one might miss targets which are regulated more at the translational level. To directly compare the level of mRNA decay with the level of protein repression, two groups used a proteomic approach [Selbach et al., 2008; Baek et al., 2008]. While Baek et al. observe a strong correlation between decrease in mRNA and protein abundance, Selbach et al. argued that for certain genes, the protein levels were significantly more reduced than the levels of corresponding mRNAs. Another recent approach looked at the ribosome association of targets regulated by miRNAs [Guo et al., 2010]. Using profiling of ribosome protected fragments, Guo et al. show that „changes in mRNA levels closely reflect the impact of miRNAs on gene expression and indicate that destabilization of target mRNAs is the predominant reason for

reduced protein output“. However, despite arguing for a predominant role of mRNA decay, these global studies also demonstrate a regulation of target mRNAs that cannot be accounted for by mRNA decay and is therefore likely to happen at the level of translation [Djuranovic et al., 2011].

Notwithstanding the fact that mRNA decay is a crucial element of miRNA-mediated repression, the temporal course of events during repression is not yet clear. There is a certain evidence that mRNA decay may be happening after translational repression [Djuranovic et al., 2011]: First, at earlier timepoints in the proteomic study, mRNA does not seem to be downregulated as strongly as at later time points [Selbach et al., 2008]. Second, kinetic studies on deadenylation *in vitro* showed that deadenylation happens after translational repression [Fabian et al., 2009].

Upon realizing the connection between miRNA translational repression and mRNA degradation, its molecular basis has been investigated more closely. Genetic and biochemical studies tried to understand which components of the various mRNA decay machineries are responsible for mRNA destabilization. Depletion of the CCR4–NOT complex as well as of the decapping enzymes DCP1 and DCP2 prevented miRNA-mediated degradation [Rehwinkel et al., 2005; Behm-Ansmant et al., 2006; Eulalio et al., 2007c; Piao et al., 2010]. Surprisingly, these studies revealed the importance of a new class of proteins in miRNA-mediated silencing: the GW182 proteins.

1.5 TNRC6 Proteins in Gene Silencing

GW182 proteins have recently emerged as key players in miRNA-mediated silencing. The first member of this family, a protein of 182 kDa, was discovered as the antigen recognized by a serum from a patient suffering from motor and sensory neuropathy [Eystathioy et al., 2002]. The discrete cytoplasmic speckles stained by the serum were termed GW-bodies, which are similar to P-bodies [Eystathioy et al., 2003]. Importantly, the report already proposed „that the GW ribonucleoprotein complex is involved in the posttranscriptional regulation of gene expression“.

Members of the GW182 protein family are characterized by the presence of multiple Gly-Trp (GW) repeats. There exist three paralogues in vertebrates, TNRC6A/GW182 (Trinucleotide repeat-containing gene 6A protein), TNRC6B and TNRC6C, with two, three and one isoforms each, respectively, and one orthologue in insects (GW182 or Gawky [Schneider et al., 2006]). All members of the GW182 protein family share a common domain organization (Figure 1.4). Two distinct structural domains are annotated, a central ubiquitin associated (UBA) domain and a C-terminal RNA recognition motif (RRM) [Ding and Han, 2007]. The RRM is flanked by regions predicted to be unstructured, called M2 and Cterm. In vertebrates, both unstructured regions contain GW repeats, in *D. melanogaster*

only the M2 region contains GW motifs. At its N-terminal side, the M2 region is adjacent to a conserved motif called PAM2. A glutamine-rich (Q-rich) region is located between the UBA domain and the PAM2. The highest number of GW repeats (GW or WG) occurs in the N-terminal region of the protein and their numbers vary between different family members.

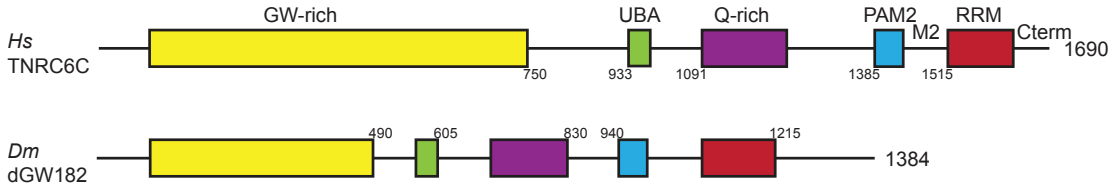

Figure 1.4: Domain organization of GW proteins. All members of the GW182 protein family share a similar domain composition. GW repeats are mainly situated in the N-terminus of the protein. Positions of Q-rich region, UBA (ubiquitin-associated) domain, PAM2 motif, and RRM are indicated.

Two GW-like proteins are found in *C. elegans*, AIN-1 and AIN-2. They share the common N-terminal GW-rich region but lack the UBA domain, the PAM2 motif, and the RRM. Therefore, they are sometimes considered not to belong to the GW protein family, even though they share the GW repeats, bind to Ago proteins and are essential for repression of protein synthesis [Zhang et al., 2007; Ding and Grosshans, 2009].

In studies following their discovery, GW182 proteins were shown to interact with Ago proteins [Meister et al., 2005; Behm-Ansmant et al., 2006]. Interactions of Ago proteins have also been investigated in *Schizosaccharomyces pombe* with Ago1 and Tas3. Till et al. found a amino acid sequence in Tas3 which binds to the PIWI domain of Ago. This conserved motif has been termed „Argonaute hook“ and it includes several GW repeats. They reported a similar short linear peptide motif in human TNRC6B which is sufficient for an *in vitro* Ago association [Till et al., 2007]. In a broader approach in plants, El-Shami et al. [2007] noted that „reiterated WG/GW motifs form evolutionarily and functionally conserved Argonaute-binding platforms in RNA interference-related components“.

As mentioned before, GW182 proteins are part of and localize to P-bodies in both mammals and *D. melanogaster* [Eystathioy et al., 2003; Schneider et al., 2006]. P-bodies have been connected with mRNA decay but are also known as storage sites for repressed mRNAs (see Chapter 1.3.3 on page 18). Knocking down GW182 proteins using RNAi severely impairs the formation of P-bodies [Jakymiw et al., 2005], however, microscopically visible P-bodies are not required for miRNA-mediated gene silencing [Eulalio et al., 2007b].

GW182 proteins have been connected to miRNA function and have been shown to be crucial components for miRNA-mediated gene silencing [Rehwinkel et al., 2005; Liu et al., 2005a; Behm-Ansmant et al., 2006]. Depletion of GW182 in *D. melanogaster* cells relieves repression of miRNA targets [Rehwinkel et al., 2005], and, more importantly, tethering GW182 proteins to an mRNA silenced the reporter independently of Ago protein [Behm-Ansmant et al., 2006]. The bypassing of Ago requirement in miRNA-mediated silencing suggested that GW182 proteins function downstream of Ago and are the effector proteins of the miRNA machinery.

1.6 Aim of this Study

miRNAs have been discovered only less than two decades ago and turned out to be major players in the regulation of gene expression in nearly all cellular processes. Since their discovery, an enormous effort by many laboratories shed light on miRNA pathway and function. They are transcribed in the nucleus and undergo two processing steps (one in the nucleus and one in the cytoplasm) until the mature miRNA is incorporated into the RISC. Argonaute proteins are the key components of the RISC and have been originally thought to mediate the effect of repression. However, recent findings suggested that members of the GW182 protein family function downstream of Ago proteins and are crucial for miRNA-mediated gene silencing.

Functional studies on miRNA mechanism revealed many discrepancies about their mode of repression. To date, multiple possibilities have been proposed, the most prominent being repression at translation initiation, repression at translation elongation or repression by target degradation. The aim of this study was to shed light on the diverse modes of action by miRNAs.

Since GW182 proteins were suggested to mediate miRNA-induced repression, our main objective was the elucidation of the role of GW182 family proteins in miRNA-mediated gene silencing. To get a better understanding of how these proteins mediate repression, we decided to perform deletion mutagenesis of the protein and see which domains contribute to silencing. If miRNA-mediated repression works by multiple mechanisms, we thought we might be able to uncouple deadenylation from the effect on translation by analyzing separate domains of TNRC6C. At that time we chose rather TNRC6C than TNRC6A or TNRC6B since most earlier studies focused on TNRC6A and TNRC6C is the only paralogue with only one isoform. To analyze the mechanistic details of different regions, we made extensive use of the tethering assay which was introduced to miRNA studies in this laboratory. Tethering a protein (or a protein fragment) to a reporter construct enabled us to measure the effect on mRNA activity [Pillai et al., 2004].

We decided to use different assays such as immunoprecipitations, mass spec-

trometry analysis, and yeast two-hybrid system to identify protein interactions with TNRC6C. Furthermore, point mutations of conserved amino acids or sequences should lead to a more detailed insight into the molecular mechanism. To differentiate between the two most prominent mechanisms – target degradation and translational repression – we planned to use reporters that are either polyadenylated or poly(A)-free. Reporters without poly(A) tail do not undergo deadenylation. Tethering fragments of TNRC6C to poly(A)$^-$ reporters should provide a measure of the effect on translational repression.

This study characterizes the human GW family member TNRC6C and describes its specific interaction partners. It further identifies two unstructured regions in the C-terminal domain as the main effectors of repression. This work will lead to a better understanding of the diverse modes of repression by miRNAs and will provide novel molecular insights ons miRNA-mediated gene silencing.

2

Results

In this study, we investigated the role of the human TNRC6C protein in gene silencing mediated by miRNAs. The first part of the result section is represented by the attached paper published in RNA [Zipprich et al., 2009] (section 2.1). It is mainly concerned with the general role of TNRC6 proteins in miRNA-mediated repression and also identifies the C-terminal domain of TNRC6C as the main mediator of repression.

In the second part, we further characterized the C-terminal domain of TNRC6C. Surprisingly, exhaustive dissection of this domain led to the identification of two unstructured protein regions which are responsible for mediating the effect. Furthermore, we could identify two specific interaction partners of the C-terminal domain, PABP and CNOT1. We were able to determine specific sequences in the two C-terminal regions which are necessary to contact downstream factors and thereby are crucial for repression. Finally, the importance of the poly(A)-tail for TNRC6C mediated repression was investigated, using poly(A)$^+$ and poly(A)$^-$ reporters.

2.1 Identification of The C-terminal Domain of TNRC6C

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Importance of the C-terminal domain of the human GW182 protein TNRC6C for translational repression

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ABSTRACT

Proteins of the GW182 family play an important role in the execution of microRNA repression in metazoa. They interact directly with Argonaute proteins, components of microRNPs, and also form part of P-bodies, structures implicated in translational repression and mRNA degradation. Recent results demonstrated that *Drosophila* GW182 has the potential to both repress translation and accelerate mRNA deadenylation and decay. In contrast to a single GW182 protein in *Drosophila*, the three GW182 paralogs TNRC6A, TNRC6B, and TNRC6C are encoded in mammalian genomes. In this study, we provide evidence that TNRC6C, like TNRC6A and TNRC6B, is important for efficient miRNA repression. We further demonstrate that tethering of each of the human TNRC6 proteins to a reporter mRNA has a dramatic inhibitory effect on protein synthesis. The repression is due to a combination of effects on the mRNA level and mRNA translation. Through deletion and mutagenesis, we identified the C-terminal part of TNRC6C encompassing the RRM RNA-binding motif as a key effector domain mediating protein synthesis repression by TNRC6C.

Keywords: GW182; miRNA; RNA stability; translation; polyadenylation

INTRODUCTION

MicroRNAs (miRNAs) are 20- to 22-nucleotide (nt)-long noncoding RNAs regulating gene expression post-transcriptionally by base-pairing to target mRNAs. In animals, most investigated miRNAs form imperfect hybrids with sequences in the 3′-untranslated region (3′-UTR), with the miRNA 5′-proximal "seed" region (positions 2–8) providing most of the pairing specificity (for review, see Bartel 2004; Bushati and Cohen 2007; Filipowicz et al. 2008). Generally, the miRNA association results in translational repression, frequently accompanied by considerable degradation of mRNA (Nilsen 2007; Standart and Jackson 2007; Eulalio et al. 2008a; Filipowicz et al. 2008; Wu and Belasco 2008). More recently, however, miRNAs were also found to have the potential to activate translation (Vasudevan et al. 2007, 2008; Orom et al. 2008). For example, in nonproliferating cells or cells in the G_0 cell cycle phase, miRNAs were reported to stimulate rather than inhibit protein synthesis (Vasudevan et al. 2007, 2008).

miRNAs function as components of ribonucleoprotein (RNP) complexes, miRNPs. The best-characterized constituents of miRNPs are proteins of the Argonaute (AGO) family. Their function in miRNA-mediated repression is well documented in many organisms (Peters and Meister 2007; Tolia and Joshua-Tor 2007). Mammals contain four AGO proteins, AGO1-4, associating with similar sets of miRNAs and participating in translational repression (Liu et al. 2004; Meister et al. 2004). In *Drosophila*, Ago1 is dedicated to the miRNA pathway while Ago2 mainly functions in RNA interference (RNAi) (Peters and Meister 2007; Tolia and Joshua-Tor 2007). The Ago proteins repress protein synthesis when artificially tethered to the mRNA 3′-UTR, indicating that they function as downstream effectors in the repression, with miRNAs mainly acting as guides bringing the proteins to mRNA targets (Pillai et al. 2004, 2005; Rehwinkel et al. 2005; Wu et al. 2008).

Argonautes are not the only proteins required for the miRNA-mediated repression. Several components of P-bodies (known also as GW-bodies), which are cytoplasmic structures involved in the degradation and storage of translationally repressed mRNAs (Eulalio et al. 2007a; Parker and Sheth 2007), also function in the miRNA pathway and, consistently, repressed mRNAs, miRNAs, and Ago proteins are enriched in P-bodies (Liu et al. 2005; Pillai et al. 2005; Sen and Blau 2005; Huang et al. 2007; for review, see Jakymiw

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et al. 2007; Eulalio et al. 2008a; Filipowicz et al. 2008). Of the P-body components, proteins of the GW182 family play a particularly important role in the execution of miRNA repression. GW182 proteins, characterized by the presence of multiple Gly-Trp (GW) repeats (Eystathioy et al. 2002; Ding et al. 2005; Rehwinkel et al. 2005; Schneider et al. 2006), interact with Argonautes through their GW-rich domain (Behm-Ansmant et al. 2006; Till et al. 2007; Eulalio et al. 2008b). Recent work carried out in the *Drosophila* system demonstrated that this interaction is essential for the repression (Till et al. 2007; Eulalio et al. 2008b). Importantly, tethering of GW182 to the mRNA bypassed the Ago1 requirement for repression in *Drosophila* cells, demonstrating that GW182 functions in the same pathway but downstream from Ago1 (Behm-Ansmant et al. 2006).

Despite a considerable research effort, the mechanistic details of miRNA function in repressing protein synthesis are still poorly understood. Moreover, the results from studies conducted in different systems and different laboratories have often been contradictory, making it difficult to obtain a lucid picture of the repression (Nilsen 2007; Standart and Jackson 2007; Eulalio et al. 2008a; Filipowicz et al. 2008; Wu and Belasco 2008). Although many experiments investigating miRNA function in metazoan cells or in vitro point to the initiation of translation as a target of miRNA repression (Humphreys et al. 2005; Pillai et al. 2005; Bhattacharyya et al. 2006; Chendrimada et al. 2007; Kiriakidou et al. 2007; Mathonnet et al. 2007; Wakiyama et al. 2007), there is also considerable evidence that miRNAs inhibit translation at post-initiation steps (Olsen and Ambros 1999; Maroney et al. 2006; Nottrott et al. 2006; Petersen et al. 2006; Lytle et al. 2007). Although reports aimed at the reconciliation of some conflicting data have appeared recently (Kong et al. 2008), the question of whether the disparities represent artifacts of different experimental approaches or whether miRNAs are indeed able to repress protein synthesis by different mechanisms remains one of the key problems to be resolved (Nilsen 2007; Eulalio et al. 2008a; Filipowicz et al. 2008).

Another important and unanswered issue is the relative contribution of translational inhibition and mRNA degradation to the final outcome of the repression. Most investigated mRNAs undergo moderate or substantial degradation, which appears to be initiated by removal of the poly(A) tail in response to miRNP association with the mRNA 3′-UTR (Bagga et al. 2005; Lim et al. 2005; Wu and Belasco 2005; Behm-Ansmant et al. 2006; Giraldez et al. 2006; Rehwinkel et al. 2006; Schmitter et al. 2006; Eulalio et al. 2007b). In *Drosophila*, the GW182 protein is implicated in the recruitment of deadenylating enzymes to the mRNA, although the protein also functions in translational repression independently of its role in deadenylation (Behm-Ansmant et al. 2006). However, many mRNAs repressed by miRNAs are resistant to degradation (for a comprehensive list, see Filipowicz et al. 2008). Which features of mRNA or of the mRNA–miRNA interaction determine whether repression follows translational inhibition or mRNA decay? Is the latter a consequence of translation being repressed, or does it occur independently of the translational status of the mRNA?

In contrast to the single GW182 protein expressed in *Drosophila*, three GW182 paralogs, TNRC6A, TNRC6B, and TNRC6C, are encoded in mammalian genomes (for review, see Ding and Han 2007; Jakymiw et al. 2007). Evidence has already been presented that the two human proteins TNRC6A and TNRC6B function in the miRNA pathway and are important for effective miRNA repression (Jakymiw et al. 2005; Liu et al. 2005; Meister et al. 2005; Till et al. 2007). However, mechanistic details of the repression have not been investigated for any of the mammalian proteins. In this study, we provide evidence that TNRC6C, like TNRC6A and TNRC6B, is essential for efficient miRNA repression and demonstrate that tethering of each of the human GW182 proteins to reporter mRNA has a dramatic effect on protein synthesis, with only a moderate effect on mRNA stability. Finally, we identify the C-terminal fragment of TNRC6C, encompassing the RNA-binding RRM motif, as a region mediating the repression. Two other domains, GW-rich and Q-rich, also repress protein synthesis upon tethering, but their effects are much less pronounced than that of the C-terminal region.

RESULTS

Human GW182 protein TNRC6C is involved in miRNA-mediated repression

The three GW182 protein paralogs encoded in mammalian genomes, TNRC6A, TNRC6B, and TNRC6C, have a domain organization similar to *Drosophila* GW182 (also known as Gawky). At the N-proximal part, they contain a domain rich in GW or WG repeats followed by a glutamine (Q)-rich region of unknown function, hereafter referred to as DUF, and an RNA-binding domain, RRM. The *Drosophila* GW182 and mammalian TNRC6C also contain a central ubiquitin-associated (UBA) domain (Fig. 1A; for review, see Ding and Han 2007). Two homologs of GW182 proteins, AIN-1 and AIN-2, were characterized in *Caenorhabditis elegans.* While AIN-1 and AIN-2 both contain GW- and Q-rich sequences, they lack other domains present in mammalian proteins (Ding et al. 2005; Zhang et al. 2007). Interestingly, through database searches, we have identified a likely homolog of TNRC6 and AIN proteins in the nematode *Brugia malayi*. In addition to GW-containing and Q-rich sequences, this protein includes a DUF domain (Fig. 1A,B). Hence, the *B. malayi* protein likely represents an evolutionary link between TNRC6 and AIN proteins.

The TNRC6A and TNRC6B proteins were demonstrated previously to play a role in the miRNA pathway in mammalian cells (Jakymiw et al. 2005; Liu et al. 2005; Meister et al. 2005), but the expression and function of TNRC6C have not been investigated. We raised polyclonal

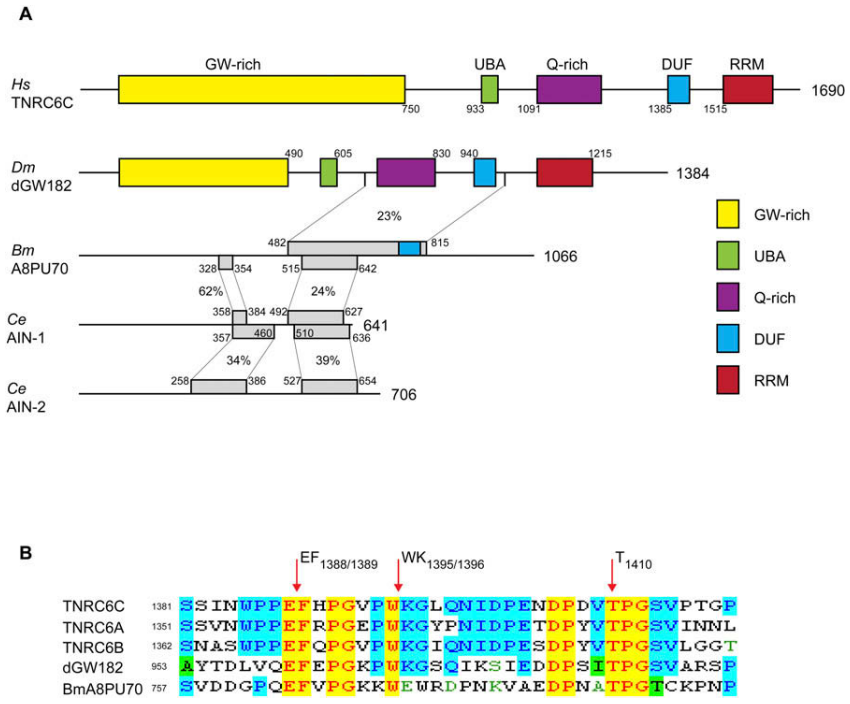

FIGURE 1. Domain structure of selected GW-182-like proteins. (*A*) Schematic representation of human (Hs) TNRC6A, TNRC6B, and TNRC6C proteins, the *Drosophila* (Dm) GW182 (dGW182), *C. elegans* (Ce) AIN-1 and AIN-2, and a candidate GW182 ortholog of *Brugia malayi* (Bm). Positions of GW-rich, Q-rich, UBA (ubiquitin-associated), DUF (domain of unknown function), and RRM domains are indicated. The percentage of amino acid identity between highlighted regions of *Drosophila*, *C. elegans*, and *B. malayi* proteins is indicated. (*B*) Amino acid alignments of DUF domains of selected GW proteins. Positions of amino acids that have been mutated to alanines, either singly (T1410) or in the combination of two (EF1388/1389 and WK1395/1396), are indicated. (Blue) Amino acids identical in more than 50% of proteins; (green) conservative substitutions by related amino acids.

antibodies (Abs) against peptides with sequence present in TNRC6C but not two other mammalian GW182 proteins. In Western analysis, the affinity-purified Ab recognized a protein of the expected size in lysates prepared from HEK293 and HeLa cells. The intensity of the recognized band was weaker in lysates of cells in which TNRC6C was knocked down by RNAi (Fig. 2A, lanes 7–10). Overexpression of the HA-tagged version of the protein further confirmed that the visualized band corresponds to TNRC6C (lane 6). The Ab did not recognize overexpressed TNRC6A and TNRC6B (Fig. 2A, lanes 4,5), consistent with it being specific for TNRC6C. RT-PCR analysis with primers specific for individual GW182 genes revealed that all three TNRC6 genes are expressed in both HEK293 and HeLa cells (data not shown).

To find out whether TNRC6C, like TNRC6A and TNRC6B (Jakymiw et al. 2005; Liu et al. 2005; Meister et al. 2005), is required for miRNA-mediated repression, we knocked it down using RNAi. As controls, TNRC6A and TNRC6B were also individually depleted using gene-specific siRNAs. The efficacy of the knockdowns was monitored by following the levels of either ectopically expressed HA-tagged TNRC6 proteins (Fig. 2B) or the endogenous TNRC6C (Fig. 2A). For siRNAs directed at TNRC6B and TNRC6C, we verified that their effects were target-specific (data not shown). As illustrated in Figure 2C, down-regulation of each TNRC6 protein partially rescued repression of the *Renilla* luciferase (RL) reporter, RL-3xBulgeB. RL-3xBulgeB harbors in its 3′-UTR three sites specific for let-7b miRNA (Pillai et al. 2005; Schmitter et al. 2006), which is abundantly expressed in HeLa cells. We conclude that TNRC6C plays a role similar to those of TNRC6A and TNRC6B, although the observation that knockdown of each individual protein had a marked effect on miRNA repression leaves open the possibility that the functions of individual TNRC6 paralogs in mediating miRNA-mediated inhibition do not entirely overlap.

Tethering of TNRC6 proteins to mRNA causes repression of protein synthesis

We used a tethering approach to investigate the effect of individual mammalian TNRC6 paralogs on protein synthesis. In this assay, which was used successfully to study functions of AGO proteins (Pillai et al. 2004; Rehwinkel et al. 2005; Kiriakidou et al. 2007; Wu et al. 2008) and the *Drosophila* GW182 (dGW182) (Behm-Ansmant et al. 2006), the proteins are expressed as fusions with the HA-tag and the phage λ N-peptide, which specifically recognizes box B hairpins inserted into the 3′-UTR of RL-5BoxB reporter. The RL expression was normalized to the activity of firefly luciferase (FL) expressed from the co-transfected plasmid bearing no 5BoxB hairpins (FL-Con). As shown in Figure 3, expression of the NHA version of each of the three TNRC6 proteins strongly repressed activity of the RL-5BoxB reporter when compared to control TNRC6 proteins containing the HA-tag but lacking the N peptide. Tethering of NHA-LacZ, used as another control, yielded RL activity similar to that measured in the presence of HA-TNRC6C (see also Figs. 6A and 8, below; data not shown). Together with Western analysis, which revealed similar expression levels of NHA- and HA-tagged proteins (Fig. 3A), the data demonstrate that the repression of protein synthesis is a result of the TNRC6 proteins tethering to mRNA.

To find out whether the tethered TNRC6 proteins repress RL activity by inhibiting translation or destabilizing the mRNA, we quantified RL-5BoxB mRNA levels and, as a reference, the levels of GFP mRNA coexpressed in transfected

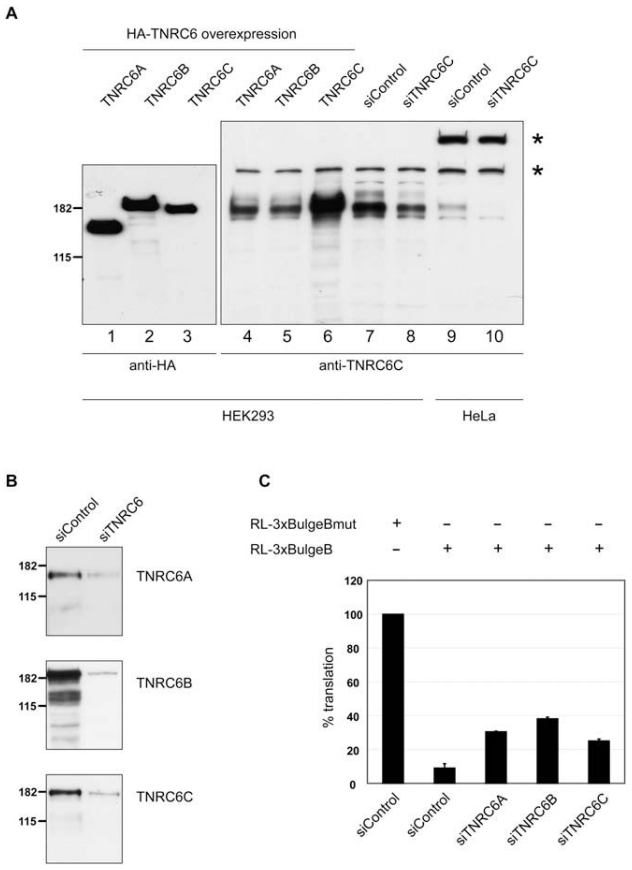

FIGURE 2. Expression of TNRC6C protein in HEK293 and HeLa cells and its importance for effective miRNA-mediated repression. (*A*) Anti-TNRC6C Abs specifically recognize endogenous TNRC6C in HEK293 and HeLa cell extracts and do not cross-react with overexpressed TNRC6A and TNRC6B proteins. (Lanes *1–6*) Extracts prepared from HEK293 cells overexpressing indicated HA-TNRC6 proteins; (lanes *7–10*) extracts of HEK293 or HeLa cells transfected with either control or anti-TNRC6 siRNAs. Abs used for Western analysis and positions of protein size markers are indicated. (*) Non-specific proteins cross-reacting with anti-TNRC6C Ab. Note that overexpression of TNRC6A or TNRC6B proteins slightly decreases the level of endogenous HTNRC6C (cf. lanes *4,5* and lane *7*). (*B*) Knockdown of individual TNRC6 proteins by specific siRNAs. Cells were cotransfected with constructs expressing indicated NHA-tagged TNRC6 proteins and either gene-specific or control siRNAs. One siRNA was used in the case of TNRC6A, and mixtures of two in the case of TNRC6B and TNRC6C (Materials and Methods). Anti-HA Ab was used for Western blot analysis. (*C*) TNRC6C, similarly to TNRC6A and TNRC6B, is required for efficient repression of RL-3xBulgeB reporter by endogenous let-7 in HeLa cells. Down-regulation of each protein partially rescues repression of RL-3xBulgeB. RL-3xBulgeBmut, containing mutations in the seed sequence of the let-7 binding that prevent the repression (Pillai et al. 2005; Schmitter et al. 2006), was used as a control reporter. SiRNAs used for knockdowns are indicated. The data represent means from three independent experiments.

cells. Comparison of RL activity (Fig. 3A) and Northern blot data (Fig. 3B) revealed that tethering of TNRC6 proteins to RL-5BoxB mRNA had a much stronger effect on protein expression (10- to 20-fold) than on mRNA levels (approximately threefold), indicating that all three TNRC6 proteins not only trigger marked mRNA destabilization but also directly affect the translation process itself.

Identification of repressive domains of TNRC6C by deletion analysis

To gain insight into the mechanism of TNRC6C-mediated repression, we generated a collection of deletion mutants of the protein and tested their effects on protein synthesis using the tethering assay. Progressive deletions from the N terminus of the protein (Fig. 4A) revealed that a fragment bearing only the C-proximal domains DUF and RRM (mutant ΔN1370) retained the potential to inhibit RL activity upon tethering to mRNA (Fig. 4B). Quantification of the data derived from many experiments in which effects of NHA-TNRC6C and NHA-ΔN1370 were compared indicated that repression by NHA-ΔN1370 was actually stronger that that by a full-length NHA-TNRC6C (for significance of the difference, see Fig. 5C and its legend), raising a possibility that the N-terminal portion of TNRC6C may modulate inhibitory activity of the C-terminal ΔN1370. Further truncation of NHA-ΔN1370, leading to the removal of DUF (mutant ΔN1471), decreased the repressive activity, but this mutant still inhibited protein synthesis approximately fourfold compared with the more than 10-fold repression seen with ΔN1370 (Fig. 4B). In the analysis of mutants with progressive N-terminal deletions and of most of other mutants described below, care was taken to assess mutant proteins expressed at similar levels (Fig. 4B). This sometimes required the adjustment of amounts of mutant-encoding plasmids transfected into cells (see Materials and Methods). However, within the range of plasmid concentrations used for transfections, the extent of repression caused by individual mutants was generally independent of the amount of transfected plasmid.

In a further set of mutants, progressive deletions were carried out from the C terminus (Fig. 4A). As expected, deletion of the C-terminal portion of TNRC6C containing the DUF and RRM domains strongly affected the repressive potential of the protein. Interestingly, analysis of other mutants revealed that the N-terminal half of the GW-rich domain (Fig. 4A, mutant 1–405) and a fragment encompassing the entire GW-rich domain and the UBA domain (Fig. 4A, mutant 1–1034) each had some repressive activity: their tethering inhibited protein synthesis ~40% (Fig. 4C). Tethering of the Q-rich domain alone (Fig. 4A, mutant 1080–1245) also caused an ~65% repression of RL activity. The repressive activity of a fragment encompassing both the GW- and Q-rich domains (Fig. 4A, mutant 1–1368) was not stronger than the individual domains alone.

The integrity of the ΔN1370 fragment is important for effective repression

Since deletion analysis revealed that the C-terminal fragment of TNRC6C (Fig. 5C, mutant ΔN1370) repressed

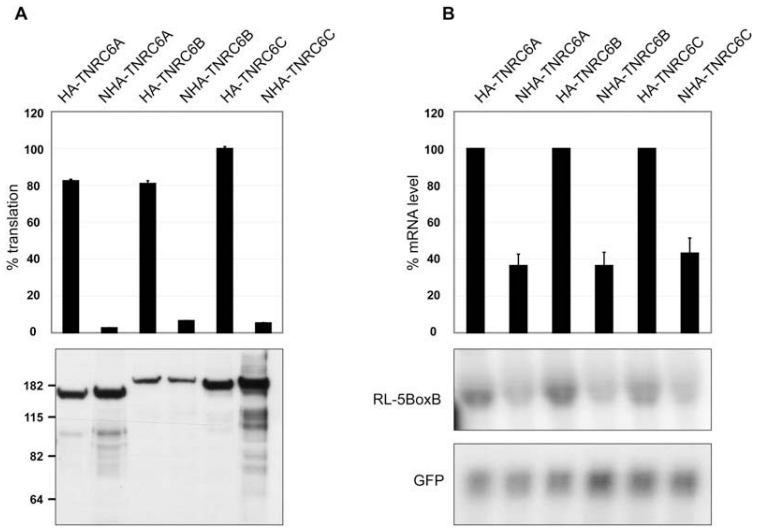

FIGURE 3. Tethering of TNRC6 proteins to mRNA causes strong repression of protein synthesis and partial mRNA degradation. (*A*) Indicated HA- or NHA-tagged TNRC6 proteins were coexpressed into HEK293 cells with RL-5BoxB, FL-Con, and (in some transfections) GFP reporters. (*Upper* panel) RL expression was normalized to the activity of FL and is shown as the percentage of activity seen in the presence of HA-TNRC6C. Tethering of NHA-LacZ protein, frequently used as an additional control (see Figs. 6 and 8), did not repress protein synthesis. (*Lower* panel) Representative Western analysis of expressed proteins, performed with anti-HA Ab. (*B*) Northern blot analysis of RL-5BoxB and GFP mRNAs levels. (*Upper* panel) PhosphorImaging quantification of RL-5BoxB mRNA, normalized to GFP mRNA. (*Bottom* panels) Representative Northern blot analysis. Values in *A* and *B* are means from three independent experiments. Values for cells expressing HA-tagged proteins were set to 100%.

protein synthesis even more effectively than the full-length protein, we focused our attention on this region. Repressive activity of GW- and Q-rich domains was relatively small and was not further investigated. The integrity of the ΔN1370 fragment appeared to be important since deletion of either the N-proximal DUF domain or 80 C-terminal amino acids resulted in a significant decrease of the repression. In addition, isolated RRM domain (Fig. 5C, fragment NHA-1505–1610) was devoid of repressive potential (Fig. 5C, left part).

RNP1 and RNP2 motifs present in the RRM domains of many characterized RNA-binding proteins contain aromatic residues involved in stacking interactions with RNA ligands (Clery et al. 2008). Similarly, the RRM domains of GW182 proteins contain several conserved aromatic amino acids, both within and outside of RNP1 and RNP2 motifs (Fig. 5B). Residues W1515, H1537, F1543, Y1556, and F1583 were individually mutated to alanine in the context of the ΔN1370 fragment. In another mutant, residues H1537 and Y1556 were simultaneously replaced with alanine. Several of the RRM mutants had a significantly lower activity in repressing protein synthesis than the wild-type ΔN1370 fragment (Fig. 5C, right part).

We also generated single or double amino acid mutations in some conserved residues of the DUF domain (for the identities of the mutated amino acids, see Fig. 1B). However, these mutations had no appreciable effect on the ability of the ΔN1370 fragment to repress protein synthesis in the tethering assay (data not shown).

The ΔN1370 fragment acts mainly as a translational repressor

To find out whether the ΔN1370 fragment acts similarly to the full-length TNRC6C and affects both mRNA translation and stability, we examined the level of RL-5BoxB reporter repressed by tethering of the ΔN1370 fragment or its RRM domain mutants. Comparison of Northern blotting and RL activity data originating from the same transfection experiments indicated that tethering of ΔN1370 results in an approximately twofold decrease in mRNA level under conditions leading to an approximately 20-fold drop in RL activity (Fig. 6A). Hence, the inhibitory effect of ΔN1370 on RL expression is due mainly to repression of translation. After correction for differences in mRNA levels, the net effect of the tethering of ΔN1370 on translation was 10-fold (Fig. 6A). Tethering of the two tested ΔN1370 RRM domain mutants also decreased the RL-5BoxB mRNA level twofold but did not inhibit protein synthesis as much as the wild-type ΔN1370. The net effect of the F1543A and H1537A/Y1556A mutants on translation was only approximately 2.5-fold, compared with the 10-fold effect of ΔN1370 (Fig. 6A). This suggests that the RRM domain functions in translational repression rather than in mRNA destabilization.

We investigated whether the repressive effect on translation seen upon tethering of TNRC6C and its ΔN1370 deletion mutants could be due to mRNA deadenylation. Total RNA isolated from cells transfected with vectors expressing different proteins was subjected to RNase H treatment in the presence or the absence of oligo(dT). Incubation in the presence of oligo(dT) should result in removal of poly(A) from mRNA and, consequently, in its faster mobility in an agarose gel. Where the mRNA has been deadenylated already in the cell, no major difference in its mobility would be expected upon RNase H digestion. As shown in Figure 6B, control RL-Con RNA isolated from cells co-transfected with NHA-TNRC6C or RL-5BoxB RNA isolated from cells co-transfected with HA-TNRC6C contained poly(A) tracts since their mobility increased upon oligo(dT) addition. Likewise, the mobility of β-actin mRNA, analyzed as an additional control, increased upon removal of poly(A) in vitro. Importantly, RL-5BoxB RNA preparations isolated from cells transfected with either NHA-TNRC6C or NHA-ΔN1370 deletion

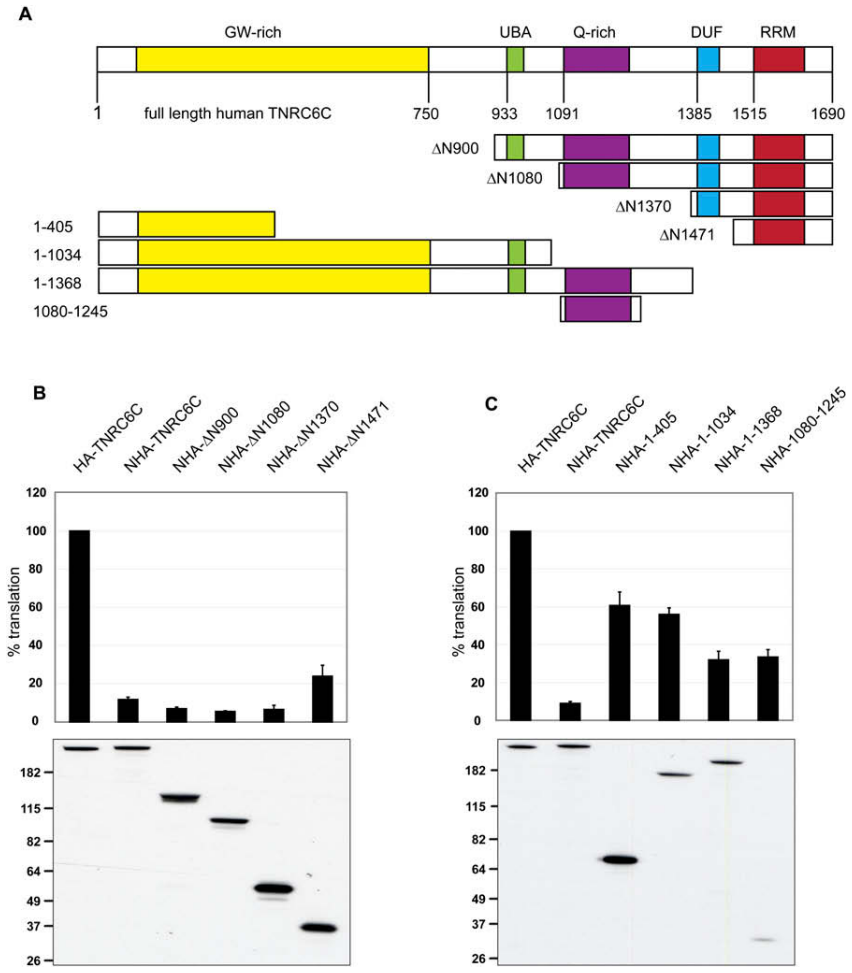

FIGURE 4. Characterization of the TNRC6C deletion mutants. (*A*) Schematic representation of TNRC6C and its deletion mutants. Mutants with progressive deletions from the (*upper* part of scheme) N terminus and (*lower* part of scheme) C terminus. Numbers correspond to amino acid positions. (*B*) The C-terminal domain of TNRC6C is sufficient to effectively repress protein synthesis when tethered to mRNA. (*Upper* panel) Repressive activity of TNRC6C and its N-terminal deletion mutants. HEK293 cells were co-transfected with plasmids expressing N-HA fusions of TNRC6C or its fragments and plasmids encoding RL-5BoxB and FL reporters. HA-TNRC6C served as a negative control. Activity of RL was normalized for expression of FL. Values represent relative RL activities normalized to FL, with translation in the presence of HA-TNRC6C set as 100%. (*Lower* panel) Expression levels of HA-TNRC6C and NHA-TNRC6C and its mutants as assessed by Western blotting using anti-HA Ab. Positions of protein size markers are indicated. (*C*) Analysis of progressive deletion mutants from the C terminus and the 1080–1245 mutant reveals only moderate repressive activity of GW- and Q-rich domains. Details of experiments are identical to those given in the legend to Figure 4B. The values in *B* and *C* are means (±SEM) from four to 12 independent experiments. Expression of the Q-rich domain was reproducibly weaker than of other domains.

mutants also changed their mobility after incubation with oligo(dT), consistent with them retaining the poly(A) tail (Fig. 6B). These data indicate that the inhibitory effect of tethering TNRC6C or ΔN1370 on translation is not due to elimination of a stimulatory role of the poly(A)-binding protein PABP on translation initiation (Kahvejian et al. 2005) or due to disruption of mRNA "circularization" (Wells et al. 1998) potentially caused by mRNA deadenylation.

The ΔN1370 fragment does not interact with endogenous AGO or TNRC6C proteins

We considered the possibility that the inhibitory effect of ΔN1370 on protein synthesis is due to the interaction of this fragment with endogenous TNRC6 or AGO proteins. If this were the case, the inhibition would not be due to the downstream function of ΔN1370 in miRNA-mediated repression but due to recruitment of the endogenous miRNP complex to the reporter mRNA. Using immunoprecipitation (IP) assays, we first determined whether NHA-ΔN1370 expressed in HEK293 cells interacts with endogenous AGO proteins or TNRC6C. Neither AGO proteins nor TNRC6C were pulled down with the anti-HA Ab but, as expected (Behm-Ansmant et al. 2006; Till et al. 2007), the full-length NHA-TNRC6C and its N-terminal GW-rich fragment NHA-1–1034 very effectively coimmunoprecipitated the endogenous Argonautes (Fig. 7A). Since specific Abs recognizing TNRC6A and TNRC6B proteins are not available, we have coexpressed HA-tagged versions of these proteins together with either Flag-HA-tagged ΔN1370 or Flag-HA-tagged AGO2, the latter protein used as a control. IP experiments with anti-Flag Abs revealed that AGO2 but not ΔN1370 is able to interact with TNRC6A and TNRC6B proteins (Fig. 7B). We conclude that the ΔN1370 fragment functions as an autonomous repressive domain, the inhibitory effect of which is not caused by interaction with Argonautes or full-length TNRC6 proteins.

