The role of GW182 proteins in microRNA-mediated gene silencing

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Abstract

MicroRNAs are endogenous approximately 21-nucleotide-long non-coding RNAs that act as post-transcriptional regulators of gene expression by base pairing to target mRNAs. Mature miRNAs form part of ribonucleoprotein complexes, called miRNA-induced silencing complexes (miRISCs), that contain Argonaute (AGO) and GW182 as core proteins. *Drosophila melanogaster* contains only one GW182 protein (DmGW182) but there are three GW182 paralogs, TNRC6A, TNRC6B, and TNRC6C, encoded in mammalian genomes. Proteins of the GW182 family play an important role in the execution of miRNA-mediated repression. However, the molecular mechanism of GW182-mediated repression is not entirely understood.

In order to get a more comprehensive understanding of the mechanism of miRNA-mediated repression, we studied the function of GW182 proteins using human HEK293 cells and *Drosophila* S2 cells as model systems. As a result of these investigations, we identified the C-terminal fragment of the human GW182 protein TNRC6C (CED) as a key region mediating miRNA-induced repression by interacting with PABP via its PAM2 motif and by recruiting the PAN2-PAN3 and CCR4-CAF1-NOT deadenylase complexes via conserved tryptophan-containing motifs (W-motifs).

In addition, tethering assays in HEK293 cells and *Drosophila* S2 cells revealed that the C-terminal regions of GW182 proteins are able to repress not only polyadenylated but also poly(A)-free mRNAs. Interestingly, the W-motifs which are essential for interaction of the CED with the CCR4-CAF1-NOT complex, were also required for the repression of poly(A)-free mRNAs by the tethered CEDs of human TNRC6C and DmGW182. Indeed, direct tethering of CCR4-CAF1-NOT complex components in HEK293 or S2 cells repressed not only polyadenylated but also poly(A)-free mRNAs and the RNA levels of poly(A)-free mRNAs were either not affected or only slightly reduced, indicating that the major part of the repression was due to inhibition of translation. Finally, repression of poly(A)-free mRNAs in *Drosophila* S2 cells by tethered DmGW182 or its CED depended on NOT1 but repression by tethered CAF1 or CNOT1 was independent of GW182, indicating that NOT1 acts downstream of GW182 in the repression of poly(A)-free mRNAs.

Taken together, these data indicate that recruitment of the CCR4-CAF1-NOT complex mediated by W-motifs of GW182 proteins, in addition to inducing deadenylation, also contributes to translational repression.

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Abbreviations

Abbreviations

5`-TOP	5`-terminal oligopyrimidine tract
Acvr2a	Activin receptor 2a
AGO	Argonaute
Atx2	Ataxin-2
CAF1	CCR4-associated factor 1
CAT-1	Cationic amino acid transporter 1
CCR4	Carbon catabolite repressor protein 4 homolog
CeALG-1	*Caenorhabditis elegans* Argonaute-like 1
CED	C-terminal effector domain
CNOT1	CCR4-NOT transcription complex subunit 1
CrPV	Cricket paralysis virus
DAZL	Deleted in azoospermia-like
DCP1	mRNA-decapping enzyme 1
Dgcr8	DiGeorge syndrome critical region gene 8
Dhh1	DEAD box helicase homolog 1
DmGW182	*Drosophila melanogaster* GW182
DNA	Deoxyribonucleic acid
Dnd1	Dead-end 1
DUF	Domain of unknown function
EDC3	Enhancer of mRNA-decapping protein 3
EDD	E3 ubiquitin-protein ligase UBR5
eIF3	Eukaryotic translation initiation factor 3
eIF4A	Eukaryotic translation initiation factor 4A
eIF4E	Eukaryotic translation initiation factor 4E
eIF4G	Eukaryotic translation initiation factor 4G
eIF6	Eukaryotic translation initiation factor 6
EMCV	Encephalomyocarditis virus
ER	Endoplasmatic reticulum
ES cells	Embryonic stem cells
ESCC miRNAs	ES cell-specific cell cycle-regulating miRNAs
ESCRT complex	Endosomal sorting complex required for transport complex
FL	Firefly luciferase