Cross-species repressive activity of GW proteins and their mutants

In the accompanying manuscript, Chekulaeva et al. (2009) have identified three nonoverlapping regions of the *Drosophila* GW182 (dGW182) protein that are able to repress protein synthesis effectively (five- to sixfold) upon tethering to mRNA: the N-terminal GW-rich domain, the Q-rich domain, and the C-proximal fragment containing DUF and RRM domains (for a scheme of dGW182, see Fig. 1A). We tested the potential of the full-length dGW182 and its active subfragments to inhibit the activity of the RL-5BoxB reporter in HEK293 cells. Tethering of a full-length

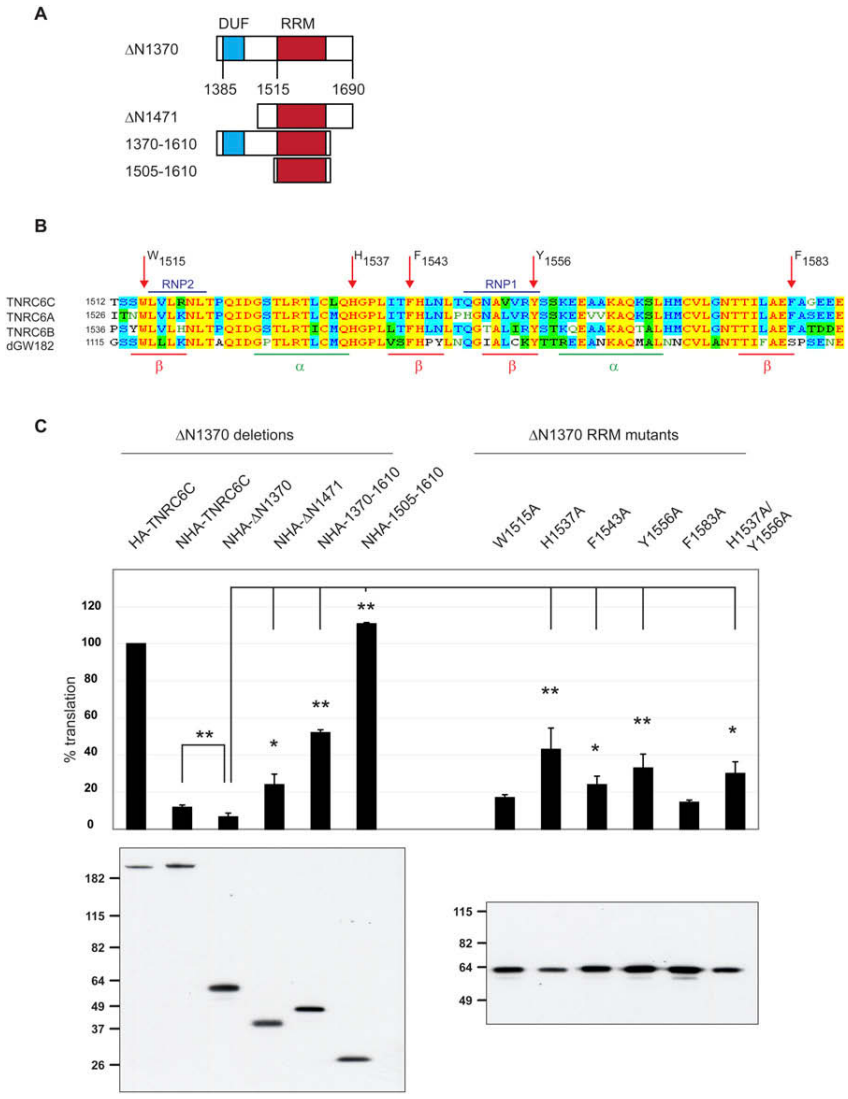

FIGURE 5. Detailed characterization of the C-terminal ΔN1370 fragment of TNRC6C. (*A*) Schematic representation of deletion mutants of the C-terminal ΔN1370 fragment of TNRC6C. (*B*) Sequence alignment of RRM domains of selected GW182 proteins. Aromatic amino acids mutated to alanines, either singly or in combination (mutant H1537/Y1556), are indicated. RNP1 and RNP2 motifs are overlined. Positions of α-helices and β-sheets predicted for the TNRC6C RRM using Phyre (http://www.sbg.bio.ic.ac.uk/phyre/) are shown *below* the alignment. (*C*, *upper* panel) Repressive activity of mutants of the ΔN1370 fragment shown in panel *A*. Cells were co-transfected with plasmids expressing NHA fusions of TNRC6C or the ΔN1370 fragment and its mutants, and the reporter plasmids. Values represent the percent of translation as measured by normalized RL activity, with translation in the presence of HA-TNRC6C taken as 100%. Error bars show standard error (n = 3–12). Statistical significance (NHA-TNRC6C versus NHA-ΔN1370 and NHA-ΔN1370 versus other deletion and RRM amino acid mutants) was calculated using the nonparametric Mann–Whitney–Wilcoxon test (NHA-TNRC6C versus NHA-ΔN1370) or paired two-tailed Student's t-test (all other comparisons); (*) $P < 0.05$; (**) $P < 0.01$. (*Lower* panels) Expression levels of HA-TNRC6C, NHA-TNRC6C, and the C-terminal ΔN1370 fragment and its mutants as assessed by Western blotting using anti-HA Ab.

NHA-dGW182 repressed RL activity as efficiently as the mammalian NHA-TNRC6C; expression of HA-dGW182 had no inhibitory effect. Interestingly, the N-terminal GW-rich domain (mutant 1–605) of dGW182 was the most active repressor in human cells; its repressive activity (approximately sevenfold) was comparable to that seen in *Drosophila* S2 cells (approximately sixfold). In contrast, three further dGW182 fragments tested (Q-rich domain, mutant 605–803, and two C-terminal fragments encompassing DUF and RRM domains, mutants 940–1385 and 940–1215) repressed RL activity in HEK293 cells only approximately two-fold (Fig. 8).

In a reciprocal cross-species experiment, different domains of TNRC6C characterized in this work were tested in *Drosophila* S2 cells. A full-length TNRC6C inhibited activity of the tethering reporter approximately sixfold, while the GW-rich (1–1034), Q-rich (1080–1245), and the C-terminal ΔN1370 fragment repressed protein synthesis ~1.5-fold, eightfold, and 20-fold, respectively (Chekulaeva et al. 2009). Hence, although the full-length dGW182 and TNRC6C proteins exerted a similar strong repressive effect irrespective of whether they were tested in the homologous or heterologous system, the contribution of individual domains to this effect differed between human and *Drosophila* proteins and cells (see Discussion).

DISCUSSION

Proteins of the GW182 family play an important role in the miRNA-mediated repression in metazoa. They directly interact with AGO proteins and appear to function as downstream effectors in the miRNA pathway, responsible for inhibition of translation and acceleration of mRNA decay (Jakymiw et al. 2005; Liu et al. 2005; Meister et al. 2005; Rehwinkel et al. 2005; Behm-Ansmant et al. 2006; Till et al. 2007; Zhang et al. 2007). In contrast to a single GW182 protein expressed in *Drosophila*, three paralogs, TNRC6A, TNRC6B, and TNRC6C, are encoded in mammalian genomes but little is known about their functions. In this study, we provide evidence that TNRC6C, like TNRC6A and TNRC6B studied previously, is expressed in HEK293 and HeLa cells and is essential for the efficient repression of a target mRNA reporter by endogenous let-7 miRNP. More important, we demonstrate that tethering of each human

TNRC6 protein to reporter mRNA strongly affects a process of translation, with a more moderate effect on mRNA stability. We show that the effect on translation is not due to the remaining mRNA being deadenylated. We identify ΔN1370, the C-terminal fragment of TNRC6C including the RRM RNA-binding motif, as a key region mediating the translational repression of TNRC6C. Two other domains, GW-rich and Q-rich, also repress protein synthesis upon tethering but only approximately twofold. The ΔN1370 fragment appears to function as an autonomous domain, the inhibitory function of which does not involve interaction with AGO or TNRC6 family proteins.

Human TNRC6A and TNRC6B were previously identified as AGO-interacting proteins, and their knockdown was shown to affect the efficiency of miRNA-mediated repression (Jakymiw et al. 2005; Liu et al. 2005; Meister et al. 2005; Till et al. 2007). Results of knockdown and co-IP experiments presented in this report extend these conclusions to TNRC6C. Involvement of TNRC6C in miRNA regulation is also supported by recent IP experiments of Landthaler et al. (2008). Our demonstration that individual tethering of each of the three TNRC6 proteins dramatically inhibits mRNA translation adds further evidence to the repressive functions of these proteins. It is intriguing that individual knockdown of each of the three TNRC6 proteins markedly interferes with miRNA repression despite their similar domain organizations. It remains to be established whether this is due to not entirely overlapping functions of individual TNRC6 paralogs in miRNA repression or is a consequence of the decreased total pool of TNRC6 proteins in the cell. Following submission of our manuscript, Li et al. (2008) reported that tethering of TNRC6A also represses translation of FL reporter in HEK293 cells but only by approximately threefold. In a total of 15 independent transfection experiments performed by us in HEK293 cells, inhibition of RL reporter by tethering of TNRC6C varied between 6.5-fold and 18-fold. In HeLa cells, the effect varied between six- and 12-fold (H. Mathys and W. Filipowicz, unpubl.).

Previous analyses of GW182 proteins identified domains responsible for interaction with Argonautes or localization to P-bodies (Behm-Ansmant et al. 2006; Till et al. 2007). However, no information was available about domains mediating the repression of protein synthesis. Deletion analysis combined with tethering assays identified ΔN1370, the C-terminal fragment of TNRC6C encompassing DUF and RRM motifs, as a region with a dramatic, up to 20-fold, repressive effect on the activity of an mRNA reporter. Noteworthy, repression of protein synthesis by ΔN1370 was even stronger from that observed when a full-length TNRC6C was tethered to mRNA, raising a possibility that the N-terminal portion of TNRC6C may modulate inhibitory activity of the C-terminal part. The integrity of ΔN1370 was essential to achieve maximal repression, since deletion of either the DUF domain or 80 C-terminal amino acids downstream from RRM decreased its inhibitory activity severalfold. Likewise, mutation of evolutionarily conserved aromatic residues of the RRM significantly lowered its inhibitory potential.

Previous work has shown that the GW182 protein in *Drosophila* S2 cells stimulates mRNA deadenylation and decay, but also has a direct inhibitory effect on mRNA translation (Behm-Ansmant et al. 2006; Eulalio et al. 2008b). We found that the inhibitory outcome of the tethering of each human TNRC6C paralog is also a combination of effects on mRNA translation and mRNA level. More detailed analysis of TNRC6C and its ΔN1370 fragment showed that mRNA escaping the degradation remained polyadenylated. Thus the inhibitory effect on translation is not due to elimination of a stimulatory role of the poly(A)-binding protein PABP on translation initiation (Kahvejian et al. 2005) or mRNA "circularization" (Wells

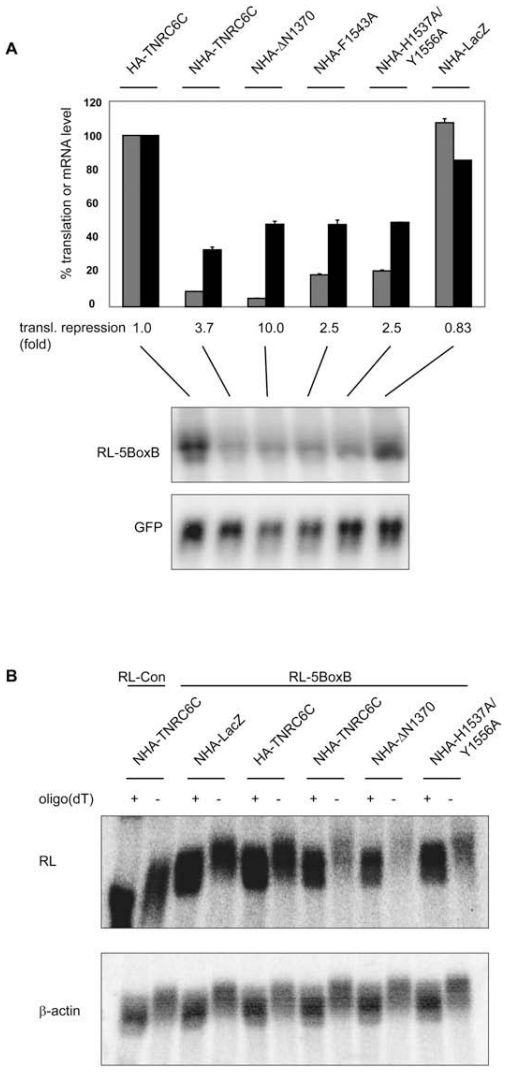

FIGURE 6. (Legend on next page)

et al. 1998), but rather results from a more direct interference with the translation process. Interestingly, comparison of the repressive effects of ΔN1370 and its mutants bearing amino acid substitutions in the RRM indicated that introduced mutations partially mitigate translational repression but have no appreciable effect on mRNA level. Hence, the RRM domain may play a more important role in translational repression rather than in mRNA destabilization. Our finding that ΔN1370 does not interact with the endogenous TNRC6 or AGO proteins also indicates that this fragment functions as an autonomous inhibitory domain and not by recruiting the endogenous miRNP complex to the reporter mRNA. This is consistent with the findings of Behm-Ansmant et al. (2006) that GW182 in *Drosophila* functions downstream from Ago1 and does not require Ago1 for inducing repression.

The ΔN1370 fragment contains two domains, DUF and RRM, that are conserved in GW182-like proteins in many but not all metazoan organisms. The DUF domain is present in proteins of vertebrates, insects, and the worm *B. malayi* (Fig. 1) but not in *Caenorhabditis elegans* (Zhang et al. 2007). The function of the DUF domain is unknown, and limited mutagenesis of the domain carried out within the context of the ΔN1370 fragment failed to identify amino acids important for the repression (Fig. 1B; data not shown). The RRM domain is conserved in all GW182 proteins of vertebrates and insects but is absent from the worm proteins. RRM domains are found in many RNA-binding proteins and are directly involved in the recognition of specific RNA substrates, primarily via aromatic amino acids of RNP1 and RNP2 motifs and via residues in loops interconnecting structural elements of the RRM (Clery et al. 2008). Several possible functions of the GW182 RRM in translational repression could be envisaged. The RRM may interact with the mRNA target and induce repression by contacting the cap or AUG regions of mRNA. Alternatively, the RRM could contact other RNA components participating in translation, such as initiator tRNA or ribosomal RNA. However, RRM domains were also shown to participate in protein–protein interactions (Clery et al. 2008). Hence, it is possible that a primary role of the GW182 RRM is to contact protein factors involved in mRNA translation. In future, it will be interesting to identify components of mRNA translation and/or decay machineries that interact with ΔN1370.

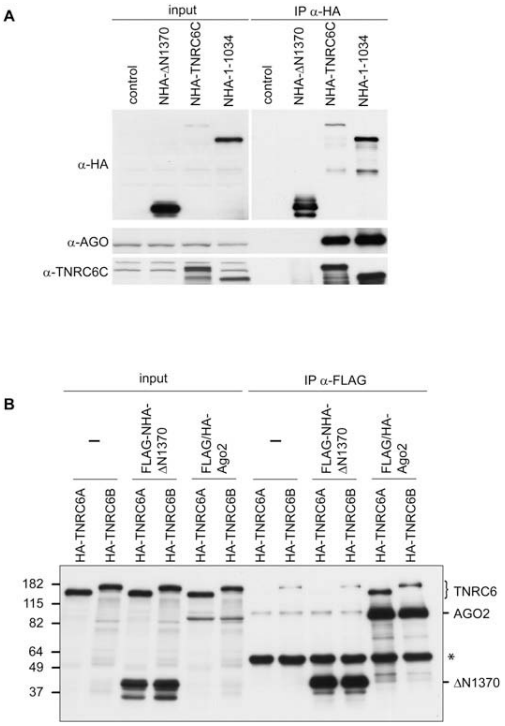

FIGURE 7. ΔN1370 does not interact with endogenous Ago and TNRC6C proteins. Cell extracts of HEK293 cells transiently expressing the indicated fusion proteins were incubated with anti-HA Affinity Matrix (Roche), and immunoprecipitated proteins (45% of the total immunoprecipitate) were analyzed by Western blotting using the indicated Abs. Note that anti-AGO mAb 2A8 recognizes all human AGO proteins (Nelson et al. 2007). Inputs represent 1% (detection of Ago) and 5% (detection of TNRC6C) of the cell extract used for IP. Nontransfected cells served as a control. (*B*) ΔN1370 does not interact with TNRC6A and TNRC6B proteins. Cell extracts of HEK293 cells transiently expressing indicated epitope-tagged proteins were incubated with anti-Flag M2-Agarose Affinity Gel (Sigma), and immunoprecipitated proteins (45% of the total immunoprecipitate) were analyzed by Western blotting using anti-HA 3F10 mAb. Inputs represent 2% of the cell extract used for IP. Note that HA-TNRC6B unspecifically binds to α-Flag beads and traces of it are present in IPs from both ΔN1370-expressing and control cells. (*) The band most probably represents the IgG heavy chain.

FIGURE 6. The ΔN1370 fragment acts mainly as a translational repressor. (*A*) Tethering of ΔN1370 causes strong repression of translation that is partially relieved by mutations in the RRM domain. Indicated proteins were coexpressed with reporter plasmids in HEK293 cells, and their effect on RL activity and RL-5BoxB mRNA stability was analyzed using extracts originating from the same transfections. (*Upper* panel) Effect of tethering on RL activity ([gray bars] normalized to FL) and RL-5BoxB mRNA level ([black bars] normalized to GFP mRNA). Values for transfection of HA-TNRC6C were set to 100%. Calculated net repressive effects on translation are shown *below* bars ($n = 3$, with exception of Northern analysis for NHA-H1537A/Y1566A and NHA-LacZ performed only twice and once, respectively). (*Lower* panels) Representative Northern analyses. (*B*) Treatment with RNase H in the presence of oligo(dT) results in faster mobility of both control mRNAs and mRNAs repressed by tethering. RL-Con and RL-5BoxB mRNAs were coexpressed in HEK293 cells with proteins indicated *above* the panels. RNA isolated from transfected cells was incubated with RNase H in the absence or presence of oligo(dT) and analyzed by Northern blotting. The same blot was consecutively hybridized with probes specific for RL and β-actin mRNAs. Note that RL-Con mRNA is 220 nt shorter than RL-5BoxB. Hybridization signals (as measured by PhosphorImaging) in lanes representing incubations without oligo(dT) were found to be reproducibly weaker than those in the lanes with oligo(dT). This is more pronounced for RL mRNAs than β-actin mRNA, and in the case of RL mRNAs, it applies to the same extent to mRNAs that do and do not undergo repression.

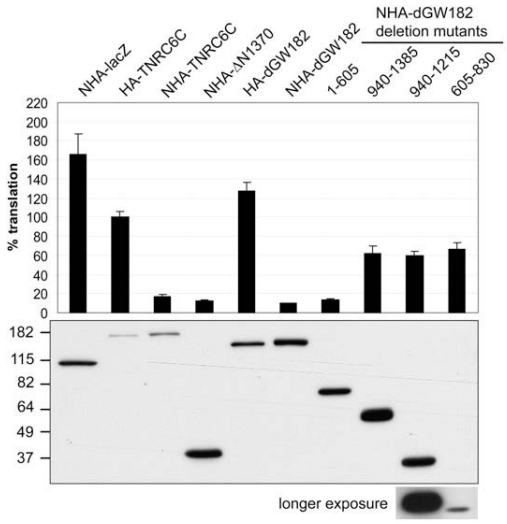

FIGURE 8. Effect of tethering of dGW182 and its deletion mutants on activity of RL-5BoxB reporter in human cells. (*Upper* panel) Tethering of dGW182 and its deletion mutants represses activity of RL-5boxB reporter in HEK293 cells. Indicated plasmids expressing human TNRC6C or *Drosophila* dGW182, or their mutants, were transfected to cells together with RL-5boxB and FL-Con. Normalized RL activity is indicated as the percentage of activity in cells expressing HA-TNRC6C set as 100%. (*Lower* panel) Expression of fusion proteins analyzed by Western blotting using anti-HA Ab. (*Inset* at the *bottom*) Shows stronger exposure of the two lanes at far *right*, indicating that the Q-rich domain (mutant 605-830) is expressed at a much lower level than the remaining proteins. However, this low level of NHA-605-830 appears to be sufficient to achieve maximal repression since transfection of higher amounts of the plasmid encoding NHA-605-830 did not result in stronger repression (data not shown). The data represent means from three independent experiments. We note that transfection of control NHA-lacZ plasmid occasionally results in RL expression that is stronger (although not significantly) than that of another control reporter, HA-TNRC6C. The data were always normalized to RL expression in the presence HA-TNRC6C, which we consider as a more appropriate control than NHA-lacZ.

In contrast to the C-terminal ΔN1370 fragment, which repressed activity of the target mRNA 10- to 20-fold, tethering of GW-rich and Q-rich domains had only an approximately twofold inhibitory effect on protein synthesis. This differs substantially from the situation in *Drosophila* cells, where three nonoverlapping regions of dGW182, GW-rich and Q-rich domains, and the C-terminal fragment equivalent to ΔN1370 were identified as regions repressing protein synthesis fivefold to sixfold upon tethering (Chekulaeva et al. 2009). The results of cross-species experiments indicated that tethering of a full-length dGW182 repressed protein synthesis in HEK293 cells as efficiently as TNRC6C (Fig. 8). Likewise, repression by full-length TNRC6C in *Drosophila* S2 cells was comparable to that of dGW182 (Chekulaeva et al. 2009). However, the contributions of individual domains to repression differed substantially between human and *Drosophila* proteins and cells. The GW-rich domain of dGW182 was the strongest repressor in human cells and was probably responsible for most of the activity of intact dGW182; the effects of the remaining domains were very limited. When different domains of human TNRC6C were tested in *Drosophila* S2 cells, the Q-rich domain and the C-terminal ΔN1370 fragment acted as strong repressors, with the GW-domain having the least effect (Chekulaeva et al. 2009). Hence, the N-terminal GW-rich domain of dGW182 is a strong repressor in both S2 and HEK293 cells, while the analogous domain of TNRC6C has little effect in either cell type. In contrast, Q-rich domains from both dGW182 and TNRC6C were strongly inhibitory in *Drosophila* but not human cells, likely reflecting some specific aspects of the repression pathway in fly cells. Interestingly, the human ΔN1370 fragment was strongly repressive in both systems, but its dGW182 counterpart had a major effect only in homologous *Drosophila* cells. The reasons for these protein-specific and cell-specific differences remain to be established, but the observations are consistent with a model proposed for *Drosophila* dGW182 according to which individual repressive domains of dGW182 contribute additively or cooperatively to the assembly of a larger repressive complex acting downstream from miRNPs (Chekulaeva et al. 2009). It will be interesting to dissect the repressive potential of the two other TNRC6 paralogs TNRC6A and TNRC6B. The relative contributions to the repression of individual domains of these two proteins may be different to those established for TNRC6C.

MATERIAL AND METHODS

Cell culture, transfection, and luciferase assays

Human HEK293T cells (hereafter referred to as HEK293) were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL) supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal calf serum (FCS). Generally, transfections were performed in triplicates in six-well plates with ~60% confluent cells using Nanofectin (PAA Laboratories), following the manufacturer's instructions. Unless indicated otherwise, amounts of transfected plasmids per well were 50 ng of indicated RL reporter, 300 ng of FL-Con, and 20-100 ng of plasmid expressing indicated HA- or NHA-tagged proteins; when indicated, in transfections simultaneously used for Northern analysis, 200 ng of peGFP-C1 (Clonetech) was also included. In some experiments, amounts of plasmids expressing TNRC6C deletion mutants were adjusted to obtain comparable levels of overexpressed proteins. Cells were lyzed 24 h post-transfection in Passive Lysis Buffer (PLB; Promega) to measure RL and FL activities by Dual-Luciferase Assay (Promega).

HeLa S3 cells were grown under similar conditions, but their transfection with siRNAs and reporter plasmids was performed in 24-well plates using Lipofectamine 2000 (Invitrogen) with proportionally lower amounts of indicated plasmids. Cells were trypsinized 24 h post-transfection and seeded into wells of a 6-well plate. After 48 h, cells were lyzed as described above.

For RNAi, 100 nM a single siRNA (GCCUAAUCUCCGUGCU CAATT and UUGAGCACGGAGAUUAGGCTG; sense and antisense

strands, respectively) were used in the case of TNRC6A, and mixtures of two siRNAs, each 50 nM, were used for silencing of TNRC6B (GGCCUUGUAUUGCCAGCAATT, UUGCUGGCAAUACAAGGC CTT and GGAGUGCCAUGGAAAGGUATT, UACCUUUCCAUG GCACUCCTT) and TNRC6C (GCAUUAAGUGCUAAACAAATT, UUUGUUUAGCACUUAAUGCTT and CCAAGAGUUCUGUCU AAUATT, UAUUAGACAGAACUCUUGGTT). All siRNAs were obtained from Microsynth. Allstars Negative Control siRNA was purchased from QIAGEN.

Plasmids

RL-5BoxB, RL-3xBulgeB, RL-3xBulgeBmut, RL-Con, and FL-Con reporters (Pillai et al. 2005; Schmitter et al. 2006) and plasmid expressing NHA-LacZ (Pillai et al. 2004) were previously described. The plasmid encoding Flag/HA-Ago2 was a kind gift of Gunter Meister (Max Planck Institute for Biochemistry) (Meister et al. 2004).

Plasmids expressing HA- and NHA-tagged TNRC6A, TNRC6B, and TNRC6C were prepared as follows. For TNRC6A, the XhoI–NotI fragment excised from plasmid phrGFP/N1-GW182-A (kindly provided by E. Chan, Department of Oral Biology, University of Florida) (Eystathioy et al. 2002) was cloned into XhoI–NotI-digested pCI-NHA or pCI-HA vector (pCI-NHA or pCI-HA contain sequences encoding NHA or HA tags in pCIneo) (Pillai et al. 2004) to yield pCI-NHA-TNRC6A and pCI-HA-TNRC6A, respectively. The TNRC6A clones lack the N-terminal 312 amino acids (Eystathioy et al. 2002). For TNRC6B, the SalI–NotI fragment from the plasmid pDEST/Myc-GW182-B (kindly provided by G. Meister, Max Planck Institute for Biochemistry) (Meister et al. 2005) was cloned into SalI–NotI-digested pCI-NHA or pCI-HA vector to yield pCI-NHA-TNRC6B and pCI-HA-TNRC6B, respectively. Plasmids expressing HA- and NHA-tagged TNRC6C, pHA-TNRC6C, and pNHA-TNRC6C were prepared as follows: The EST clone KIAA1582 (from Kazusa DNA Research Institute, Japan) was digested with BstEII and NotI, and a 4.2-kb fragment corresponding to the downstream ORF part was eluted from agarose gel. The N-terminal ORF fragment was PCR-amplified using CGGAAT TCATGGCTACAGGGAGTGCCCAGGG and TGACTGAACCCAG AATTGCTATTTCC oligonucleotides as primers and digested with EcoRI and BstEII. The two fragments were inserted into a pCI-NHA vector pre-cut with EcoRI and NotI to yield pCI-NHA-TNRC6C. pCI-NHA-TNRC6C has an XhoI site between sequences encoding N and HA peptides, and two NheI sites: one upstream of the N-peptide-encoding sequence and another in the ORF. The plasmid was partially digested with NheI and the linearized DNA eluted from a gel. The DNA was then digested with XhoI. The desired 10.4-kb XhoI fragment was purified, the NheI and XhoI overhang sequences filled in with Klenow polymerase, and the plasmid religated.

Deletion mutants of TNRC6C were designed taking into account structure propensity calculations (http://bioinf.cs.ucl.ac.uk/disopred/). Mutants were obtained using the In-Fusion 2.0 Dry-Down PCR Cloning Kit (Clontech) and pCI-NHA-TNRC6C as a template. PCR products were cloned into linearized pCI-NHA. To prepare pFLAG-NHA-ΔN1370, sequence encoding NHA-ΔN1370 was PCR-amplified using pCl-NHA-ΔN1370 as a template and AGGCT AGTCGACATGGACGCACAAACACGACG and AACCCTCACT AAAGGGAAGC oligonucleotides as primers. Following digestion with SalI and NotI, the fragment was inserted into SalI/NotI-digested expression plasmid pCIneo1FLAG (kindly provided by Michael Doyle of this laboratory).

Site-directed mutagenesis was performed by PCR using a pCI-NHA-ΔN1370 plasmid and partially overlapping primers containing desired mutations as described (Zheng et al. 2004). The original template was digested by the methylation-dependent enzyme DpnI and the PCR product was transformed into competent cells.

To generate pCI-NHA-dGW182, the sequence encoding NHA-dGW182 in a modified version of plasmid pAC5.1-λN-HA-GW182 (Behm-Ansmant et al. 2006) was PCR-amplified and cloned into pCIneo digested with NheI and NotI. Plasmids encoding deletion mutants were generated in a similar way as pCI-NHA-dGW182, using *Drosophila* plasmids expressing corresponding dGW182 mutants as templates (Chekulaeva et al. 2009). pCI-HA-dGW182 plasmid was generated from a pCI-NHA-dGW182 plasmid by PCR amplification of the HA-dGW182 region, its digestion with SmaI and NotI, and cloning into pCIneo digested with NheI and NotI.

Correctness of all plasmids was verified by sequencing.

Northern and RNase H analyses

Total RNA was isolated from cells 24 h post-transfection using Trizol reagent (Invitrogen). Twelve micrograms of total RNA from each transfection was resolved in a formaldehyde–1% agarose gel and blotted to the Nylon membrane for 48 h using 10× SSC. The RL- GFP- and β-actin-specific DNA probes (0.9, 0.75, and 1.0 kb long, respectively) were ^{32}P-labeled using Random-primed DNA labeling Kit (Roche) and used for hybridization. Radioactivity was quantified with a PhosphorImager (Storm 860; Molecular Dynamics).

To analyze the polyadenylation status of mRNA, 20 μg of total RNA isolated from transfected cells was annealed in the presence or absence of 2 μg of oligo(dT) for 15 min at room temperature and then treated with RNase H (New England Biolabs) in the presence of RNasin Plus (Promega; 1 μL per reaction) for 45 min at 37°C, following the manufacturer's recommendations. RNA was purified with Trizol LS (Invitrogen), separated on a denaturing 1% agarose gel, and analyzed by Northern blotting.

Antibodies, Western blotting, and immunoprecipitations

Antibodies against human TNRC6C were raised in rabbits by Eurogentec, using a mixture of two peptides, TGSAQGNFTGHTKKT and TTIQDVNRYLLKSGG. The Abs were affinity-purified using individual peptides coupled to Sepharose. For Western analysis, aliquots of cell lysates in PLB were subjected to SDS-PAGE using a pre-cast 4%–12% gradient (Invitrogen) (Figs. 4 and 5) or 6% linear polyacrylamide gels (Figs. 2, 3; 10% for Fig. 8). Note that migration of investigated proteins in relation to protein size markers differs between these two types of gels. Anti-HA mAb 3F10 (Roche; 1:1000 dilution) or a combination of anti-TNRC6C rabbit Abs (1:1000) were used as primary antibodies, and goat anti-rat Ab coupled to HRP (MP Biochemicals; 1:8000) and anti-rabbit Ab (GE Healthcare, 1:10,000) as secondary Abs. Proteins were detected using ECL (GE Healthcare).

For HA epitope IP reactions, cells were lysed with 50 mM Tris-HCl (pH 7.5) containing 150 mM KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5% Triton X-100, 2 mM DTT, 40 U/mL RNaseOUT

Recombinant Ribonuclease Inhibitor (Invitrogen), and EDTA-free Protease Inhibitor Cocktail (Roche). The cleared lysate was incubated with anti-HA Affinity Matrix (Roche). After washing with 10 mM Tris-HCl (pH 7.5) containing 200 mM KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, proteins associated with the beads were analyzed by Western blotting using anti-TNRC6C Abs, anti-HA mAb 42F13 (FMI Monoclonal Antibody Facility), and mAb 2A8 (Nelson et al. 2007) recognizing human AGO proteins (kindly provided by Z. Mourelatos, University of Pennsylvania School of Medicine). Flag IPs were performed using the Flag Tagged Protein Immunoprecipitation Kit (Sigma) according to the manufacturer's protocol. Immunoprecipitated proteins and input fractions were analyzed by Western blotting using anti-HA 3F10 antibody (Roche).

Statistical analysis

Data were tested for Normality using the Shapiro test. The null hypothesis for the Shapiro test is Normal data. Statistical significances were calculated on the Normally distributed data sets using a two-tailed paired Student's *t*-test. When the Shapiro test reported a *P*-value close to or below 0.05 (data are non-Normal), we performed the nonparametric Mann–Whitney–Wilcoxon test. The error bars plotted throughout show the standard error of the mean (SEM). It follows from the central limit theorem that the distribution of sample means will be Normal even if the underlying sample distribution is not. So even for these cases, the error of the sample means will still be correct.

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REFERENCES

Bagga, S., Bracht, J., Hunter, S., Massirer, K., Holtz, J., Eachus, R., and Pasquinelli, A.E. 2005. Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. *Cell* **122:** 553–563.

Bartel, D.P. 2004. MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell* **116:** 281–297.

Behm-Ansmant, I., Rehwinkel, J., Doerks, T., Stark, A., Bork, P., and Izaurralde, E. 2006. mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. *Genes & Dev.* **20:** 1885–1898.

Bhattacharyya, S.N., Habermacher, R., Martine, U., Closs, E.I., and Filipowicz, W. 2006. Relief of microRNA-mediated translational repression in human cells subjected to stress. *Cell* **125:** 1111–1124.

Bushati, N. and Cohen, S.M. 2007. MicroRNA functions. *Annu. Rev. Cell Dev. Biol.* **23:** 175–205.

Chekulaeva, M., Filipowicz, W., and Parker, R. 2009. Multiple independent domains of dGW182 function in miRNA-mediated repression in *Drosophila*. RNA (this issue). doi: 10.1261/rna.1364809.

Chendrimada, T.P., Finn, K.J., Ji, X., Baillat, D., Gregory, R.I., Liebhaber, S.A., Pasquinelli, A.E., and Shiekhattar, R. 2007. MicroRNA silencing through RISC recruitment of eIF6. *Nature* **447:** 823–828.

Clery, A., Blatter, M., and Allain, F.H. 2008. RNA recognition motifs: Boring? Not quite. *Curr. Opin. Struct. Biol.* **18:** 290–298.

Ding, L. and Han, M. 2007. GW182 family proteins are crucial for microRNA-mediated gene silencing. *Trends Cell Biol.* **17:** 411–416.

Ding, L., Spencer, A., Morita, K., and Han, M. 2005. The developmental timing regulator AIN-1 interacts with miRISCs and may target the argonaute protein ALG-1 to cytoplasmic P bodies in *C. elegans*. *Mol. Cell* **19:** 437–447.

Eulalio, A., Behm-Ansmant, I., and Izaurralde, E. 2007a. P bodies: At the crossroads of post-transcriptional pathways. *Nat. Rev. Mol. Cell Biol.* **8:** 9–22.

Eulalio, A., Rehwinkel, J., Stricker, M., Huntzinger, E., Yang, S.F., Doerks, T., Dorner, S., Bork, P., Boutros, M., and Izaurralde, E. 2007b. Target-specific requirements for enhancers of decapping in miRNA-mediated gene silencing. *Genes & Dev.* **21:** 2558–2570.

Eulalio, A., Huntzinger, E., and Izaurralde, E. 2008a. Getting to the root of miRNA-mediated gene silencing. *Cell* **132:** 9–14.

Eulalio, A., Huntzinger, E., and Izaurralde, E. 2008b. GW182 interaction with Argonaute is essential for miRNA-mediated translational repression and mRNA decay. *Nat. Struct. Mol. Biol.* **15:** 346–353.

Eystathioy, T., Chan, E.K., Tenenbaum, S.A., Keene, J.D., Griffith, K., and Fritzler, M.J. 2002. A phosphorylated cytoplasmic autoantigen, GW182, associates with a unique population of human mRNAs within novel cytoplasmic speckles. *Mol. Biol. Cell* **13:** 1338–1351.

Filipowicz, W., Bhattacharyya, S.N., and Sonenberg, N. 2008. Mechanisms of post-transcriptional regulation by microRNAs: Are the answers in sight? *Nat. Rev. Genet.* **9:** 102–114.

Giraldez, A.J., Mishima, Y., Rihel, J., Grocock, R.J., Van Dongen, S., Inoue, K., Enright, A.J., and Schier, A.F. 2006. Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. *Science* **312:** 75–79.

Huang, J., Liang, Z., Yang, B., Tian, H., Ma, J., and Zhang, H. 2007. Derepression of microRNA-mediated protein translation inhibition by apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G (APOBEC3G) and its family members. *J. Biol. Chem.* **282:** 33632–33640.

Humphreys, D.T., Westman, B.J., Martin, D.I., and Preiss, T. 2005. MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function. *Proc. Natl. Acad. Sci.* **102:** 16961–16966.

Jakymiw, A., Lian, S., Eystathioy, T., Li, S., Satoh, M., Hamel, J.C., Fritzler, M.J., and Chan, E.K. 2005. Disruption of GW bodies impairs mammalian RNA interference. *Nat. Cell Biol.* **7:** 1267–1274.

Jakymiw, A., Pauley, K.M., Li, S., Ikeda, K., Lian, S., Eystathioy, T., Satoh, M., Fritzler, M.J., and Chan, E.K. 2007. The role of GW/P-bodies in RNA processing and silencing. *J. Cell Sci.* **120:** 1317–1323.

Kahvejian, A., Svitkin, Y.V., Sukarieh, R., M'Boutchou, M.N., and Sonenberg, N. 2005. Mammalian poly(A)-binding protein is a eukaryotic translation initiation factor, which acts via multiple mechanisms. *Genes & Dev.* **19:** 104–113.

Kiriakidou, M., Tan, G.S., Lamprinaki, S., De Planell-Saguer, M., Nelson, P.T., and Mourelatos, Z. 2007. An mRNA m^7G cap binding-like motif within human Ago2 represses translation. *Cell* **129:** 1141–1151.

Kong, Y.W., Cannell, I.G., de Moor, C.H., Hill, K., Garside, P.G., Hamilton, T.L., Meijer, H.A., Dobbyn, H.C., Stoneley, M., Spriggs, K.A., et al. 2008. The mechanism of micro-RNA-mediated translation repression is determined by the promoter of the target gene. *Proc. Natl. Acad. Sci.* **105:** 8866–8871.

Landthaler, M., Gaidatzis, D., Rothballer, A., Chen, P.Y., Soll, S.J., Dinic, L., Ojo, T., Hafner, M., Zavolan, M., and Tuschl, T. 2008. Molecular characterization of human Argonaute-containing

ribonucleoprotein complexes and their bound target mRNAs. *RNA* **14:** 2580–2596.

Li, S., Lian, S.L., Moser, J.J., Fritzler, M.L., Fritzler, M.J., Satoh, M., and Chan, E.K. 2008. Identification of GW182 and its novel isoform TNGW1 as translational repressors in Ago2-mediated silencing. *J. Cell Sci.* **121:** 4134–4144.

Lim, L.P., Lau, N.C., Garrett-Engele, P., Grimson, A., Schelter, J.M., Castle, J., Bartel, D.P., Linsley, P.S., and Johnson, J.M. 2005. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature* **433:** 769–773.

Liu, J., Carmell, M.A., Rivas, F.V., Marsden, C.G., Thomson, J.M., Song, J.J., Hammond, S.M., Joshua-Tor, L., and Hannon, G.J. 2004. Argonaute2 is the catalytic engine of mammalian RNAi. *Science* **305:** 1437–1441.

Liu, J., Rivas, F.V., Wohlschlegel, J., Yates III, J.R., Parker, R., and Hannon, G.J. 2005. A role for the P-body component GW182 in microRNA function. *Nat. Cell Biol.* **7:** 1261–1266.

Lytle, J.R., Yario, T.A., and Steitz, J.A. 2007. Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5′ UTR as in the 3′ UTR. *Proc. Natl. Acad. Sci.* **104:** 9667–9672.

Maroney, P.A., Yu, Y., Fisher, J., and Nilsen, T.W. 2006. Evidence that microRNAs are associated with translating messenger RNAs in human cells. *Nat. Struct. Mol. Biol.* **13:** 1102–1107.

Mathonnet, G., Fabian, M.R., Svitkin, Y.V., Parsyan, A., Huck, L., Murata, T., Biffo, S., Merrick, W.C., Darzynkiewicz, E., Pillai, R.S., et al. 2007. MicroRNA inhibition of translation initiation in vitro by targeting the cap-binding complex eIF4F. *Science* **317:** 1764–1767.

Meister, G., Landthaler, M., Patkaniowska, A., Dorsett, Y., Teng, G., and Tuschl, T. 2004. Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Mol. Cell* **15:** 185–197.

Meister, G., Landthaler, M., Peters, L., Chen, P.Y., Urlaub, H., Luhrmann, R., and Tuschl, T. 2005. Identification of novel argonaute-associated proteins. *Curr. Biol.* **15:** 2149–2155.

Nelson, P.T., De Planell-Saguer, M., Lamprinaki, S., Kiriakidou, M., Zhang, P., O'Doherty, U., and Mourelatos, Z. 2007. A novel monoclonal antibody against human Argonaute proteins reveals unexpected characteristics of miRNAs in human blood cells. *RNA* **13:** 1787–1792.

Nilsen, T.W. 2007. Mechanisms of microRNA-mediated gene regulation in animal cells. *Trends Genet.* **23:** 243–249.

Nottrott, S., Simard, M.J., and Richter, J.D. 2006. Human let-7a miRNA blocks protein production on actively translating polyribosomes. *Nat. Struct. Mol. Biol.* **13:** 1108–1114.

Olsen, P.H. and Ambros, V. 1999. The lin-4 regulatory RNA controls developmental timing in *Caenorhabditis elegans* by blocking LIN-14 protein synthesis after the initiation of translation. *Dev. Biol.* **216:** 671–680.

Orom, U.A., Nielsen, F.C., and Lund, A.H. 2008. MicroRNA-10a binds the 5′UTR of ribosomal protein mRNAs and enhances their translation. *Mol. Cell* **30:** 460–471.

Parker, R. and Sheth, U. 2007. P bodies and the control of mRNA translation and degradation. *Mol. Cell* **25:** 635–646.

Peters, L. and Meister, G. 2007. Argonaute proteins: mediators of RNA silencing. *Mol. Cell* **26:** 611–623.

Petersen, C.P., Bordeleau, M.E., Pelletier, J., and Sharp, P.A. 2006. Short RNAs repress translation after initiation in mammalian cells. *Mol. Cell* **21:** 533–542.

Pillai, R.S., Artus, C.G., and Filipowicz, W. 2004. Tethering of human Ago proteins to mRNA mimics the miRNA-mediated repression of protein synthesis. *RNA* **10:** 1518–1525.

Pillai, R.S., Bhattacharyya, S.N., Artus, C.G., Zoller, T., Cougot, N., Basyuk, E., Bertrand, E., and Filipowicz, W. 2005. Inhibition of translational initiation by Let-7 MicroRNA in human cells. *Science* **309:** 1573–1576.

Rehwinkel, J., Behm-Ansmant, I., Gatfield, D., and Izaurralde, E. 2005. A crucial role for GW182 and the DCP1:DCP2 decapping complex in miRNA-mediated gene silencing. *RNA* **11:** 1640–1647.

Rehwinkel, J., Natalin, P., Stark, A., Brennecke, J., Cohen, S.M., and Izaurralde, E. 2006. Genome-wide analysis of mRNAs regulated by Drosha and Argonaute proteins in *Drosophila melanogaster*. *Mol. Cell. Biol.* **26:** 2965–2975.

Schmitter, D., Filkowski, J., Sewer, A., Pillai, R.S., Oakeley, E.J., Zavolan, M., Svoboda, P., and Filipowicz, W. 2006. Effects of Dicer and Argonaute down-regulation on mRNA levels in human HEK293 cells. *Nucleic Acids Res.* **34:** 4801–4815.

Schneider, M.D., Najand, N., Chaker, S., Pare, J.M., Haskins, J., Hughes, S.C., Hobman, T.C., Locke, J., and Simmonds, A.J. 2006. Gawky is a component of cytoplasmic mRNA processing bodies required for early *Drosophila* development. *J. Cell Biol.* **174:** 349–358.

Sen, G.L. and Blau, H.M. 2005. Argonaute 2/RISC resides in sites of mammalian mRNA decay known as cytoplasmic bodies. *Nat. Cell Biol.* **7:** 633–636.

Standart, N. and Jackson, R.J. 2007. MicroRNAs repress translation of m7Gppp-capped target mRNAs in vitro by inhibiting initiation and promoting deadenylation. *Genes & Dev.* **21:** 1975–1982.

Till, S., Lejeune, E., Thermann, R., Bortfeld, M., Hothorn, M., Enderle, D., Heinrich, C., Hentze, M.W., and Ladurner, A.G. 2007. A conserved motif in Argonaute-interacting proteins mediates functional interactions through the Argonaute PIWI domain. *Nat. Struct. Mol. Biol.* **14:** 897–903.

Tolia, N.H. and Joshua-Tor, L. 2007. Slicer and the argonautes. *Nat. Chem. Biol.* **3:** 36–43.

Vasudevan, S., Tong, Y., and Steitz, J.A. 2007. Switching from repression to activation: microRNAs can up-regulate translation. *Science* **318:** 1931–1934.

Vasudevan, S., Tong, Y., and Steitz, J.A. 2008. Cell-cycle control of microRNA-mediated translation regulation. *Cell Cycle* **7:** 1545–1549.

Wakiyama, M., Takimoto, K., Ohara, O., and Yokoyama, S. 2007. Let-7 microRNA-mediated mRNA deadenylation and translational repression in a mammalian cell-free system. *Genes & Dev.* **21:** 1857–1862.

Wells, S.E., Hillner, P.E., Vale, R.D., and Sachs, A.B. 1998. Circularization of mRNA by eukaryotic translation initiation factors. *Mol. Cell* **2:** 135–140.

Wu, L. and Belasco, J.G. 2005. Micro-RNA regulation of the mammalian lin-28 gene during neuronal differentiation of embryonal carcinoma cells. *Mol. Cell. Biol.* **25:** 9198–9208.

Wu, L. and Belasco, J.G. 2008. Let me count the ways: Mechanisms of gene regulation by miRNAs and siRNAs. *Mol. Cell* **29:** 1–7.

Wu, L., Fan, J., and Belasco, J.G. 2008. Importance of translation and nonnucleolytic ago proteins for on-target RNA interference. *Curr. Biol.* **18:** 1327–1332.

Zhang, L., Ding, L., Cheung, T.H., Dong, M.Q., Chen, J., Sewell, A.K., Liu, X., Yates III, J.R., and Han, M. 2007. Systematic identification of *C. elegans* miRISC proteins, miRNAs, and mRNA targets by their interactions with GW182 proteins AIN-1 and AIN-2. *Mol. Cell* **28:** 598–613.

Zheng, L., Baumann, U., and Reymond, J.L. 2004. An efficient one-step site-directed and site-saturation mutagenesis protocol. *Nucleic Acids Res.* **32:** e115.

2.2 M2 and Cterm are Sufficient for mRNA Repression

The preceding dissection of the human protein TNRC6C strongly highlighted the importance of the C-terminal effector domain (CED) in mRNA repression. The most conserved region of the CED is the RRM (RNA Recognition Motif) domain. However, the RRM alone has no function in repression, as shown in Figure 5 of Zipprich et al. [2009], presented above in section 2.1. To establish which part of the CED is responsible for the repression or if its sub-fragments function by distinct mechanisms, we analyzed activity of smaller fragments of the CED (see Figure 2.1).

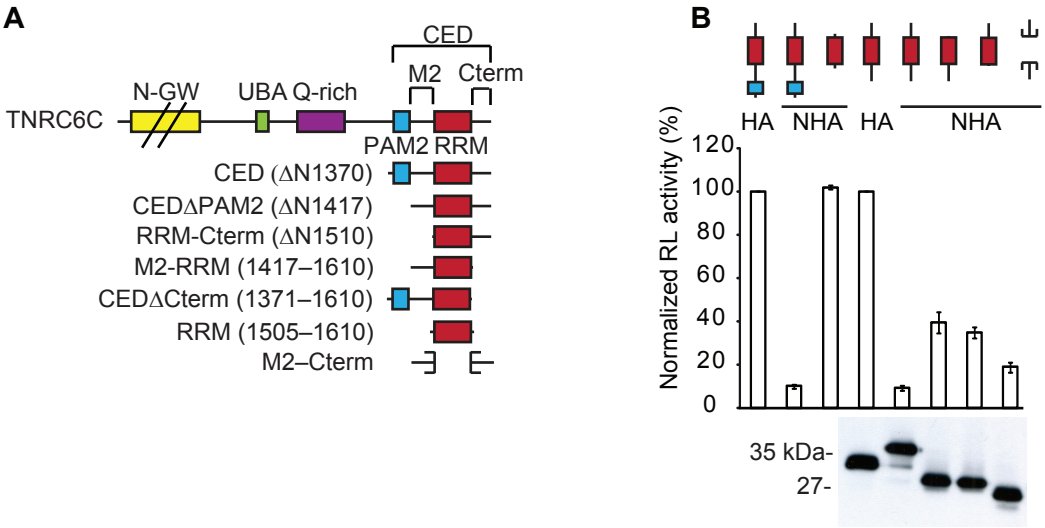
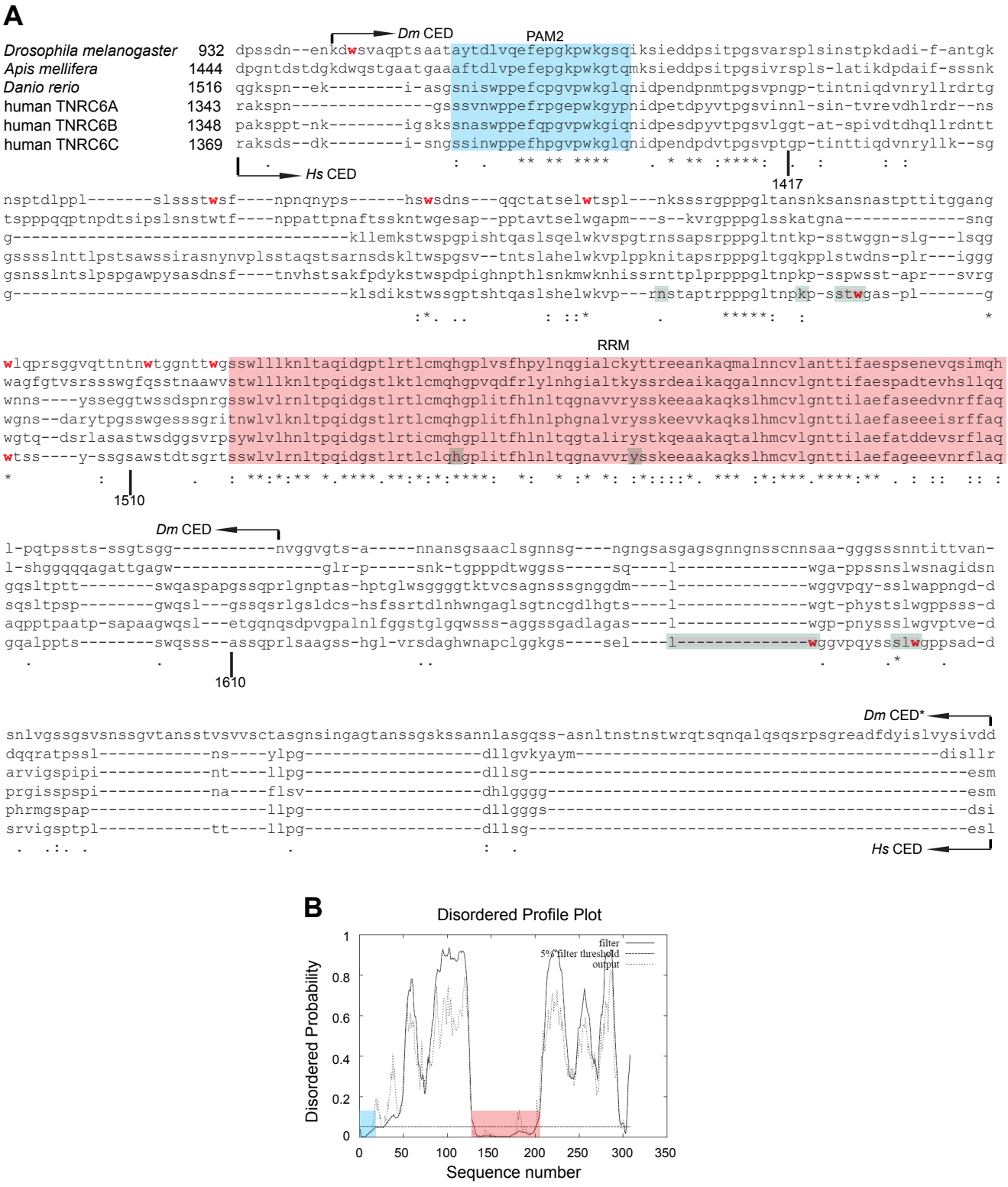


Figure 2.1: Dissection of the CED. (A) Schematic representation of human TNRC6C and fragments analyzed in the study. Individual domains and regions of TNRC6C are indicated: N-GW, GW-repeat-rich region; UBA, ubiquitin associated-like domain; RRM, RNA recognition motif; M2 and Cterm2, regions flanking RRM, constituting – together with PAM2 and RRM – the CED region. **(B)** M2 and Cterm regions of TNRC6C mediate repression of tethered mRNA. HEK293T cells were co-transfected with plasmids encoding NHA-CED or indicated fragments, RL-5BoxB, and firefly luciferase (FL) reporters to control transfection efficiency. As negative controls, untethered HA-CED and tethered NHA-RRM (where „N“ stands for tethering λ peptide) were expressed. Values represent percentage of Renilla luciferase (RL) activity (normalized to FL activity) in the presence of nontethered HA-CED or HA-CEDΔPAM2. Figure modified from Chekulaeva et al. [2011], see appendix B.

To get an insight into the activity of specific sub-fragments, we constructed deletion constructs of the full CED (ΔN1370) according to the four distinguishable regions, PAM2, M2, RRM and Cterm. CEDΔPAM2 (ΔN1417) comprises the full CED without the PAM2 motif. M2-RRM (1417-1610) and RRM-Cterm (ΔN1510) represent constructs comprising the RRM with the flanking M2 or Cterm region, respectively. CEDΔCterm (1371-1610) includes the PAM2 motif, the M2 region and the RRM. RRM alone (1505-1610) served as a negative control. We further constructed an M2-Cterm fusion, which comprises the M2 and the Cterm region without the RRM in between. Two different variants of the M2-Cterm fusion were cloned, with five or seven amino acids between both regions. No difference in activity was observed between the two constructs. The combination of our constructs allowed us to perform a more detailed analysis of each of the four regions in the CED. All constructs were cloned into the pCI-neo vector bearing an HA- or NHA-tag (see section 4.2).

Like the full length construct, tethering the CED or CEDΔPAM2 represses RL expression by approximately ten times if compared to a non-tethered control or to the RRM alone. Significantly, constructs lacking either M2 or Cterm region showed reduced repression, hinting to an importance of both regions. Both, M2 and Cterm show very little conservation and are predicted to be unstructured (analyzed with http://dis.embl.de and http://bioinf.cs.ucl.ac.uk/disopred/), see Figure

Figure 2.2 *(following page)*: Alignment of the CED of GW182 proteins from different species. (A) Alignment was performed using the T-Coffee tool (http://tcoffee.vital-it.ch/cgi-bin/Tcoffee/tcoffee_cgi/index.cgi). The CED regions used throughout the work are delineated with bent arrows. PAM2 and RRM motifs are highlighted in blue and pink, respectively. Mutations in human M2 and Cterm region are highlighted in gray with tryptophan residues marked in red. In *Drosophila* GW182, red tryptophan residues correspond to mutations used in the yeast two-hybrid system (compare page 51). Mutations in the RRM were performed in Zipprich et al. (see section 2.1) and are highlighted in gray. Amino acids were always mutated to alanines. The numbers correspond to amino acid positions in full-length GW182 proteins. Numbers of TNRC6A correspond to the protein described in Zipprich et al. (see section 2.1). Asterisks mark residues identical in all sequences, colons mark conservative substitutions, and dots mark semi-conservative substitutions. **(B)** Disordered profile plot of TNRC6C CED. Prediction was performed using the DISOPRED2 prediction (http://bioinf.cs.ucl.ac.uk/disopred/). PAM2 and RRM motifs are highlighted in blue and pink, respectively. Amino acids with a probability higher than 0.05 (dotted line) are predicted to be disoredered.


A
Dm CED
PAM2
Drosophila melanogaster 932 dpssdn--enkdwsvaqptsaataytdlvqefepgkpwkgsqiksieddpsitpgsvarsplsinstpkdadi-f-antgk
Apis mellifera 1444 dpgntdstdgkdwqstgaatgaaaftdlvpefepgkpwkgtqmksieddpsitpgsivrspls-latikdpdaif-sssnk
Danio rerio 1516 qgkspn--ek--------i-asgsniswppefcpgvpwkglqnidpendpnmtpgsvpngp-tintniqdvnryllrdrtg
human TNRC6A 1343 rakspn----------gsssvnwppefrpgepwkgypnidpetdpyvtpgsvinnl-sin-tvrevdhlrdr--ns
human TNRC6B 1348 paksppt-nk--------igskssnaswppefqpgvpwkgiqnidpesdpyvtpgsvlggt-at-spivdtdhqllrdntt
human TNRC6C 1369 raksds--dk--------i-sngssinwppefhpgvpwkglqnidpendpdvtpgsvptgp-tinttiqdvnryllk--sg
Hs CED
1417
nsptdlppl-------slsssttwsf----npnqnyps------hswsdns---qqctatselwtspl---nksssrgpppgltansnksansnastpttitggang
tspppqqptnpdtsipslsnstwtf----nppattpnaftsskntwgesap--pptavtselwgapm---s--kvrgpppglsskatgna----------sng
g-----------------------------------kllemkstwspgpishtqaslsqelwkvspgtrnssapsrpppgltntkp-sstwggn-slg---lsqg
gsssslnttlpstsawssirasnynvplsstaqstsarnsdskltwspgsv--tntslahelwkvplppknitapsrpppgltgqkpplstwdns-plr---iggg
gsnsslntslpspgawpysasdnsf----tnvhstsakfpdykstwspdpighnpthlsnkmwknhissrnttplprpppgltnpkp-sspwsst-apr---svrg
g-----------------------------------klsdikstwssgptshtqaslshelwkvp---rnstaptrpppgltnpkp-sstwgas-pl-------g
RRM
wlqprsggvqttntnwtggnttwgsswlllknltaqidgptlrtlcmqhgplvsfhpylnqgialckyttreeankaqmalnncvlanttifaespsenevqsimqh
wagfgtvsrssswgfqsstnaawvstwlllknltpqidgstlktlcmqhgpvqdfrlylnhgialtkyssrdeaikaqgalnncvlgnttifaespadtevhsllqq
wnns----ysseggtwssdspnrgsswlvlrnltpqidgstlrtlcmqhgplitfhlnltqgnavvrysskeeaakaqkslhmcvlgnttilaefaseedvnrffaq
wgns--darytpgsswgesssgritnwlvlknltpqidgstlrtlcmqhgplitfhlnlphgnalvrysskeevvkaqkslhmcvlgnttilaefaseeeisrffaq
wgtq--dsrlasastwsdggsvrpsywlvlhnltpqidgstlrticmqhgplltfhlnltqgtalirystkqeaakaqtalhmcvlgnttilaefatddevsrflaq
wtss----y-ssgsawstdtsgrtsswlvlrnltpqidgstlrtlclqhgplitfhlnltqgnavvrysskeeaakaqkslhmcvlgnttilaefageeevnrflaq
1510
Dm CED
l-pqtpssts-ssgtsgg----------nvggvgts-a-----nnansgsaaclsgnnsg----ngngsasgagsgnngnsscnnsaa-gggsssnntittvan-
l-shggqqqagattgagw----------------glr-p-----snk-tgpppdtwggss-----sq----l--------------wga-ppssnslwsnagidsn
gqsltptt--------swqaspapgssqprlgnptas-hptglwsggggtktvcsagnsssgnggdm----l--------------wggvpqy-sslwappngd-d
sqsltpsp--------gwqsl---gssqsrlgsldcs-hsfssrtdlnhwngaglsgtncgdlhgts----l--------------wgt-phystslwgppsss-d
aqpptpaatp-sapaagwqsl---etgqnqsdpvgpalnlfggstglgqwsss-aggssgadlagas----l--------------wgp-pnysslwgvptve-d
gqalppts--------swqsss--assqprlsaagss-hgl-vrsdaghwnapclggkgs----sel----l--------------wggvpqyssslwgppsad-d
1610
Dm CED*
snlvgssgsvsnssgvtansstvsvvsctasgnsingagtanssgskssannlasgqss-asnltnstnstwrqtsqnqalqsqsrpsgreadfdyislvysivdd
dqqratpssl----------ns----ylpg----------dllgvkyaym----------disllr
arvigspipi----------nt----llpg----------dllsg----------esm
prgisspspi----------na----flsv----------dhlgggg----------esm
phrmgspap----------llpg----------dllgggs----------dsi
srvigsptpl----------tt----llpg----------dllsg----------esl
Hs CED
B
Disordered Profile Plot
filter
5% filter threshold
output
Disordered Probability
Sequence number
0
0.2
0.4
0.6
0.8
1
0
50
100
150
200
250
300
350

2.2). Interestingly, the M2-Cterm fusion repressed almost as well as CEDΔPAM2. This result further strengthens the idea that neither the PAM2 motif nor the RRM play a considerable role in mediating repression and that the combination of M2 and Cterm is sufficient for effective mRNA repression.

2.3 The CED interacts with PABP and CNOT1

The dissection of the CED region of the human protein TNRC6C surprisingly indicates an important function for the two unstructured regions flanking the RRM. We assumed that the most likely way for those regions to mediate repression is by interacting with other proteins.

To identify interacting proteins, a GST-fusion construct of the CED was overexpressed in HEK293T cells and was pulled-down via the GST-tag. The protein was then eluted from the beads and the pulled-down material was separated by SDS-PAGE and analyzed by mass spectrometry. Mass spectrometry analysis identified several promising candidates which co-purified with the CED of TNRC6C(Figure 2.3 A). The appearance of PABP and components of the CCR4–NOT complex was especially intriguing since they both play important roles in mRNA translation and mRNA regulation.

With subsequent pull-down experiments, we could confirm the interaction of PABP and CNOT1 with TNRC6C and were able to map the interaction to different regions within the CED (see Figure 2.3 B). In pull-down experiments, the RRM alone did not pull down CNOT1 or CAF1, whereas a fusion of M2 and Cterm regions pulled them down with an efficiency similar to that of CEDΔPAM2. Deleting either one of the two unstructured regions clearly reduced the interaction with CNOT1 or CAF, showing a strong correlation with the repressive activity of the fragments (compare with Figure 2.1). The interaction with PABP, however, seems not to be mediated by the two regions flanking the RRM but rather by the PAM2 motif. This finding is consistent with data of Fabian et al. [2009], see Appendix A and Jinek et al. [2010]. Importantly, the observed interactions were not mediated by RNA as all pull-down experiments included treatment with micrococcal nuclease.

2.4 GW Repeats Play a Role in Repression

The M2 and the Cterm regions of the CED are predicted to be unstructured and show relatively little conservation compared with the highly conserved RRM (see Figure 2.2). However, they seem to be responsible for mediating the repression via the interaction with the CCR4–NOT machinery. We wanted to test if M2 and Cterm execute different functions or if both regions take part in the same

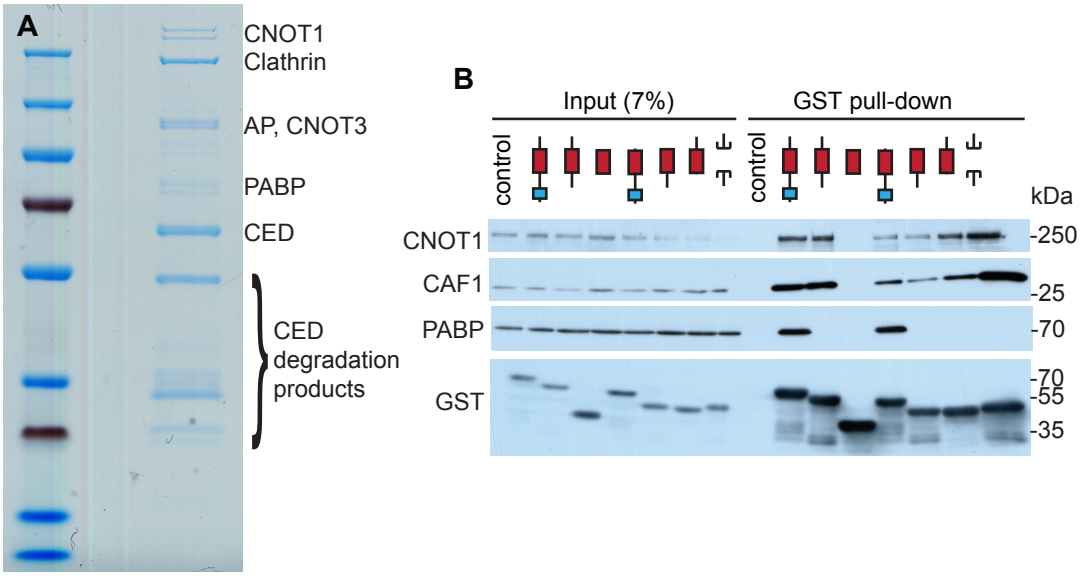


Figure 2.3: The CED interacts with PABP and the CCR4–NOT complex. (A) Mass spec analysis of purified CED. CED fragment was overexpressed in HEK293T cells and purified via GST-tag. The most prominent bands include CNOT1, Clathrin, PABP and components of the adaptor protein (AP) complex which links Clathrin to distinct receptors. **(B)** M2 and Cterm regions of the CED interact with components of the CCR4–NOT complex but not with PABP. TNRC6C CED and its subfragments were used for GST pull-down assays. Inputs (7%) and pull-down assays were analyzed by western blotting. Extracts from nontransfected cells were used as controls. Pull-down experimnets were performed by Hansruedi Mathys, Figure modified from Chekulaeva et al. [2011], see appendix B.

mechanism. To analyze a single region (either M2 or Cterm), we duplicated the sequence and fused it together, resulting in an M2-M2 and a Cterm-Cterm construct. We could not test single regions since they did not express in HEK293T cells. Both constructs were then cloned into pCi-neo vectors and compared to the M2-Cterm fusion (Figure 2.4, panel A). Our analysis revealed considerable redundancy of repressive sequences in both regions since a fusion of M2 (M2-M2) or Cterm (Cterm-Cterm) is nearly as active as the M2-Cterm fusion. The strong redundancy probably implies that both regions contain similar elements for contacting a single distinct downstream factor. Since the deletion of either region strongly impairs repression and interaction with CNOT1, we concluded that M2 and Cterm region have an additive effect on repression.

To identify important amino acids in M2 and Cterm, we performed mutagenesis of selected short amino acid stretches in the context of CEDΔPAM2 sub-fragments,

M2-RRM or RRM-Cterm. Mutagenesis of the same amino acids in the context of full-length CED or CEDΔPAM2 had a very limited effect on repression, likely due to the redundancy of sequence elements present in M2 and Cterm regions (data not shown). Amino acids to be mutated were chosen according to conservation, even though there is very little general sequence conservation in both flanking regions (see Figure 2.2). Furthermore, tryptophan residues in GW/WG repeats were selected for mutation.

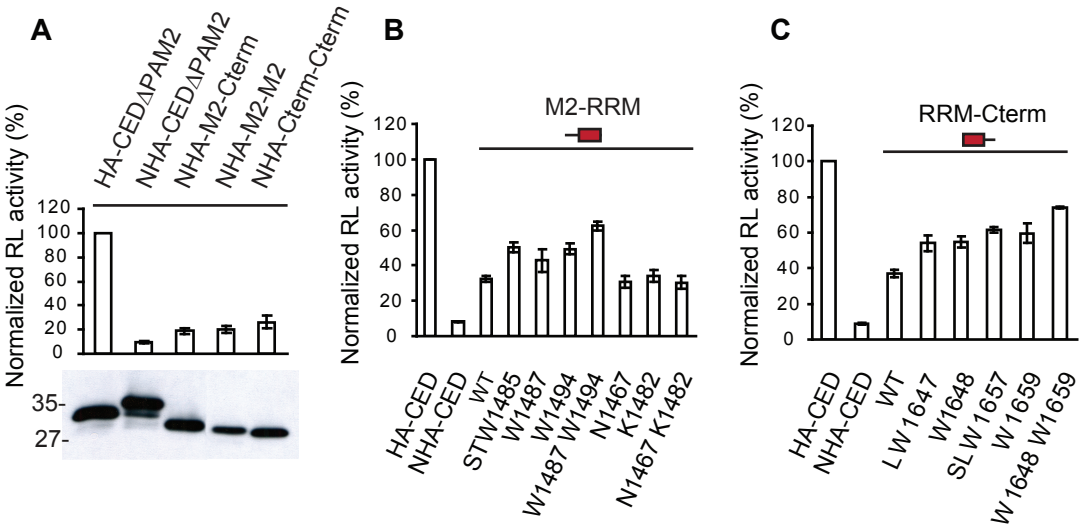


Figure 2.4: GW repeats are important for repression. (A) Duplicated M2 or Cterm regions retain the full repressive potential of the M2-Cterm fusion. Analyzed constructs are indicated at the top. Values are normalized to activity of HA-CEDΔPAM2 which is taken as 100%. **(B)** Mutations of W residues in M2 lead to alleviation of repression by M2-RRM. Tethering assay with indicated M2-RRM mutants was performed as previously described. **(C)** Point mutants of RRM-Cterm showed similar alleviation of repression as M2-RRM constructs. WT, wild-typr control. Figure modified from Chekulaeva et al. [2011], see appendix B.

Importantly, our results showed that all mutations significantly affecting repression were in elements containing tryptophan residues (Figure 2.4). In the Cterm region, two stretches of amino acids mutated were mutated, LW1647 and SLW1657 (Figure 2.4 C, compare also Figure 2.2 on page 47) (mutations were always to alanine; if several consecutive amino acids were mutated, the number corresponds to the first residue in the mutated stretch). Both mutations had a significant effect on repression, and both motifs include W residues in a context of GW or WG dipeptides. Consequent mutations of only W residues alone had a similar effect

and mutating W residues in both elements (mutant W1648W1659) resulted in a stronger reduction in repression. Similar effects could be observed for mutants of the M2-RRM fragment (Figure 2.4 B). Mutant STW1485 significantly released repression, and single point mutations of tryptophans (W1487 and W1494) had a similar effect. Combining both mutations (mutant W1487W1494) resulted in an even stronger release of repression. As a control, mutations of N1467 or K1482, or the combination of both, had no effect on repression. We observed that mutating tryptophans also strongly affected the activity of specific fragments to pull down CNOT1 and CAF1 (data of Hansruedi Mathys, shown in supplementary data of Chekulaeva et al. [2011], see appendix B).

We conclude that the Ws in M2 and Cterm are important for both mRNA repression and interaction with the CCR4–NOT complex. M2 and Cterm both contain sequence elements which, when mutated, affect repression and interaction, pointing towards a considerable redundancy. Furthermore, our data suggests a critical role for W residues in repression in a manner that involves recruitment of the CCR4–NOT complex.

2.5 The CED interacts with CNOT1 in yeast two-hybrid system

Mass spectrometry and pull-down experiments showed that the CED region interacts with several components of the CCR4–NOT complex. It could be expected that only one of the components interacts directly while the others are pulled-down indirectly. Since CNOT1 was the most effectively pulled down CCR4–NOT complex component, we assumed it to interact directly with the CED.

To investigate this hypothesis, we made use of the yeast two-hybrid system. In this system, the two proteins of interest are fused to the DNA-binding domain and the activation domain of a transcription factor, respectively. Only if the two proteins interact with each other, the close proximity of DNA-binding and activation domains allows a transcription of the reporter gene expressing β-Galactosidase (β-Gal). In this study, we used the yeast strain W303-1B with the reporter plasmid pSH18-34 (see section 4.4). Different components of the CNOT–CCR4 complex were fused to the LexA DNA binding domain and the CED was fused to the B42 activation domain. The CCR4–NOT complex is strongly conserved between many species, including yeast, fly, and mammals. Therefore, an interaction between *D. melanogaster* CED and human CNOT1 was also expected to occur. We could show previously in cross-species experiments that human full length TNRC6C and CED have an effect in *D. melanogaster*, and vice versa (compare Figure 8 of section 2.1 [Zipprich et al., 2009] and Chekulaeva et al. [2009]).

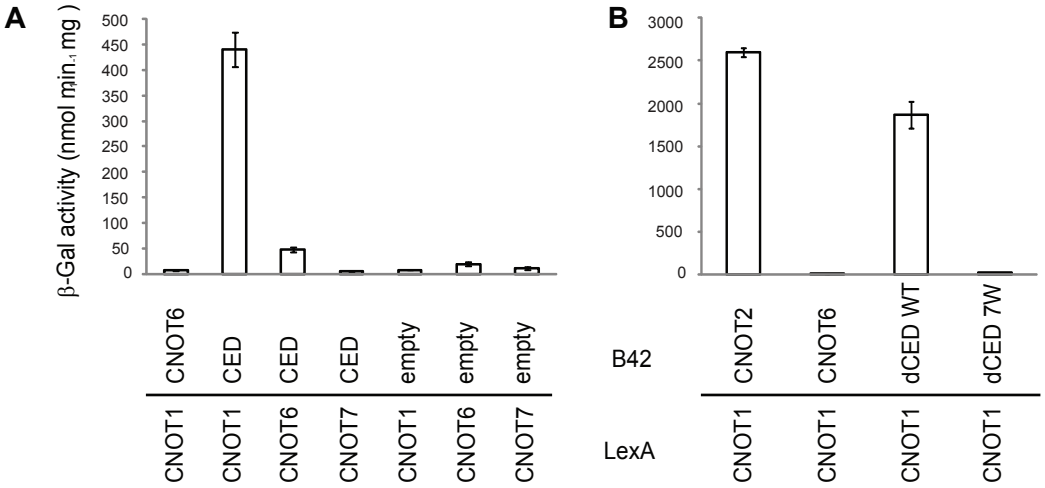


Figure 2.5: The CED interacts with CNOT1 in yeast two-hybrid system. (A) Indicated LexA- and B42-fusion expression plasmids (or empty pJG4-5 vector) were transformed into yeast strain W303-1B together with a LacZ reporter gene containing eight LexA operator sites. Empty pJG4-5 vector and the CNOT1-CNOT6 combination served as as control for lack of self-activation and as negative control. The graph shows a representative experiment (of four experiments performed), values represent mean ± SEM of β-Gal activity. **(B)** Same experimental setup as in (A), CNOT1-CNOT2 and CNOT1-CNOT6 served as positive and negative control, respectively. *D. melanogaster* CED (dCED) either wild type (WT) or mutant with seven tryptophans mutated to alanines (7W) was tested for interaction with CNOT1.

We observed a strong β-Gal activity for the CNOT1-CED pair while the negative control (CNOT1-CNOT6) and the empty vector control (CNOT1-empty) did not show any signal (panel A in Figure 2.5). This result strongly suggests a direct interaction between CED and CNOT1. While an indirect interaction via endogenous yeast components cannot be excluded, it is highly unlikely. No β-Gal activity could be observed for CED-CNOT6 and CED-CNOT7. These negative results prove that at least for those two components of the complex, there is neither a direct nor an indirect interaction in the tested yeast two-hybrid system. The interaction between CNOT1 and CED was also established for the *D. melanogaster* CED. This is consistent with the known conservation of the complex between species. More importantly, this interaction is completely abolished if all tryptophans in the CED are mutated to alanines (Figure 2.5, B). This result reinforces the suggested direct

interaction between CNOT1 and CED and the crucial role of tryptophan motifs.

2.6 Poly(A) Tail is not Required for Repression by the CED

The identification of the CCR4–NOT deadenylase complex as a downstream factor active in miRNA mediated repression could explain how miRNAs induce deadenylation and mRNA decay. However, the question remains if the CED can also induce repression at the level of translation, in a PABP and poly(A) independent way. To investigate this, we tested whether the TNRC6C CED or CEDΔPAM2 can repress mRNA expression if tethered to a reporter without a poly(A) tail. Repression of a poly(A)$^-$ mRNA would indicate that the observed effect is not due to the removal of the poly(A) tail and the subsequent inhibition of circularization.

To generate a poly(A) free reporter, we initially replaced the cleavage and polyadenylation signal in the RL-5BoxB reporter with sequences comprising a histone stem-loop and the histone downstream element (together referred to as HSL). This feature is responsible for formation of the 3' end of histone mRNAs, which do not undergo polyadenylation. Since in transfected HEK293 cells only about 50% of the transcript was cleaved at the HSL element (data not shown), we additionally introduced a hammerhead ribozyme (HhR) in a region downstream of HSL. The resulting reporter (RL-5BoxB-HSL+HhR, Figure 2.6) has been confirmed to be completely poly(A) free (experiments performed by Hansruedi Mathys, data shown in supplementary information of Chekulaeva et al. [2011], see appendix B).

Repression of poly(A)$^+$ mRNA by tethered CED or CEDΔPAM2 is approximately ten times when compared to the CED lacking the N-peptide (Figure 2.6, see also Figure 2.1 and Figure 4 of the paper in section 2.1). Surprisingly, tethering of CED or CEDΔPAM2 also repressed expression of a poly(A) free reporter (RL-5BoxB-HSL+HhR) about five times (Figure 2.6, panel B). To estimate the contribution of degradation to the repression, we monitored our reporters by Northern blotting. Northern analysis revealed that whereas tethering of the TNRC6C fragments CED or CEDΔPAM2 induced marked degradation of the poly(A)$^+$ RNA, the level of RNA transcribed from RL-5BoxB-HSL+HhR plasmid remained unaffected (Figure 2.6, panel C and D). These results suggest that the CED represses function of both poly(A)$^+$ and poly(A)$^-$ mRNAs.

In addition to the expected band at the size of RL-5BoxB-HSL+HhR RNA, Northern analysis also revealed larger RNA fragments cross-reacting with the RL probe. These bands were more prominent for samples isolated from cells transfected with the RL-5BoxB-HSL+HhR plasmid, raising the possibility that RL activity measured in cells expressing the RL-5BoxB-HSL+HhR reporter might originate, at

least partially, from transcripts other than those processed at HSL and HhR sites. To get additional evidence that the CED can repress translation independent of poly(A) tail, Hansruedi Mathys transfected HEK293T cells with *in vitro* transcribed poly(A) free RL-5BoxB RNA. Importantly, tethering of either CED or CEDΔPAM2 repressed RL activity in cells transfected with non-polyadenylated RL-5BoxB RNA (data not shown). This additional evidence clearly supports the conclusion that the CED and CEDΔPAM2 region of TNRC6C can repress RNA independently of a poly(A) tail, hence very likely at the level of translation.

We previously showed that the CED recruits the CCR4–NOT complex to repress protein synthesis. To address the question if the inhibitory effect on poly(A)$^-$ mRNAs also depends on CCR4–NOT, Hansruedi Mathys in our laboratory tested the *D. melanogaster* GW182 CED fragment in S2 cells depleted of NOT1. He indeed could show that repression of poly(A)$^-$ mRNAs depends on the CCR4–NOT complex (see Figure 6 of appendix B, [Chekulaeva et al., 2011])), consistent with the demonstrated role of CAF1, a CCR4–NOT complex component, in inducing inhibition of translation in microinjected *Xenopus* oocytes [Cooke et al., 2010].

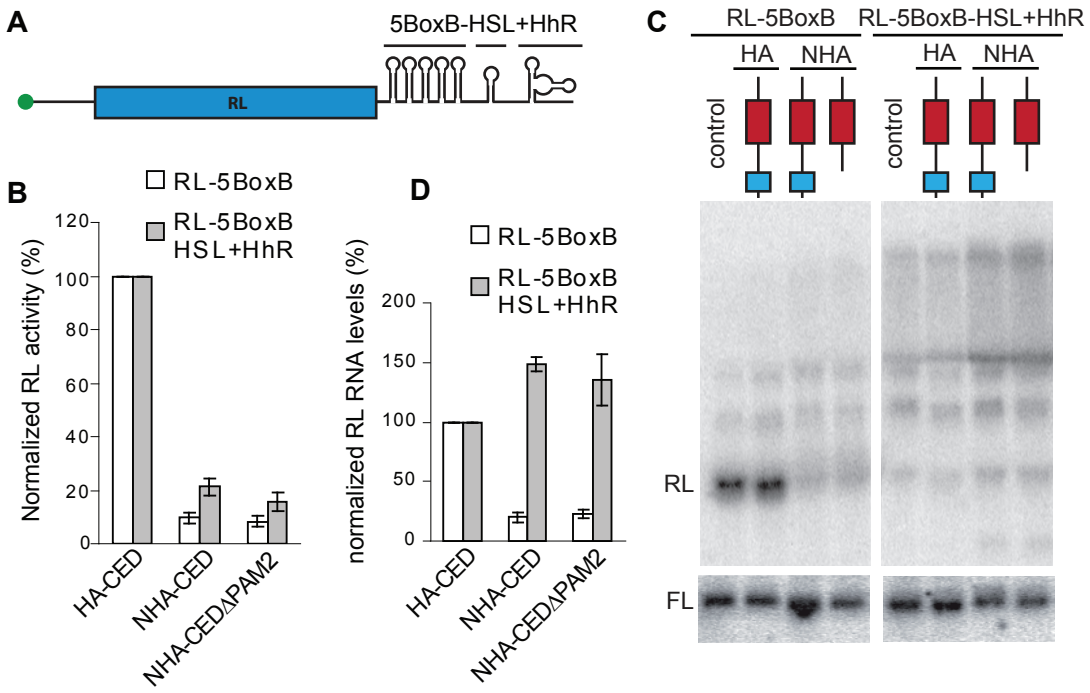


Figure 2.6: Tethering of the CED represses function of both poly(A)$^+$ and poly(A)$^-$ mRNAs. (A) Schematic representation of the RL-5BoxB-HSL+HhR reporter construct used for the experiments. **(B)** Effect of tethering the CED or CEDΔPAM2 on activity of reporters with and without poly(A) tail. HEK293T cells were cotransfected with plasmids expressing NHA-CED or NHA-CEDΔPAM2, and plasmids encoding either RL-5BoxB or RL-5BoxB-HSL+HhR, and also with FL-Con. HA-CED served as a negative control. Normalized RL activity is indicated as the percentage of activity in cells expressing HA-CED set as 100%. **(C)** Analysis of RL-5BoxB and RL-5BoxB-HSL+HhR RNA levels by Northern blotting in an experiment performed as described in panel (B). Total RNA isolated from cells transfected with indicated plasmids was analyzed using RL- or FL-specific probes. Cells transfected only with RL and FL reporters served as control. Positions of RL and FL mRNAs are indicated. **(D)** Quantification of Northern blots was performed using a PhosphorImager and the Image-Quant software. Normalized values represent means ± SE (n = 3).

3

Discussion

In the first part of this work (section 2.1), we characterized the human GW182 protein TNRC6C. In human, the C-terminal domain (CED) showed the most prominent effect in repression (about ten times inhibition), while the N-terminal GW-rich domain and the Q-rich domain only showed a repression of approximately 50%. This situation differs from *D. melanogaster* as shown in Chekulaeva et al. [2009, in the same issue of RNA]. In this report, Chekulaeva et al. from our laboratory concluded that in *D. melanogaster*, multiple independent domains (the N-terminal effector domain (NED), the Q-rich region and the CED) function in miRNA-mediated repression. We also performed site directed mutagenesis on the RRM in the CED (Figure 5 of Zipprich et al. [2009], see section 2.1). Two single point mutations showed a significant release of repression, H1537A and Y1556A (see Figure 2.2). A later report from Izaurralde's group presented a 3D structure of the *D. melanogaster* GW182 RRM and revleaed that both amino acids reside in the core of the RRM [Eulalio et al., 2009]. The implications of this finding are discussed below. Furthermore, we could show that the repression mediated by the CED is due to a combination of effects on the mRNA level and mRNA translation. While Northern blots revealed only a ∼50% reduction of mRNA levels, repression of RL activity was about 20 times. In cross-species experiments, we tested the potential of the full-length *Drosophila* GW182 and its active subfragments to inhibit the activity of the RL-5BoxB reporter in HEK293 cells. Interestingly, only the full-length dGW182 and the N-terminal GW rich domain showed an effective repression while the Q-rich domain and the C-terminal fragment repressed RL activity in HEK293 cells only approximately two times (Figure 8 of Zipprich et al. [2009], section 2.1). Activity of different domains of human TNRC6C was also tested in *Drosophila* S2 cells [Chekulaeva et al., 2009]. Full-length TNRC6C inhibited activity of the tethering reporter approximately six times. The most significant effect could be observed for the C-terminal domain (CED), which showed a repression of 20 times. The N-terminal GW-rich region and the Q-rich region repressed protein

synthesis ∼1.5 times and eight times, respectively. We concluded that although the full-length GW182 proteins showed a strong repression in either organism, the contribution of individual domains to this effect differed between human and *D. melanogaster* proteins and cells. These results – the implication of the C-terminal domain and the general role of TNRC6 proteins in miRNA-mediated repression – have already been discussed in the discussion section of section 2.1.

In the second part of this study, we further characterized the C-terminal domain, CED. The two regions flanking the RRM, M2 and Cterm, were found to mediate the repressive effect. The PAM2 motif and the RRM did not contribute to the repression. Both M2 and Cterm regions are predicted to be unstructured and show significantly less conservation than the RRM or the PAM2 motif (see Figure 2.2 on page 47). We could further identify two direct interaction partners of the CED, PABP and CNOT1. Both proteins interact with the CED, however with its different sub-fragments. We also investigated the relevance of a poly(A)-tail of the repressed reporter and therefore the importance of deadenylation in repression mediated by miRNAs.

Below we first discuss the contribution of individual regions to the repression and their role in interacting with other proteins, including the role of GW repeats. This part is followed by a short discussion of the importance of deadenylation for repression. Finally, we comment on the role of the CCR4–NOT complex in miRNA-mediated gene silencing.

The PAM2 Motif Interacts with PABP

The relatively conserved PAM2 motif of TNRC6C has been shown to be important for the interaction with PABP [Fabian et al., 2009; Jinek et al., 2010; Tritschler et al., 2010]. It shares sequence similarity with the PAM2 motif of PAIP1 and PAIP2, two proteins known for their interaction with PABP. The PAM2 motif is recognized by the MLLE motif in the C-terminal domain of PABP (PABC). Surprisingly, in *D. melanogaster*, the PAM2 motif seems to be dispensable for PABP binding in cell lysates [Zekri et al., 2009]. This report instead identified the M2 and Cterm sequences as PABP interacting regions and showed that all four RRMs from PABP are necessary for the interaction. Zekri et al. also argue that dGW182 competes with eIF4G for binding to PABP and interferes with the formation of an mRNA closed loop, which leads to less efficient translation. A more recent study from the same laboratory, however, identified two PABP-interacting regions, the PAM2 motif and the M2 and Cterm regions in dGW182 [Huntzinger et al., 2010]. In contrast to human GW182 proteins, where PAM2 motif is essential for binding to PABP, in dGW182, both regions seem to contribute to the PABP binding. This leads to the conclusion that while both human and fly GW182 proteins bind to PABP, the relative strength of the PABP-binding sites seem to be species-specific.

The role of the GW182 binding to PABP is not yet entirely clear. Fukaya and Tomari [2011] proposed that the GW182-PABP interaction is not essential for miRNA-mediated repression *in vitro*. They added GST-PAIP2 to the lysates to block PABP function and saw no effect on translational silencing or deadenylation. This is consistent with our observations that CEDΔPAM2 is still active in repression. On the other hand, Fabian et al. [2011] reported that the PAN2/PAN3 deadenylase associates with the human GW182 PAM2 motif, possibly through the interaction with PABP. It is known that PABP interacts with the PAN2/PAN3 deadenylase via the PAN3 subunit [Siddiqui et al., 2007]. Taken together, these results suggest that the GW182 protein may interact with PABP to recruit the PAN2–PAN3 deadenylase complex. This however does not contradict the PABP-independent repression we observed for the CEDΔPAM2 fragment, since the PAN2–PAN3 complex has been shown not to be essential for miRNA-mediated deadenylation [Behm-Ansmant et al., 2006].

These results are in agreement with reports from the Shyu laboratory, which suggest a biphasic deadenylation. Yamashita et al. propose that the first deadenylation step is mediated by the PAN2–PAN3 complex and shortens the poly(A) tail to $\sim$110 nt. The second phase involves nearly complete deadenylation of mRNA with the CCR4–NOT complex being the responsible catalytic enzyme. While this puts the PAN2–PAN3 complex at the starting point of deadenylation, they also suggest that „endogenous CCR4 may take over cytoplasmic deadenylation when endogenous PAN2 activity is impeded“ [Yamashita et al., 2005]. In a more recent report, Chen et al. show that repression by tethered human Ago proteins and TNRC6C recapitulates the two deadenylation steps [Chen et al., 2009].

The RRM Does Not Directly Contribute to Repression

The function of the highly conserved and structured RRM present in GW182 proteins remains unclear. In tethering assays, deleting the RRM has no effect on repression. All binding sites of proteins known to interact with TNRC6C have been mapped to regions other than RRM, mainly the N-terminal GW-rich region (AGO), the two RRM flanking regions (CNOT1) and the PAM2 motif (PABP). Furthermore, the RRM does not bind RNA (Eulalio et al. [2009] and H. Mathys, unpublished results). This could be explained by the presence of the additional C-terminal α-helix, which folds on top of the β-sheet surface generally used by RRMs to bind RNA [Eulalio et al., 2009]. The NMR structure was solved for the dGW182 RRM, but due to the high degree of conservation, it can be assumed that the RRM domain adopts a similar fold in vertebrates. While Eulalio et al. could show that the RRM is dispensable for P-body localization, they also concluded that the domain contributes to silencing since its deletion impairs the silencing

activity of GW182 in a miRNA target-specific manner. We could not confirm this result for experiments in which repression is mediated by tethering.

One has to keep in mind that RRM is flanked by unstructured regions, which are responsible for mediating the downstream effect via GW repeats. It is possible that the RRM serves as a scaffold to organize the tryptophans and so contributes to the interaction. The hydrophobic cleft of the RRM [Eulalio et al., 2009] has been suggested to interact with other proteins. Protein-protein interactions mediated by RRMs are not uncommon [Clery et al., 2008]. Supporting evidence for a function of the RRM in repression comes from the single point mutations described in Zipprich et al. [2009] (see Figure 5 of section 2.1 and compare Figure 2.2 on page 47). Even though the mutated amino acids are probably in the core of the RRM and are not actively involved in any interaction, one can hypothesize that mutagenesis of these residues might disturb the tertiary structure of the RRM which could in turn have an effect on the organization of the two flanking regions. However, the observation that full repression by tethering occurred even if the RRM was not present as shown for the M2-RRM fusion construct speaks against this model. It is interesting to note that the point mutations in the RRM only had an effect on translational repression and not on deadenylation; interpretation of this observation is difficult at present.

Importance of M2 and Cterm Regions

A. GW Repeats in M2 and Cterm Mediate Repression

In this study, we showed that the two unstructured and not particularly conserved regions flanking the RRM, M2 and Cterm, are responsible for mediating the repressive effect. The deletion of either one of the flanking regions (constructs M2-RRM or RRM-Cterm) led to a significant reduction in repression. This result was rather surprising since we expected the structured and far more conserved RRM domain to mediate the effect. It was commonly believed that a functionality of a given protein is determined by its specific three-dimensional structure. However, the validity of this 'lock and key' model has been challenged by the discovery of a significant number of unstructured proteins [see Uversky and Dunker, 2010, for a comprehensive review]. A model which is now more fitting to our case is the idea of the 'induced fit' mechanism. In such a scenario, an unstructured region can either fold or fit into a given pocket, establishing an interaction between two proteins. A prominent example is the C-terminal domain of the large subunit of RNA polymerase II [Noble et al., 2005]. It consists of up to 52 repetitive sequences and despite being disordered in solution, this region servers as a platform for many

transcription and RNA processing factors. If we apply this idea to our case, the two unstructured domains could indeed operate as a binding platform for their protein partners.

We tried to examine how two unstructured and non-conserved protein regions are able to mediate repression. Point mutations of partially conserved sequences revealed the importance of tryptophans in the context of GW/WG repeats. In human TNRC6C, M2 and Cterm contain five and four tryptophans, respectively. Subsequent single point mutations of tryptophans present in the GW/WG context in M2-RRM or RRM-Cterm had a significant effect on repression. Combinations of single Trp→Ala mutations showed an additive effect even in the context of the full CED [Chekulaeva et al., 2011], see Figure 2 of Appendix B. Since the regions are predicted to be unstructures, the Trp→Ala substitutions most likely do not affect the folding of the CED. The M2-M2 and the Cterm-Cterm fusion constructs had similar repressive activity when compared to a M2-Cterm fusion. This as well hints at an additive and redundant effect of GW repeats.

B. The CED Interacts with the CCR4–NOT Complex via GW repeats

The fact that the GW182 proteins mediate deadenylation via the CCR4–NOT complex has been proposed earlier [Behm-Ansmant et al., 2006; Zekri et al., 2009; Piao et al., 2010]. We could show in this work that TNRC6C interacts directly with CNOT1 and thereby recruits the deadenylation machinery. In contrast to the interaction with PABP, this binding to the CCR4–NOT complex is not mediated by PAM2 but rather by the two regions flanking the RRM, M2 and Cterm. Deletion of PAM2 or its mutation that disrupts the interaction between CED and PABP did not affect the CED association with CCR4–NOT. This supports the idea that the repression is mediated by the two regions flanking the RRM. Importantly, we could show that single Trp→Ala mutations significantly decreased the interaction of M2-RRM or RRM-Cterm with the CCR4–NOT complex (Hansruedi Mathys, data shown in supplementary data of Chekulaeva et al. [2011], see appendix B). Since it is unlikely that mutations have an effect on folding, the mutated amino acids probably actively take part in the interaction.