Ge-1/Hedls	Autoantigen Ge1/Human enhancer of decapping large subunit
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
HA	Hemagglutinin
HCV	Hepatitis C virus
HEAT	Huntingtin, elongation factor 3 (EF3), protein phosphatase 2A (PP2A), and the yeast PI3-kinase TOR1
HEK293 cells	Human embryonic kidney 293 cell line
HeLa cells	Cervical carcinoma cell line derived from Henrietta Lacks
HhR	Hammerhead ribozyme
HO mRNA	*HO* (“homothallism”) gene encoded mRNA
HPat1	Human PAT1-like protein 1
HSL	Histone stem-loop
Huh7 cells	Human hepatocellular carcinoma cell line Huh7
HuR	Hu-antigen R
IgG	Immunoglobulin G
IP	Immunoprecipitation
IRES	Internal ribosomal entry site
ITAFs	Internal ribosome-entry site (IRES) trans-acting factors
KLF4	Krueppel-like factor 4
LSm1-7	U6 snRNA-associated Sm-like protein 1-7
Me31b	Maternal expression at 31B protein
miR	MicroRNA
miRNA	MicroRNA
Mpt5p	Multicopy suppressor of Pop2 5 protein
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MVBs	Multivesicular bodies
NED	N-terminal effector domain
OCT4	Octamer-binding transcription factor 4
ORF	Open reading frame
PABP	Polyadenylate-binding protein
PAIP2	PABP-interacting protein 2
PAM2	PABP-interacting motif 2
PAN2	PABP-dependent poly(A)-specific ribonuclease subunit 2
PAN3	PABP-dependent poly(A)-specific ribonuclease subunit 3

PAZ domain	Piwi-Argonaute-Zwille domain
piRNA	Piwi-interacting RNA
Pop2p	PGK promoter directed over production 2 protein
RCK	Oncogene RCK
REST	RE1-silencing transcription factor
RISC	RNA-induced silencing complex
RL	Renilla luciferase
RNA	Ribonucleic acid
RNAi	RNA interference
RRM	RNA recognition motif
SCP1	Small C-terminal domain phosphatase 1
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SF3b155	Splicing factor 3b 155kDa subunit
Sic1p	Substrate/Subunit inhibitor of Cyclin-dependent kinase 1 protein
SOX2	(Sex determining region Y)-box 2
TGFbeta	Transforming growth factor beta
TNRC6A	Trinucleotide repeat containing gene 6A protein
TNRC6B	Trinucleotide repeat containing gene 6B protein
TNRC6C	Trinucleotide repeat containing gene 6C protein
TRBP	TAR RNA-binding protein
tRNA	Transfer ribonucleic acid
U2AF65	U2 small nuclear RNA auxiliary factor 65
UTR	Untranslated region
XRN1	5’-3’ exoribonuclease 1

Units

°C	° Celsius
bp	Base pair
h	Hour
kb	Kilobase
kDa	Kilodalton
mg	Milligram

min	Minute
mM	Millimolar
ng	Nanogram
nt	Nucleotide
μg	Microgram
μl	Microliter
μM	Micromolar

1. Introduction

How can a single cell develop into an organism as complex as a human being? Although we are far from understanding this amazing process completely, it is apparent, that what begins as a fertilized oocyte transforms into an organism with approximately 10^{14} cells and about 400 different cell types (Azevedo et al., 2009; Vickaryous and Hall, 2006). This transformation is achieved by many cell divisions during which the DNA of a cell is faithfully replicated and is equally distributed so that each daughter cell receives one copy of the complete genome. Thus a neuron and a liver cell contain the same DNA. Why then, are these two cell types so different? The answer is: “Development is, by definition, epigenetic.” (Reik, 2007). In other words, development is possible because the readout of the genome is regulated epigenetically. Due to epigenetic regulation of gene expression different cell types can express a specific set of genes required for their functions.