The question remains if each GW motif interacts with different CNOT1 molecules or if several motifs bind to one CNOT1 protein? The spliceosome component SF3b155 interacts with the U2 snRNP factor $U2AF^{65}$. SF3b155 contains seven RWD/E repeats with sequence similarity to the previously characterized $U2AF^{65}$-binding domain of SF1 [Thickman et al., 2006]. The repeats are located in a region that lacks any detectable secondary structure and five of the seven tryptophan-containing sites have the potential to bind to the $U2AF^{65}$ homology motif. Thickman et al. proposed that the multiple $U2AF^{65}$ binding sites of SF3b155 provide a regulatory mechanism. It is tempting to speculate that such a scenario also applies

to the GW repeats of GW182 proteins.

Poly(A)$^-$ RNA is Repressed by the CED

An important observation of this work is that the CED is able to repress reporters without a poly(A) tail. The poly(A)$^-$ RNA is insusceptible to deadenylation. We could show by Northern blotting that repressed poly(A)$^-$ reporters do not undergo degradation. Although it is now widely accepted that mRNA decay plays an important role in miRNA-mediated repression [Huntzinger and Izaurralde, 2010], our results clearly suggest that the CED also works via a mechanism which represses RNA translation in a deadenylation independent manner. We identified two downstream factors of the CED, PABP and CNOT1. This findings open several possibilities for a translational repression. First, the interaction with PABP could disturb the eIF4G-PABP interaction, loosening the closed loop of translated RNAs. The model that miRNAs interfere with the closed loop of mRNAs has been proposed previoulsy [Beilharz et al., 2010; Zekri et al., 2009]. The disturbance of the closed loop would lead to a less efficient recycling of ribosome subunits and to less efficient initiation, and consequently to a decrease in translation. The second possibility is that the effect on translation is mediated by the interacting protein complex, the CCR4–NOT complex. This implication is discussed in the next paragraph.

Function of the CCR4–NOT Complex in Translational Repression

The recruitment of the CCR4–NOT deadenylation machinery by the RISC plausibly explains the induced deadenylation but could it also explain the repression of poly(A)$^-$ reporters? Cooke et al. showed that tethering of CAF1 to the microinjected reporter mRNA can repress translation at the initiation step in *Xenopus* oocytes. Consistent with this finding, Hansruedi Mathys has found that tethering of the components of the CCR4–NOT complex even in cells in which endogenous GW182 has been knocked down repressed activity of poly(A)$^-$ reporters (data shown in Figure 6 of appendix B, [Chekulaeva et al., 2011]). This indicated that repression by tethering of CCR4–NOT proteins is dGW182-independent and that CCR4–NOT proteins are involved directly in translational repression induced by miRNAs. We can only speculate about which component of the CCR4–NOT complex is responsible for the translational effect and what exactly is its mechanism. TNRC6C interacts directly with CNOT1 but it is unlikely that CNOT1 mediates the effect. A more likely candidate is CAF1, which represses mRNA function when tethered to it in *Drosophila* S2 cells (Figure 6 of appendix B, [Chekulaeva et al., 2011]) and in *Xenopus* oocytes [Cooke et al., 2010]. However, the exact molecular

details of how CAF1 might interfere with translation, and which step is being regulated, remains to be elucidated.

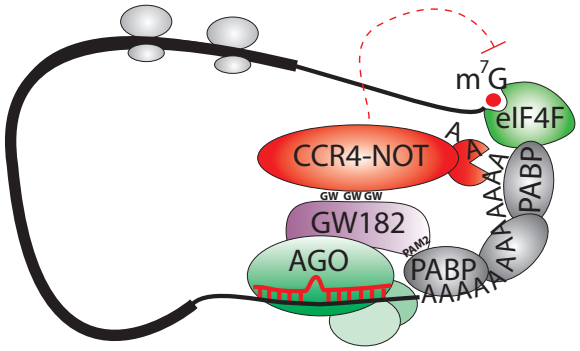


Figure 3.1: Scheme of Possible GW182 Mediated Repression. GW182 proteins are recruited to mRNA through direct interaction with the miRNA-AGO complex. The PAM2 motif of GW182 interacts with PABP. The GW182 protein also recruits, through the GW-motifs, the CCR4–NOT complex that catalyzes mRNA deadenylation. The CCR4–NOT complex most likely also represses translation, in all likelihood at the level of initiation (dotted red line).

Concluding Remarks

In this work, we provide additional information about the role of GW182 proteins in miRNA-mediated gene silencing. Five main conclusions can be drawn from this study:

1. The C-terminal GW182 region, CED, is the main effector region mediating the repression.

2. The two unstructured regions flanking the RRM mediate translational repression. None of the known protein interactions maps to the RRM, but the RRM might play a role in organizing its unstructured flanking regions.

3. The PAM2 motif of the TNRC6 CED interacts with PABP. This interaction is not crucial for miRNA-mediated repression in the tethering assay. However, it may help to stabilize the interaction with the CCR4–NOT complex and, in addition, probably recruits the PAN2–PAN3 deadenylase complex. Furthermore, it may interfere with the PABP–eIF4G association, thus contributing to both translational inhibition and mRNA deadenylation.

4. The CCR4–NOT complex is recruited to the RISC via GW-motifs in the GW182 CED that interacts directly with CNOT1. GW-repeats work in an additive way and tryptophan residues are crucial for the interaction with the CCR4–NOT complex and for mediating the repression.

5. Components of the CCR4–NOT complex can also repress activity of poly(A)$^-$ RNA. The exact mode of their action is not yet known. However, it is likely that the CCR4–NOT complex interferes with translation initiation.

Taken together, our data support and define the important role of GW182 proteins. The current model (Figure 3.1) explains how RNA degradation is induced by miRNAs. The fact that the CCR4–NOT complex also induces translational repression might help to understand at which step of translation mRNA expression is being regulated. It is interesting to note that in *S. cerevisiae* and in *D. melanogaster*, the CCR4–NOT complex is known to interact with the translational repressor Dhh1 [Coller et al., 2001]. Dhh1 has a clear role in decapping and in translation inhibition [Coller and Parker, 2005]. Moreover, evidence exists that its orthologues in other organisms are required for miRNA-mediated repression [Chu and Rana, 2006; Eulalio et al., 2007c], suggesting a possible mechanism by which the CCR4–NOT complex could repress translation. More studies will be needed to clarify the role of the CCR4–NOT complex in deadenylation-independent gene silencing.

4

Materials and Methods

4.1 Cell Culture, Transfections and Luciferase Assays

HEK293T cells were grown as described in the Methods section of Zipprich et al. [2009], see 2.1 on page 31. Transfections were done in 6-well plates as described and in 24-well plates using proportionally adjusted amounts of plasmid. Luciferase activities were measured with the Dual-Luciferase Reporter Assay System (Promega) as described. In all luciferase assays, values represent means ± S.E.M..

4.2 DNA constructs and protein mutants

Reporter plasmids RL-5BoxB and FL-Con [Pillai et al., 2004], plasmids encoding TNRC6A, TNRC6C and its deletion fragments [Zipprich et al., 2009](see section 2.1) as well as the the pEBG-Δ1370 plasmid encoding TNRC6C GST-CED [Fabian et al., 2009] have been described previously. To obtain the RL-5BoxB-HSL+HhR reporter, the HhR motif was PCR amplified from the FL-5BoxB-HhR reporter described in Eulalio et al. [2008b]. The HSL sequence was a kind gift from W.F. Marzluff. Both fragments were cloned into the standard RL-5BoxB reporter [Zipprich et al., 2009]. The TNRC6A clone lacks the first 312 amino acids [Eystathioy et al., 2002]. Point mutations in TNRC6C and its fragments were introduced by site-directed mutagenesis according to Zheng et al. [2004]. The 7W mutant of dGW182 used for the yeast two-hybrid system is described in Chekulaeva et al. [2011], see appendix B. Sub-fragments of TNRC6C were generated by PCR-amplification of the corresponding regions and cloning them into pCI-neo vector (Promega) bearing an HA- or NHA-tag [Pillai et al., 2004]. To construct the plasmid expressing the M2-Cterm fusion, the M2 and Cterm encoding regions were separately amplified

by PCR and consecutively cloned into pCI-NHA vector. Two different variants of the M2-Cterm fusion were cloned, with either AADGG or AAAGGGG as linker between both regions.

4.3 Pull-down assays and Western Blotting

For GST pull-down assays, HEK293T cells grown in a 10 cm dish were transfected with 5 µg plasmid expressing GST-TNRC6C CED. Cells were lysed 24 h after transfection and GST-fusions were pulled down as described [Fabian et al., 2009]. Cells were lysed in buffer A (50 mM Tris-HCl, pH 7.5, 150 mM KCl, 0.5% (v/v) Triton X-100, 1x complete EDTA-free protease inhibitor mix (Roche)), and cleared lysates were treated with micrococcal nuclease ($10\,{}^{ng}/_{\mu L}$) for 25 min at 20 °C. The lysates were incubated with glutathione (GSH)-Sepharose beads (GE Healthcare) for 2 h at 4 °C; beads were washed 3x with buffer A containing 0.1% (v/v) Triton X-100, and GST-fusions were eluted with 50 mM GSH.

For western blotting analysis, input and pulled-down or immunoprecipitated material were separated by SDS-PAGE using 10% linear polyacrylamid gels or NuPAGE Novex 4-12% Bis-Tris Gels (Invitrogen). For estimating the expression level of HA-fusion proteins in tethering assays, aliquots of cell lysates in Passive Lysis Buffer (PLB, Promega) were analyzed by SDS-PAGE as described above. Proteins were detected using ECL (GE Healthcare) or SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific). The following primary antibodies were used: anti-CNOT1, 1:250 dilution (provided by M. Collart); anti-CAF1 (Abnova), 1:1,000; anti-PABP (Cell Signaling Technology), 1:5,000; anti-GST (GE Healthcare), 1:10,000; anti-α-tubulin (Sigma T5168), 1:10,000; anti-HA tag (Roche 3F10, 1:5,000 or Santa Cruz sc-7392, 1:2,000); and anti-LexA (Santa Cruz sc-7544), 1:2,000.

For MS analysis, cells in one 10 cm cell culture dish were transfected with 6 µg plasmid expressing GST-TNRC6C CED. The lysate was cleared and incubated with glutathione (GSH)-Sepharose beads as described above. After separating the bands by SDS-PAGE, the Coomassie-stained bands were digested with trypsin. Tryptic peptides were analyzed by nano-HPLC (Agilent 1100 nanoLC system, Agilent Technologies, Santa Clara, CA) coupled to a 4000 Q TRAP mass spectrometer (Applied Biosystems, Foster City, CA). Peptides were identified searching UniProt database (version 13.8) restricted to *H. sapiens* using Mascot (version 2.1, Matrix Science, London).

4.4 Yeast two-hybrid assays

For all experiments, the strain W303-1B [MATα *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*] was used. LexA-CNOT1L (amino acids 648-2376), CNOT6 and CNOT7 constructs (in pEG202 backbone) were a kind gift from H.T.M. Timmers (University of Utrecht) as was B42-CNOT2 (all described in Lau et al. [2009]). B42-CED was cloned by amplifying the CED ORF by PCR with oligonucleotides ATGATGCCCGGGCTCGTGCCAAATCTGACAGTGAT and GTCTGCTCGAAGCATTAACCC used as forward and reverse primers, respectively. The PCR product was cloned into the pJG4-5 vector, also provided by H.T.M. Timmers. *Drosophila* CED wild type and mutant were kindly provided by M. Chekulaeva and are described in Chekulaeva et al. [2011], see appendix B. Both plasmids were cloned as a B42-fusion into the pJG4-5 vector. The reporter plasmid pSH18-34 was used to measure β-Gal activity [Sato et al., 1994]. Transformations with different plasmids were carried out according to the protocol described in Amberg [2005]. For measurements of β-Gal activity, 100 µL synthetic complete (SC) medium [-Trp -His -URA, containing 2% (w/v) lactate, 3% (v/v) glycerol and 2% (w/v) glucose] was inoculated with a single colony and grown for 6-7 h. The preculture was diluted 1:100 in 5 mL SC without glucose and grown overnight. Galactose was added to a final concentration of 2% (w/v) and the culture was grown for 5 more hours. Cells were pelleted and stored at -80 °C. The β-Gal-activity was analyzed according to the protocol described in Amberg [2005].

4.5 Northern Blotting

10-20 µg of total RNA isolated from HEK293T cells using Trizol Reagent (Invitrogen) was resolved in a denaturing 1% (w/v) agarose gel and transferred to Hybond-N^+ membrane (GE Healthcare Life Sciences) using 10x SSC. RL- or FL-specific probes internally labelled with [α-^{32}P]UTP using Random-primed DNA labeling Kit (Roche) were hybridized to the RNA on the membrane in ULTRAhyb Ultrasensitive Hybridization Buffer (Ambion) at 68 °C. After washing the membrane with 0.2x SSC containing 0.1% (v/v) SDS at 68 °C, the signal was detected using a PhosphorImager screen and a GE TyphoonTM 9400 scanner.

Appendix A

Mammalian miRNA RISC Recruits CAF1 and PABP

Marc R. Fabian, Géraldine Mathonnet, Thomas Sundermeier, Hansruedi Mathys, Jakob T. Zipprich, Yuri V. Svitkin, Fabiola Rivas, Martin Jinek, James Wohlschlegel, Jennifer A. Doudna, Chyi-Ying A. Chen, Ann-Bin Shyu, John R. Yates Ill, Gregory J. Hannon, Witold Filipowicz, Thomas F. Duchaine and Nahum Sonenberg

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Molecular Cell
Article

Mammalian miRNA RISC Recruits CAF1 and PABP to Affect PABP-Dependent Deadenylation

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SUMMARY

MicroRNAs (miRNAs) inhibit mRNA expression in general by base pairing to the 3′UTR of target mRNAs and consequently inhibiting translation and/or initiating poly(A) tail deadenylation and mRNA destabilization. Here we examine the mechanism and kinetics of miRNA-mediated deadenylation in mouse Krebs-2 ascites extract. We demonstrate that miRNA-mediated mRNA deadenylation occurs subsequent to initial translational inhibition, indicating a two-step mechanism of miRNA action, which serves to consolidate repression. We show that a let-7 miRNA-loaded RNA-induced silencing complex (miRISC) interacts with the poly(A)-binding protein (PABP) and the CAF1 and CCR4 deadenylases. In addition, we demonstrate that miRNA-mediated deadenylation is dependent upon CAF1 activity and PABP, which serves as a bona fide miRNA coactivator. Importantly, we present evidence that GW182, a core component of the miRISC, directly interacts with PABP via its C-terminal region and that this interaction is required for miRNA-mediated deadenylation.

INTRODUCTION

MicroRNAs (miRNAs) are short single-stranded RNAs (~21 nt in length) encoded within the genome of species ranging from protozoans to plants to mammals (Bartel, 2004; Molnar et al., 2007). miRNAs play key roles in a broad range of biological processes including hematopoiesis, insulin secretion, apoptosis, and organogenesis (Bartel, 2004). When assembled together with Argonaute (Ago) proteins into the miRNA-induced silencing complex (miRISC), miRNAs base pair with and repress mRNA expression through mechanisms that are not fully understood (Eulalio et al., 2008a; Filipowicz et al., 2008).

miRNAs were reported to employ different mechanisms to inhibit expression of targeted mRNAs (Eulalio et al., 2008a; Filipowicz et al., 2008). Some data indicate that miRNAs interfere with mRNA translation at the initiation step (Chendrimada et al., 2007; Ding and Grosshans, 2009; Humphreys et al., 2005; Mathonnet et al., 2007; Pillai et al., 2005; Thermann and Hentze, 2007; Wang et al., 2008), whereas other studies concluded that the miRNA machinery represses translation at postinitiation steps (Gu et al., 2009; Lytle et al., 2007; Maroney et al., 2006; Nottrott et al., 2006; Olsen and Ambros, 1999; Petersen et al., 2006). miRNAs have been observed, although not in every study, to mediate deadenylation and/or decay of targeted mRNAs (Behm-Ansmant et al., 2006; Giraldez et al., 2006; Wakiyama et al., 2007; Wu et al., 2006).

In addition to Ago proteins, GW182 proteins also play key roles in miRNA-mediated repression. One GW182 protein (Gawky) exists in *Drosophila*, and three GW182 paralogs (TNRC6A, TNRC6B, and TNRC6C) are present in mammals. Direct interaction of GW182 with Ago proteins is critical for miRNA-mediated translation repression and mRNA decay (Eulalio et al., 2008b). Studies conducted with either TNRC6C or Gawky artificially tethered to a reporter mRNA demonstrated that a region within their C termini is required for repression of translation (Chekulaeva et al., 2009; Eulalio et al., 2009b; Zipprich et al., 2009).

Cell culture-based assays invariably measure miRNA effects hours or days after the initial mRNA target site recognition, making it difficult to ascertain the temporal order and contribution of the different proposed mechanisms to mRNA repression. Moreover, RNAi-based approaches for identifying miRNA-associated factors may perturb cellular transcriptional programs in such a way that it becomes difficult to determine direct contributions. Thus, developing an in vitro system that faithfully

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recapitulates all aspects of miRNA-mediated repression is necessary to elucidate the biochemistry of miRNA mechanisms of action, especially at early time points. Such systems have recently been reported (Mathonnet et al., 2007; Thermann and Hentze, 2007; Wakiyama et al., 2007; Wang et al., 2006).

To explore the mechanisms that miRNAs utilize to repress mRNA expression in mammals, we utilized an in vitro translation extract from mouse Krebs-2 ascites cells (referred to throughout as Krebs extract). We showed before that the earliest detectable effect of miRNA action is the inhibition of cap-dependent translation initiation (Mathonnet et al., 2007). We demonstrate here that miRNA-mediated deadenylation follows the initial inhibition of cap-dependent translation. We further show that Ago2 interacts with the CNOT7/CAF1 (hereafter referred to as CAF1) deadenylase and poly(A)-binding protein (PABP) in an RNA-independent manner, and that both proteins are required to facilitate miRNA-mediated deadenylation. Importantly, we show that PABP physically interacts with the miRISC by directly binding the C terminus of GW182 and is required for deadenylation.

RESULTS

miRNA-Mediated Deadenylation Follows Initial Translation Inhibition

We previously described an in vitro translation extract derived from Krebs-2 ascites cells that contains high levels (~150 pM) of the let-7a (referred throughout as let-7) miRNA and displays a faithful let-7 miRNA response (Mathonnet et al., 2007). The Krebs extract manifests reduced translation initiation of in vitro-transcribed let-7-targeted mRNAs starting within the first 15 min of incubation without detectable mRNA degradation (Mathonnet et al., 2007). Since miRNAs were also reported to induce mRNA deadenylation (Eulalio et al., 2009a; Giraldez et al., 2006; Wakiyama et al., 2007; Wu et al., 2006), and since deadenylation generally results in translational repression (Wormington, 1993), we wished to determine whether miRNA-mediated deadenylation can be recapitulated in a Krebs extract and study the temporal relationship between translation inhibition and deadenylation. A polyadenylated RL-6xB mRNA (Figure 1A), labeled uniformly with ^{32}P-UTP, was incubated in a Krebs extract, and its integrity was analyzed by denaturing polyacrylamide-gel electrophoresis (PAGE) followed by autoradiography. A new RNA band migrating faster than the full-length mRNA was detected after ~1 hr of incubation (Figure 1B, lanes 3–7, and see Figure S1 available online). Formation of the new RNA species was dependent on let-7 miRNA as (1) inclusion of anti-let-7 2′-*O*-methylated oligonucleotide (2′-*O*-Me), but not anti-miR122 2′-*O*-Me, in the Krebs extract blocked the generation of this product (lanes 8 and 9, respectively); and (2) a reporter containing mutations in nucleotides complementary to the let-7 "seed" sequence (RL-6xBMut-pA; see Figure 1A and Figure 1B, lanes 1–7), and a reporter devoid of let-7 sites (RL-pA; Figure S1) failed to give rise to this band. Cloning and sequencing of the new RNA species using an oligonucleotide-ligation RT-PCR strategy (Figure S2) demonstrated that it represents a deadenylation product of the RL-6xB-pA mRNA. Thus, let-7 miRNA mediates deadenylation of the targeted mRNA in the Krebs extract, but with the earliest detection only after 1.3 hr of incubation. As translational inhibition (~55%) occurs within the first hour of incubation in the same Krebs extract in which deadenylation has been monitored (Figure 1C; see also Mathonnet et al. [2007]), it appears that miRNA-mediated inhibition of cap-dependent translation precedes mRNA deadenylation. When translation of RL-6xB-pA mRNA was allowed to proceed for longer times, the degree of translation repression increased from ~55% at 1 hr to ~77% at 2 hr (Figure 1C; three different experiments). These data indicate that deadenylation may consolidate the initial inhibition of cap-dependent translation.

Next, we asked whether deadenylation is dependent on translation. To this end, translation was inhibited in the Krebs extract by the addition of either cycloheximide (Figure 1B, lanes 10–12), which blocks translation elongation, or hippuristanol (lanes 13–15), which inhibits translation initiation (Bordeleau et al., 2006). Inhibiting either step of translation failed to block let-7-induced deadenylation of RL-6xB-pA mRNA. We then examined whether the m^{7}GpppG-cap structure is required for miRNA-mediated deadenylation. Deadenylation assays were conducted with RL-6xB-pA and RL-6xBMUT-pA mRNAs possessing an ApppG-cap, which cannot be bound by eIF4E but protects the RNA against degradation by 5′-3′ exonucleases. Neither the time course nor the extent of deadenylation of A-capped RL-6xB-pA significantly differed from RL-6xB-pA bearing an m^{7}GpppG structure (Figure 1B). Since miRNA-mediated deadenylation is an m^{7}GpppG-cap- and translation-independent event, we examined whether any RNA element upstream of the RL-6xB-pA 3′UTR is required for miRNA-mediated deadenylation. ApppG-capped 3′UTR transcripts were generated that lack an open reading frame and contain six either functional (6xB-3′UTR) or mutated (6xBMUT-3′UTR) let-7 sites and a 98 nt poly(A) tail (Figure 1D). The 6xB-3′UTR RNA recapitulated both the time course and the deadenylation pattern observed for the full-length RL-6xB-pA mRNA (Figure 1D). Deadenylation was dependent on let-7 miRNA as (1) addition of anti-let-7a 2′*O*-Me oligonucleotide, but not a nonspecific anti-miR122 2′-*O*-Me oligonucleotide (Figure 1D, lanes 12 and 13, respectively), abrogated the deadenylation of 6xB-3′UTR RNA; and (2) the 6xBMUT-3′UTR RNA was not deadenylated (Figure 1D). A 6xB-3′UTR RNA with a longer poly(A) tail (150 nt, 6xB-3′UTR*) behaved similarly to the 6xB-3′UTR RNA vis-a-vis the time course and the deadenylation pattern (Figure 1E). Taken together, our findings demonstrate that no RNA determinant other than the let-7 target sites is required for miRNA-mediated deadenylation.

Argonaute Proteins Interact with CAF1 and CCR4 Deadenylases

We used several approaches to identify the deadenylase(s) involved in the miRNA-mediated deadenylation. In one approach, Myc-tagged Ago1 and Ago2 were stably transfected into HEK293 cells. Tagged Ago proteins were immunopurified, and the associated proteins were identified by using multidimensional protein identification technology (MuDPIT) (Washburn et al., 2001; Wolters et al., 2001). This method was validated by the identification of known Ago2-interacting proteins such as HSP90, DICER, TRBP, and GW182 (Figure 2A) (Chendrimada et al., 2005; Landthaler et al., 2008; Liu et al., 2005; Meister

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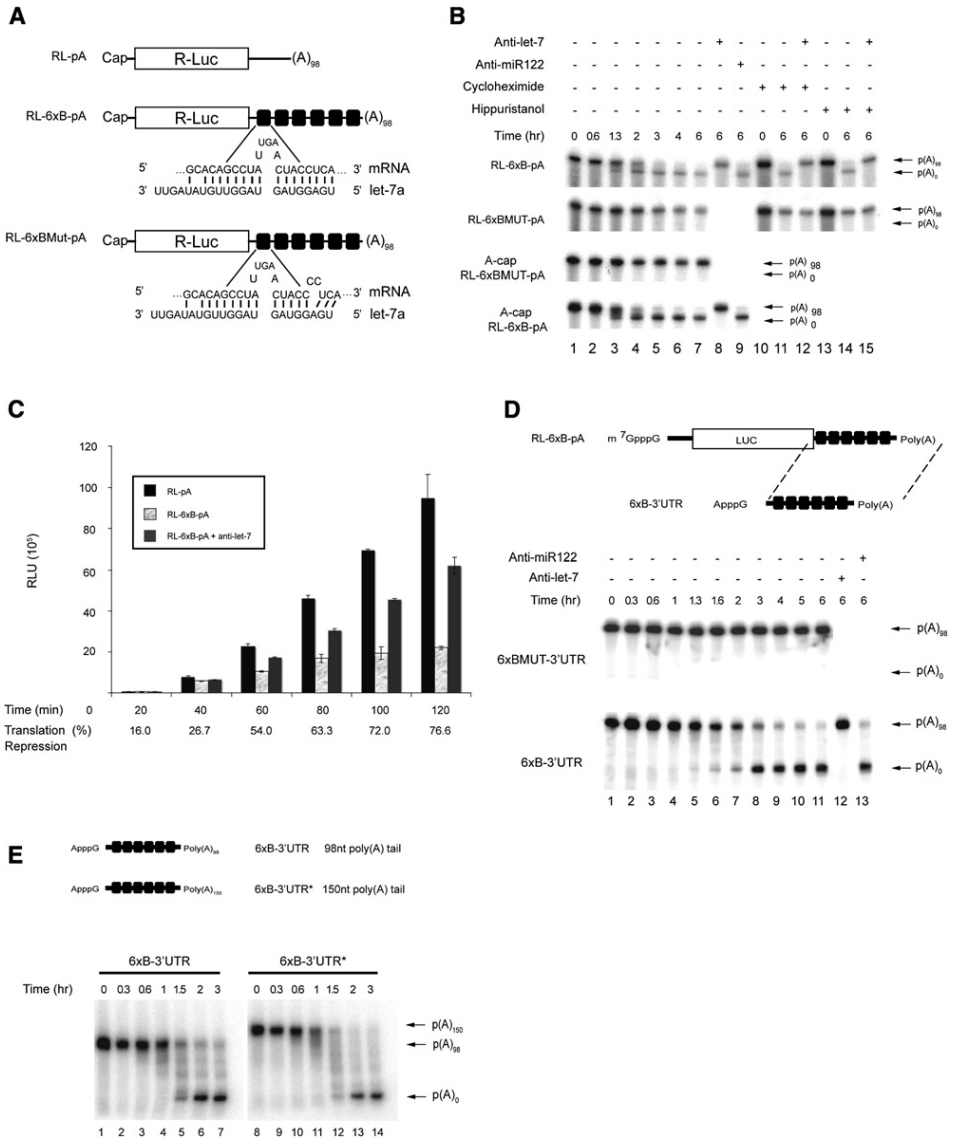

Figure 1. Deadenylation Mediated by let-7 miRNA in a Krebs Extract

(A) Schematic representation of the *Renilla* luciferase (Rluc) reporter mRNAs. Sequences of the let-7-binding sites (RL-6xB) and mutated seed sites (RL-6xBMut) are shown below the drawings.

(B) Time course of RL-6xB-pA and RL-6xBMUT-pA mRNA deadenylation as determined by autoradiography. Reporter mRNAs were incubated in the presence or absence of 10 μM cycloheximide, 1 mM hippuristanol, or 10 nM 2′-*O*-Me oligonucleotide (either anti-let-7a or anti-miR122).

(C) A time course of translation of RL-pA, RL-6xB-pA, and RL-6xB-pA in the presence of anti-let-7 2′-*O*-Me. Average percentage repression is labeled below each time point. Error bars represent the standard deviation of three independent experiments.

(D) Schematic representation of the 6xB-3′UTR reporter RNA and time course of 6xB-3′UTR and 6xBMUT-3′UTR RNA deadenylation in a Krebs extract as determined by autoradiography. Reporters were incubated in the presence or absence of 10 nM 2′-*O*-Me oligonucleotide (either anti-let-7a or anti-miR122), and their stability was monitored by autoradiography.

(E) Schematic representation of the 6xB-3′UTR reporter RNAs with either 98As or 150As (*). Time course of 6xB-3′UTR and 6xB-3′UTR* deadenylation in a Krebs extract as determined by autoradiography. Polyadenylated and deadenylated mRNAs are marked on the right of the figure.

et al., 2005). In addition, PABP was identified in both Ago1 and Ago2 immunopurifications (Figure 2A) (Hock et al., 2007; Landthaler et al., 2008). One identified protein that was not reported before to interact with Ago proteins was CAF1 deadenylase. To validate this interaction, we performed coimmunoprecipitation experiments using a micrococcal nuclease-treated Krebs extract. When endogenous Ago2 was immunoprecipitated from the Krebs extract, the precipitated fraction contained Ago2 and CAF1, but not eIF4E (Figure 2B). When endogenous CAF1 was immunoprecipitated from Krebs extracts, the precipitated fraction contained CAF1, CCR4 (a CAF1-associated deadenylase [Tucker et al., 2001]), and Ago2, but not eIF4E (Figure 2C).

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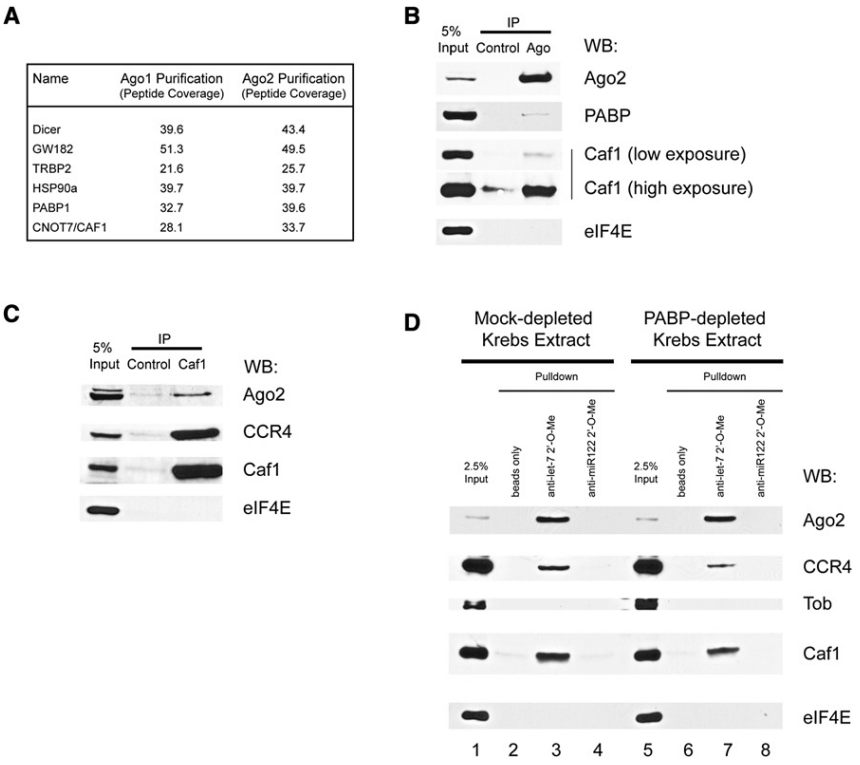

Figure 2. Ago Proteins Interact with PABP and the CAF1/CCR4 Deadenylase Complex

(A) MuDPIT analysis of Ago1- and Ago2-interacting proteins. Identified proteins are listed along with corresponding peptide coverage for Ago1 and Ago2 coimmunoprecipitations.

(B) Immunoprecipitation of endogenous Ago2 protein from micrococcal nuclease-treated Krebs extract using anti-Ago2 antibody. Immunoprecipitated complexes were subjected to SDS-PAGE and probed with anti-Ago2 antibody, anti-CAF1 antibody, anti-PABP antibody, or anti-eIF4E antibody.

(C) Immunoprecipitation of endogenous CAF1 protein from micrococcal nuclease-treated Krebs extract using anti-CAF1 antibody. Immunoprecipitated complexes were subjected to SDS-PAGE and probed with anti-Ago2 antibody, anti-CAF1 antibody, anti-CCR4 antibody, or anti-eIF4E antibody.

(D) Pulldown of Ago2, CCR4, and CAF1 from micrococcal nuclease-treated Krebs extracts using biotin-conjugated anti-let-7 2′-O-Me oligonucleotide and streptavidin Dynabeads. Isolated complexes were subjected to SDS-PAGE and probed with anti-Ago2 antibody, anti-CAF1 antibody, anti-CCR4 antibody, anti-Tob antibody, or anti-eIF4E antibody.

To determine whether the CAF1 and CCR4 deadenylases can be recruited by the let-7-loaded Ago2, we used a 2′-*O*-Me RNA target "bait" pulldown assay (Hutvagner et al., 2004). Biotinylated 2′-*O*-Me oligonucleotides, which mimic partially complementary mRNA target sites for let-7 or miR122 (a liver-specific miRNA [Lagos-Quintana et al., 2002] that can pull down Ago2 from lysates derived from Huh7 liver cells [Figure S3]), were incubated in Krebs extract and pulled down using streptavidin beads. The associated proteins were eluted and analyzed by western blotting. Ago2 bound specifically to the anti-let-7 2′-*O*-Me beads and failed to bind to control beads or anti-miR122 2′-*O*-Me beads (Figure 2D, lanes 2–4). Importantly, in these pulldown experiments a similar pattern of enrichment was observed for CAF1 and CCR4, but not for eIF4E or Tob (a protein that can associate with CAF1 to enhance deadenylation [Ezzeddine et al., 2007; Mauxion et al., 2008]). These results demonstrate that CAF1 and CCR4 can be specifically recruited to the target-bound let-7-loaded Ago2.

miRNAs Require CAF1 Activity to Promote Deadenylation

To determine whether CAF1 is required for miRNA-mediated deadenylation, it was immunodepleted (~80%) from the Krebs extract using an affinity-purified CAF1 antibody (Figure S4). Analysis of the depleted extract (Figure 3) demonstrated that miRNA-mediated translation inhibition is partially relieved in both CAF1- and Ago2-depleted extracts (37.8% [Figure 3B] and 14.9% repression [Figure 3D], respectively, after 3 hr incubation) when compared to the corresponding control-depleted extracts (68.9% [Figure 3A] and 54.5% [Figure 3C] repression after 3 hr incubation). The Ago2-depleted extract was dramatically impaired in its ability to deadenylate the 6xB-3′UTR RNA, inasmuch as deadenylation was barely detectable even after 6 hr of incubation (Figure 3E, lane 5). A similar decrease in deadenylation was detected in a Krebs extract depleted of CAF1 (Figure 3F, lane 10). These deadenylation defects were specific, because in a mock-depleted extract, 6xB-3′UTR RNA was deadenylated in a let-7-dependent manner (Figures 3E and 3F, lanes 1–3). miRNA-mediated deadenylation was modestly restored (2.2-fold increase; from ~5% to ~12% deadenylation) by the addition of affinity-purified wild-type HA-CAF1 to the CAF1-depleted extract (Figure 3F, lane 11), while wild-type HA-CAF1 had no noticeable effect on mock-depleted extract (lane 4). Modest restoration was most likely due to a small fraction of affinity-purified wild-type HA-CAF1 being bound to let-7-loaded miRISC. In contrast, addition of affinity-purified catalytically inactive HA-CAF1 mutant (D40A) (Zheng et al., 2008) decreased miRNA-induced deadenylation in both mock- and CAF1-depleted extracts (Figure 3F, lanes 5 and 12, respectively). This is likely due to HA-CAF1(D40A) acting as a dominant-negative mutant in both mock- and CAF1-depleted extracts. Taken together, these results show that miRNA-induced deadenylation is executed, at least in part, by the CAF1 deadenylase.

Ago2-GW182 Interaction Is Essential for miRNA-Mediated Deadenylation

The Ago-binding protein GW182 is required for efficient miRNA-mediated silencing in *C. elegans* and in *Drosophila* S2 cells (Behm-Ansmant et al., 2006; Ding and Han, 2007; Eulalio et al., 2008b). GW182 is required for the assembly of P bodies, protein-RNA assemblies thought to contribute to translation

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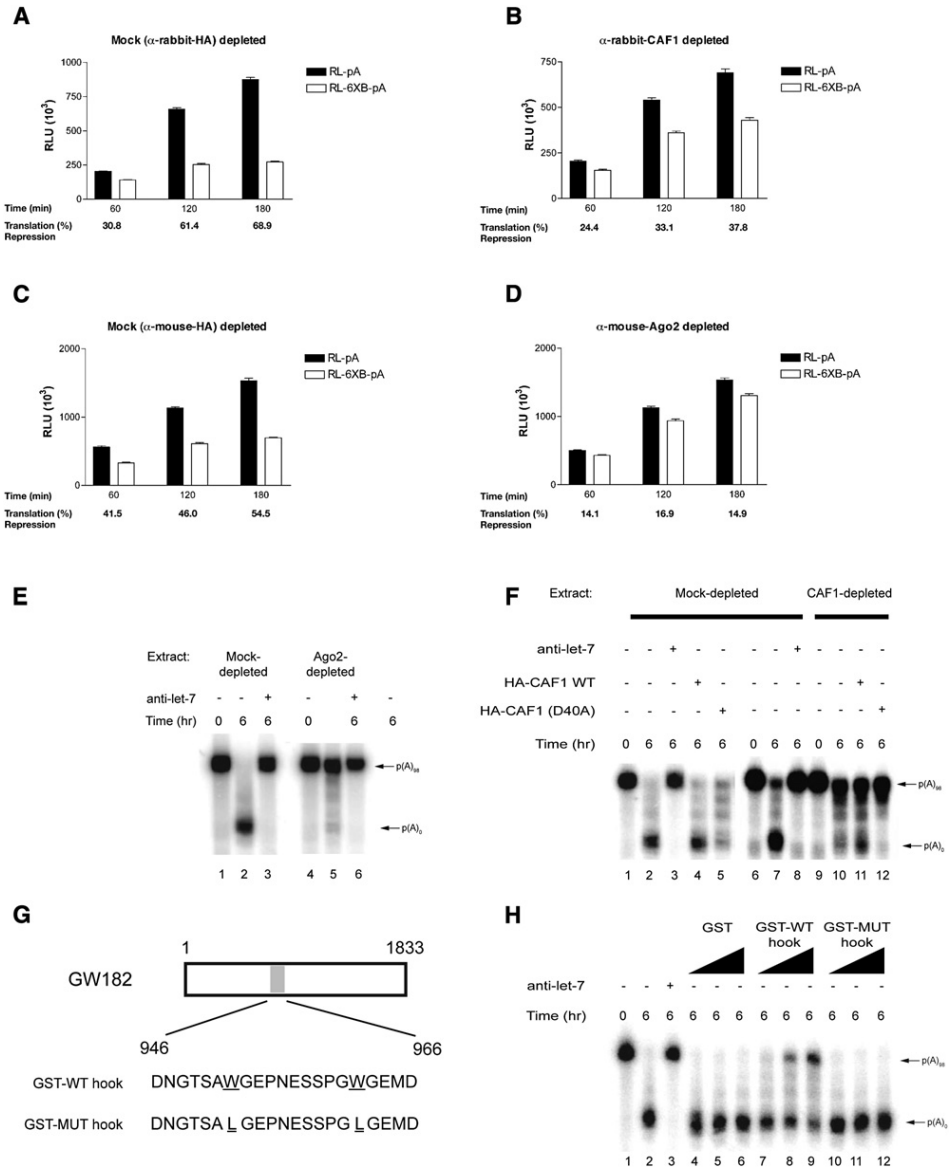

Figure 3. let-7-Mediated Deadenylation Requires CAF1, Ago2, and GW182

(A–D) Time course of RL-pA and RL-6xB-pA translation in rabbit anti-HA- (A), rabbit anti-CAF1- (B), mouse anti-HA- (C), and mouse anti-Ago2-depleted Krebs extracts (D). Average percentage repression is labeled below each time point. Error bars represent the standard deviation of three independent experiments.

(E) 6xB-3′UTR RNA deadenylation in the presence or absence of 10 nM anti-let-7a 2′-*O*-Me in control (mouse anti-HA) or anti-Ago2-depleted Krebs extract. 6xB-3′UTR RNA deadenylation was followed by autoradiography. Polyadenylated and deadenylated mRNAs are marked on the right of the figure.

(F) 6xB-3′UTR RNA deadenylation in control (rabbit anti-HA) or anti-CAF1-depleted extract in the presence or absence of either 10 nM anti-let-7a 2′-O-Me oligonucleotide, or WT or D40A HA-CAF1 protein.

(G) Wild-type and mutant hook peptides derived from GW182.

(H) 6xB-3′UTR RNA deadenylation in Krebs extract in the presence or absence of either GST or GST hook peptides at concentrations ranging from 0.1 to 2.0 μg per reaction, respectively.

inhibition and mRNA destabilization (Behm-Ansmant et al., 2006; Ding and Han, 2007; Jakymiw et al., 2007; Liu et al., 2005; Pillai et al., 2005; Rehwinkel et al., 2005). CAF1 also localizes to P bodies in mammalian cells (Zheng et al., 2008). We therefore investigated whether the GW182 interaction with Ago2 plays a role in miRNA-mediated deadenylation in vitro. To this end, we used a 22 amino acid fragment of GW182 (called "Ago hook") (Figure 3G) that competes with GW182 for Ago binding and inhibits miRNA-mediated repression in vivo (Till et al., 2007). A Krebs extract was incubated with either GST alone, GST fused to Ago hook (GST-WT hook), or GST fused to a mutant hook (GST-MUT hook) containing two Trp to Leu mutations that abrogate the

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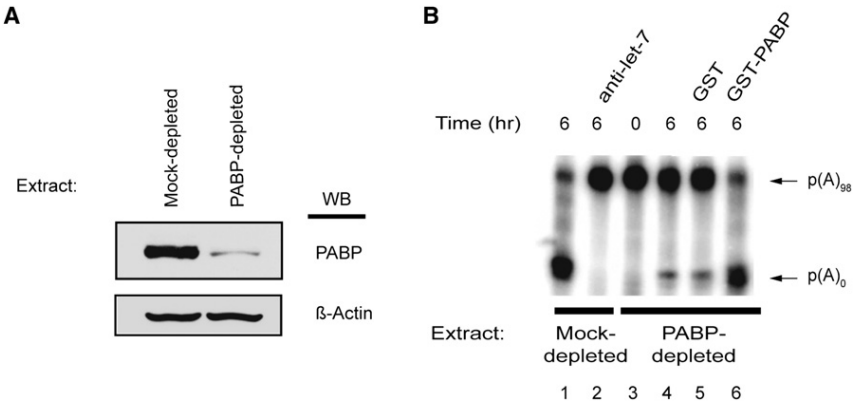

Figure 4. let-7-Dependent Deadenylation Requires PABP

(A) Western blot analysis of Krebs-2 extracts depleted with either GST (Control Extract) or GST-Paip2 (PABP-depleted Extract) probed with anti-PABP antibody and anti-β-actin antibody.

(B) A-capped 6xB-3′UTR RNA incubated in either mock-depleted (lanes 1–2) or PABP-depleted extract (lanes 3–6). PABP-depleted extract was supplemented with recombinant GST, GST-PABP (100 ng, which is the equivalent of roughly 50% of endogenous PABP present in an in vitro reaction), and RNA stability was monitored by autoradiography. Polyadenylated and deadenylated mRNAs are marked on the right of the panel.

ability of the hook to bind to Ago (Till et al., 2007) (Figure S5). Addition of a recombinant GST-WT hook, but not GST alone or GST-MUT hook to the Krebs extract, impaired the deadenylation of 6xB-3′UTR RNA in a concentration-dependent manner (Figure 3H, lanes 7–9 compared to lanes 4–6 and 10–12, respectively). These findings indicate that miRNA-mediated deadenylation in vitro requires GW182 contact with Ago2 at the hook site.

PABP Is Required for miRNA-Mediated Deadenylation

Since the MuDPIT analysis identified PABP as an Ago-interacting protein, it was pertinent to determine whether PABP is necessary for miRNA-induced deadenylation. A Krebs extract was depleted (>95%) of endogenous PABP using a GST-tagged PABP-interacting protein 2 (Paip2) affinity matrix (Figure 4A). Paip2 is a strong translational inhibitor and acts by sequestering PABP and blocking PABP-poly(A) tail and PABP-eIF4G interactions in vitro (Karim et al., 2006; Khaleghpour et al., 2001). GST-Paip2 coupled to a resin was previously used to efficiently deplete PABP from a Krebs extract, resulting in reduced translation (Kahvejian et al., 2005). Strikingly, the PABP-depleted extract was severely impaired in its ability to deadenylate the 6xB-3′UTR RNA (Figure 4B, compare lane 4 to lane 1). This was a specific consequence of PABP depletion as a mock-depleted extract still deadenylated the reporter RNA and was responsive to the addition of anti-let-7 2′-*O*-Me oligonucleotide (Figure 4B, lanes 1 and 2, respectively). Moreover, addition of recombinant GST-PABP (50% of endogenous PABP levels in a Krebs extract [Figure S6]) to the PABP-depleted extract (lane 6), but not GST alone (lane 5), completely rescued miRNA-mediated deadenylation of 6xB-3′UTR RNA. The rescue was prevented by the addition of anti-let-7 2′-*O*-Me oligonucleotide (Figure S7, lane 8). These findings clearly show that PABP is essential for miRNA-mediated deadenylation in vitro.

PABP Function in miRNA-Mediated Deadenylation Is Antagonized by eIF4G

How does PABP facilitate miRNA-mediated deadenylation? PABP is probably not required for miRISC target site recognition, as the let-7-loaded Ago2 can be pulled down with anti-let-7 2′-*O*-Me oligonucleotide from a PABP-depleted Krebs extract almost as well as from a nondepleted extract (Figure 2D, compare lanes 3 and 7). Moreover, PABP is required for recruiting neither CAF1 nor CCR4 as they are pulled down in similar amounts from PABP-depleted extracts with anti-let-7 2′-*O*-Me oligonucleotide (Figure 2D, compare lanes 3 and 7). It is unlikely that PABP's role is to compete with other proteins for poly(A) tail binding, as adding free poly(A) to PABP-depleted extracts (Figure S8) does not rescue miRNA-mediated deadenylation.

The N-terminal region of PABP can interact with the translation initiation factor eIF4G, and this interaction stimulates translation (Imataka et al., 1998; Wakiyama et al., 2000). To determine whether this interaction might antagonize deadenylation, Krebs extract was incubated with increasing concentrations of either an N-terminal eIF4G fragment (GST-eIF4G 41-244wt) that binds the N terminus of PABP or a mutant eIF4G fragment (GST-eIF4G 41-244mut) that does not bind to PABP (Kahvejian et al., 2005) (Figures 5A and S9). Addition of a wild-type (lanes 3–6), but not the mutant eIF4G fragment (lanes 7–10), impaired the deadenylation of 6xB-3′UTR RNA in a concentration-dependent manner.

We next examined whether the effect of GST-eIF4G 41-244wt on miRNA-mediated deadenylation was a result of its binding to PABP. PABP-depleted extracts were supplemented with either wild-type or PABP M161A that cannot bind eIF4G (Groft and Burley, 2002) (Figures 5B, 5C, and S9). miRNA-mediated deadenylation in PABP-depleted extracts can be rescued equally well with PABP M161A as compared to wild-type PABP (Figure 5B, compare lanes 5–7 with lanes 10–12). Addition of GST-eIF4G 41-244wt blocked deadenylation in a PABP-depleted extract supplemented with wild-type PABP (lane 8) but decreased it only minimally when supplemented with PABP M161A (lane 13). These findings suggest that the eIF4G-PABP interaction is not required for, but rather interferes with, miRNA-mediated deadenylation.

PABP Interacts with the C-Terminal Region of GW182

GW182 is a core component of miRISC, and its contact with Ago is required for miRNA-mediated repression (Eulalio et al., 2008b; Till et al., 2007). Mammalian and *C. elegans* GW182 protein orthologs were previously shown to coimmunoprecipitate with PABP (Landthaler et al., 2008; Zhang et al., 2007), but whether these associations were direct has not been determined. To test for a direct interaction between GW182 and PABP, we performed GST pulldown experiments using recombinant His-tagged PABP and four GST- and FLAG-tagged partially overlapping fragments (covering amino acids 1–500, 400–900, 800–1360, and 1260–1690) of the human GW182 protein TNRC6C (Figure 6A). GST on its own and fusions with TNRC6C fragments

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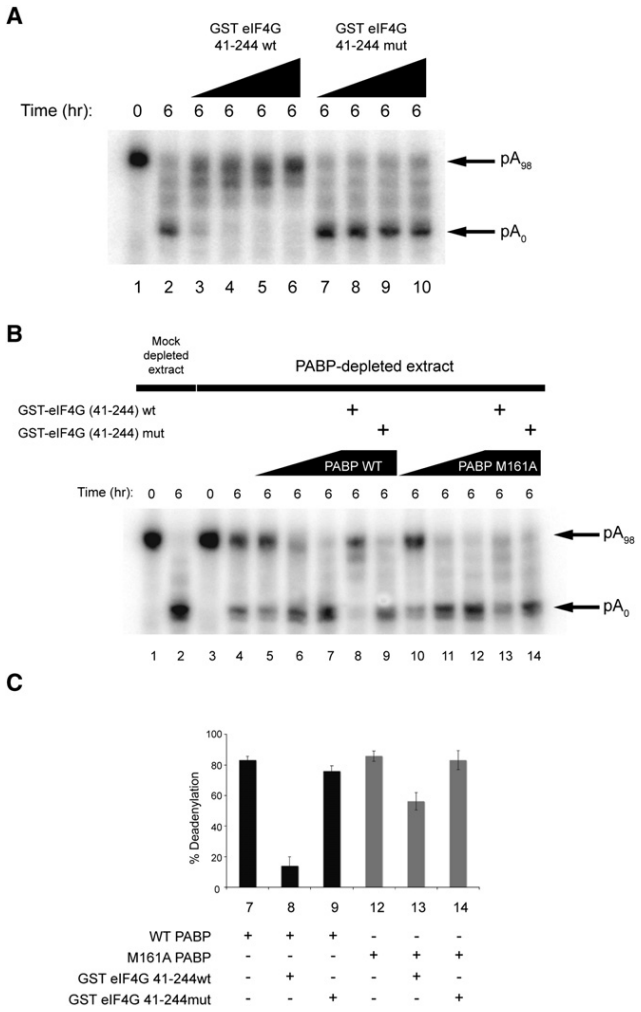

Figure 5. eIF4G Contact with PABP Antagonizes miRNA-Mediated Deadenylation

(A) 6xB-3′UTR RNA deadenylation in Krebs extract in the presence or absence of increasing concentrations (0.15, 0.5, 1.0, and 3.0 μg per reaction) of wild-type or mutant GST-eIF4G 41-244.

(B) 6xB-3′UTR RNA deadenylation in mock- or PABP-depleted Krebs extract in the presence or absence of increasing concentrations (25, 50, or 100 ng per reaction, respectively) of either wild-type (lanes 5–7) or M161A PABP (lanes 10–12) and/or wild-type (lanes 8 and 13) or mutant (lanes 9 and 14) GST-eIF4G (41-244).

(C) Quantification of deadenylated bands as a percentage of total RNA in (B) is shown in bar graphs (with standard deviations).

1–500, 400–900, and 800–1360 did not interact with PABP. In contrast, the C-terminal 1260–1690 of TNRC6C, which harbors both the domain of unknown function (DUF; Zipprich et al., 2009) and RRM domains of TNRC6C pulled down PABP very efficiently (20% of input; Figure 6A). GST pulldown experiments using overlapping fragments of another human GW182 paralog (TNRC6A) and PABP yielded similar results (data not shown).

We next investigated whether the C-terminal region of TNRC6C interacts with PABP in transfected HEK293 cells. Of the HA-tagged fragments spanning different regions of TNRC6C (Figure 6B), only the C-terminal fragment, ΔN1370, encompassing residues 1370–1690, pulled down endogenous PABP (Figure 6B, lane 8). In additional experiments, lysates from cells expressing GST-ΔN1370 were used for GST pulldowns. In the absence of micrococcal nuclease treatment, GST-ΔN1370 pulled down both PABP and eIF4G. However, in nuclease-treated lysates GST-ΔN1370 pulled down only PABP (Figure 6C), demonstrating the RNA independence of the interaction between TNRC6C and PABP. Taken together, these data indicate that the C-terminal region of the GW182 protein TNRC6C interacts directly with PABP in an RNA-independent manner.

GW182 Contact with the PABP C-Terminal Domain Is Required for Maximal miRNA-Mediated Deadenylation

We next performed a sequence analysis of the C terminus of GW182 proteins to identify any potential PABP-interacting motifs. We observed a short sequence within the DUF that shows similarity to the Paip2 PAM2 motif (Figure 7A) that is required for Paip2 to bind the second half of the PABP C terminus (C2) (Khaleghpour et al., 2001; Kozlov et al., 2004). GST pulldown experiments were subsequently carried out using recombinant GST-tagged C-terminal PABP fragments (GST-C1 and GST-C2) and the FLAG-tagged TNRC6C C terminus (covering amino acids 1260–1690 [Figure 7B]). The PABP GST-C1 fusion did not pull down the GW182 1260–1690 fusion. In contrast, GST-C2 pulled down the TNRC6C C-terminal fragment very efficiently (~40% of input).

To determine whether miRNA-mediated deadenylation requires GW182 contact with the PABP C2 domain, a Paip2-derived PAM2 peptide that specifically binds the C2 domain (Figure 7C) was used. Addition of increasing concentrations of wild-type, but not mutant PAM2 peptide (F117A [Kozlov et al., 2004]) to GST-PABP incubated with TNRC6C 1260–1690 fragment, blocked, albeit not completely, the binding of the TNRC6C C terminus to PABP (lanes 6–8 compared to lanes 9–11). Consistently, addition of the wild-type, but not the mutant PAM2 peptide to a Krebs extract, interfered with miRNA-mediated deadenylation in vitro in a dose-dependent manner (Figure 7D, lanes 3–6 compared to lanes 7–10). Taken together, these findings demonstrate that maximal miRNA-mediated deadenylation in vitro requires GW182 contact with the PABP C2 domain.

To further assess the function of the GW182 C terminus in miRNA-mediated deadenylation, we added the C-terminal recombinant 1260–1690 fragment to in vitro deadenylation reactions. The fragment dramatically enhanced miRNA-mediated deadenylation in vitro (Figure 7E, lanes 7–10 as compared to lane 2). The enhancement is specific, since adding a TNRC6C fragment 800–1360 that overlaps the 1260–1690 fragment but cannot bind PABP inhibited rather than enhanced the deadenylation in the same assays (lanes 11–14 as compared to lane 2). These data demonstrate the key role that the PABP-GW182 interaction plays in miRNA-mediated deadenylation.

DISCUSSION

In this report we used a mammalian cell-free extract to demonstrate that miRNAs mediate deadenylation of a target mRNA subsequent to initial inhibition of cap-dependent translation.

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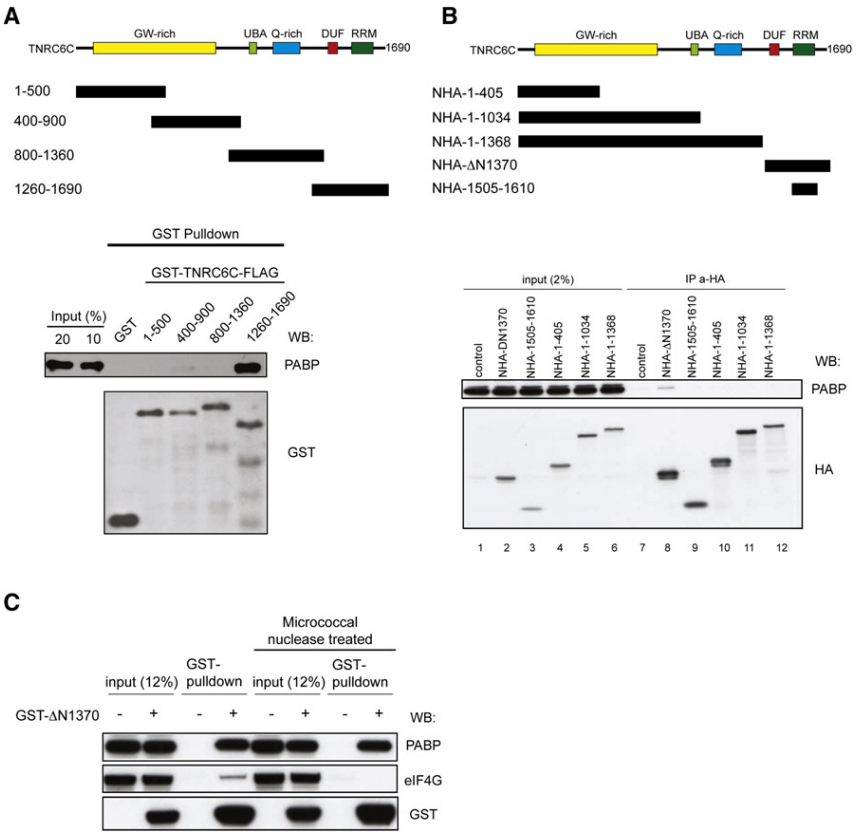

Figure 6. The C Terminus of TNRC6C Directly Binds PABP

(A) Schematic representation of human TNRC6C and GST- and FLAG-tagged recombinant protein fragments. Western blot analysis of GST pulldowns of PABP incubated with GST or various fragments of GST-TNRC6C-FLAG and probed with anti-PABP and anti-GST antibodies.

(B) Schematic representation of human TNRC6C HA-tagged fragments transfected into HEK293 cells. Cell extracts of HEK293 cells, transiently expressing the indicated fusion proteins, were incubated with Anti-HA Affinity Matrix (Roche), and immunoprecipitated proteins were analyzed by western blotting using the indicated antibodies. Inputs represent 1% of the cell extract used for IP. Nontransfected cells served as a control.

(C) Cell extracts of HEK293 cells transiently expressing GST-ΔN1370 were pulled down using glutathione Sepharose resin in the presence or absence of micrococcal nuclease. GST pulldowns were analyzed by western blotting using anti-PABP, anti-eIF4G, and anti-GST antibodies. Nontransfected cells served as a control.

Biochemical methods and functional assays in this in vitro system elucidated some of the protein and RNA requirements for miRNA-mediated mRNA deadenylation.

CAF1 and CCR4 Are Mammalian miRISC-Associated Deadenylases

miRNAs have previously been implicated in the deadenylation of targeted mRNAs in mammalian cells (Wu et al., 2006). One major deadenylase complex in mammals is the multisubunit CCR4-NOT complex, which contains two proteins having deadenylase activity, CCR4 and CAF1 (Yamashita et al., 2005; Zheng et al., 2008). Although CCR4 is the active deadenylase in the yeast CCR4-NOT complex (Tucker et al., 2001, 2002), mammalian CAF1 is also a processive deadenylase that regulates mRNA decay (Bianchin et al., 2005; Funakoshi et al., 2007; Schwede et al., 2008; Viswanathan et al., 2004; Zheng et al., 2008). Previous work carried out in *Drosophila* S2 cells demonstrated that the CCR4-NOT complex (which contains CAF1) facilitates miRNA-mediated deadenylation (Behm-Ansmant et al., 2006). Our results bolster these findings and show that the association of the miRISC with the deadenylase complex is conserved between *Drosophila* and mammals. Moreover, we provide biochemical evidence that both deadenylases physically interact with the mammalian miRISC, and that CAF1 activity is responsible, at least in part, for miRNA-mediated deadenylation. As CAF1 interacts with both Ago1 and Ago2 in HEK293 cells, this suggests that both Ago proteins are involved in facilitating miRNA-mediated deadenylation in mammals.

PABP as a Coactivator of miRNA-Mediated Deadenylation

Studies aimed at characterizing miRISC-associated proteins have previously identified PABP by mass spectrometry of immunoprecipitates not subjected to ribonuclease treatment (Hock et al., 2007; Landthaler et al., 2008; Zhang et al., 2007). We show that PABP is required for miRNA-mediated deadenylation and physically interacts with the miRISC via direct contact with GW182. Moreover, our results suggest that PABP-GW182 interaction is required to facilitate miRNA-mediated deadenylation. Previous studies have shown that PABP augments the activity of different deadenylases. PABP helps to recruit the PAN2/3 deadenylase complex to poly(A) tails in both yeast and mammalian systems via a direct interaction between the PAN3 subunit and the PABP C-terminal domain (Lowell et al., 1992; Uchida et al., 2004). The PABP C-terminal domain directly binds to the CAF1-interacting protein Tob, which may contribute to the CCR4-CAF1-mediated deadenylation of some mRNAs (Ezzeddine et al., 2007; Simon and Seraphin, 2007). In contrast to these modes of PABP-dependent deadenylation, our data show that PABP is not required for recruitment of either the miRISC or the miRISC-associated deadenylase complex to miRNA-targeted mRNAs (Figure 2D). Furthermore, while CAF1 is recruited to the miRISC, Tob is not (Figure 2D).

PABP-GW182 Interaction and miRNA-Mediated Repression

GW182 is a core component of the miRISC and is critical for miRNA-mediated repression. All three mammalian paralogs of GW182 (TNRC6A, TNRC6B, and TNRC6C) are involved in miRNA-mediated repression (Jakymiw et al., 2007; Lazzaretti et al., 2009; Liu et al., 2005; Till et al., 2007; Zipprich et al.,

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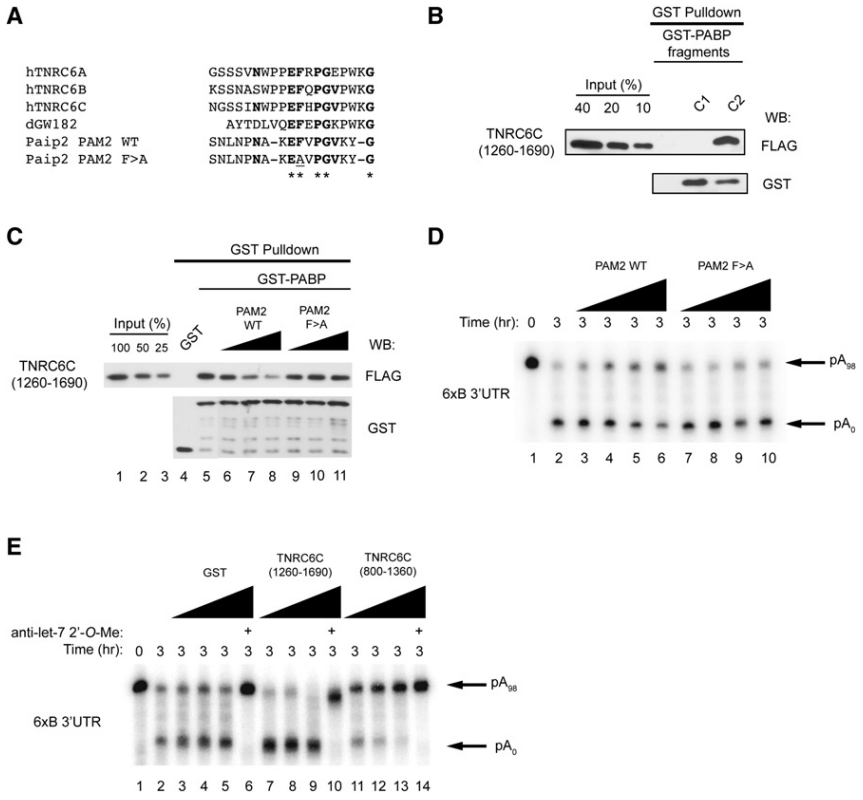

Figure 7. GW182 Binding to PABP Is Required for miRNA-Mediated Deadenylation

(A) Alignment of GW182 DUF sequences with Paip2 PAM2 motif.

(B) Western blot analysis of GST pulldowns of TNRC6C (1260-1690)-FLAG incubated with various C-terminal (C1 and C2) fragments of GST-PABP and probed with anti-FLAG and anti-GST antibodies.

(C) Western blot analysis of GST pulldowns of GST-PABP incubated with TNRC6C (1260-1690)-FLAG and/or wild-type or mutant PAM2 peptide and probed with anti-FLAG and anti-GST antibodies.

(D) 6xB-3′UTR RNA deadenylation in Krebs extract in the presence of increasing concentrations (1, 10, 50, and 100 μM) of wild-type or mutant (F > A) Paip2 PAM2 peptides.

(E) 6xB-3′UTR RNA deadenylation in Krebs extract, as determined by autoradiography, in the presence of increasing concentrations (0.5, 1.0, and 2.0 μg per reaction) of GST, TNRC6C (1260-1690), or TNRC6C (800-1360).

2009). Tethering experiments of different TNRC6C fragments to a reporter mRNA demonstrated that a C-terminal fragment of TNRC6C, harboring both the DUF and RRM domains, represses protein synthesis as effectively (>10-fold) as a full-length TNRC6C protein (Zipprich et al., 2009). Experiments performed with *Drosophila* GW182 protein in S2 cells also pointed to the importance of the protein C terminus for repression of protein synthesis (Chekulaeva et al., 2009; Eulalio et al., 2009b), implying functional conservation. We demonstrate that the mammalian GW182 C terminus directly binds PABP in an RNA-independent manner. Importantly, we show that GW182-PABP contact through the PABP C2 domain is required for efficient miRNA-mediated deadenylation. Because PABP functions as a bona fide translation initiation factor (Kahvejian et al., 2005), these data provide evidence that the mammalian miRISC directly interacts with a component of the translation initiation machinery. It is possible that PABP binding to GW182 may compete with eIF4G binding, as adding an eIF4G fragment that binds to the N terminus of PABP blocks miRNA-mediated deadenylation in vitro (Figure 5). In addition, it is conceivable that PABP binding to GW182 may function to juxtapose the poly(A) tail against the miRISC-associated deadenylase complex (see model, Figure 8). Although intriguing, these possibilities are still speculative at this point and await future experimental validation.

Temporal Mode of miRNA Action

miRNAs inhibit translation and/or mediate deadenylation and decay of target mRNAs (Filipowicz et al., 2008). In previous studies, mostly carried out in cultured cells, it was impossible to determine the earliest events leading to the miRNA-mediated repression (Behm-Ansmant et al., 2006; Giraldez et al., 2006; Humphreys et al., 2005; Petersen et al., 2006; Pillai et al., 2005). We have demonstrated that miRNAs inhibit translation initiation as early as 15–40 min after addition of mRNA to the Krebs extract (Mathonnet et al., 2007, and this study). As shown in this work, the miRNA-induced deadenylation of targeted mRNAs in vitro is a slower event, which follows the miRISC-mediated repression of translation initiation. These results indicate that miRNAs can function by two complementary and likely sequential mechanisms, first by inhibiting initiation of cap-dependent translation, which is then followed by the deadenylation of the target mRNA. As miRNA-mediated repression in Krebs extract further increases between 1 and 2 hr of incubation and miRNA-mediated translation repression is partially inhibited in CAF1-depleted extract, it is possible that deadenylation has an additional repressive effect supplementary to the initial inhibition of cap-dependent translation.

EXPERIMENTAL PROCEDURES

DNA Constructs and Protein Purification

Myc-Ago1 and Ago2 DNA constructs have been described (Liu et al., 2005). HA-CAF1 wild-type and HA-CAF1 D40A constructs have been described (Zheng et al., 2008). pGST-Paip2 and pGST-PABP full-length and fragments C1 and C2 have been described (Khaleghpour et al., 2001). Plasmids encoding wild-type and mutant HA-fused CAF1 proteins were transfected into HeLa cells and proteins were eluted with HA peptide (Anaspec). Eluted proteins were analyzed by western blot analysis using CAF1 and Ago2 antibodies. The plasmids pCI-NHA-1-405, pCI-NHA-1-1034, pCI-NHA-1-1368, pCI-NHA-ΔN1370, and pCI-NHA-1505-1610 were previously described (Zipprich et al., 2009). To generate the plasmid pEBG-ΔN1370, the sequence encoding a C-terminal part of TNRC6C was PCR amplified using CCCGTCG GATCCCGTGCCAAATCTGACAG TGA and AACCCTCACTAAAGGGAAGC

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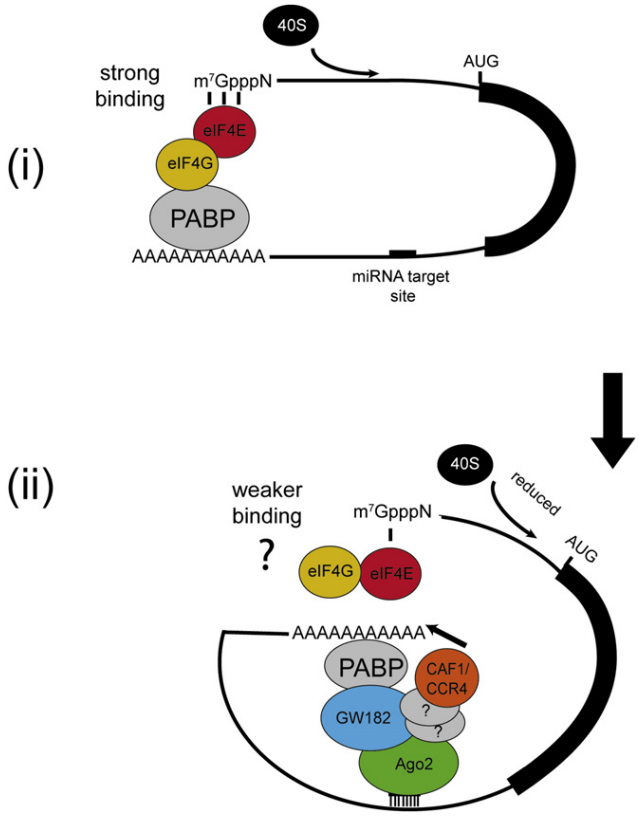

Figure 8. Model for Temporal Stepwise miRNA-Mediated Gene Silencing

(i) mRNA circularization via eIF4G-PABP interaction stimulates cap-dependent translation by enhancing eIF4E's binding to the mRNA 5′ cap structure (strong binding [Kahvejian et al., 2005]).

(ii) miRISC binds to its target site in the 3′UTR. GW182 binds to PABP, hypothetically inhibiting its interaction with eIF4G, thereby repressing cap-dependent translation by decreasing eIF4E's binding to the 5′ cap structure (weaker binding), and sequestering the poly(A) tail into the vicinity of CAF1 and CCR4 deadenylases (illustrated by an arrow) to facilitate deadenylation of the mRNA. The interaction between CAF1/CCR4 and Ago2 is probably indirect through other proteins (depicted as question marks).

oligonucleotides as primers and pCI-NHA-ΔN1370 as template. The fragment was digested with BamHI and NotI and inserted into pEBG-Piwi (Tahbaz et al., 2004; Zipprich et al., 2009) precut with BamHI and NotI.

To generate the plasmids used for bacterial expression of GST-FLAG-TNRC6C fragments (pGST-TNRC6C1-500, pGST-TNRC6C400-900, pGST-TNRC6C800-1360, and pGST-TNRC6C1260-1690), the appropriate DNA was amplified by PCR using pCI-NHA-TNRC6C as template and the following primer pairs: GGCCGGCCGTCGACTCATGGC TACAGGGAGTGCCCAGGG CAAC and CTTGTCATCGTCGTCCTTGTAGTCAGCA CTGTTCATGATGGAC CCATCGTTCTTC (1–500), GGCCGGCCGTCGACTCAGTG ATGGTTCTGGC AACCACAATGAAG and CTTGTCATCGTCGTCCTTGTAGTCAG CCACGTC CCCTTCTTCATCCTCCCACTG (400–900), GGCCGGCCGTCGACTCTC ATC AGGCTGGGGAGAAATGCCTAATG and CTTGTCATCGTCGTCCTTGTAGTC AGCGGGAGGACTGGCTGGTGACTCACTGTTC (800–1360), and GGCCGGC CGTC GACTCAACACCTTTGCTCCTTACCCTCTCGCTG and CTTGTCATCG TCGTCCTT GTAGTCAGCCAGGGACTCCCCGCTGAGCAGGTCCCC (1260–1690). These PCR products were subjected to a second round of PCR amplification using the original forward primer and a new reverse primer (CCGGC CGCGGCCGCTCACTTGTCATCGT CGTCCTTGTAGTCAGC). The product of these PCR reactions was then gel purified, digested with SalI and NotI restriction enzymes, and ligated into similarly digested pGEX-6P-1 expression vector (GE Healthcare). This strategy resulted in constructs that express the appropriate fragment of TNRC6C carrying N-terminal GST and C-terminal FLAG epitopes. TNRC6C fragments were expressed in Rosetta-2(DE3) *E. coli* cells (EMD Biosciences) and purified by two sequential affinity chromatography steps, first over glutathione Sepharose 4B resin (GE), followed by M2-FLAG affinity resin (Sigma).

In Vitro Transcription

Plasmids that lack or contain six let-7 target sites (RL and RL-6xB, respectively) were described (Pillai et al., 2005). A 98 base pair poly(A) sequence was added to the 3′UTR of both constructs. RL-6xBMut was constructed as previously published (Mathonnet et al., 2007; Pillai et al., 2005). A 150 base pair poly(A) sequence was synthesized (IDT) and added to the 3′UTR of RL-6xB (RL-6xB-pA*). For in vitro transcription, plasmids were linearized with ApaI and filled in using the Klenow fragment. Transcription reactions were performed using MAXIscript In Vitro Transcription Kit (Ambion) in 20 μl at 37°C according to the manufacturer's protocol in the presence of the cap analog m^7(3′-O-methyl)(5′)Gppp(5′)G (anti-reverse cap analog, ARCA; New England Biolabs). ApppG-capped mRNAs were synthesized using ApppG (New England Biolabs) instead of ARCA. 6xB-3′UTR and 6xBMUT-3′UTR transcripts were generated from PCR products derived from RL-6xB-pA and RL-6xBMUT-pA templates and T7-3′UTR (GGCGCCTAATACGACTCACTAT AGGGGTAAGTACATCAAGAGCTTCG) and Oligo 3R(−) (GGTGACACTATAGA ATAGGGCCC) primers. PCR products were digested with ApaI and filled in using the Klenow fragment. To synthesize radiolabeled mRNAs, UTP was substituted with [α-^{32}P]UTP (800 Ci/mmol, 10 mCi/ml; PerkinElmer) according to the manufacturer's protocol. The mRNA was loaded on a mini Quick Spin RNA Column (Roche) to remove unincorporated nucleotides.

In Vitro Translation Assays

Krebs-2 ascites cell extract was prepared as previously described (Svitkin and Sonenberg, 2004). Translation reactions were performed in a total volume of 10 μl at 30°C. A typical reaction mixture contained 7 μl extract, 1 μl mRNA, and, where indicated, 2′-*O*-Me oligonucleotide complementary to let-7a or miR-122a or poly$(A)_{30}$ oligonucleotide (Dharmacon) in water. The mixture was preincubated for 20 min at 16°C and then at 30°C for 120 min. When the 2′-*O*-Me oligonucleotide was added, the extract was first incubated at 30°C for 20 min in the absence of mRNA to allow for the annealing of the oligonucleotide with its target miRNA. The reaction was stopped by addition of 20 μl cold 1 × PBS. For time course experiments, the reaction was scaled up to 80 μl, and 10 μl was withdrawn at each time point. Luciferase activity was measured by using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol.

mRNA Stability Assay

Radiolabeled RNA (0.1 ng) was incubated in Krebs-2 ascites in a total volume of 10 μl in the absence or presence of 10 nM let-7 2′-*O*-Me oligonucleotide. Aliquots of the reaction mixture were withdrawn at specific intervals, and the RNA was extracted using TRIzol reagent (Invitrogen) and loaded on a 4% or 4.5% polyacrylamide/urea gel. The gel was dried and analyzed using a Typhoon PhosphorImager (GE Healthcare).

Oligonucleotide Ligation-Mediated Cloning of RNA

Radiolabeled RNA from Krebs extract was extracted with TRIzol and loaded on a 4% polyacrylamide/urea gel. Specific RNA bands were cut from the gel and eluted in 2x proteinase K buffer (100 mM Tris-HCl, pH 8.3; 25 mM EDTA, pH 8.0; 300 mM NaCl; 2% (w/v) SDS), purified and ligated to a miRNA universal linker (NEB) using T4 RNA ligase 1 in the absence of ATP. Ligation products were purified and reverse transcribed with Superscipt III (Invitrogen), and amplified using Titanium DNA polymerase (Clontech). PCR products were cloned and sequenced.

Immunodepletion Assay, GST Pulldown Assay, Western Blotting, and Antibodies

protein G-Sepharose (GE Healthcare) (20 μl) was washed and incubated in 100 μl of Krebs extract with 6 μg of either mouse monoclonal anti-HA

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(Covance), rabbit anti-HA (Sigma), mouse monoclonal anti-Ago2 (Wako Chemicals), or affinity-purified rabbit polyclonal anti-CAF1 with gentle agitation for 2 hr at 4°C. The resin was then centrifuged at 500 × g, and the supernatant was collected. GST pulldown assays of Krebs extract have been described (Kahvejian et al., 2005). Antibodies and their working dilutions for western blotting were as follows: rabbit polyclonal anti-Ago2, 1:1000; rabbit polyclonal anti-PABP, 1:1000 (Cell Signaling Technologies); mouse monoclonal anti-Actin, 1:5000 (Sigma); mouse monoclonal anti-FLAG, 1:5000 (Sigma); mouse monoclonal anti-CAF1, 1:1000; mouse monoclonal anti-Tob 4B1, 1:1000 (Sigma); and mouse monoclonal anti-CCR4, 1:1000. For the GST pulldown assay with HEK293 cell extracts, cells were lysed with 50 mM Tris-HCl (pH 7.5) containing 150 mM KCl, 0.5% Triton X-100, 2 mM DTT, and complete EDTA-free protease inhibitor cocktail (Roche). The cleared lysate was incubated with glutathione Sepharose 4B (GE Healthcare) followed by washing with 50 mM Tris-HCl (pH 7.5), containing 150 mM KCl, 0.1% Triton X-100, 2 mM DTT, and complete protease inhibitor cocktail (Roche). Proteins associated with glutathione Sepharose beads were eluted with 50 mM glutathione in the same buffer as used for washing the beads and analyzed by western blotting using anti-PABP1 antibody (Cell Signaling Technology), anti-eIF4GI antibody (Gradi et al., 1998), and anti-GST antibody (GE Healthcare). To examine RNA dependence of protein-protein interactions, cleared lysates were treated with micrococcal nuclease (Roche) (10 μg/ml) for 25 min at room temperature in the presence of 1 mM $CaCl_2$ before incubation with glutathione Sepharose 4B beads.

Anti-let-7 2′-*O*-Me Oligonucleotide Biotin Pulldown Assay

M-280 streptavidin magnetic Dynabeads (Invitrogen) were washed three times in buffer D (25 mM HEPES-KOH [pH 7.3], 2 mM $MgCl_2$, 50 mM KCl, 75 mM KOAc) and resuspended in buffer D with 2 mM DTT and 1 M NaCl and incubated with biotin-labeled anti-let-7 2′-*O*-Me, anti-miR122 2′-*O*-Me, or anti-miR35 2′-*O*-Me oligonucleotide (Integrated DNA Technologies) for 60 min at 4°C. 2′-*O*-Me-bound beads were washed three times in buffer D and then incubated in aliquots of Krebs extract containing protease inhibitors at 30°C for 60 min. Beads were washed three times in buffer D with 0.5% NP-40 and boiled in SDS sample buffer and analyzed by SDS-PAGE and western blotting.

Cell Lines

HEK293 cells were transfected with Myc-Ago1 and Myc-Ago2 DNA constructs using LT-1 transfection reagent according to the manufacturer's instructions (Mirus). All constructs contain a G418 resistance cassette. Stable transfectants were selected with 500 μg/ml G418 (Roche) for at least 2 weeks prior to being used in experiments.

Other HEK293T cells were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL) supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal calf serum (FCS). Transfections were performed in 10 cm cell culture dishes with ~60% confluent cells using Nanofectin (PAA Laboratories), following the manufacturer's instructions. For mass spectrometry analysis, cells in one 10 cm cell culture dish were transfected with 6 μg of the plasmid pEBG-DN1370. For IP experiments, cells in 10 cm cell culture dishes were transfected with 6 μg of the plasmids pCI-NHA-1505-1610 and pCI-NHA-ΔN1370 and 20 μg of the plasmids pCI-NHA-1-405, pCI-NHA-1-1034, and pCI-NHA-1-1368. For the GST pulldown experiment cells, 10 cm cell culture dishes were transfected with 4 μg of the plasmid pEBG-ΔN1370.

Mass Spectrometry Analysis

Cells were lysed with 50 mM Tris-HCl (pH 7.5) containing 150 mM KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5% Triton X-100, 2 mM DTT, and EDTA-free protease inhibitor cocktail (Roche). The cleared lysate was incubated with glutathione Sepharose 4B (GE Healthcare) followed by washing with 50 mM Tris-HCl (pH 7.5) containing 150 mM KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5% Triton X-100, 2 mM DTT, and EDTA-free protease inhibitor cocktail (Roche). Cysteine residues of proteins associated with the beads were reduced and alkylated prior to gel separation. The Coomassie-stained bands were digested with trypsin, and tryptic peptides were analyzed by nano-HPLC (Agilent 1100 nanoLC system, Agilent Technologies, Santa Clara, CA) coupled to a 4000 Q TRAP mass spectrometer (Applied Biosystems, Foster City, CA). Peptides were identified searching UniProt database (version 13.8) restricted to *human* using Mascot (version 2.1, Matrix Science, London).

MuDPIT and Coimmunoprecipitation Analysis

Samples were prepared as follows: HEK293 cells were harvested and washed with phosphate-buffered saline (PBS). Cells were washed once in hypotonic lysis buffer (10 mM Tris-HCl [pH 7.5], 10 mM KCl, 1.5 mM $MgCl_2$, 5 mM DTT, and EDTA-free protease inhibitor cocktail [Roche]) and allowed to swell for 20 min on ice prior to homogenization. Cell extracts were centrifuged in a tabletop centrifuge at 10,000 rpm for 30 min at 4°C to clarify the lysate. The salt concentration in the extract was raised to 100 mM KCl. To immunoprecipitate Ago and Ago-interacting proteins, Myc-agarose beads (Sigma) were added to the extract and allowed to incubate for 6 hr with gentle rotation. Immunoprecipitates were washed (wash buffer, 10 mM Tris-HCl [pH 7.5], 100 mM KCl, 1.5 mM $MgCl_2$, 5 mM DTT, and EDTA-free protease inhibitor cocktail [Roche]) four times for 30 min each. Immunocomplexes were eluted from Myc-agarose beads by two serial washes in elution buffer (100 mM Tris-HCl [pH 8.0], 8M urea). Proteins in eluates were precipitated with trichloroacetic acid and submitted for MuDPIT analysis (Washburn et al., 2001). Samples analyzed for coimmunoprecipitation of Ago and Ago-interacting proteins from HEK293 cells were prepared as above. In cases in which immunoprecipitates were subjected to RNase A treatment, immunoprecipitation was performed as described, but the next to last washing step was done in the presence of RNase A (10 units/ml in wash buffer). Samples were washed an additional two times prior to SDS-PAGE and western blot analysis. Samples analyzed for coimmunoprecipitation of Ago2- and CAF1-interacting proteins from Krebs extracts were prepared as follows: Krebs extracts were treated with micrococcal nuclease (Roche) in the presence of $CaCl_2$ for 30 min at 20°C and subsequently with EGTA as previously described (Svitkin and Sonenberg, 2004). Krebs extracts were then mixed with protein G Dynabeads (Invitrogen) already bound to either mouse monoclonal anti-HA (Covance), rabbit anti-HA (Sigma), mouse monoclonal anti-Ago2 (Wako Chemicals), or affinity-purified rabbit anti-CAF1 and gently mixed at 30°C for 60 min. Immunoprecipitates were washed five times with buffer D containing 0.5% NP-40 prior to SDS-PAGE and western blot analysis. For HA epitope IP reactions, cells were lysed with 50 mM Tris-HCl (pH 7.5) containing 150 mM KCl, 0.5% Triton X-100, 2 mM DTT, and protease inhibitor cocktail (Roche). The cleared lysate was incubated with Anti-HA Affinity Matrix (Roche). After washing with 10 mM Tris-HCl (pH 7.5) containing 200 mM KCl, proteins associated with the beads were analyzed by western blotting using anti-HA 3F10 antibody (Roche) and PABP1 antibody (Cell Signaling Technology) (Polacek et al., 2009).

SUPPLEMENTAL DATA

Supplemental Data include ten figures and can be found with this article online at http://www.cell.com/molecular-cell/supplemental/S1097-2765(09)00550-4.

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REFERENCES

Bartel, D.P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell *116*, 281–297.

Behm-Ansmant, I., Rehwinkel, J., Doerks, T., Stark, A., Bork, P., and Izaurralde, E. (2006). mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. Genes Dev. *20*, 1885–1898.

Bianchin, C., Mauxion, F., Sentis, S., Seraphin, B., and Corbo, L. (2005). Conservation of the deadenylase activity of proteins of the Caf1 family in human. RNA *11*, 487–494.

Bordeleau, M.E., Mori, A., Oberer, M., Lindqvist, L., Chard, L.S., Higa, T., Belsham, G.J., Wagner, G., Tanaka, J., and Pelletier, J. (2006). Functional characterization of IRESes by an inhibitor of the RNA helicase eIF4A. Nat. Chem. Biol. *2*, 213–220.

Chekulaeva, M., Filipowicz, W., and Parker, R. (2009). Multiple independent domains of dGW182 function in miRNA-mediated repression in Drosophila. RNA *15*, 794–803. Published online March 20, 2009. 10.1261/rna.1364909.

Chendrimada, T.P., Gregory, R.I., Kumaraswamy, E., Norman, J., Cooch, N., Nishikura, K., and Shiekhattar, R. (2005). TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. Nature *436*, 740–744.

Chendrimada, T.P., Finn, K.J., Ji, X., Baillat, D., Gregory, R.I., Liebhaber, S.A., Pasquinelli, A.E., and Shiekhattar, R. (2007). MicroRNA silencing through RISC recruitment of eIF6. Nature *447*, 823–828.

Ding, X.C., and Grosshans, H. (2009). Repression of C. elegans microRNA targets at the initiation level of translation requires GW182 proteins. EMBO J. *28*, 213–222.

Ding, L., and Han, M. (2007). GW182 family proteins are crucial for microRNA-mediated gene silencing. Trends Cell Biol. *17*, 411–416.

Eulalio, A., Huntzinger, E., and Izaurralde, E. (2008a). Getting to the root of miRNA-mediated gene silencing. Cell *132*, 9–14.

Eulalio, A., Huntzinger, E., and Izaurralde, E. (2008b). GW182 interaction with Argonaute is essential for miRNA-mediated translational repression and mRNA decay. Nat. Struct. Mol. Biol. *15*, 346–353.

Eulalio, A., Huntzinger, E., Nishihara, T., Rehwinkel, J., Fauser, M., and Izaurralde, E. (2009a). Deadenylation is a widespread effect of miRNA regulation. RNA *15*, 21–32.

Eulalio, A., Tritschler, F., Buttner, R., Weichenrieder, O., Izaurralde, E., and Truffault, V. (2009b). The RRM domain in GW182 proteins contributes to miRNA-mediated gene silencing. Nucleic Acids Res. *37*, 2974–2983. Published online March 18, 2009. 10.1093/nar/gkp173.

Ezzeddine, N., Chang, T.-C., Zhu, W., Yamashita, A., Chen, C.-Y.A., Zhong, Z., Yamashita, Y., Zheng, D., and Shyu, A.-B. (2007). Human TOB, an antiproliferative transcription factor, is a poly(A)-binding protein-dependent positive regulator of cytoplasmic mRNA deadenylation. Mol. Cell. Biol. *27*, 7791–7801.

Filipowicz, W., Bhattacharyya, S.N., and Sonenberg, N. (2008). Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat. Rev. Genet. *9*, 102–114.

Funakoshi, Y., Doi, Y., Hosoda, N., Uchida, N., Osawa, M., Shimada, I., Tsujimoto, M., Suzuki, T., Katada, T., and Hoshino, S. (2007). Mechanism of mRNA deadenylation: evidence for a molecular interplay between translation termination factor eRF3 and mRNA deadenylases. Genes Dev. *21*, 3135–3148.

Giraldez, A.J., Mishima, Y., Rihel, J., Grocock, R.J., Van Dongen, S., Inoue, K., Enright, A.J., and Schier, A.F. (2006). Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. Science *312*, 75–79.

Gradi, A., Svitkin, Y.V., Imataka, H., and Sonenberg, N. (1998). Proteolysis of human eukaryotic translation initiation factor eIF4GII, but not eIF4GI, coincides with the shutoff of host protein synthesis after poliovirus infection. Proc. Natl. Acad. Sci. USA *95*, 11089–11094.

Groft, C.M., and Burley, S.K. (2002). Recognition of eIF4G by rotavirus NSP3 reveals a basis for mRNA circularization. Mol. Cell *9*, 1273–1283.

Gu, S., Jin, L., Zhang, F., Sarnow, P., and Kay, M.A. (2009). Biological basis for restriction of microRNA targets to the 3′ untranslated region in mammalian mRNAs. Nat. Struct. Mol. Biol. *16*, 144–150.

Hock, J., Weinmann, L., Ender, C., Rudel, S., Kremmer, E., Raabe, M., Urlaub, H., and Meister, G. (2007). Proteomic and functional analysis of Argonaute-containing mRNA-protein complexes in human cells. EMBO Rep. *8*, 1052–1060.

Humphreys, D.T., Westman, B.J., Martin, D.I., and Preiss, T. (2005). MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function. Proc. Natl. Acad. Sci. USA *102*, 16961–16966.

Hutvagner, G., Simard, M.J., Mello, C.C., and Zamore, P.D. (2004). Sequence-specific inhibition of small RNA function. PLoS Biol. *2*, E98. 10.1371/journal.pbio.0020098.

Imataka, H., Gradi, A., and Sonenberg, N. (1998). A newly identified N-terminal amino acid sequence of human eIF4G binds poly(A)-binding protein and functions in poly(A)-dependent translation. EMBO J. *17*, 7480–7489.

Jakymiw, A., Pauley, K.M., Li, S., Ikeda, K., Lian, S., Eystathioy, T., Satoh, M., Fritzler, M.J., and Chan, E.K. (2007). The role of GW/P-bodies in RNA processing and silencing. J. Cell Sci. *120*, 1317–1323.

Kahvejian, A., Svitkin, Y.V., Sukarieh, R., M'Boutchou, M.N., and Sonenberg, N. (2005). Mammalian poly(A)-binding protein is a eukaryotic translation initiation factor, which acts via multiple mechanisms. Genes Dev. *19*, 104–113.

Karim, M.M., Svitkin, Y.V., Kahvejian, A., De Crescenzo, G., Costa-Mattioli, M., and Sonenberg, N. (2006). A mechanism of translational repression by competition of Paip2 with eIF4G for poly(A) binding protein (PABP) binding. Proc. Natl. Acad. Sci. USA *103*, 9494–9499.

Khaleghpour, K., Kahvejian, A., De Crescenzo, G., Roy, G., Svitkin, Y.V., Imataka, H., O'Connor-McCourt, M., and Sonenberg, N. (2001). Dual interactions of the translational repressor Paip2 with poly(A) binding protein. Mol. Cell. Biol. *21*, 5200–5213.