Clearly, sophisticated regulation of gene expression is of immense importance for the phenomenon of life. Therefore it is not surprising that a massive number of different regulatory mechanisms has evolved. Gene expression is a multistep process that involves the transcription, translation and turnover of messenger RNAs and proteins (Schwanhausser et al., 2011) and each of these steps has been demonstrated to be targeted by regulatory processes (Hochstrasser, 1996; Kadonaga, 2004; Parker and Song, 2004; Sonenberg and Hinnebusch, 2009).

The central dogma of molecular biology states that information flows from DNA to RNA, which is then translated into protein (Crick, 1970). Crick`s statement was often interpreted in the sense that RNA only plays a role as an intermediate messenger whereas cellular functions are transacted by proteins. The central role of proteins as regulators of gene expression was supported by studies of the lac operon which lead to the identification of the lac repressor (Gilbert and Muller-Hill, 1966; Jacob and Monod, 1961) and by the subsequent discovery of protein transcription factors (reviewed in Zamore and Haley, (2005)). On the other hand, the composition and expression of the human genome may point to an important role of RNA as an end-point product of gene expression. Although 98.8% of the human genome consists of non-protein-coding DNA, nonetheless approximately 90% of the genome is transcribed into non-coding RNA (Amaral et al., 2008; Birney et al., 2007). Francis Crick could not know about these numbers but nonetheless he noted that some RNAs could be end-point products of gene expression and indeed, ribosomal RNA, tRNA, and spliceosomal and small nucleolar RNAs were found to act in fundamental

cellular processes (Mattick, 2011). Furthermore, as early as in 1969, Britten and Davidson proposed that RNAs could also function in the regulation of gene expression (Britten and Davidson, 1969).

Nowadays it is well established that RNAs do regulate gene expression (Zamore and Haley, 2005). One class of small regulatory RNAs, the microRNAs, is predicted to regulate more than 50% of all human genes (Friedman et al., 2009b). Although microRNAs may affect nearly all biological processes in human cells, they escaped notice of researchers for a surprisingly long time (Bartel, 2009). The first microRNA was discovered in 1993 by Victor Ambros and Gary Ruvkun (Lee et al., 1993; Wightman et al., 1993). Studying the *lin-4* gene, which had been shown to control the timing of larval development in *Caenorhabditis elegans* (Ambros and Horvitz, 1984), Ambros and Ruvkun demonstrated that *lin-4* does not code for a protein (Lee et al., 1993). Instead, it codes for a short RNA species, 22 nucleotides in length, that was realized to have multiple complemetary sites in the 3`UTR of *lin-14* mRNA (Wightman et al., 1993). Ambros and Ruvkun proposed a model in which the short *lin-4* RNA base pairs to sites in the *lin-14* 3`UTR to repress *lin-14* translation (Lee et al., 1993; Wightman et al., 1993). Nearly a decade later, the Ruvkun lab found that *let-7*, another gene involved in developmental timing of *C. elegans*, encodes a small RNA which negatively regulates the *lin-41* gene (Reinhart et al., 2000). Based on their similarity, it was apparent that lin-4 and let-7 belong to the same class of post-transcriptional regulators. They are both 21-22 nucleotides in length, both originate from RNA precursors forming a stem-loop structure, and both act as negative regulators of gene expression by binding to partially complementary sites in the 3`UTR of a regulated RNA. When Pasquinelli and colleagues found let-7 RNAs in samples from various animal species, including humans, it became apparent that these small RNAs are more than a peculiarity of the worm (Pasquinelli et al., 2000). Subsequent studies by the Ambros, Bartel and Tuschl labs revealed that there a hundreds of small RNAs with the characteristics of lin-4 and let-7 expressed in metazoans (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). The class of small RNAs founded by lin-4 and let-7 was named microRNAs (miRNAs).

1.1 Biological functions of microRNAs

1.1.1 Biological functions in normal conditions

Given the widespread impact of miRNAs on gene expression it is not surprising that miRNAs have important roles in a wide range of biological processes (Huntzinger and Izaurralde, 2011). The following sections describe a set of well characterized examples of miRNA-mediated regulation of biological processes.