Kozlov, G., De Crescenzo, G., Lim, N.S., Siddiqui, N., Fantus, D., Kahvejian, A., Trempe, J.F., Elias, D., Ekiel, I., Sonenberg, N., et al. (2004). Structural basis of ligand recognition by PABC, a highly specific peptide-binding domain found in poly(A)-binding protein and a HECT ubiquitin ligase. EMBO J. *23*, 272–281.

Lagos-Quintana, M., Rauhut, R., Yalcin, A., Meyer, J., Lendeckel, W., and Tuschl, T. (2002). Identification of tissue-specific microRNAs from mouse. Curr. Biol. *12*, 735–739.

Landthaler, M., Gaidatzis, D., Rothballer, A., Chen, P.Y., Soll, S.J., Dinic, L., Ojo, T., Hafner, M., Zavolan, M., and Tuschl, T. (2008). Molecular characterization of human Argonaute-containing ribonucleoprotein complexes and their bound target mRNAs. RNA *14*, 2580–2596.

Lazzaretti, D., Tournier, I., and Izaurralde, E. (2009). The C-terminal domains of human TNRC6A, TNRC6B, and TNRC6C silence bound transcripts independently of Argonaute proteins. RNA *15*, 1059–1066.

Liu, J., Rivas, F.V., Wohlschlegel, J., Yates, J.R., 3rd, Parker, R., and Hannon, G.J. (2005). A role for the P-body component GW182 in microRNA function. Nat. Cell Biol. *7*, 1261–1266.

Lowell, J.E., Rudner, D.Z., and Sachs, A.B. (1992). 3′-UTR-dependent deadenylation by the yeast poly(A) nuclease. Genes Dev. *6*, 2088–2099.

Lytle, J.R., Yario, T.A., and Steitz, J.A. (2007). Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5′ UTR as in the 3′ UTR. Proc. Natl. Acad. Sci. USA *104*, 9667–9672.

Maroney, P.A., Yu, Y., Fisher, J., and Nilsen, T.W. (2006). Evidence that microRNAs are associated with translating messenger RNAs in human cells. Nat. Struct. Mol. Biol. *13*, 1102–1107.

Mathonnet, G., Fabian, M.R., Svitkin, Y.V., Parsyan, A., Huck, L., Murata, T., Biffo, S., Merrick, W.C., Darzynkiewicz, E., Pillai, R.S., et al. (2007). MicroRNA inhibition of translation initiation in vitro by targeting the cap-binding complex eIF4F. Science *317*, 1764–1767.

Mauxion, F., Faux, C., and Seraphin, B. (2008). The BTG2 protein is a general activator of mRNA deadenylation. EMBO J. *27*, 1039–1048.

Meister, G., Landthaler, M., Peters, L., Chen, P.Y., Urlaub, H., Luhrmann, R., and Tuschl, T. (2005). Identification of novel argonaute-associated proteins. Curr. Biol. *15*, 2149–2155.

Molnar, A., Schwach, F., Studholme, D.J., Thuenemann, E.C., and Baulcombe, D.C. (2007). miRNAs control gene expression in the single-cell alga Chlamydomonas reinhardtii. Nature *447*, 1126–1129.

Nottrott, S., Simard, M.J., and Richter, J.D. (2006). Human let-7a miRNA blocks protein production on actively translating polyribosomes. Nat. Struct. Mol. Biol. *13*, 1108–1114.

Olsen, P.H., and Ambros, V. (1999). The lin-4 regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation. Dev. Biol. *216*, 671–680.

Petersen, C.P., Bordeleau, M.E., Pelletier, J., and Sharp, P.A. (2006). Short RNAs repress translation after initiation in mammalian cells. Mol. Cell *21*, 533–542.

Pillai, R.S., Bhattacharyya, S.N., Artus, C.G., Zoller, T., Cougot, N., Basyuk, E., Bertrand, E., and Filipowicz, W. (2005). Inhibition of translational initiation by Let-7 MicroRNA in human cells. Science *309*, 1573–1576.

Polacek, C., Friebe, P., and Harris, E. (2009). Poly(A)-binding protein binds to the non-polyadenylated 3′ untranslated region of dengue virus and modulates translation efficiency. J. Gen. Virol. *90*, 687–692.

Rehwinkel, J., Behm-Ansmant, I., Gatfield, D., and Izaurralde, E. (2005). A crucial role for GW182 and the DCP1:DCP2 decapping complex in miRNA-mediated gene silencing. RNA *11*, 1640–1647.

Schwede, A., Ellis, L., Luther, J., Carrington, M., Stoecklin, G., and Clayton, C. (2008). A role for Caf1 in mRNA deadenylation and decay in trypanosomes and human cells. Nucleic Acids Res. *36*, 3374–3388.

Simon, E., and Seraphin, B. (2007). A specific role for the C-terminal region of the poly(A)-binding protein in mRNA decay. Nucleic Acids Res. *35*, 6017–6028.

Svitkin, Y.V., and Sonenberg, N. (2004). An efficient system for cap- and poly(A)-dependent translation in vitro. Methods Mol. Biol. *257*, 155–170.

Tahbaz, N., Kolb, F.A., Zhang, H., Jaronczyk, K., Filipowicz, W., and Hobman, T.C. (2004). Characterization of the interactions between mammalian PAZ PIWI domain proteins and Dicer. EMBO Rep. *5*, 189–194.

Thermann, R., and Hentze, M.W. (2007). Drosophila miR2 induces pseudo-polysomes and inhibits translation initiation. Nature *447*, 875–878.

Till, S., Lejeune, E., Thermann, R., Bortfeld, M., Hothorn, M., Enderle, D., Heinrich, C., Hentze, M.W., and Ladurner, A.G. (2007). A conserved motif in Argonaute-interacting proteins mediates functional interactions through the Argonaute PIWI domain. Nat. Struct. Mol. Biol. *14*, 897–903.

Tucker, M., Valencia-Sanchez, M.A., Staples, R.R., Chen, J., Denis, C.L., and Parker, R. (2001). The transcription factor associated Ccr4 and Caf1 proteins are components of the major cytoplasmic mRNA deadenylase in Saccharomyces cerevisiae. Cell *104*, 377–386.

Tucker, M., Staples, R.R., Valencia-Sanchez, M.A., Muhlrad, D., and Parker, R. (2002). Ccr4p is the catalytic subunit of a Ccr4p/Pop2p/Notp mRNA deadenylase complex in Saccharomyces cerevisiae. EMBO J. *21*, 1427–1436.

Uchida, N., Hoshino, S., and Katada, T. (2004). Identification of a human cytoplasmic poly(A) nuclease complex stimulated by poly(A)-binding protein. J. Biol. Chem. *279*, 1383–1391.

Viswanathan, P., Ohn, T., Chiang, Y.-C., Chen, J., and Denis, C.L. (2004). Mouse CAF1 can function as a processive deadenylase/3′-5′-exonuclease in vitro but in yeast the deadenylase function of CAF1 is not required for mRNA poly(A) removal. J. Biol. Chem. *279*, 23988–23995.

Wakiyama, M., Imataka, H., and Sonenberg, N. (2000). Interaction of eIF4G with poly(A)-binding protein stimulates translation and is critical for Xenopus oocyte maturation. Curr. Biol. *10*, 1147–1150.

Wakiyama, M., Takimoto, K., Ohara, O., and Yokoyama, S. (2007). Let-7 microRNA-mediated mRNA deadenylation and translational repression in a mammalian cell-free system. Genes Dev. *21*, 1857–1862.

Wang, B., Love, T.M., Call, M.E., Doench, J.G., and Novina, C.D. (2006). Recapitulation of short RNA-directed translational gene silencing in vitro. Mol. Cell *22*, 553–560.

Wang, B., Yanez, A., and Novina, C.D. (2008). MicroRNA-repressed mRNAs contain 40S but not 60S components. Proc. Natl. Acad. Sci. USA *105*, 5343–5348.

Washburn, M.P., Wolters, D., and Yates, J.R., 3rd. (2001). Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nat. Biotechnol. *19*, 242–247.

Wolters, D.A., Washburn, M.P., and Yates, J.R., 3rd. (2001). An automated multidimensional protein identification technology for shotgun proteomics. Anal. Chem. *73*, 5683–5690.

Wormington, M. (1993). Poly(A) and translation: development control. Curr. Opin. Cell Biol. *5*, 950–954.

Wu, L., Fan, J., and Belasco, J.G. (2006). MicroRNAs direct rapid deadenylation of mRNA. Proc. Natl. Acad. Sci. USA *103*, 4034–4039.

Yamashita, A., Chang, T.C., Yamashita, Y., Zhu, W., Zhong, Z., Chen, C.Y., and Shyu, A.B. (2005). Concerted action of poly(A) nucleases and decapping enzyme in mammalian mRNA turnover. Nat. Struct. Mol. Biol. *12*, 1054–1063.

Zhang, L., Ding, L., Cheung, T.H., Dong, M.Q., Chen, J., Sewell, A.K., Liu, X., Yates, J.R., 3rd, and Han, M. (2007). Systematic identification of C. elegans miRISC proteins, miRNAs, and mRNA targets by their interactions with GW182 proteins AIN-1 and AIN-2. Mol. Cell *28*, 598–613.

Zheng, D., Ezzeddine, N., Chen, C.Y., Zhu, W., He, X., and Shyu, A.B. (2008). Deadenylation is prerequisite for P-body formation and mRNA decay in mammalian cells. J. Cell Biol. *182*, 89–101.

Zipprich, J.T., Bhattacharyya, S., Mathys, H., and Filipowicz, W. (2009). Importance of the C-terminal domain of the human GW182 protein TNRC6C for translational repression. RNA *15*, 781–793. Published online March 20, 2009. 10.1261/rna.1448009.

Appendix B

MiRNA Repression Involves W-containing Motifs

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miRNA repression involves GW182-mediated recruitment of CCR4–NOT through conserved W-containing motifs

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miRNA-mediated repression in animals is dependent on the GW182 protein family. GW182 proteins are recruited to the miRNA repression complex through direct interaction with Argonaute proteins, and they function downstream to repress target mRNA. Here we demonstrate that in human and *Drosophila melanogaster* cells, the critical repressive features of both the N-terminal and C-terminal effector domains of GW182 proteins are Gly/Ser/Thr-Trp (G/S/TW) or Trp-Gly/Ser/Thr (WG/S/T) motifs. These motifs, which are dispersed across both domains and act in an additive manner, function by recruiting components of the CCR4–NOT deadenylation complex. A heterologous yeast polypeptide with engineered WG/S/T motifs acquired the ability to repress tethered mRNA and to interact with the CCR4–NOT complex. These results identify previously unknown effector motifs functioning as important mediators of miRNA-induced silencing in both species, and they reveal that recruitment of the CCR4–NOT complex by tryptophan-containing motifs acts downstream of GW182 to repress mRNAs, including inhibiting translation independently of deadenylation.

MicroRNAs (miRNAs) are small, ~21-nt–long RNAs that post-transcriptionally regulate gene expression in eukaryotes. In animals, miRNAs bind to partially complementary sites in mRNAs, leading to translational repression and mRNA deadenylation and degradation[1–4]. miRNAs function as part of ribonucleoprotein complexes, miRNPs, with Argonaute (AGO) and GW182 family proteins being the crucial components. GW182s interact directly with AGO proteins and function downstream as effectors mediating mRNA repression. Hence, understanding the function of GW182 proteins is critical for understanding miRNA-mediated repression.

GW182 functional regions have been mapped in *D. melanogaster* and mammalian proteins. In *D. melanogaster,* three regions were found to repress tethered mRNA to a similar extent[5]: the N-terminal effector domain (NED) having multiple GW-repeats, the middle Q-rich region, and the C-terminal effector domain (CED) containing the poly(A) binding protein (PABP)-interacting motif 2 (PAM2) and the RNA-recognition motif (RRM). The role of the CED in repression was also previously established by others[6–8]. In mammals, tethering of the three regions mentioned above also represses reporter mRNA, with the major contribution being provided by the CED[9–11]. The mechanism by which GW182 domains repress mRNA function appears to be evolutionarily conserved, as dGW182 can repress mRNA function in mammalian cells, and human TNRC6 proteins (mammals express three counterparts of dGW182: TNRC6A, B and C) act as repressors in *D. melanogaster* cells[5,8,9].

The CED of both human and fly GW182s interacts with PABP, and this interaction, possibly by interfering with the PABP-eIF4G association, promotes target mRNA deadenylation by recruiting, through PABP, the components of the CCR4–NOT deadenylation complex[7,8,12,13]. In addition, others[14–16] have demonstrated the role of CCR4–NOT and PAN2–PAN3 deadenylation complexes in the deadenylation of miRNA targets. It is unclear how GW182 proteins recruit these deadenylase complexes and how translation repression is modulated. One possible model is that the interaction of CED with PABP interferes with the PABP-eIF4G association and reduces translation[7,12,13]. However, interfering with eIF4G-PABP interaction and binding of the CCR4–NOT complex through PABP cannot explain the repression of mRNAs bearing no poly(A) tails (reviewed in refs. 2,3), nor can it explain the repression by GW182 domains other than CED.

Previous work on the fly GW182 and human NED indicated a role for glycine-tryptophan (GW) repeats as effector motifs contributing to miRNA-mediated silencing[17,18]. Here we set out to investigate how the GW182 CED and NED regions bring about mRNA repression. We found that motifs bearing tryptophan residues also in contexts other than GW or WG function as important repressive sequences in the CED, both in human and *D. melanogaster* cells. The effector G/S/TW and WG/S/T motifs in the NED and CED recruit the components of CCR4–NOT and PAN2–PAN3 complexes in a PABP-independent manner to repress function of both poly(A)$^+$ and poly(A)$^-$ mRNAs. These results identify the recruitment of the CCR4–NOT complex

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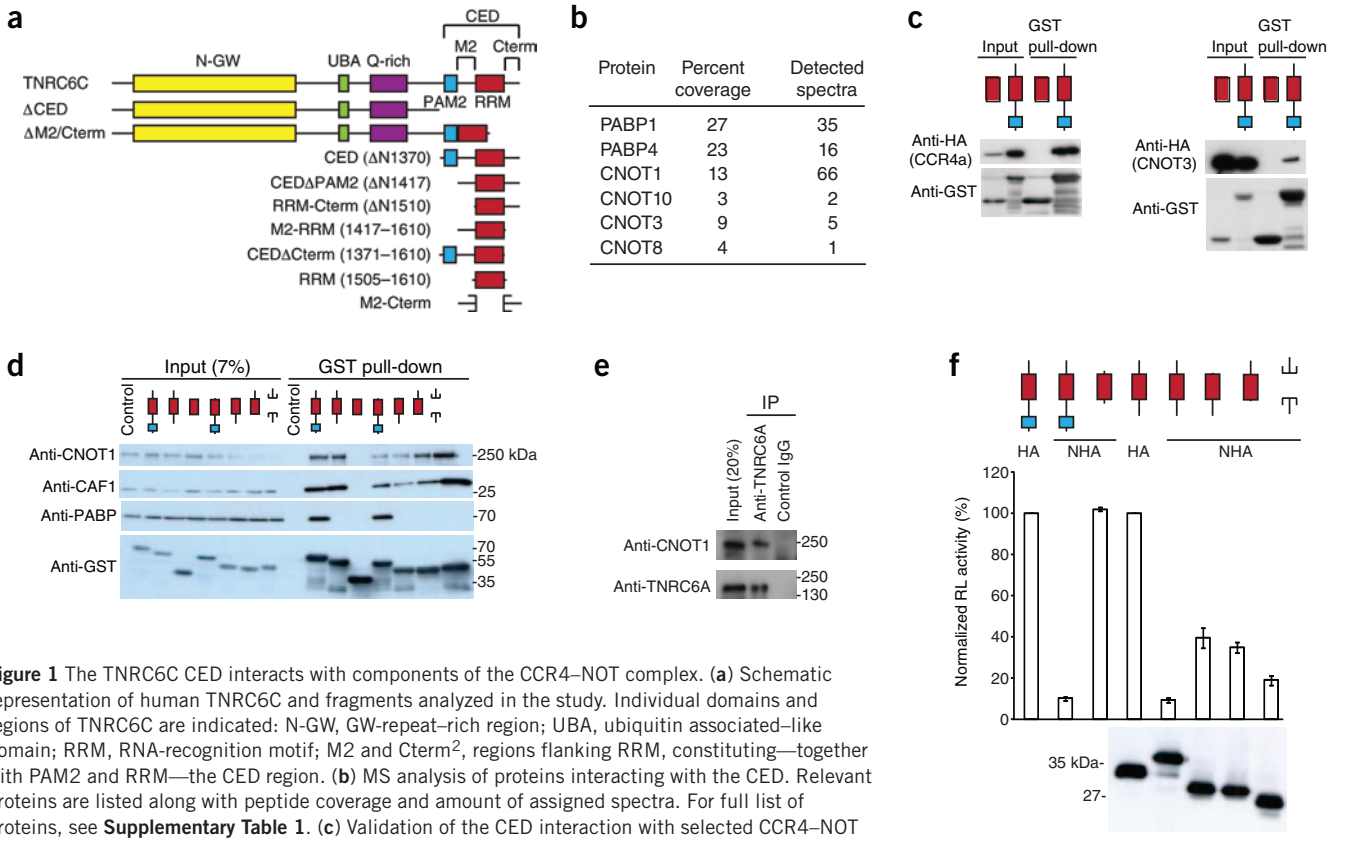

Figure 1 The TNRC6C CED interacts with components of the CCR4–NOT complex. (**a**) Schematic representation of human TNRC6C and fragments analyzed in the study. Individual domains and regions of TNRC6C are indicated: N-GW, GW-repeat–rich region; UBA, ubiquitin associated–like domain; RRM, RNA-recognition motif; M2 and Cterm[2], regions flanking RRM, constituting—together with PAM2 and RRM—the CED region. (**b**) MS analysis of proteins interacting with the CED. Relevant proteins are listed along with peptide coverage and amount of assigned spectra. For full list of proteins, see **Supplementary Table 1**. (**c**) Validation of the CED interaction with selected CCR4–NOT components by GST pull-down assays and western blotting. GST-RRM was used as a control. (**d**) M2 and Cterm regions of the CED interact with components of the CCR4–NOT complex but not with PABP. TNRC6C CED and its subfragments were used for GST pull-down assays. Inputs (7%) and pull-down assays were analyzed by western blotting. Extracts from nontransfected cells were used as controls. (**e**) CNOT1 co-immunoprecipitates with endogenous TNRC6A. (**f**) M2 and Cterm regions of TNRC6C mediate repression of tethered mRNA. HEK293T cells were co-transfected with plasmids encoding NHA-CED or indicated fragments, and RL-5BoxB and firefly luciferase–transfection control (FL-Con) reporters. As negative controls, untethered hemagglutinin-CED (HA-CED) and tethered NHA-RRM (where 'N' stands for tethering λ peptide; see **Supplementary Fig. 2a**) were expressed. Values represent percentage of *Renilla* luciferase activity (normalized to firefly luciferase activity) in the presence of nontethered HA-CED or HA-CEDΔPAM2. In all luciferase assays presented in this work, values represent means ± s.e.m. from three to six experiments. Expression levels of HA- or NHA-fusion proteins were estimated by western blotting.

as a critical event for miRNA-mediated mRNA degradation and translation repression.

RESULTS

The CED of TNRC6C interacts with the CCR4–NOT complex

The CED of human TNRC6C (ΔN1370 fragment; **Fig. 1a**) functions as an autonomous repressive domain, inducing both translational inhibition and mRNA degradation[9]. To elucidate how the CED induces the repression of target mRNAs, it was expressed as a glutathione *S*-transferase (GST) fusion in HEK293T cells and used for pull-down experiments. Among the pulled-down proteins, MS identified several components of the CCR4–NOT complex, including CNOT1, its scaffolding component and CNOT8, a paralog of the deadenylase CNOT7/CAF1 (**Fig. 1b**). PABP was also among the interacting proteins, consistent with previous findings[8,12,13]. The interaction of the CED with different components of CCR4–NOT, either endogenous or ectopically expressed, was confirmed by western blotting (**Fig. 1c**,**d**). Notably, endogenous TNRC6A could also co-immunoprecipitate CNOT1 (**Fig. 1e**).

CAF1 was reported to interact with PABP through the TOB1 protein[19], raising the possibility that the CED recruits CCR4–NOT through PABP. The PAM2 motif (**Fig. 1a** and **Supplementary Fig. 1**) represents the main region in the CED responsible for its interaction with PABP in human cells[8,13]. Deletion of PAM2 (CEDΔPAM2) abrogated the association with PABP without affecting the interaction with CNOT1 and CAF1, suggesting that the CED interaction with CCR4–NOT is PABP-independent (**Fig. 1d**). Moreover, the observed interactions were not mediated by RNA, as they were resistant to micrococcal nuclease treatment (**Fig. 1d** and Online Methods).

To identify sequences in CEDΔPAM2 responsible for the CCR4–NOT interaction, we did pull-down assays with CEDΔPAM2 subfragments (see **Fig. 1a**). Deleting either M2 or C-terminal (Cterm) regions reduced the interaction with CNOT1 and CAF1. The RRM alone did not pull down CNOT1 or CAF1, whereas a fusion of M2 and Cterm regions pulled them down with an efficiency similar to that of CEDΔPAM2 (**Fig. 1d**).

Repression by the CED correlates with CCR4–NOT interaction

The CED domain and its subfragments were tested for activity in repressing protein synthesis in an mRNA-tethering assay (**Supplementary Fig. 2a**). Tethering of the CED or CEDΔPAM2 repressed *Renilla* luciferase expression by approximately ten times, when compared to proteins lacking the N-peptide (**Fig. 1f**). Constructs lacking either M2 or Cterm regions showed reduced repression, whereas the M2-Cterm fusion repressed almost as well as CEDΔPAM2 (**Fig. 1f**). Hence, similarly to their requirement for the interaction with the CCR4–NOT complex, the combined M2 and Cterm regions are sufficient for effective mRNA repression[8].

When analyzed in the context of full-length TNRC6C, deletion of M2 and Cterm regions alleviated mRNA repression to a level comparable to that seen when the entire CED is deleted (**Supplementary Fig. 2b**). Similarly, both TNRC6C deletion mutants interacted less strongly with CAF1 and CNOT1 (**Supplementary Fig. 2c**). The ability of both mutants to still partially repress mRNA function and associate with CCR4–NOT is readily explained by observations that, in addition to the CED, N-proximal regions of GW182s have the potential to repress mRNAs[5,9,17,18] and associate with CCR4–NOT components (see below).

To determine the features of M2 and Cterm regions that repress mRNA function, we identified conserved regions of two to six amino acids by alignment of different GW182 proteins (**Supplementary Fig. 1**). Because their mutagenesis in the context of CEDΔPAM2 had a very limited effect (data not shown), we tested the mutations in the context of CEDΔPAM2 subfragments, M2-RRM or RRM-Cterm (**Fig. 1a** and **Supplementary Fig. 2d–h**). This analysis revealed considerable redundancy of the CED sequences responsible for mediating both the interaction with CCR4–NOT and repression of mRNA function. Unexpectedly, our results also showed that all mutations appreciably affecting both activities were in elements containing tryptophan residues, and those tryptophan residues were important for the repressive activity, in a manner that involved recruitment of CCR4–NOT (**Supplementary Figs. 2d–h** and **3a**,**b** and **Supplementary Results**).

W-motifs represent signals recruiting deadenylase complexes

When inspecting the alignment of the CED across different species, we noted that GW or WG repeats in one GW182 homolog often align with the S/TW or WS/T repeats in other homologs (**Supplementary Fig. 1**). We hypothesized that reiterated G/S/TW or WG/S/T repeats (referred to as W-motifs), rather than only GW or WG repeats, must have a role in repression. The TNRC6C CED contains eight W-motifs (**Fig. 2a** and **Supplementary Fig. 1**). We analyzed the effect of Trp→Ala mutations in W-motifs on expression of the tethered mRNA (**Fig. 2a**). Notably, although single Trp→Ala mutations had no marked effect on repression by the CED, their combinations had a progressive additive effect. Notably, when all eight tryptophans were mutated (W8), repression by the CED was fully alleviated. We observed no alleviation when other conserved amino acid stretches were mutated in either PAM2 or M2 regions. Western blot analysis showed that the differences in repressive potential could not be explained by differences in expression levels (**Fig. 2a**). The most conserved tryptophan residue, Trp1515, did not contribute to repression (8W and 7W mutants differ only in the Trp1515 mutation). Trp1515 participates in the RRM structure[6], whereas other W-motifs reside in regions predicted as disordered (http://dis.embl.de). Otherwise, W-motifs seem to contribute to repression independently of the degree of conservation and the context; that is, whether they are located next to glycine, serine or threonine residues (**Fig. 2a**).

Because the CED Trp→Ala mutants relieve repression activity, we determined, by MS, how these mutations affect the interaction of proteins with the CED (**Supplementary Fig. 4a**). As expected, the wild-type CED associated with different components of the CCR4–NOT complex. However, none of them associated with the 7W mutant, indicating that the CED interacts with CCR4–NOT in a W-dependent manner. As both wild-type and 7W mutant CEDs contain the PAM2 region, each associated with PABP. We also observed that the PAN2–PAN3 deadenylase complex components were present among proteins bound by wild-type but not 7W mutant fusions, though PAN2 and PAN3 were found in smaller amounts than CCR4–NOT proteins.

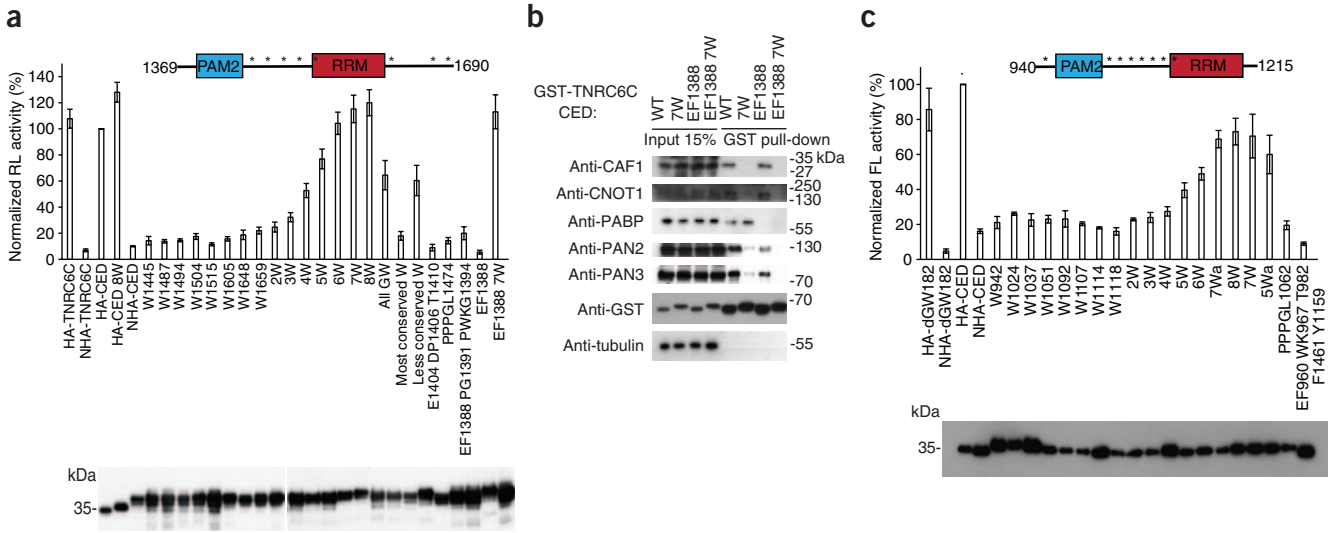

Figure 2 W-motifs in GW182 proteins mediate mRNA repression by recruiting CCR4–NOT and PAN2–PAN3 deadenylation complexes. (**a**) Mutations of tryptophan residues in W-motifs alleviate repression by the TNRC6C CED. Schematic representation of the TNRC6C CED with positions of W-motifs marked with asterisks is shown above the graph. Plasmids encoding either wild-type NHA-CED or its mutants (mutations always to alanine; when several consecutive amino acids are mutated, the number corresponds to the first residue in the mutated stretch) were co-transfected to HEK293T cells, together with RL-5BoxB and FL-Con. As negative controls, plasmids encoding untethered HA-TNRC6C or HA-CED were used. Mutants 2W through 8W contain Trp→Ala mutations in W-motifs (for details, see Online Methods). All GW, W1487 W1494 W1648 W1659; most conserved tryptophan, W1504 W1515; less conserved tryptophan, W1487 W1605 W1648 W1659. Values represent percentages of *Renilla* luciferase produced in the presence of untethered HA-CED control. Expression of HA- or NHA- fusion proteins was estimated by western blotting (lower panel). (**b**) Proteins identified as interacting with the CED in a tryptophan-dependent manner by MS (**Supplementary Fig. 4a**) were validated by GST pull-down assays and western blotting. Positions of protein size markers are indicated. (**c**) W-motifs are required for repression by the *D. melanogaster* GW182 CED. NHA-dGW182 CED, either wild-type or with mutations, were co-transfected with FL-5BoxB and RL-Con in S2 cells. As negative controls, plasmids encoding HA-dGW182 and HA-dGW182 CED were used. Mutants 2W through 8W contain mutations in W-motifs, with some (5Wa and 7Wa) having different combinations of mutated tryptophans (positions of W-motifs are marked with asterisks in the scheme above; for details, see Online Methods). Expression of firefly luciferase was normalized to *Renilla* luciferase. Values represent percentages of firefly luciferase produced in the presence of HA-CED. Expression of HA-fusions was estimated by western blotting.

We also analyzed the pull-down assays by western blotting (**Fig. 2b**). Both CNOT1 and CAF1 interacted with wild-type CED but not with its 7W mutant. Mutations of W-motifs also strongly affected association with PAN2 and PAN3 but had no major effect on interaction with PABP. In two out of four experiments, however, PABP binding was slightly affected in the 7W mutant (1.5-fold to two-fold; not shown). This could be explained by the secondary weak PABP binding site located in the M2 or Cterm regions[7,13]. Interactions with PABP through this site seemed to be indirect[8], suggesting that they occur through components of the CCR4–NOT or PAN2–PAN3 complexes.

We have mapped regions in the CED required for PABP and CCR4–NOT interactions, so we were able to determine the interdependence of these interactions. Mutations in PAM2 that disrupted the CED-PABP interaction (mutant EF1388; mutations are always to alanine; when several consecutive amino acids are mutated, the number corresponds to the first residue in the mutated stretch) did not affect the association of CED with CCR4–NOT, whereas the 7W mutant that did not interact with CCR4–NOT still interacted with PABP (**Fig. 2b**). Hence, the CED interactions with CCR4–NOT and PABP are independent. The PAN2–PAN3 interactions were more complex: mutation of PAM2 somewhat reduced binding of PAN2 and PAN3, though not as strongly as mutations of W-motifs, and the double EF1388 7W mutant showed no PAN2–PAN3 binding (**Fig. 2b**). These results suggest that PAN2–PAN3 is primarily recruited through the function of W-motifs but that it can also weakly interact with the CED through PABP, which is consistent with the direct PAN3-PABP interaction previously described[20].

To investigate whether the role of W-motifs in repression is conserved across the species, we also introduced Trp→Ala mutations into the eight W-motifs in the dGW182 CED (**Fig. 2c** and **Supplementary Fig. 1**). The mutant proteins were tethered to the firefly luciferase reporter FL-5BoxB, expressed in fly S2 cells. As in the case of the TNRC6C CED, mutations alleviated repression in an additive manner, leading to almost no repression when all tryptophans were mutated. In contrast, mutation of other conserved sequences had no appreciable effect (**Fig. 2c**).

Taken together, our data indicate that the role of W-motifs in mRNA repression is evolutionarily conserved and that W-motifs function by recruiting CCR4–NOT and PAN2–PAN3 complexes independently of PABP.

Repression by NED and CED follows a similar mechanism

To test if the recruitment of the CCR4–NOT complex represents a mechanism conserved across different effector domains and across species, we analyzed the function of the dGW182 NED in human HEK293T cells. Our previous work demonstrated that the dGW182 NED is able to repress the tethered mRNA in human cells[9], and we investigated whether mutations in W-motifs in that region would affect its repressive potential. Because the 205–490 dGW182 fragment, studied previously in S2 cells, was less effective in human cells (data not shown),

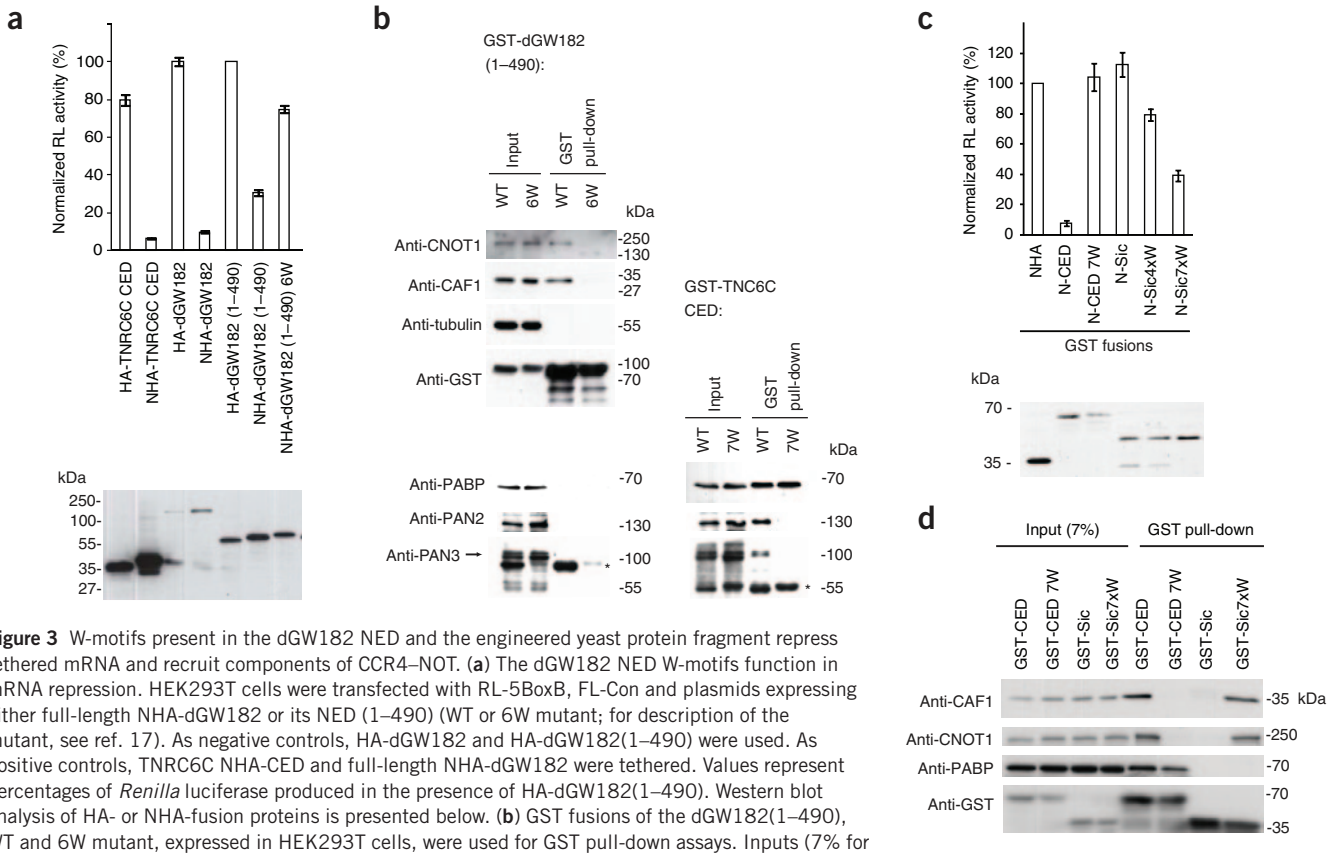

Figure 3 W-motifs present in the dGW182 NED and the engineered yeast protein fragment repress tethered mRNA and recruit components of CCR4–NOT. (**a**) The dGW182 NED W-motifs function in mRNA repression. HEK293T cells were transfected with RL-5BoxB, FL-Con and plasmids expressing either full-length NHA-dGW182 or its NED (1–490) (WT or 6W mutant; for description of the mutant, see ref. 17). As negative controls, HA-dGW182 and HA-dGW182(1–490) were used. As positive controls, TNRC6C NHA-CED and full-length NHA-dGW182 were tethered. Values represent percentages of *Renilla* luciferase produced in the presence of HA-dGW182(1–490). Western blot analysis of HA- or NHA-fusion proteins is presented below. (**b**) GST fusions of the dGW182(1–490), WT and 6W mutant, expressed in HEK293T cells, were used for GST pull-down assays. Inputs (7% for anti-CNOT1, anti-CAF1, anti-tubulin and anti-GST; 15% for anti-PABP, anti-PAN2 and anti-PAN3) and the pulled-down material were analyzed by western blotting, using indicated antibodies. Additional western blots (on the right) for PABP, PAN2 and PAN3 represent pull-down assays done with the TNRC6C GST-CED analyzed in parallel on the same gel. Anti-PAN3 antibody cross-reacts with GST (asterisk). (**c**) W-motifs are sufficient to induce repression of tethered mRNA. HEK293T cells were transfected with RL-5BoxB, FL-Con and plasmids encoding engineered N-Sic-GST protein fusions having either four (N-Sic4xW-GST) or seven (N-Sic7xW-GST) W-motifs. N-Sic-GST containing no tryptophan residues, and NHA-GST, served as controls; plasmids encoding TNRC6C N-CED-GST, WT and 7W mutant were transfected for comparison. (**d**) GST pull-down assays with GST-Sic7xW, GST-CED (positive control), and GST-CED 7W and GST-Sic (negative controls), were done as in **Figure 1d**. The pulled-down material was analyzed by western blotting, using indicated antibodies.

a longer 1–490 fragment was used instead. We observed that six Trp→Ala mutations in GW repeats in the 205–490 region (mutant NHA-dGW182(1–490)6W) led to a marked alleviation of repression (**Fig. 3a**), similar to that observed in *D. melanogaster* S2 cells[17].

Analysis of interaction partners of the dGW182 NED(1–490) in HEK293T cells revealed that it interacts with CNOT1 and CAF1 in a W-dependent manner (**Fig. 3b**), suggesting that the mechanism of mRNA repression by different GW182 domains is similar and involves the recruitment of CCR4–NOT through W-motifs. Neither PABP nor PAN2–PAN3 was detected in the NED GST pull-down assays, whereas they were pulled down with the TNRC6C CED (**Fig. 3b**, lower panels). Thus, interaction with PABP and PAN2–PAN3 may not be required for repression by the NED.

Engineered W-motifs are sufficient to induce repression

We investigated whether W-motifs are not only required but also sufficient to induce mRNA repression. We introduced X→Trp mutations (with X corresponding to any amino acid) to the unstructured fragment of the yeast protein Sic1p[21]. The resulting engineered proteins, having either four (Sic4xW) or seven (Sic7xW, **Supplementary Fig. 4b** and **Supplementary Methods**) sequences resembling the W-motifs, were fused to N- and GST polypeptides and their activity tested in the tethering assay. Notably, the proteins containing W-motifs were able to repress *Renilla* luciferase-5BoxB (RL-5BoxB) mRNA, with the degree of repression being dependent on the number of motifs (**Fig. 3c**). Moreover, GST pull-down experiments revealed that both CAF1 and CNOT1, but not PABP, were bound by Sic7xW but not the control tryptophan-free fragment (**Fig. 3d**). Hence, W-motifs are not only necessary but also sufficient to induce mRNA repression by recruiting CCR4–NOT.

W-motifs function in a genuine miRNA-mediated repression

We next investigated whether W-motifs also function in the context of full-length GW182 proteins. Mutation of tryptophan residues in W-motifs of the CED strongly compromised the repressive potential of TNRC6C in HEK293T cells (**Fig. 4a**, mutants 7W and 8W, ~four-fold effect; for clarity, the data are also shown as fold derepression in the right panels of **Fig. 4a**,**b**). A more marked effect (~ten-fold) of tryptophan mutations on activity of the CED alone (**Fig. 4a**; see also **Fig. 2a**) is readily explained by the potential of the TNRC6 N-proximal sequences to partially repress the tethered mRNA[9,18]. In the context of the full-length TNRC6C, the PAM2 mutation EF1388 led to moderate alleviation of repression, consistent with previous data[8].

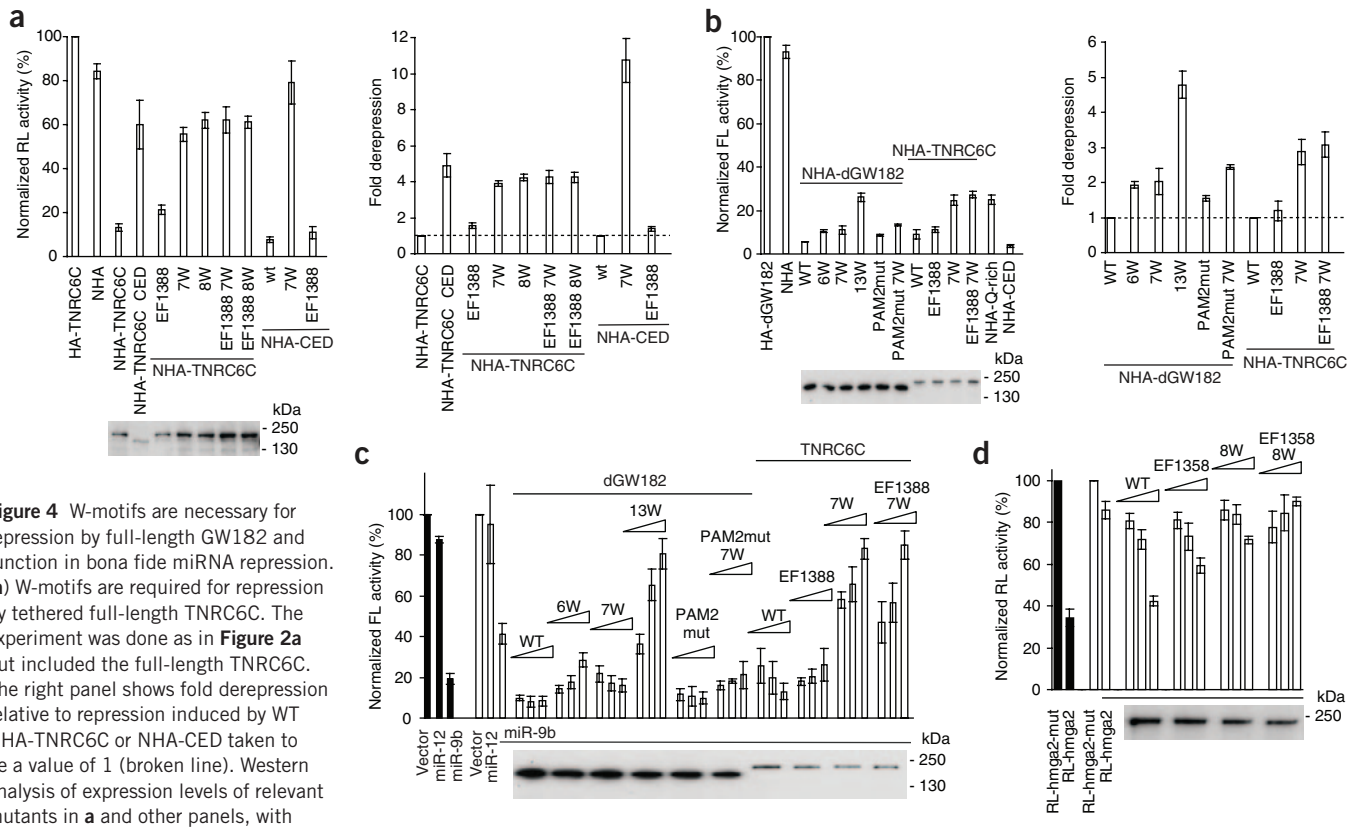

Figure 4 W-motifs are necessary for repression by full-length GW182 and function in bona fide miRNA repression. (**a**) W-motifs are required for repression by tethered full-length TNRC6C. The experiment was done as in **Figure 2a** but included the full-length TNRC6C. The right panel shows fold derepression relative to repression induced by WT NHA-TNRC6C or NHA-CED taken to be a value of 1 (broken line). Western analysis of expression levels of relevant mutants in **a** and other panels, with anti-HA antibody, is shown below the graphs. (**b**) Mutations in W-motifs lead to partial derepression of tethered mRNAs in *D. melanogaster* S2 cells. The assay was done as in **Figure 2c** but with the full-length dGW182 and TNRC6C. 6W, 7W and EF1388 mutations were described in **Figures 2** and **3a** but are here introduced into the full-length proteins. 13W mutant combines 6W and 7W; PAM2mut has EF960 WK967 Thr982 mutated. NHA-Q–rich (1080–1245) and NHA-CED represent TNRC6C fragments. In the right panel, data are presented as in **a**. (**c**) W-motifs are required to rescue depletion of endogenous dGW182. Endogenous dGW182 was depleted in *D. melanogaster* S2 cells with dsRNA (open bars); a batch of cells was treated with GFP-specific dsRNA as a control (black bars). Cells were transfected with RL-Con, FL-nerfin, and plasmids encoding miR-9b or miR-12, or the empty vector. To rescue depletion of dGW182, increasing amounts of plasmids encoding NHA-dGW182, NHA-TNRC6C or their mutants were co-transfected. In panels **c** and **d**, extracts from cells transfected with highest plasmid concentrations were used for western blotting. (**d**) W-motifs are necessary to complement the knockdown of endogenous TNRC6 proteins. HeLa cells were transfected with siRNAs targeting three endogenous TNRC6 proteins (open bars) or AllStars siRNA (negative control, black bars), RL-hmga2 reporter containing let-7 sites or its mutant version (RL-hmga2 mut), and increasing amounts of plasmids expressing NHA-TNRC6A or its mutants: 8W has Trp→Ala mutations in W-motifs within the CED region (**Supplementary Fig. 1** and Online Methods); EF1358 has PAM2 mutated.

In *D. melanogaster* S2 cells, mutating W-motifs also led to alleviation of repression induced by either dGW182 or TNRC6C, though the effects were less pronounced than in human cells (**Fig. 4b**). This can be explained by a marked contribution of the Q-rich domains of these proteins to the repression in S2 cells (**Fig. 4b**, NHA-Q-rich and ref. 5). For dGW182, mutating W-motifs in either NED (mutant 6W) or CED (7W) alone had only a mild effect (~two-fold), but combining these mutations (13W) led to more than four-fold alleviation of repression. Mutating seven tryptophans within the CED of TNRC6C alleviated repression ~three-fold, with mutations in PAM2 having no effect (**Fig. 4b**).

Having demonstrated that W-motifs function in the context of full-length GW182 proteins, we analyzed their importance in a bona fide miRNA repression assay. We depleted S2 cells of the endogenous dGW182 and tested tryptophan mutants of dGW182 for activity to rescue miRNA repression. To assess miRNA-mediated silencing, cells were co-transfected with the firefly luciferase–nerfin (FL-nerfin) reporter and the plasmid expressing miR-9b, which targets the FL-nerfin 3′ UTR. miR-9b efficiently repressed FL-nerfin mRNA in control cells (**Fig. 4c**, black bars), and depletion of dGW182 (open bars) partially alleviated miR-9b–induced repression; as expected, transfection of a plasmid encoding wild-type dGW182 resistant to RNAi rescued the repression. Mutations of tryptophans in either NED (6W) or CED (7W) had only a minor effect on the functionality of dGW182 in the rescue, consistent with independent repression by NED and CED domains[5]. However, combining the tryptophan mutations in both regions led to a strong alleviation of repression, demonstrating the role of W-motifs in miRNA-mediated silencing. Mutation of the PAM2 motif had no appreciable effect.

Because GW repeats present in the N-terminal part of dGW182 contribute to dAGO1 binding[22], we tested if mutations of tryptophans introduced into dGW182 affect its interaction with dAGO1. We found that whereas the 7W mutant interacted with dAGO1 as efficiently as wild-type dGW182, the 6W and 13W mutants showed lower levels of binding (**Supplementary Fig. 4c**). Consequently, it is possible that tryptophan residues in the NED contribute to the rescue not only by enhancing the CCR4–NOT interaction (**Fig. 3b**) but also by increasing the affinity of dGW182 for dAGO1. However, as 6W and 13W mutants have similar dAGO1-binding properties (**Supplementary Fig. 4c**), we can conclude that W-motifs in the CED are required for the dGW182 function in miRNA repression (**Fig. 4c**).

Because human TNRC6C is able to complement the knockdown of dGW182 in S2 cells[8] (**Fig. 4c**), we tested the effect of tryptophan mutations on its function in rescue experiments. Notably, mutations of the W-motifs within the CED region (7W) strongly alleviated repression by TNRC6C. This is consistent with findings that the CED represents the major repressive region of human GW182 proteins[6,9,11]. To test the requirement of W-motifs for miRNA repression in human cells, we used a reporter having the 3′ UTR of the *HMGA2* gene (RL-hmga2), which is targeted by let-7 miRNA[23,24]. This miRNA is expressed endogenously in HeLa cells, and it represses RL-hmga2 by about three times when compared with its mutant version that has disabled let-7 sites (**Fig. 4d**, black bars). Depletion of all three TNRC6 proteins by RNAi led to almost full alleviation of the repression (**Fig. 4d**, open bars), which could be rescued with the wild-type TNRC6A (we used a TNRC6A paralog, as it functions most efficiently in the complementation assay[8]). Mutation of PAM2 (EF1358) partially interfered with the rescue, consistent with the previous report[8]. Notably, mutations of W-motifs either alone (8W) or in combination with PAM2 mutation (EF1358 8W) led to a nearly complete loss of TNRC6A function in miRNA repression. We conclude that W-motifs of both *D. melanogaster* and human GW182s are important for bona fide miRNA-mediated silencing.

Role of W-motifs and CCR4–NOT in poly(A)$^-$ mRNA repression

Recruitment of the CCR4–NOT deadenylase explains how miRNAs and tethered GW182 silencing domains induce deadenylation and mRNA decay[2,3]. Indeed, we observed that tethering of the dGW182 CED induces deadenylation of the FL-5BoxB reporter and that this effect is dependent on W-motifs (**Supplementary Fig. 5**). Do the CED and CCR4–NOT also mediate the translational repression known to be induced by miRNA machinery[2–4]? To address this question we first tested whether the dGW182 CED can repress, in a W-motif–dependent manner, tethered mRNAs in which the polyadenylation signal is substituted by either a histone stem loop (HSL) or a hammerhead ribozyme (HhR). These mRNAs, FL-5BoxB-HSL and FL-5BoxB-HhR, were previously shown to have no poly(A) and to undergo translational repression in S2 cells in response to tethered dGW182, without changes in mRNA levels[25]. Tethering of dGW182 to FL-5BoxB-HSL and FL-5BoxB-HhR repressed their activity by four and two times, respectively (**Fig. 5a**), as reported[25]. Tethering of the dGW182 CED or its longer version extending to the dGW182 C terminus (CED*) was slightly less inhibitory, but, notably, the inhibition was nearly fully relieved by mutating W-motifs. Similarly to the effect of CED domains, direct tethering of the fly Caf1 (dCAF1) and human CNOT1 (the *D. melanogaster* clone is not available) reduced, by 55% to 75%, activity of both poly(A)$^+$ and poly(A)$^-$ reporters in S2 cells (**Fig. 5b**). Although the inhibition of poly(A)$^+$ RNA by either the dGW182 CED domain or CCR4–NOT components was associated with a decrease of approximately two times in mRNA levels, repression of poly(A)$^-$ mRNAs was not accompanied by pronounced mRNA degradation (**Fig. 5b**).

We also investigated whether human TNRC6C CED and human CCR4–NOT proteins can repress tethered mRNA independently of poly(A) in HEK293T cells. We found that both classes of proteins repress activity of the poly(A)$^-$ reporter that was either expressed from plasmids or transfected as *in vitro* transcribed mRNA, the latter bearing the cordycepin residue at the 3′ end to prevent its potential adenylation in the cell. Inhibition of the poly(A)$^-$ mRNAs was not accompanied by their degradation (**Supplementary Figs. 6a–e** and **7a–d** and **Supplementary Results**).

Collectively, these results show that recruitment of the GW182 CED or components of CCR4–NOT also induces silencing of poly(A)$^-$ mRNAs, without any accompanying RNA degradation, suggesting that the CCR4–NOT complex mediates not only mRNA deadenylation but also translational repression.

Repression of poly(A)$^-$ RNA by GW182 depends on CCR4–NOT

If the CCR4–NOT complex functions downstream of GW182 during repression of poly(A)$^-$ mRNAs, the inhibitory effect of GW182 should be dependent on CCR4–NOT. To address this assumption, dGW182 and its fragments were tested for their ability to repress the poly(A)$^-$ mRNA in S2 cells depleted of NOT1, a large CCR4–NOT complex scaffolding protein[26]. Depletion of NOT1 resulted in a marked alleviation of repression, more pronounced for the fragments of dGW182 (2.5-fold to three-fold) than the full-length dGW182 (two-fold) (**Fig. 6a**). This is probably due to dGW182 also containing domains (for example, Q-rich[5]) that may repress mRNA by a CCR4–NOT–independent mechanism.

The observation that repression of poly(A)$^-$ RNA by tethering dGW182 and its fragments depends on NOT1 suggested that the CCR4–NOT complex also acts downstream of GW182 in translational repression. Consistently, repression caused by tethering of the CCR4–NOT proteins dCAF1 and CNOT1 to FL-5BoxB-HSL RNA was not affected by depletion of endogenous dGW182 (**Fig. 6b**). Of note, the dGW182 depletion resulted in partial (30–40%) alleviation of the repression of the poly(A)$^+$ FL-5BoxB reporter (**Fig. 6b**). This is consistent with results indicating that GW182 affects repression not only through the recruitment

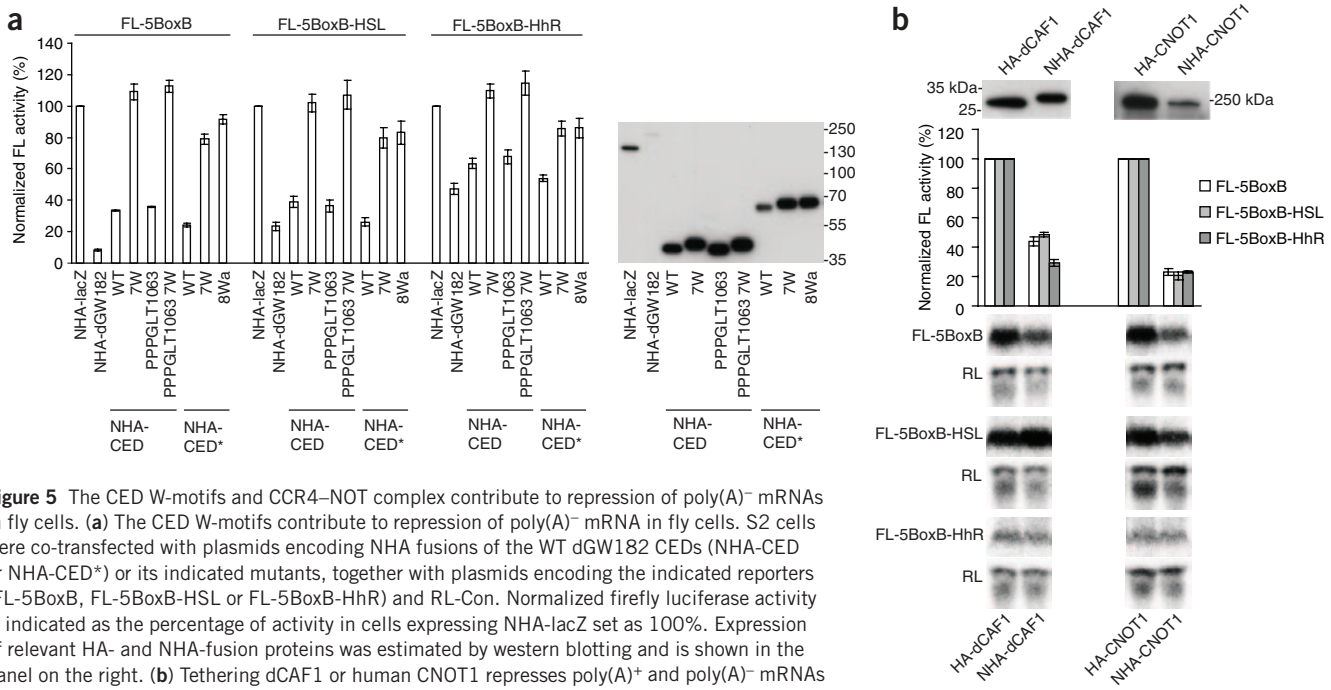

Figure 5 The CED W-motifs and CCR4–NOT complex contribute to repression of poly(A)⁻ mRNAs in fly cells. (**a**) The CED W-motifs contribute to repression of poly(A)⁻ mRNA in fly cells. S2 cells were co-transfected with plasmids encoding NHA fusions of the WT dGW182 CEDs (NHA-CED or NHA-CED*) or its indicated mutants, together with plasmids encoding the indicated reporters (FL-5BoxB, FL-5BoxB-HSL or FL-5BoxB-HhR) and RL-Con. Normalized firefly luciferase activity is indicated as the percentage of activity in cells expressing NHA-lacZ set as 100%. Expression of relevant HA- and NHA-fusion proteins was estimated by western blotting and is shown in the panel on the right. (**b**) Tethering dCAF1 or human CNOT1 represses poly(A)⁺ and poly(A)⁻ mRNAs in fly cells. Cells were co-transfected with plasmids expressing HA or NHA fusions of dCAF1 or human CNOT1 and plasmids encoding indicated reporters. Normalized firefly luciferase activity is indicated as the percentage of activity in cells expressing HA fusions of dCAF1 or human CNOT1 set as 100%. Expression levels of HA- and NHA-fusion proteins were estimated by western blotting (shown above the graph). HA- and NHA-CNOT1 were only detectable after enrichment by anti-HA antibody immunoprecipitation. Lower signal of the NHA-tagged, compared to HA-tagged protein, may be partially due to the lower reactivity of anti-HA antibody with the internally located epitope. Analysis of mRNA levels by northern blotting is shown below the graph. Identity of analyzed reporters (including *Renilla* luciferase mRNA as a reference) is shown on the left, and the co-transfected CCR4–NOT complex components are indicated at the bottom.

of CCR4–NOT but also through the association with PABP, and the latter interaction has been shown to be important for miRNA-induced deadenylation[7,12,13]. We conclude that the CCR4–NOT complex also functions downstream of GW182 during repression of poly(A)⁻ mRNAs, consistent with its role in mediating inhibition of translation.

DISCUSSION

We here provide evidence that human and *D. melanogaster* GW182 proteins repress mRNAs by recruiting the CCR4–NOT complex to the mRNA, in a PABP-independent manner. This recruitment specificity comes from W-motifs that are dispersed throughout the N- and C-terminal regions of the proteins and that act in an additive manner. Moreover, we found that recruitment of CCR4–NOT represses both poly(A)⁺ and poly(A)⁻ mRNAs, arguing that this complex, in addition to catalyzing mRNA deadenylation, also mediates miRNA-induced translational repression.

The following evidence supports the conclusion that W-motifs represent critical signals for recruiting CCR4–NOT and inducing mRNA repression. (i) Exhaustive mutagenesis of the CED identified redundant W-containing elements in the CED M2 and Cterm regions and demonstrated a strong correlation between repression and interaction with CCR4–NOT. (ii) Introduction of an increasing number of Trp→Ala mutations, in both GW (or WG) and S/TW (or WS/T) contexts, across the CED regions of either TNRC6C or dGW182, had an additive effect on alleviating repression, regardless of whether these substitutions were tested in the CED or full-length proteins. (iii) W-motifs present in the NED and CED regions functioned in an additive manner and by similar mechanisms that involved the recruitment of the CCR4–NOT complex. (iv) In the assay measuring bona fide miRNA repression, the activity of dGW182, TNRC6C and TNRC6A to rescue miRNA-mediated silencing in GW182-depleted cells was strongly compromised upon mutation of W-motifs. (v) Finally, fragments of the yeast protein Sic1p having engineered W-motifs acquired the ability to repress mRNA and to interact with the CCR4–NOT components. Hence, W-motifs are not only required but also sufficient to induce repression by recruitment of the CCR4–NOT complex. Notably, two motifs in TNRC6C, identified in an accompanying paper[27] as important for mediating deadenylation and CCR4–NOT interaction *in vitro*, also contain tryptophan residues.

It is unlikely that alleviation of mRNA repression by Trp→Ala substitutions is due to perturbation by the higher-order structure of the polypeptides or by their folding upon binding to target proteins. First, the mutated W-motifs are located in the NED and CED regions that are predicted to be disordered (http://dis.embl.de). Indeed, NMR analysis of the TNRC6C NED confirmed its disordered character (F. Laughlin, M. Chekulaeva, W.F. and F. Allain, unpublished data). Second, in the case of the CED 'half' regions—that is, the M2-RRM and RRM-Cterm regions—mutating even one or two tryptophan residues had an appreciable effect on repression. Third, the Sic1p protein fragment used for the gain-of-repression experiments is known to be unstructured[21] and, apart from engineered W-motifs, shows no sequence similarity to repressive GW182 fragments.

We also observed that the CED domain interacts with the PAN2–PAN3 complex in a manner dependent on W-motifs. Others[15] have previously shown that PAN2 contributes to miRNA-mediated deadenylation, most probably at its initial stage. Our data indicate that PAN2–PAN3 is primarily recruited through the function of the W-motifs in the CED, but it can also weakly interact with the CED through PABP (**Fig. 2c**), consistent with the previously described direct PAN3-PABP interaction[20].

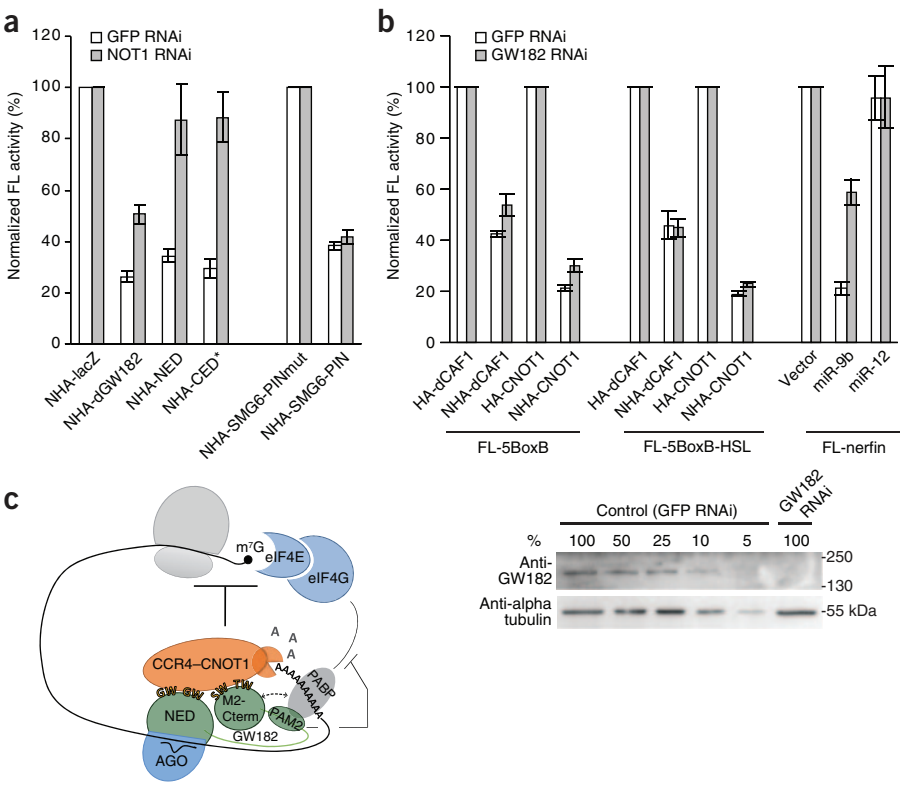

Figure 6 Repression of poly(A)⁻ RNA by tethering dGW182 or its fragments depends on NOT1, but repression by tethered CCR4–NOT components is dGW182-independent. (**a**) Repression of FL-5BoxB-HSL reporter by tethering dGW182 or its fragments is alleviated in S2 cells depleted of NOT1. S2 cells treated with dsRNA targeting GFP or NOT1 were co-transfected with plasmids expressing either NHA fusions of dGW182 and its fragments or the PIN domain (either WT or a catalytic mutant thereof) of the endonuclease SMG6, and also reporter plasmids FL-5BoxB-HSL and RL-Con. Normalized firefly luciferase activity is indicated as percentage of the activity in cells expressing NHA-lacZ or SMG6-PINmut, set as 100%. The NOT1 depletion affected the repression by dGW182 and its fragments but had no effect on repression by SMG6-PIN that targets mRNA for endonucleolytic degradation[35], supporting the specificity of the effect. (**b**) Repression of FL-5BoxB and FL-5BoxB-HSL reporters by tethered dCAF1 and human CNOT1 is unaffected in S2 cells depleted of dGW182. Normalized firefly luciferase activity is indicated as the percentage of activity in cells expressing HA-dCAF1 or HA-CNOT1, or cells transfected with pAC5.1 (empty vector), each set as 100%. The efficiency of GW182 depletion was analyzed by western blotting (lower panel). Lanes 1–5, dilutions of the extract from S2 cells treated with GFP-specific (control) dsRNA. (**c**) Scheme illustrating a possible mode of action of GW182 proteins in miRNA-mediated repression. GW182 proteins are recruited to mRNA through direct interaction with the miRNA–AGO complex. The GW182 NED and CED regions both recruit, through the W-motifs, the CCR4–NOT complex that represses translation and leads to mRNA deadenylation. Interaction of the GW182 PAM2 motif with PABP may interfere with the PABP-eIF4G association, thus contributing to translational inhibition and mRNA deadenylation. The PABP interaction with the CED M2/C-term regions (broken line) may be mediated by the CCR4–NOT complex (see text).

The additive contribution of W-motifs, distributed in disordered protein regions, raises the question of how these motifs promote the interaction of GW182 and CCR4–NOT. Does the sheer quantity of the motifs just increase the probability of initial productive interactions? Do the tryptophan-containing regions recruit more than one CCR4–NOT complex at a time? One model of GW182 function is reminiscent of protein-protein interactions reported for the U2AF homology motif (UHM) of the U2 snRNP factor U2AF[65] (ref. 28). In that case, the spliceosome component SF3b155 binds to the U2AF[65] UHM through motifs having an essential tryptophan and consensus RWD/E. Similarly to GW182 proteins, SF3b155 contains an unstructured region with seven RWD/E repeats[28].

The CCR4–NOT components CAF1 and CNOT1 were previously identified as important for miRNA-mediated deadenylation in both flies and mammals, and it has been suggested that the interaction of GW182 with PABP might lead to the recruitment of CCR4–NOT to mRNA[7,12,14–16]. Our data indicate that recruitment of CCR4–NOT by W-motifs present in CED and NED regions is independent of PABP and represents either a complementary or alternative mechanism for repression. The critical observation in our study was that deletion of PAM2 or its mutation that disrupts CED-PABP interaction did not affect the CED association with CCR4–NOT and mRNA repression, whereas the CED 7W mutant, which still interacted with PABP but not with CCR4–NOT, was inactive in repression (**Fig. 2**). Moreover, the dGW182 NED region, which is repressive in both S2 and HEK293T cells, interacted with the CCR4–NOT complex components but not with PABP (**Fig. 3b**). Similarly, the repressive yeast Sic1p fragment associated with the CCR4–NOT proteins but not PABP (**Fig. 3d**). The association between the TNRC6C CED and CCR4–NOT most probably occurs through the CNOT1 subunit of the complex, because human CNOT1, but not CNOT6 or CNOT7/CAF1, interacted with the CED in the yeast two-hybrid system (**Supplementary Fig. 8**). CNOT1 was also by far the most effectively pulled down CCR4–NOT complex component identified by MS (**Fig. 1b** and **Supplementary Fig. 4a**).

One of the most important findings of our work is that components of the CCR4–NOT complex are able to repress not only polyadenylated but also poly(A)-free mRNAs. The observation that repression of poly(A)⁻ RNA by dGW182 and its fragments depends on CCR4–NOT, whereas repression by tethering of CCR4–NOT proteins is dGW182-independent, indicates that the CCR4–NOT complex acts downstream of GW182 proteins also during repression of poly(A)⁻ mRNAs. Together with the finding that the CCR4–NOT repression of poly(A)⁻ RNAs is not associated with a decrease in mRNA levels, these data strongly implicate the CCR4–NOT proteins in mediating translational repression induced by miRNAs. These results are consistent with recent work[29] showing that tethering of CAF1 to the microinjected reporter mRNA can repress translation at the initiation step in *Xenopus laevis* oocytes. Our experiments extend these results by demonstrating that the CCR4–NOT complex may be responsible for translational repression induced by miRNAs. We also found that in HEK293T and S2 cells, the tethering of CAF1 and, notably, other subunits of the CCR4–NOT complex, repressed mRNA activity (**Supplementary Fig. 7c**), without affecting the levels of poly(A)⁻ mRNA (**Fig. 5b** and **Supplementary Fig. 7b**). Jointly, these observations indicate that W-motif–mediated recruitment of the CCR4–NOT complex causes both translational repression and deadenylation of target mRNAs

(see model in **Fig. 6c**). We find it interesting that in yeast and in fly, the CCR4–NOT complex is known to interact with the translational repressor Dhh1/Me31b[30,31], whose orthologs in other organisms are known to be required for miRNA-mediated repression[32–34], suggesting a possible mechanism by which the CCR4–NOT complex could repress translation.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/nsmb/.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

M. Chekulaeva, H.M., J.T.Z., J.A., M. Colic, R.P. and W.F. designed the experiments. M. Chekulaeva, H.M., J.T.Z., J.A. and M. Colic conducted the experiments. M. Chekulaeva, H.M., R.P. and W.F. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1. Bartel, D.P. MicroRNAs: target recognition and regulatory functions. *Cell* **136**, 215–233 (2009).
2. Huntzinger, E. & Izaurralde, E. Gene silencing by microRNAs: contributions of translational repression and mRNA decay. *Nat. Rev. Genet.* **12**, 99–110 (2011).
3. Fabian, M.R., Sonenberg, N. & Filipowicz, W. Regulation of mRNA translation and stability by microRNAs. *Annu. Rev. Biochem.* **79**, 351–379 (2010).
4. Djuranovic, S., Nahvi, A. & Green, R. A parsimonious model for gene regulation by miRNAs. *Science* **331**, 550–553 (2011).
5. Chekulaeva, M., Filipowicz, W. & Parker, R. Multiple independent domains of dGW182 function in miRNA-mediated repression in *Drosophila*. *RNA* **15**, 794–803 (2009).
6. Eulalio, A. *et al.* The RRM domain in GW182 proteins contributes to miRNA-mediated gene silencing. *Nucleic Acids Res.* **37**, 2974–2983 (2009).
7. Zekri, L., Huntzinger, E., Heimstadt, S. & Izaurralde, E. The silencing domain of GW182 interacts with PABPC1 to promote translational repression and degradation of miRNA targets and is required for target release. *Mol. Cell. Biol.* **23**, 6220–6231 (2009).
8. Huntzinger, E., Braun, J.E., Heimstadt, S., Zekri, L. & Izaurralde, E. Two PABPC1-binding sites in GW182 proteins promote miRNA-mediated gene silencing. *EMBO J.* **29**, 4146–4160 (2010).
9. Zipprich, J.T., Bhattacharyya, S., Mathys, H. & Filipowicz, W. Importance of the C-terminal domain of the human GW182 protein TNRC6C for translational repression. *RNA* **15**, 781–793 (2009).
10. Lazzaretti, D., Tournier, I. & Izaurralde, E. The C-terminal domains of human TNRC6A, TNRC6B, and TNRC6C silence bound transcripts independently of Argonaute proteins. *RNA* **15**, 1059–1066 (2009).
11. Baillat, D. & Shiekhattar, R. Functional dissection of the human TNRC6 (GW182-related) family of proteins. *Mol. Cell. Biol.* **29**, 4144–4155 (2009).
12. Fabian, M.R. *et al.* Mammalian miRNA RISC recruits CAF1 and PABP to affect PABP-dependent deadenylation. *Mol. Cell* **35**, 868–880 (2009).
13. Jinek, M., Fabian, M.R., Coyle, S.M., Sonenberg, N. & Doudna, J.A. Structural insights into the human GW182-PABC interaction in microRNA-mediated deadenylation. *Nat. Struct. Mol. Biol.* **17**, 238–240 (2010).
14. Behm-Ansmant, I. *et al.* mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. *Genes Dev.* **20**, 1885–1898 (2006).
15. Chen, C.Y., Zheng, D., Xia, Z. & Shyu, A.B. Ago-TNRC6 triggers microRNA-mediated decay by promoting two deadenylation steps. *Nat. Struct. Mol. Biol.* **16**, 1160–1166 (2009).
16. Piao, X., Zhang, X., Wu, L. & Belasco, J.G. CCR4-NOT deadenylates mRNA associated with RNA-induced silencing complexes in human cells. *Mol. Cell. Biol.* **30**, 1486–1494 (2010).
17. Chekulaeva, M., Parker, R. & Filipowicz, W. The GW/WG repeats of *Drosophila* GW182 function as effector motifs for miRNA-mediated repression. *Nucleic Acids Res.* **38**, 6673–6683 (2010).
18. Yao, B. *et al.* Divergent GW182 functional domains in the regulation of translational silencing. *Nucleic Acids Res.* **39**, 2534–2547 (2011).
19. Ezzeddine, N. *et al.* Human TOB, an antiproliferative transcription factor, is a poly(A)-binding protein-dependent positive regulator of cytoplasmic mRNA deadenylation. *Mol. Cell. Biol.* **27**, 7791–7801 (2007).
20. Siddiqui, N. *et al.* Poly(A) nuclease interacts with the C-terminal domain of polyadenylate-binding protein domain from poly(A)-binding protein. *J. Biol. Chem.* **282**, 25067–25075 (2007).
21. Mittag, T. *et al.* Structure/function implications in a dynamic complex of the intrinsically disordered Sic1 with the Cdc4 subunit of an SCF ubiquitin ligase. *Structure* **18**, 494–506 (2010).
22. Eulalio, A., Helms, S., Fritzsch, C., Fauser, M. & Izaurralde, E. A C-terminal silencing domain in GW182 is essential for miRNA function. *RNA* **15**, 1067–1077 (2009).
23. Höck, J. *et al.* Proteomic and functional analysis of Argonaute-containing mRNA-protein complexes in human cells. *EMBO Rep.* **8**, 1052–1060 (2007).
24. Mayr, C., Hemann, M.T. & Bartel, D.P. Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation. *Science* **315**, 1576–1579 (2007).
25. Eulalio, A. *et al.* Deadenylation is a widespread effect of miRNA regulation. *RNA* **15**, 21–32 (2009).
26. Collart, M.A. & Timmers, H.T. The eukaryotic Ccr4-not complex: a regulatory platform integrating mRNA metabolism with cellular signaling pathways? *Prog. Nucleic Acid Res. Mol. Biol.* **77**, 289–322 (2004).
27. Fabian, M.R. *et al.* miRNA-mediated deadenylation is orchestrated by GW182 through two conserved motifs that interact with CCR4–NOT. *Nat. Struct. Mol. Biol.* doi:10.1038/nsmb.2149 (published online 7 Oct 2011).
28. Thickman, K.R., Swenson, M.C., Kabogo, J.M., Gryczynski, Z. & Kielkopf, C.L. Multiple U2AF65 binding sites within SF3b155: thermodynamic and spectroscopic characterization of protein-protein interactions among pre-mRNA splicing factors. *J. Mol. Biol.* **356**, 664–683 (2006).
29. Cooke, A., Prigge, A. & Wickens, M. Translational repression by deadenylases. *J. Biol. Chem.* **285**, 28506–28513 (2010).
30. Coller, J.M., Tucker, M., Sheth, U., Valencia-Sanchez, M.A. & Parker, R. The DEAD box helicase, Dhh1p, functions in mRNA decapping and interacts with both the decapping and deadenylase complexes. *RNA* **7**, 1717–1727 (2001).
31. Temme, C. *et al.* Subunits of the *Drosophila* CCR4-NOT complex and their roles in mRNA deadenylation. *RNA* **16**, 1356–1370 (2010).
32. Chu, C.Y. & Rana, T.M. Translation repression in human cells by microRNA-induced gene silencing requires RCK/p54. *PLoS Biol.* **4**, e210 (2006).
33. Barbee, S.A. *et al.* Staufen- and FMRP-containing neuronal RNPs are structurally and functionally related to somatic P bodies. *Neuron* **52**, 997–1009 (2006).
34. Eulalio, A. *et al.* Target-specific requirements for enhancers of decapping in miRNA-mediated gene silencing. *Genes Dev.* **21**, 2558–2570 (2007).
35. Glavan, F., Behm-Ansmant, I., Izaurralde, E. & Conti, E. Structures of the PIN domains of SMG6 and SMG5 reveal a nuclease within the mRNA surveillance complex. *EMBO J.* **25**, 5117–5125 (2006).

ONLINE METHODS

Cell culture, transfections, RNAi and luciferase assays. Human HEK293T cells were grown in DMEM (GIBCO BRL) supplemented with 2 mM L-glutamine and 10% (v/v) FCS buffer. Transfections were done in 6-, 12-, 24- and 96-well plates with nanofectin (PAA Laboratories), according to manufacturer's instructions. In tethering experiments, cells were transfected with 1 ng RL-5BoxB, 20 ng FL-Con and 20–30 ng HA- or NHA-fusion constructs per well in a 96-well plate. For other formats, the amount of plasmids was adjusted proportionally. Cells were lysed 24 h after transfection. For TNRC6 rescue experiments, HeLa cells stably expressing Tet-On machinery[36] were transfected using attractene reagent (Qiagen). Per well of the 96-well plate, transfection mixtures contained 10 ng of the let-7 reporter plasmid, increasing amounts of NHA-TNRC6A or its point mutants (20, 60 and 180 ng), and either siRNAs specific to TNRC6A, B and C (5′-GCCUAAUCUCCGUGCUCAATT-3′, 5′-GGCCUUGUAUUGCCAGCAATT-3′ and 5′-GCAUUAAGUGCUAAACAA-ATT-3′ (Microsynth; sequences represent sense strands), 0.53 pmol each; or 1.6 pmol AllStars siRNA negative control (Qiagen). TNRC6A plasmids were made resistant to siRNA by introducing silent point mutations. Let-7 reporter plasmids (kindly provided by J. Béthune) encoded *Renilla* luciferase fused to the human *HMGA2* 3′ UTR, either WT with sites recognized by let-7 (RL-hmga2), or mutant in which let-7 sites were mutated (RL-hmga2 mut)[37,38], as well as FL-Con, both under control of the tetracycline-responsive element. Expression of reporters was induced with 1 μg ml^{-1} doxycycline 2 d after transfection and cells were lysed 4 h after induction. *D. melanogaster* S2 cells were transfected in 96-well plates with Cellfectin II and PLUS reagents (Invitrogen). In tethering experiments, we transfected 5 ng FL-5BoxB plasmid, 30 ng RL-Con, and 20–30 ng plasmid encoding HA- or NHA-fusion protein per well. Cells were lysed 3 d after transfection. In rescue experiments, transfection mixtures contained 5 ng FL-nerfin reporter plasmid, 30 ng RL-Con and 5 ng of either an empty vector or a plasmid encoding miR-9b or miR-12 per well of a 96-well plate; plasmids encoding dGW182, TNRC6C and their mutants were added in increasing amounts from 3–30 ng. RNAi experiments were conducted as described[39] using dsRNA targeting the dGW182 3′ UTR or the coding region of NOT1. S2 cells were treated with dsRNA twice, on days 1 and 4, transfected on day 6 and lysed on day 9.

Luciferase activities were measured with the Dual-Luciferase Reporter Assay System (Promega). In all luciferase assays, values represent means ± s.e.m. from three to six independent experiments.

CED mutants containing mutations in W-motifs. Positions of single tryptophan mutations are as indicated in **Figure 2a**,**c**. Other mutants in the TNRC6C CED are designated as follows: 2W stands for W1445 W1487; 3W, W1445 W1487 W1494; 4W, W1445 W1487 W1494 W1659; 5W, W1445 W1487 W1494 W1648 W1659; 6W, W1445 W1487 W1494 W1605 W1648 W1659; 7W, W1445 W1487 W1494 W1504 W1605 W1648 W1659; 8W, W1445 W1487 W1494 W1504 W1515 W1605 W1648 W1659; all GW, W1487 W1494 W1648 W1659; most conserved, W1504 W1515; and less conserved, W1487 W1605 W1648 W1659.

For selecting most conserved and less conserved W-motifs mutated in the last two mutants, the protein alignment included sequences of more GW182 proteins than the one shown in **Supplementary Figure 1** (data not shown).

The mutants in the dGW182 CED are designated as follows: 2W stands for W1107 W1114; 3W, W1107 W1114 W1118; 4W, W1092 W1107 W1114 W1118; 5W, W1051 W1092 W1107 W1114 W1118; 6W, W1037 W1051 W1092 W1107 W1114 W1118; 7Wa, W1024 W1037 W1051 W1092 W1107 W1114 W1118; 8W, W942 W1024 W1037 W1051 W1092 W1107 W1114 W1118A; 8Wa, W942 W1024 W1037 W1051 W1092 W1107 W1114 W1350; 7W, W942 W1024 W1037 W1051 W1092 W1107 W1114; and 5Wa, W942 W1024 W1037 W1051 W1092.

The 8W mutant of the TNRC6A contains the following mutations: W1420A W1450A W1494A W1505A W1518A W1619A W1666A W1676A (see **Supplementary Fig. 1**).

Pull-down assays and western blotting. For GST pull-down assays, HEK293T cells grown in a 10-cm dish were transfected with 5 μg plasmid expressing GST-TNRC6C CED, GST-dGW182(1–490) (or mutants thereof), GST-Sic or GST-Sic7xW. Cells were lysed 24 h after transfection and GST-fusions were pulled down as described[40]. In short, cells were lysed in buffer A (50 mM Tris-HCl, pH 7.5, 150 mM KCl, 0.5% (v/v) Triton X-100, 1× complete EDTA-free protease inhibitor mix (Roche)), and cleared lysates were treated with micrococcal nuclease (10 ng μl^{-1}) for 25 min at 20 °C. We have verified that this treatment eliminates RNA-dependent interactions (see, for example, **Fig. 6c** in ref. 40). The lysates were incubated with glutathione (GSH)-Sepharose beads (GE Healthcare) for 2 h at 4 °C; beads were washed 3× with buffer A containing 0.1% (v/v) Triton X-100, and GST-fusions were eluted with 50 mM GSH. For anti-TNRC6A immunoprecipitations, HeLa cells were lysed in buffer B (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM DTT, 0.5% (v/v) NP40, 1× complete EDTA-free protease inhibitor (Roche)), treated with micrococcal nuclease as described above and incubated with anti-TNRC6A antibody (Bethyl A302-330A) or, as a negative control, with rabbit IgG (Sigma) bound to Dynabeads Protein G (Invitrogen) overnight at 4 °C. Beads were washed 3× with buffer B containing 0.1% (v/v) NP-40 and boiled in Laemmli SDS-PAGE buffer.

The following primary antibodies were used for western blotting: anti-TNRC6A, 1:5000 (Bethyl A302-329A); anti-CNOT1, 1:250 dilution (provided by M. Collart); anti-CAF1 (Abnova), 1:1,000; anti-PABP (Cell Signaling Technology), 1:5,000; anti-PAN2, 1:1,000 and anti-PAN3, 1:500 (both provided by A.-B. Shyu); anti-dGW182, 1:2,000 (provided by E. Izaurralde); anti-GST (GE Healthcare), 1:10,000; anti-α-tubulin (Sigma T5168), 1:10,000; anti-HA tag (Roche 3F10), 1:5,000; anti-HA tag (Santa Cruz sc-7392), 1:2,000; and anti-LexA (Santa Cruz sc-7544), 1:2,000.

36. Weidenfeld, I. *et al.* Inducible expression of coding and inhibitory RNAs from retargetable genomic loci. *Nucleic Acids Res.* **37**, e50 (2009).
37. Höck, J. *et al.* Proteomic and functional analysis of Argonaute-containing mRNA-protein complexes in human cells. *EMBO Rep.* **8**, 1052–1060 (2007).
38. Mayr, C., Hemann, M.T. & Bartel, D.P. Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation. *Science* **315**, 1576–1579 (2007).
39. Chekulaeva, M., Filipowicz, W. & Parker, R. Multiple independent domains of dGW182 function in miRNA-mediated repression in *Drosophila*. *RNA* **15**, 794–803 (2009).
40. Fabian, M.R. *et al.* Mammalian miRNA RISC recruits CAF1 and PABP to affect PABP-dependent deadenylation. *Mol. Cell* **35**, 868–880 (2009).

References

P. P. Amaral, M. E. Dinger, T. R. Mercer, and J. S. Mattick. The eukaryotic genome as an RNA machine. *Science*, 319(5871):1787–9, 2008. 1.2

D. C. Amberg. *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory, 2005. 4.4

N. Amrani, M. S. Sachs, and A. Jacobson. Early nonsense: mRNA decay solves a translational problem. *Nature reviews. Molecular cell biology*, 7(6):415–25, 2006. 1.4.4

O. T. Avery, C. M. Macleod, and M. McCarty. Studies on the Chemical Nature of the Substance Inducing Transformation of Pneumococcal Types : Induction of Transformation by a Desoxyribonucleic Acid Fraction Isolated from Pneumococcus Type Iii. *J Exp Med*, 79(2):137–58, 1944. 1.1

D. Baek, J. Villen, C. Shin, F. D. Camargo, S. P. Gygi, and D. P. Bartel. The impact of microRNAs on protein output. *Nature*, 455(7209):64–71, 2008. 1.2, 1.3.2, 1.4.4

S. Bagga, J. Bracht, S. Hunter, K. Massirer, J. Holtz, R. Eachus, and A. E. Pasquinelli. Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. *Cell*, 122 (4):553–63, 2005. 1.4.4

V. Balagopal and R. Parker. Polysomes, P bodies and stress granules: states and fates of eukaryotic mRNAs. *Curr Opin Cell Biol*, 2009. 1.3.3

A. J. Bannister and T. Kouzarides. Regulation of chromatin by histone modifications. *Cell research*, 21(3):381–95, 2011. 1.1.2.2

D. P. Bartel. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*, 116(2): 281–97, 2004. 1.2

D. P. Bartel. MicroRNAs: target recognition and regulatory functions. *Cell*, 136(2): 215–33, 2009. 1.3.2

V. I. Bashkirov, H. Scherthan, J. A. Solinger, J. M. Buerstedde, and W. D. Heyer. A mouse cytoplasmic exoribonuclease (mXRN1p) with preference for G4 tetraplex substrates. *The Journal of cell biology*, 136(4):761–73, 1997. 1.3.3

S. Baskerville and D. P. Bartel. Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes. *RNA*, 11(3):241–7, 2005. 1.2.2

I. Behm-Ansmant, J. Rehwinkel, T. Doerks, A. Stark, P. Bork, and E. Izaurralde. mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. *Genes Dev*, 20(14):1885–98, 2006. 1.4.4, 1.5, 3, 3

T. H. Beilharz, D. T. Humphreys, and T. Preiss. miRNA Effects on mRNA closed-loop formation during translation initiation. *Progress in molecular and subcellular biology*, 50:99–112, 2010. 3

E. Bernstein and C. D. Allis. RNA meets chromatin. *Genes Dev*, 19(14):1635–55, 2005. 1.1.2.3

S. N. Bhattacharyya, R. Habermacher, U. Martine, E. I. Closs, and W. Filipowicz. Relief of microRNA-mediated translational repression in human cells subjected to stress. *Cell*, 125(6):1111–24, 2006. 1.3.3, 1.4.3

M. J. Blow, R. J. Grocock, S. van Dongen, A. J. Enright, E. Dicks, P. A. Futreal, R. Wooster, and M. R. Stratton. RNA editing of human microRNAs. *Genome biology*, 7(4):R27, 2006. 1.2.3

M. T. Bohnsack, K. Czaplinski, and D. Gorlich. Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. *RNA*, 10(2): 185–91, 2004. 1.2.2.1

R. Bonasio, S. Tu, and D. Reinberg. Molecular signals of epigenetic states. *Science*, 330 (6004):612–6, 2010. 1.1.2

G. M. Borchert, W. Lanier, and B. L. Davidson. RNA polymerase III transcribes human microRNAs. *Nat Struct Mol Biol*, 13(12):1097–101, 2006. 1.2.2

J. Bracht, S. Hunter, R. Eachus, P. Weeks, and A. E. Pasquinelli. Trans-splicing and polyadenylation of let-7 microRNA primary transcripts. *RNA*, 10(10):1586–94, 2004. 1.2.2

M. Brengues, D. Teixeira, and R. Parker. Movement of eukaryotic mRNAs between polysomes and cytoplasmic processing bodies. *Science*, 310(5747):486–9, 2005. 1.3.3

R. A. Brink. Basis of a Genetic Change Which Invariably Occurs in Certain Maize Heterozygotes. *Science*, 127(3307):1182–3, 1958. 1.1

P. Brodersen and O. Voinnet. Revisiting the principles of microRNA target recognition and mode of action. *Nat Rev Mol Cell Biol*, 10(2):141–8, 2009. 1.3.2

B. Brueckner, C. Stresemann, R. Kuner, C. Mund, T. Musch, M. Meister, H. Sultmann, and F. Lyko. The human let-7a-3 locus contains an epigenetically regulated microRNA gene with oncogenic function. *Cancer research*, 67(4):1419–23, 2007. 1.2.3

X. Cai, C. H. Hagedorn, and B. R. Cullen. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. *RNA*, 10(12): 1957–66, 2004. 1.2.2

G. A. Calin, C. D. Dumitru, and et al. Shimizu, M. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proceedings of the National Academy of Sciences of the United States of America*, 99(24):15524–9, 2002. 1.2.1

Elof Axel Carlson. *Mendel's Legacy: The Origin of Classical Genetics*. Cold Spring Harbor Laboratory Press, 2004. 1.1

M. A. Carmell and G. J. Hannon. RNase III enzymes and the initiation of gene silencing. *Nat Struct Mol Biol*, 11(3):214–8, 2004. 1.2.2.2

R. W. Carthew and E. J. Sontheimer. Origins and Mechanisms of miRNAs and siRNAs. *Cell*, 136(4):642–55, 2009. 1.2.2

M. Ceci, C. Gaviraghi, C. Gorrini, L. A. Sala, N. Offenhauser, P. C. Marchisio, and S. Biffo. Release of eIF6 (p27BBP) from the 60S subunit allows 80S ribosome assembly. *Nature*, 426(6966):579–84, 2003. 1.4.3

E. S. Cenik and P. D. Zamore. Argonaute proteins. *Current biology : CB*, 21(12):R446–9, 2011. 1.3.1

S. Chatterjee and H. Grosshans. Active turnover modulates mature microRNA activity in Caenorhabditis elegans. *Nature*, 461(7263):546–9, 2009. 1.2.3

M. Chekulaeva and W. Filipowicz. Mechanisms of miRNA-mediated post-transcriptional regulation in animal cells. *Curr Opin Cell Biol*, 21(3):452–60, 2009. 1.4

M. Chekulaeva, W. Filipowicz, and R. Parker. Multiple independent domains of dGW182 function in miRNA-mediated repression in Drosophila. *RNA*, 15(5):794–803, 2009. 2.5, 3

M. Chekulaeva, H. Mathys, J. T. Zipprich, J. Attig, M. Colic, R. Parker, and W. Filipowicz. miRNA repression involves GW182-mediated recruitment of CCR4-NOT through conserved W-containing motifs. *Nat Struct Mol Biol*, 18(11):1218–26, 2011. 2.1, 2.3, 2.4, 2.6, 2.6, 3, 3, 3, 4.2, 4.4

S. Cheloufi, C. O. Dos Santos, M. M. Chong, and G. J. Hannon. A dicer-independent miRNA biogenesis pathway that requires Ago catalysis. *Nature*, 465(7298):584–9, 2010. 1.2.2.2

C. Y. Chen, D. Zheng, Z. Xia, and A. B. Shyu. Ago-TNRC6 triggers microRNA-mediated decay by promoting two deadenylation steps. *Nat Struct Mol Biol*, 16(11):1160–6, 2009. 3

T. P. Chendrimada, R. I. Gregory, E. Kumaraswamy, J. Norman, N. Cooch, K. Nishikura, and R. Shiekhattar. TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature*, 436(7051):740–4, 2005. 1.3

T. P. Chendrimada, K. J. Finn, X. Ji, D. Baillat, R. I. Gregory, S. A. Liebhaber, A. E. Pasquinelli, and R. Shiekhattar. MicroRNA silencing through RISC recruitment of eIF6. *Nature*, 447(7146):823–8, 2007. 1.4.3

C. Y. Chu and T. M. Rana. Translation repression in human cells by microRNA-induced gene silencing requires RCK/p54. *PLoS biology*, 4(7):e210, 2006. 1.3.3, 3

D. Cifuentes, H. Xue, D. W. Taylor, H. Patnode, Y. Mishima, S. Cheloufi, E. Ma, S. Mane, G. J. Hannon, N. D. Lawson, S. A. Wolfe, and A. J. Giraldez. A novel miRNA processing pathway independent of Dicer requires Argonaute2 catalytic activity. *Science*, 328(5986):1694–8, 2010. 1.2.2.2

A. Clery, M. Blatter, and F. H. Allain. RNA recognition motifs: boring? Not quite. *Curr Opin Struct Biol*, 18(3):290–8, 2008. 3

M. A. Collart and O. O. Panasenko. The Ccr4-Not complex. *Gene*, 492(1):42–53, 2011. 1.4.4

J. Coller and R. Parker. General translational repression by activators of mRNA decapping. *Cell*, 122(6):875–86, 2005. 3

J. M. Coller, M. Tucker, U. Sheth, M. A. Valencia-Sanchez, and R. Parker. The DEAD box helicase, Dhh1p, functions in mRNA decapping and interacts with both the decapping and deadenylase complexes. *RNA*, 7(12):1717–27, 2001. 3

A. Cooke, A. Prigge, and M. Wickens. Translational repression by deadenylases. *J Biol Chem*, 285(37):28506–13, 2010. 2.6, 3

J. Couzin. MicroRNAs make big impression in disease after disease. *Science*, 319(5871): 1782–4, 2008. 1.2

A. M. Denli, B. B. Tops, R. H. Plasterk, R. F. Ketting, and G. J. Hannon. Processing of primary microRNAs by the Microprocessor complex. *Nature*, 432(7014):231–5, 2004. 1.2.2.1

S. Diederichs and D. A. Haber. Dual role for argonautes in microRNA processing and posttranscriptional regulation of microRNA expression. *Cell*, 131(6):1097–108, 2007. 1.2.2.2

L. Ding and M. Han. GW182 family proteins are crucial for microRNA-mediated gene silencing. *Trends Cell Biol*, 17(8):411–6, 2007. 1.5

S. W. Ding. RNA-based antiviral immunity. *Nat Rev Immunol*, 10(9):632–44, 2010. 1.4.4

X. C. Ding and H. Grosshans. Repression of C. elegans microRNA targets at the initiation level of translation requires GW182 proteins. *EMBO J*, 28(3):213–22, 2009. 1.4.2, 1.4.3, 1.5

S. Djuranovic, A. Nahvi, and R. Green. A parsimonious model for gene regulation by miRNAs. *Science*, 331(6017):550–3, 2011. 1.4.4

M. El-Shami, D. Pontier, S. Lahmy, L. Braun, C. Picart, D. Vega, M. A. Hakimi, S. E. Jacobsen, R. Cooke, and T. Lagrange. Reiterated WG/GW motifs form functionally and evolutionarily conserved ARGONAUTE-binding platforms in RNAi-related components. *Genes Dev*, 21(20):2539–44, 2007. 1.5

A. Eulalio, I. Behm-Ansmant, and E. Izaurralde. P bodies: at the crossroads of post-transcriptional pathways. *Nat Rev Mol Cell Biol*, 8(1):9–22, 2007a. 1.3.3

A. Eulalio, I. Behm-Ansmant, D. Schweizer, and E. Izaurralde. P-body formation is a consequence, not the cause, of RNA-mediated gene silencing. *Mol Cell Biol*, 27(11): 3970–81, 2007b. 1.3.3, 1.5

A. Eulalio, J. Rehwinkel, M. Stricker, E. Huntzinger, S. F. Yang, T. Doerks, S. Dorner, P. Bork, M. Boutros, and E. Izaurralde. Target-specific requirements for enhancers of decapping in miRNA-mediated gene silencing. *Genes Dev*, 21(20):2558–70, 2007c. 1.4.4, 3

A. Eulalio, E. Huntzinger, and E. Izaurralde. GW182 interaction with Argonaute is essential for miRNA-mediated translational repression and mRNA decay. *Nat Struct Mol Biol*, 15(4):346–53, 2008a. 1.4.3

A. Eulalio, E. Huntzinger, T. Nishihara, J. Rehwinkel, M. Fauser, and E. Izaurralde. Deadenylation is a widespread effect of miRNA regulation. *RNA*, 15(1):21–32, 2008b. 4.2

A. Eulalio, F. Tritschler, R. Buttner, O. Weichenrieder, E. Izaurralde, and V. Truffault. The RRM domain in GW182 proteins contributes to miRNA-mediated gene silencing. *Nucleic Acids Res*, 37(9):2974–83, 2009. 3, 3

T. Eystathioy, E. K. Chan, S. A. Tenenbaum, J. D. Keene, K. Griffith, and M. J. Fritzler. A phosphorylated cytoplasmic autoantigen, GW182, associates with a unique population of human mRNAs within novel cytoplasmic speckles. *Mol Biol Cell*, 13(4): 1338–51, 2002. 1.5, 4.2

T. Eystathioy, A. Jakymiw, E. K. Chan, B. Seraphin, N. Cougot, and M. J. Fritzler. The GW182 protein colocalizes with mRNA degradation associated proteins hDcp1 and hLSm4 in cytoplasmic GW bodies. *RNA*, 9(10):1171–3, 2003. 1.3.3, 1.5, 1.5

M. R. Fabian, G. Mathonnet, and et al. Sundermeier, T. Mammalian miRNA RISC Recruits CAF1 and PABP to Affect PABP-Dependent Deadenylation. *Mol Cell*, 35(6): 868–80, 2009. 1.4.4, 2.3, 3, 4.2, 4.3

M. R. Fabian, N. Sonenberg, and W. Filipowicz. Regulation of mRNA translation and stability by microRNAs. *Annu Rev Biochem*, 79:351–79, 2010. 1.4, 1.4.1

M. R. Fabian, M. K. Cieplak, F. Frank, M. Morita, J. Green, T. Srikumar, B. Nagar, T. Yamamoto, B. Raught, T. F. Duchaine, and N. Sonenberg. miRNA-mediated deadenylation is orchestrated by GW182 through two conserved motifs that interact with CCR4-NOT. *Nat Struct Mol Biol*, 18(11):1211–7, 2011. 3

M. B. Fasken and A. H. Corbett. Process or perish: quality control in mRNA biogenesis. *Nat Struct Mol Biol*, 12(6):482–8, 2005. 1.4.4

A. Fire, S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver, and C. C. Mello. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature*, 391(6669):806–11, 1998. 1.3

K. Forstemann, M. D. Horwich, L. Wee, Y. Tomari, and P. D. Zamore. Drosophila microRNAs are sorted into functionally distinct argonaute complexes after production by dicer-1. *Cell*, 130(2):287–97, 2007. 1.3.1.2

F. Frank, N. Sonenberg, and B. Nagar. Structural basis for 5'-nucleotide base-specific recognition of guide RNA by human AGO2. *Nature*, 465(7299):818–22, 2010. 1.3.1.1

R. C. Friedman, K. K. Farh, C. B. Burge, and D. P. Bartel. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res*, 19(1):92–105, 2009. 1.2, 1.3.2

T. Fukaya and Y. Tomari. PABP is not essential for microRNA-mediated translational repression and deadenylation in vitro. *The EMBO journal*, 30(24):4998–5009, 2011. 3

F. Fuks, W. A. Burgers, A. Brehm, L. Hughes-Davies, and T. Kouzarides. DNA methyltransferase Dnmt1 associates with histone deacetylase activity. *Nature genetics*, 24(1):88–91, 2000. 1.1.2.2

T. Fukuda, K. Yamagata, and et al. Fujiyama, S. DEAD-box RNA helicase subunits of the Drosha complex are required for processing of rRNA and a subset of microRNAs. *Nature cell biology*, 9(5):604–11, 2007. 1.2.3

V. K. Gangaraju and H. Lin. MicroRNAs: key regulators of stem cells. *Nat Rev Mol Cell Biol*, 10(2):116–25, 2009. 1.2

N. L. Garneau, J. Wilusz, and C. J. Wilusz. The highways and byways of mRNA decay. *Nat Rev Mol Cell Biol*, 8(2):113–26, 2007. 1.2.3, 1.4.4

D. Gatfield, G. Le Martelot, C. E. Vejnar, D. Gerlach, O. Schaad, F. Fleury-Olela, A. L. Ruskeepaa, M. Oresic, C. C. Esau, E. M. Zdobnov, and U. Schibler. Integration of microRNA miR-122 in hepatic circadian gene expression. *Genes & development*, 23 (11):1313–26, 2009. 1.2.3

D. J. Gibbings, C. Ciaudo, M. Erhardt, and O. Voinnet. Multivesicular bodies associate with components of miRNA effector complexes and modulate miRNA activity. *Nat Cell Biol*, 2009. 1.3.3

A. J. Giraldez, Y. Mishima, J. Rihel, R. J. Grocock, S. Van Dongen, K. Inoue, A. J. Enright, and A. F. Schier. Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. *Science*, 312(5770):75–9, 2006. 1.4.4

A. D. Goldberg, C. D. Allis, and E. Bernstein. Epigenetics: a landscape takes shape. *Cell*, 128(4):635–8, 2007. 1.1.1, 1.1.1

M. G. Goll and T. H. Bestor. Eukaryotic cytosine methyltransferases. *Annu Rev Biochem*, 74:481–514, 2005. 1.1.2.1

R. I. Gregory, K. P. Yan, G. Amuthan, T. Chendrimada, B. Doratotaj, N. Cooch, and R. Shiekhattar. The Microprocessor complex mediates the genesis of microRNAs. *Nature*, 432(7014):235–40, 2004. 1.2.2.1

R. I. Gregory, T. P. Chendrimada, N. Cooch, and R. Shiekhattar. Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. *Cell*, 123(4):631–40, 2005. 1.3

S. Griffiths-Jones. The microRNA Registry. *Nucleic acids research*, 32(Database issue): D109–11, 2004. 1.2.1

H. Grosshans and W. Filipowicz. Molecular biology: the expanding world of small RNAs. *Nature*, 451(7177):414–6, 2008. 1.1.2.3

S. Gu, L. Jin, F. Zhang, P. Sarnow, and M. A. Kay. Biological basis for restriction of microRNA targets to the 3' untranslated region in mammalian mRNAs. *Nat Struct Mol Biol*, 16(2):144–50, 2009. 1.3.2

H. Guo, N. T. Ingolia, J. S. Weissman, and D. P. Bartel. Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature*, 466(7308):835–40, 2010. 1.4.4

A. D. Haase, L. Jaskiewicz, H. Zhang, S. Laine, R. Sack, A. Gatignol, and W. Filipowicz. TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing. *EMBO Rep*, 6(10):961–7, 2005. 1.2.2.2

J. Han, Y. Lee, K. H. Yeom, Y. K. Kim, H. Jin, and V. N. Kim. The Drosha-DGCR8 complex in primary microRNA processing. *Genes & development*, 18(24):3016–27, 2004. 1.2.2.1

J. Han, J. S. Pedersen, S. C. Kwon, C. D. Belair, Y. K. Kim, K. H. Yeom, W. Y. Yang, D. Haussler, R. Blelloch, and V. N. Kim. Posttranscriptional crossregulation between Drosha and DGCR8. *Cell*, 136(1):75–84, 2009. 1.2.3

J. Hock, L. Weinmann, C. Ender, S. Rudel, E. Kremmer, M. Raabe, H. Urlaub, and G. Meister. Proteomic and functional analysis of Argonaute-containing mRNA-protein complexes in human cells. *EMBO Rep*, 8(11):1052–60, 2007. 1.3.1.2

R. Holliday and J. E. Pugh. DNA modification mechanisms and gene activity during development. *Science*, 187(4173):226–32, 1975. 1.1.2.1

Y. Huang, Q. Zou, S. P. Wang, S. M. Tang, G. Z. Zhang, and X. J. Shen. The discovery approaches and detection methods of microRNAs. *Molecular biology reports*, 38(6): 4125–35, 2011. 1.2.1

D. T. Humphreys, B. J. Westman, D. I. Martin, and T. Preiss. MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function. *Proc Natl Acad Sci U S A*, 102(47):16961–6, 2005. 1.4.3

E. Huntzinger and E. Izaurralde. Gene silencing by microRNAs: contributions of translational repression and mRNA decay. *Nat Rev Genet*, 12(2):99–110, 2010. 1.4, 3

E. Huntzinger, J. E. Braun, S. Heimstadt, L. Zekri, and E. Izaurralde. Two PABPC1-binding sites in GW182 proteins promote miRNA-mediated gene silencing. *EMBO J*, 29(24):4146–60, 2010. 3

G. Hutvagner and M. J. Simard. Argonaute proteins: key players in RNA silencing. *Nat Rev Mol Cell Biol*, 9(1):22–32, 2008. 1.3.1

H. W. Hwang, E. A. Wentzel, and J. T. Mendell. Cell-cell contact globally activates microRNA biogenesis. *Proc Natl Acad Sci U S A*, 2009. 1.4.3

R. S. Illingworth and A. P. Bird. CpG islands–'a rough guide'. *FEBS letters*, 583(11): 1713–20, 2009. 1.1.2.1

M. V. Iorio, C. Piovan, and C. M. Croce. Interplay between microRNAs and the epigenetic machinery: an intricate network. *Biochim Biophys Acta*, 1799(10-12):694–701, 2010. 1.2

S. Iwasaki, T. Kawamata, and Y. Tomari. Drosophila argonaute1 and argonaute2 employ distinct mechanisms for translational repression. *Mol Cell*, 34(1):58–67, 2009. 1.3.1.2

R. J. Jackson. Alternative mechanisms of initiating translation of mammalian mRNAs. *Biochemical Society transactions*, 33(Pt 6):1231–41, 2005. 1.4.1

R. J. Jackson, C. U. Hellen, and T. V. Pestova. The mechanism of eukaryotic translation initiation and principles of its regulation. *Nat Rev Mol Cell Biol*, 11(2):113–27, 2010. 1.4.1

A. Jakymiw, S. Lian, T. Eystathioy, S. Li, M. Satoh, J. C. Hamel, M. J. Fritzler, and E. K. Chan. Disruption of GW bodies impairs mammalian RNA interference. *Nat Cell Biol*, 7(12):1267–74, 2005. 1.3.3, 1.5

L. Jaskiewicz and W. Filipowicz. Role of Dicer in posttranscriptional RNA silencing. *Curr Top Microbiol Immunol*, 320:77–97, 2008. 1.2.2.2

T. Jenuwein and C. D. Allis. Translating the histone code. *Science*, 293(5532):1074–80, 2001. 1.1.2.2

M. Jinek and J. A. Doudna. A three-dimensional view of the molecular machinery of RNA interference. *Nature*, 457(7228):405–12, 2009. 1.3.1

M. Jinek, M. R. Fabian, S. M. Coyle, N. Sonenberg, and J. A. Doudna. Structural insights into the human GW182-PABC interaction in microRNA-mediated deadenylation. *Nat Struct Mol Biol*, 17(2):238–40, 2010. 2.3, 3

S. M. Johnson, S. Y. Lin, and F. J. Slack. The time of appearance of the C. elegans let-7 microRNA is transcriptionally controlled utilizing a temporal regulatory element in its promoter. *Developmental biology*, 259(2):364–79, 2003. 1.2.3

M. W. Jones-Rhoades, D. P. Bartel, and B. Bartel. MicroRNAS and their regulatory roles in plants. *Annual review of plant biology*, 57:19–53, 2006. 1.4

L. D. Kapp and J. R. Lorsch. The molecular mechanics of eukaryotic translation. *Annual review of biochemistry*, 73:657–704, 2004. 1.4.1

N. Kataoka, M. Fujita, and M. Ohno. Functional association of the Microprocessor complex with the spliceosome. *Molecular and cellular biology*, 29(12):3243–54, 2009. 1.2.2.1

V. N. Kim. MicroRNA biogenesis: coordinated cropping and dicing. *Nature reviews. Molecular cell biology*, 6(5):376–85, 2005. 1.2.2.1

V. N. Kim. Small RNAs just got bigger: Piwi-interacting RNAs (piRNAs) in mammalian testes. *Genes & development*, 20(15):1993–7, 2006. 1.3.1

V. N. Kim, J. Han, and M. C. Siomi. Biogenesis of small RNAs in animals. *Nat Rev Mol Cell Biol*, 10(2):126–39, 2009. 1.2.2

L. N. Kinch and N. V. Grishin. The human Ago2 MC region does not contain an eIF4E-like mRNA cap binding motif. *Biology direct*, 4:2, 2009. 1.4.3

M. Kiriakidou, G. S. Tan, S. Lamprinaki, M. De Planell-Saguer, P. T. Nelson, and Z. Mourelatos. An mRNA m7G cap binding-like motif within human Ago2 represses translation. *Cell*, 129(6):1141–51, 2007. 1.4.3

S. W. Knight and B. L. Bass. A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in Caenorhabditis elegans. *Science*, 293(5538):2269–71, 2001. 1.2.2.2

Y. W. Kong, I. G. Cannell, C. H. de Moor, K. Hill, P. G. Garside, T. L. Hamilton, H. A. Meijer, H. C. Dobbyn, M. Stoneley, K. A. Spriggs, A. E. Willis, and M. Bushell. The mechanism of micro-RNA-mediated translation repression is determined by the promoter of the target gene. *Proc Natl Acad Sci U S A*, 2008. 1.4.3

T. Kouzarides. Chromatin modifications and their function. *Cell*, 128(4):693–705, 2007. 1.1.2.2

A. Kozomara and S. Griffiths-Jones. miRBase: integrating microRNA annotation and deep-sequencing data. *Nucleic acids research*, 39(Database issue):D152–7, 2011. 1.2.1

J. Krol, V. Busskamp, and et al. Markiewicz, I. Characterizing light-regulated retinal microRNAs reveals rapid turnover as a common property of neuronal microRNAs. *Cell*, 141(4):618–31, 2010a. 1.2.3

J. Krol, I. Loedige, and W. Filipowicz. The widespread regulation of microRNA biogenesis, function and decay. *Nat Rev Genet*, 11(9):597–610, 2010b. 1.2, 1.2.3

J. Krutzfeldt, N. Rajewsky, R. Braich, K. G. Rajeev, T. Tuschl, M. Manoharan, and M. Stoffel. Silencing of microRNAs in vivo with 'antagomirs'. *Nature*, 438(7068):685–9, 2005. 1.4.4

M. Kulkarni, S. Ozgur, and G. Stoecklin. On track with P-bodies. *Biochem Soc Trans*, 38(Pt 1):242–51, 2010. 1.3.3

M. Lagos-Quintana, R. Rauhut, W. Lendeckel, and T. Tuschl. Identification of novel genes coding for small expressed RNAs. *Science*, 294(5543):853–8, 2001. 1.2.1

E. C. Lai. Micro RNAs are complementary to 3' UTR sequence motifs that mediate negative post-transcriptional regulation. *Nature genetics*, 30(4):363–4, 2002. 1.3.2

S. Lall, D. Grun, and et al. Krek, A. A genome-wide map of conserved microRNA targets in C. elegans. *Current biology : CB*, 16(5):460–71, 2006. 1.3.2

E. S. Lander, L. M. Linton, and et al. Birren, B. Initial sequencing and analysis of the human genome. *Nature*, 409(6822):860–921, 2001. 1.1

M. Landthaler, A. Yalcin, and T. Tuschl. The human DiGeorge syndrome critical region gene 8 and Its D. melanogaster homolog are required for miRNA biogenesis. *Current biology : CB*, 14(23):2162–7, 2004. 1.2.2.1

N. C. Lau, L. P. Lim, E. G. Weinstein, and D. P. Bartel. An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. *Science*, 294(5543): 858–62, 2001. 1.2.1

N. C. Lau, A. Kolkman, F. M. van Schaik, K. W. Mulder, W. W. Pijnappel, A. J. Heck, and H. T. Timmers. Human Ccr4-Not complexes contain variable deadenylase subunits. *The Biochemical journal*, 422(3):443–53, 2009. 1.4.4, 4.4

R. C. Lee and V. Ambros. An extensive class of small RNAs in Caenorhabditis elegans. *Science*, 294(5543):862–4, 2001. 1.2.1

R. C. Lee, R. L. Feinbaum, and V. Ambros. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell*, 75(5):843–54, 1993. 1.2.1, 1.3.2

Y. Lee, C. Ahn, J. Han, H. Choi, J. Kim, J. Yim, J. Lee, P. Provost, O. Radmark, S. Kim, and V. N. Kim. The nuclear RNase III Drosha initiates microRNA processing. *Nature*, 425(6956):415–9, 2003. 1.2.2.1

Y. Lee, M. Kim, J. Han, K. H. Yeom, S. Lee, S. H. Baek, and V. N. Kim. MicroRNA genes are transcribed by RNA polymerase II. *EMBO J*, 23(20):4051–60, 2004a. 1.2.2

Y. Lee, I. Hur, S. Y. Park, Y. K. Kim, M. R. Suh, and V. N. Kim. The role of PACT in the RNA silencing pathway. *The EMBO journal*, 25(3):522–32, 2006. 1.2.2.2

Y. S. Lee, K. Nakahara, J. W. Pham, K. Kim, Z. He, E. J. Sontheimer, and R. W. Carthew. Distinct roles for Drosophila Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell*, 117(1):69–81, 2004b. 1.2.2.2, 1.3

Y. S. Lee, S. Pressman, and et al. Andress, A. P. Silencing by small RNAs is linked to endosomal trafficking. *Nature cell biology*, 11(9):1150–6, 2009. 1.3.3

B. P. Lewis, I. H. Shih, M. W. Jones-Rhoades, D. P. Bartel, and C. B. Burge. Prediction of mammalian microRNA targets. *Cell*, 115(7):787–98, 2003. 1.3.2

B. P. Lewis, C. B. Burge, and D. P. Bartel. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*, 120 (1):15–20, 2005. 1.3.2

L. P. Lim, M. E. Glasner, S. Yekta, C. B. Burge, and D. P. Bartel. Vertebrate microRNA genes. *Science*, 299(5612):1540, 2003. 1.2

L. P. Lim, N. C. Lau, P. Garrett-Engele, A. Grimson, J. M. Schelter, J. Castle, D. P. Bartel, P. S. Linsley, and J. M. Johnson. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature*, 433(7027):769–73, 2005. 1.4.4

H. Lin and A. C. Spradling. A novel group of pumilio mutations affects the asymmetric division of germline stem cells in the Drosophila ovary. *Development*, 124(12):2463–76, 1997. 1.3.1

J. Liu, M. A. Carmell, F. V. Rivas, C. G. Marsden, J. M. Thomson, J. J. Song, S. M. Hammond, L. Joshua-Tor, and G. J. Hannon. Argonaute2 is the catalytic engine of mammalian RNAi. *Science*, 305(5689):1437–41, 2004. 1.3.1.1, 1.3.1.2

J. Liu, F. V. Rivas, J. Wohlschlegel, 3rd Yates, J. R., R. Parker, and G. J. Hannon. A role for the P-body component GW182 in microRNA function. *Nat Cell Biol*, 7(12): 1261–6, 2005a. 1.5

J. Liu, M. A. Valencia-Sanchez, G. J. Hannon, and R. Parker. MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. *Nature cell biology*, 7(7): 719–23, 2005b. 1.3.3

Q. Liu, T. A. Rand, S. Kalidas, F. Du, H. E. Kim, D. P. Smith, and X. Wang. R2D2, a bridge between the initiation and effector steps of the Drosophila RNAi pathway. *Science*, 301(5641):1921–5, 2003. 1.3

J. B. Ma, K. Ye, and D. J. Patel. Structural basis for overhang-specific small interfering RNA recognition by the PAZ domain. *Nature*, 429(6989):318–22, 2004. 1.3.1.1

E. Maniataki and Z. Mourelatos. A human, ATP-independent, RISC assembly machine fueled by pre-miRNA. *Genes & development*, 19(24):2979–90, 2005. 1.3

P. A. Maroney, Y. Yu, J. Fisher, and T. W. Nilsen. Evidence that microRNAs are associated with translating messenger RNAs in human cells. *Nat Struct Mol Biol*, 13 (12):1102–7, 2006. 1.4.2

G. Mathonnet, M. R. Fabian, Y. V. Svitkin, A. Parsyan, L. Huck, T. Murata, S. Biffo, W. C. Merrick, E. Darzynkiewicz, R. S. Pillai, W. Filipowicz, T. F. Duchaine, and N. Sonenberg. MicroRNA inhibition of translation initiation in vitro by targeting the cap-binding complex eIF4F. *Science*, 317(5845):1764–7, 2007. 1.4.3

C. Matranga, Y. Tomari, C. Shin, D. P. Bartel, and P. D. Zamore. Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. *Cell*, 123(4):607–20, 2005. 1.3

G. Meister, M. Landthaler, A. Patkaniowska, Y. Dorsett, G. Teng, and T. Tuschl. Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Mol Cell*, 15 (2):185–97, 2004. 1.3.1.2

G. Meister, M. Landthaler, L. Peters, P. Y. Chen, H. Urlaub, R. Luhrmann, and T. Tuschl. Identification of novel argonaute-associated proteins. *Curr Biol*, 15(23):2149–55, 2005. 1.5

F. Miescher. Ueber die chemische Zusammensetzung des Eiters. *Med.-Chem. Unters*, 4: 441–460, 1871. 1.1

D. Moazed. Small RNAs in transcriptional gene silencing and genome defence. *Nature*, 457(7228):413–20, 2009. 1.1.2.3

F. Moretti, R. Thermann, and M. W. Hentze. Mechanism of translational regulation by miR-2 from sites in the 5' untranslated region or the open reading frame. *RNA*, 16 (12):2493–502, 2010. 1.3.2

T. H. Morgan. Sex Limited Inheritance in Drosophila. *Science*, 32(812):120–2, 1910. 1.1

M. Morlando, M. Ballarino, N. Gromak, F. Pagano, I. Bozzoni, and N. J. Proudfoot. Primary microRNA transcripts are processed co-transcriptionally. *Nat Struct Mol Biol*, 15(9):902–9, 2008. 1.2.2.1

B. Moussian, H. Schoof, A. Haecker, G. Jurgens, and T. Laux. Role of the ZWILLE gene in the regulation of central shoot meristem cell fate during Arabidopsis embryogenesis. *The EMBO journal*, 17(6):1799–809, 1998. 1.3.1

Y. Nam, C. Chen, R. I. Gregory, J. J. Chou, and P. Sliz. Molecular Basis for Interaction of let-7 MicroRNAs with Lin28. *Cell*, 2011. 1.2.3

P. T. Nelson, A. G. Hatzigeorgiou, and Z. Mourelatos. miRNP:mRNA association in polyribosomes in a human neuronal cell line. *RNA*, 10(3):387–94, 2004. 1.4.2

T. Nissan and R. Parker. Computational analysis of miRNA-mediated repression of translation: implications for models of translation initiation inhibition. *RNA*, 14(8): 1480–91, 2008. 1.4.3

C. G. Noble, D. Hollingworth, S. R. Martin, V. Ennis-Adeniran, S. J. Smerdon, G. Kelly, I. A. Taylor, and A. Ramos. Key features of the interaction between Pcf11 CID and RNA polymerase II CTD. *Nat Struct Mol Biol*, 12(2):144–51, 2005. 3

S. Nottrott, M. J. Simard, and J. D. Richter. Human let-7a miRNA blocks protein production on actively translating polyribosomes. *Nat Struct Mol Biol*, 13(12):1108–14, 2006. 1.4.2

K. Okamura, A. Ishizuka, H. Siomi, and M. C. Siomi. Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways. *Genes & development*, 18 (14):1655–66, 2004. 1.3, 1.3.1

K. Okamura, J. W. Hagen, H. Duan, D. M. Tyler, and E. C. Lai. The mirtron pathway generates microRNA-class regulatory RNAs in Drosophila. *Cell*, 130(1):89–100, 2007. 1.2.2.1

P. H. Olsen and V. Ambros. The lin-4 regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation. *Dev Biol*, 216(2):671–80, 1999. 1.4.2

U. A. Orom, F. C. Nielsen, and A. H. Lund. MicroRNA-10a binds the 5'UTR of ribosomal protein mRNAs and enhances their translation. *Mol Cell*, 30(4):460–71, 2008. 1.4.3

J. S. Parker, S. M. Roe, and D. Barford. Crystal structure of a PIWI protein suggests mechanisms for siRNA recognition and slicer activity. *EMBO J*, 23(24):4727–37, 2004. 1.3.1.1

R. Parker and U. Sheth. P bodies and the control of mRNA translation and degradation. *Mol Cell*, 25(5):635–46, 2007. 1.3.3

R. Parker and H. Song. The enzymes and control of eukaryotic mRNA turnover. *Nat Struct Mol Biol*, 11(2):121–7, 2004. 1.4.4

A. E. Pasquinelli, B. J. Reinhart, and et al. Slack, F. Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. *Nature*, 408(6808):86–9, 2000. 1.2.1

K. M. Pauley, T. Eystathioy, A. Jakymiw, J. C. Hamel, M. J. Fritzler, and E. K. Chan. Formation of GW bodies is a consequence of microRNA genesis. *EMBO reports*, 7(9): 904–10, 2006. 1.3.3

L. Peters and G. Meister. Argonaute proteins: mediators of RNA silencing. *Mol Cell*, 26 (5):611–23, 2007. 1.3.1.2

C. P. Petersen, M. E. Bordeleau, J. Pelletier, and P. A. Sharp. Short RNAs repress translation after initiation in mammalian cells. *Mol Cell*, 21(4):533–42, 2006. 1.4.2

X. Piao, X. Zhang, L. Wu, and J. G. Belasco. CCR4-NOT deadenylates mRNA associated with RNA-induced silencing complexes in human cells. *Mol Cell Biol*, 30(6):1486–94, 2010. 1.4.4, 3

R. S. Pillai, C. G. Artus, and W. Filipowicz. Tethering of human Ago proteins to mRNA mimics the miRNA-mediated repression of protein synthesis. *RNA*, 10(10):1518–25, 2004. 1.3.1.1, 1.6, 4.2

R. S. Pillai, S. N. Bhattacharyya, C. G. Artus, T. Zoller, N. Cougot, E. Basyuk, E. Bertrand, and W. Filipowicz. Inhibition of translational initiation by Let-7 MicroRNA in human cells. *Science*, 309(5740):1573–6, 2005. 1.3.3, 1.4.2, 1.4.3

E. Piskounova, C. Polytarchou, J. E. Thornton, R. J. Lapierre, C. Pothoulakis, J. P. Hagan, D. Iliopoulos, and R. I. Gregory. Lin28A and Lin28B Inhibit let-7 MicroRNA Biogenesis by Distinct Mechanisms. *Cell*, 147(5):1066–79, 2011. 1.2.3

A. Portela and M. Esteller. Epigenetic modifications and human disease. *Nat Biotechnol*, 28(10):1057–68, 2010. 1.1.2.2

V. Ramachandran and X. Chen. Degradation of microRNAs by a family of exoribonucleases in Arabidopsis. *Science*, 321(5895):1490–2, 2008. 1.2.3

J. Rehwinkel, I. Behm-Ansmant, D. Gatfield, and E. Izaurralde. A crucial role for GW182 and the DCP1:DCP2 decapping complex in miRNA-mediated gene silencing. *RNA*, 11 (11):1640–7, 2005. 1.4.4, 1.5

J. Rehwinkel, P. Natalin, A. Stark, J. Brennecke, S. M. Cohen, and E. Izaurralde. Genome-wide analysis of mRNAs regulated by Drosha and Argonaute proteins in Drosophila melanogaster. *Mol Cell Biol*, 26(8):2965–75, 2006. 1.4.4

B. J. Reinhart, F. J. Slack, M. Basson, A. E. Pasquinelli, J. C. Bettinger, A. E. Rougvie, H. R. Horvitz, and G. Ruvkun. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. *Nature*, 403(6772):901–6, 2000. 1.2.1

M. W. Rhoades, B. J. Reinhart, L. P. Lim, C. B. Burge, B. Bartel, and D. P. Bartel. Prediction of plant microRNA targets. *Cell*, 110(4):513–20, 2002. 1.3.2

A. D. Riggs. X inactivation, differentiation, and DNA methylation. *Cytogenetics and cell genetics*, 14(1):9–25, 1975. 1.1.2.1

F. V. Rivas, N. H. Tolia, J. J. Song, J. P. Aragon, J. Liu, G. J. Hannon, and L. Joshua-Tor. Purified Argonaute2 and an siRNA form recombinant human RISC. *Nat Struct Mol Biol*, 12(4):340–9, 2005. 1.3.1.1

A. Rodriguez, S. Griffiths-Jones, J. L. Ashurst, and A. Bradley. Identification of mammalian microRNA host genes and transcription units. *Genome research*, 14(10A): 1902–10, 2004. 1.2.2

J. G. Ruby, C. H. Jan, and D. P. Bartel. Intronic microRNA precursors that bypass Drosha processing. *Nature*, 448(7149):83–6, 2007. 1.2.2.1

F. Sanger, G. M. Air, B. G. Barrell, N. L. Brown, A. R. Coulson, C. A. Fiddes, C. A. Hutchison, P. M. Slocombe, and M. Smith. Nucleotide sequence of bacteriophage phi X174 DNA. *Nature*, 265(5596):687–95, 1977. 1.1

T. Sato, M. Hanada, S. Bodrug, S. Irie, N. Iwama, L. H. Boise, C. B. Thompson, E. Golemis, L. Fong, H. G. Wang, and et al. Interactions among members of the Bcl-2 protein family analyzed with a yeast two-hybrid system. *Proceedings of the National Academy of Sciences of the United States of America*, 91(20):9238–42, 1994. 4.4

M. D. Schneider, N. Najand, S. Chaker, J. M. Pare, J. Haskins, S. C. Hughes, T. C. Hobman, J. Locke, and A. J. Simmonds. Gawky is a component of cytoplasmic mRNA processing bodies required for early Drosophila development. *J Cell Biol*, 174(3): 349–58, 2006. 1.5, 1.5

D. S. Schwarz, G. Hutvagner, T. Du, Z. Xu, N. Aronin, and P. D. Zamore. Asymmetry in the assembly of the RNAi enzyme complex. *Cell*, 115(2):199–208, 2003. 1.3

M. Selbach, B. Schwanhausser, N. Thierfelder, Z. Fang, R. Khanin, and N. Rajewsky. Widespread changes in protein synthesis induced by microRNAs. *Nature*, 455(7209): 58–63, 2008. 1.2, 1.3.2, 1.4.4

G. L. Sen and H. M. Blau. Argonaute 2/RISC resides in sites of mammalian mRNA decay known as cytoplasmic bodies. *Nat Cell Biol*, 7(6):633–6, 2005. 1.3.3

P. A. Sharp. The centrality of RNA. *Cell*, 136(4):577–80, 2009. 1.1.2.3

N. Siddiqui, D. A. Mangus, T. C. Chang, J. M. Palermino, A. B. Shyu, and K. Gehring. Poly(A) nuclease interacts with the C-terminal domain of polyadenylate-binding protein domain from poly(A)-binding protein. *The Journal of biological chemistry*, 282(34): 25067–75, 2007. 3

M. C. Siomi, K. Sato, D. Pezic, and A. A. Aravin. PIWI-interacting small RNAs: the vanguard of genome defence. *Nature reviews. Molecular cell biology*, 12(4):246–58, 2011. 1.3.1

H. O. Smith and K. W. Wilcox. A restriction enzyme from Hemophilus influenzae. I. Purification and general properties. *J Mol Biol*, 51(2):379–91, 1970. 1.1

J. J. Song, S. K. Smith, G. J. Hannon, and L. Joshua-Tor. Crystal structure of Argonaute and its implications for RISC slicer activity. *Science*, 305(5689):1434–7, 2004. 1.3.1.1

Y. H. Sun, S. Lu, R. Shi, and V. L. Chiang. Computational prediction of plant miRNA targets. *Methods in molecular biology*, 744:175–86, 2011. 1.3.2

W. S. Sutton. On the morphology of the chromosome group in Brachystola magna. *Biol Bull*, 4:24–39, 1903. 1.1

M. Tan, H. Luo, and et al. Lee, S. Identification of 67 histone marks and histone lysine crotonylation as a new type of histone modification. *Cell*, 146(6):1016–28, 2011. 1.1.2.2

R. Thermann and M. W. Hentze. Drosophila miR2 induces pseudo-polysomes and inhibits translation initiation. *Nature*, 447(7146):875–8, 2007. 1.4.3

K. R. Thickman, M. C. Swenson, J. M. Kabogo, Z. Gryczynski, and C. L. Kielkopf. Multiple U2AF65 binding sites within SF3b155: thermodynamic and spectroscopic characterization of protein-protein interactions among pre-mRNA splicing factors. *Journal of molecular biology*, 356(3):664–83, 2006. 3

M. Thomas, J. Lieberman, and A. Lal. Desperately seeking microRNA targets. *Nat Struct Mol Biol*, 17(10):1169–74, 2010. 1.2.1

S. Till, E. Lejeune, R. Thermann, M. Bortfeld, M. Hothorn, D. Enderle, C. Heinrich, M. W. Hentze, and A. G. Ladurner. A conserved motif in Argonaute-interacting proteins mediates functional interactions through the Argonaute PIWI domain. *Nat Struct Mol Biol*, 14(10):897–903, 2007. 1.5

Y. Tomari, C. Matranga, B. Haley, N. Martinez, and P. D. Zamore. A protein sensor for siRNA asymmetry. *Science*, 306(5700):1377–80, 2004. 1.3

F. Tritschler, E. Huntzinger, and E. Izaurralde. Role of GW182 proteins and PABPC1 in the miRNA pathway: a sense of deja vu. *Nat Rev Mol Cell Biol*, 11(5):379–84, 2010. 3

M. Tucker, R. R. Staples, M. A. Valencia-Sanchez, D. Muhlrad, and R. Parker. Ccr4p is the catalytic subunit of a Ccr4p/Pop2p/Notp mRNA deadenylase complex in Saccharomyces cerevisiae. *The EMBO journal*, 21(6):1427–36, 2002. 1.4.4

V. N. Uversky and A. K. Dunker. Understanding protein non-folding. *Biochimica et biophysica acta*, 1804(6):1231–64, 2010. 3

H. Valadi, K. Ekstrom, A. Bossios, M. Sjostrand, J. J. Lee, and J. O. Lotvall. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nature cell biology*, 9(6):654–9, 2007. 1.3.3

K. Van Der Kelen, R. Beyaert, D. Inze, and L. De Veylder. Translational control of eukaryotic gene expression. *Crit Rev Biochem Mol Biol*, 44(4):143–68, 2009. 1.4.1

S. Vasudevan, Y. Tong, and J. A. Steitz. Switching from repression to activation: microRNAs can up-regulate translation. *Science*, 318(5858):1931–4, 2007. 1.4.3

J. C. Venter, M. D. Adams, and et al. Myers, E. W. The sequence of the human genome. *Science*, 291(5507):1304–51, 2001. 1.1

S. R. Viswanathan and G. Q. Daley. Lin28: A microRNA regulator with a macro role. *Cell*, 140(4):445–9, 2010. 1.2.3

C.H. Waddington. The epigenotype. *Endeavour*, 1(1):18–20, 1942. 1.1.1

C.H. Waddington. *The strategy of the genes; a discussion of some aspects of theoretical biology.* Allen & Unwin, London, 1957. 1.1

M. Wakiyama, K. Takimoto, O. Ohara, and S. Yokoyama. Let-7 microRNA-mediated mRNA deadenylation and translational repression in a mammalian cell-free system. *Genes Dev*, 21(15):1857–62, 2007. 1.4.3

B. Wang, T. M. Love, M. E. Call, J. G. Doench, and C. D. Novina. Recapitulation of short RNA-directed translational gene silencing in vitro. *Molecular cell*, 22(4):553–60, 2006. 1.4.3

B. Wang, A. Yanez, and C. D. Novina. MicroRNA-repressed mRNAs contain 40S but not 60S components. *Proc Natl Acad Sci U S A*, 105(14):5343–8, 2008. 1.4.3

Z. Wang and M. Kiledjian. Functional link between the mammalian exosome and mRNA decapping. *Cell*, 107(6):751–62, 2001. 1.4.4

J. D. Watson and F. H. Crick. Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. *Nature*, 171(4356):737–8, 1953. 1.1

B. Wightman, I. Ha, and G. Ruvkun. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. *Cell*, 75(5): 855–62, 1993. 1.2.1

J. Winter, S. Jung, S. Keller, R. I. Gregory, and S. Diederichs. Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat Cell Biol*, 11(3):228–34, 2009. 1.2.3

L. Wu and J. G. Belasco. Micro-RNA regulation of the mammalian lin-28 gene during neuronal differentiation of embryonal carcinoma cells. *Mol Cell Biol*, 25(21):9198–208, 2005. 1.4.4

J. Wysocka, T. Swigut, H. Xiao, T. A. Milne, S. Y. Kwon, J. Landry, M. Kauer, A. J. Tackett, B. T. Chait, P. Badenhorst, C. Wu, and C. D. Allis. A PHD finger of NURF couples histone H3 lysine 4 trimethylation with chromatin remodelling. *Nature*, 442 (7098):86–90, 2006. 1.1.2.2

A. Yamashita, T. C. Chang, Y. Yamashita, W. Zhu, Z. Zhong, C. Y. Chen, and A. B. Shyu. Concerted action of poly(A) nucleases and decapping enzyme in mammalian mRNA turnover. *Nat Struct Mol Biol*, 12(12):1054–63, 2005. 1.4.4, 3

W. Yang, T. P. Chendrimada, Q. Wang, M. Higuchi, P. H. Seeburg, R. Shiekhattar, and K. Nishikura. Modulation of microRNA processing and expression through RNA editing by ADAR deaminases. *Nat Struct Mol Biol*, 13(1):13–21, 2006. 1.2.3

W. J. Yang, D. D. Yang, S. Na, G. E. Sandusky, Q. Zhang, and G. Zhao. Dicer is required for embryonic angiogenesis during mouse development. *The Journal of biological chemistry*, 280(10):9330–5, 2005. 1.2.1

S. Yekta, I. H. Shih, and D. P. Bartel. MicroRNA-directed cleavage of HOXB8 mRNA. *Science*, 304(5670):594–6, 2004. 1.3.2

R. Yi, Y. Qin, I. G. Macara, and B. R. Cullen. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes & development*, 17(24):3011–6, 2003. 1.2.2.1

E. Yigit, P. J. Batista, Y. Bei, K. M. Pang, C. C. Chen, N. H. Tolia, L. Joshua-Tor, S. Mitani, M. J. Simard, and C. C. Mello. Analysis of the C. elegans Argonaute family reveals that distinct Argonautes act sequentially during RNAi. *Cell*, 127(4):747–57, 2006. 1.3.1

A. Zdanowicz, R. Thermann, J. Kowalska, J. Jemielity, K. Duncan, T. Preiss, E. Darzynkiewicz, and M. W. Hentze. Drosophila miR2 primarily targets the m7GpppN cap structure for translational repression. *Mol Cell*, 35(6):881–8, 2009. 1.4.3

L. Zekri, E. Huntzinger, S. Heimstadt, and E. Izaurralde. The silencing domain of GW182 interacts with PABPC1 to promote translational repression and degradation of microRNA targets and is required for target release. *Mol Cell Biol*, 29(23):6220–31, 2009. 3, 3, 3

H. Zhang, F. A. Kolb, V. Brondani, E. Billy, and W. Filipowicz. Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP. *The EMBO journal*, 21(21):5875–85, 2002. 1.2.2.2

H. Zhang, F. A. Kolb, L. Jaskiewicz, E. Westhof, and W. Filipowicz. Single processing center models for human Dicer and bacterial RNase III. *Cell*, 118(1):57–68, 2004. 1.2.2.2

L. Zhang, L. Ding, T. H. Cheung, M. Q. Dong, J. Chen, A. K. Sewell, X. Liu, 3rd Yates, J. R., and M. Han. Systematic identification of C. elegans miRISC proteins, miRNAs, and mRNA targets by their interactions with GW182 proteins AIN-1 and AIN-2. *Mol Cell*, 28(4):598–613, 2007. 1.5

L. Zheng, U. Baumann, and J. L. Reymond. An efficient one-step site-directed and site-saturation mutagenesis protocol. *Nucleic Acids Res*, 32(14):e115, 2004. 4.2

J. T. Zipprich, S. Bhattacharyya, H. Mathys, and W. Filipowicz. Importance of the C-terminal domain of the human GW182 protein TNRC6C for translational repression. *RNA*, 15(5):781–93, 2009. 2, 2.2, 2.2, 2.5, 3, 3, 4.1, 4.2

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