1.1.1.1 Clearance of maternal mRNAs

In zebrafish, the miR-430 cluster is involved in the degradation of hundreds of maternal mRNAs during the maternal-zygotic transition (Giraldez et al., 2006). Zebrafish mutants that lack both maternal and zygotic Dicer activity show defects in embryonic morphogenesis which are rescued by the expression of mature miR-430 family members. The clearance of maternal mRNAs by miRNAs is evolutionarily conserved as it has been observed also in frogs (Lund et al., 2009), *Drosophila melanogaster* (Bushati et al., 2008) and *C. elegans* (Wu et al., 2010).

1.1.1.2 Embryonic stem cell proliferation and differentiation

miRNAs are also involved in embryonic stem (ES) cell proliferation and differentiation. Dicer1 and also Dgcr8 mutant mouse ES cells show severe growth and differentiation defects (Kanellopoulou et al., 2005; Murchison et al., 2005; Pauli et al., 2011; Tang et al., 2007; Wang et al., 2007). The proliferation defects of the Dgcr8 mutant mouse ES cells can be partially rescued by the expression of so called ES cell-specific cell-cycle-regulating (ESCC) miRNAs of the miR-290-295/302 family (Wang et al., 2008c). These miRNAs silence multiple negative regulators of the G1 to S phase transition and thereby promote a cell cycle that is characteristic for ES cells (Wang et al., 2008c). The differentiation defects of the Dgcr8 mutant ES cells is partially rescued by the expression of let-7 miRNA (Melton et al., 2010). let-7 represses genes that promote cell cycle progression and also genes that promote stem cell identity and thereby facilitates differentiation and represses self renewal (Johnson et al., 2007; Melton et al., 2010; Pauli et al., 2011). Thus, ESCC miRNAs and let-7 miRNAs seem to have opposing roles in mouse ES cell self-renewal and differentiation. Similarly to the role of let-7 in mouse ES cells, human miR-145 also

represses pluripotency in differentiating ES cells by downregulating OCT4, SOX2 and KLF4 (Xu et al., 2009).

1.1.1.3 Germ layer specification

During germ layer specification, Nodal, a member of the transforming growth factor beta (TGFbeta) family, promotes mesoderm and endoderm formation. Lefty, another member of the TGFbeta family, blocks Nodal signaling and promotes ectoderm development. Zebrafish miR-430 balances Nodal signaling by targeting the Nodal ligand Squint and its antagonist Lefty2, leading to mesoderm formation (Choi et al., 2007). Similarly, human miR-302 represses Lefty, which stimulates Nodal signaling and differentiation into mesoderm (Rosa et al., 2009). In frogs, the Nodal receptor activin receptor 2a (Acvr2a) is also regulated by miRNAs (Martello et al., 2007). *Xenopus laevis* miR-15 and miR-16 are ventrally enriched and restrict the expression of Acvr2a to the dorsal side which is thought to contribute to the dorsal-ventral gradient of the Nodal signaling pathway activity (Martello et al., 2007).

1.1.1.4 Cell fate specification

Regulation of cell fate specification by miRNAs has been observed in neurons, muscles, the haematopoietic system and other cell types (Pauli et al., 2011). The differentiation of neural progenitor cells into neurons involves the exchange of the neural progenitor specific chromatin remodeling complex BAF (npBAF) with the neuron-specific BAF (nBAF) complex. This transition is mediated by the repression of a npBAF subunit by miRNAs miR-9* and miR-124 (Yoo et al., 2009). In addition, miR-124 promotes neural differentiation by down regulating small C-terminal domain phosphatase 1 (SCP1), which acts as a cofactor of REST in suppressing transcription of genes that promote neural development (Visvanathan et al., 2007).

Another prominent example of cell fate specification by miRNAs is the formation of a highly specialized neuronal fate regulated by miRNA lsy-6 in *C. elegans* (Pauli et al., 2011). During neuronal development the gustatory neurons ASEL and ASER acquire left side and right side neuronal identity, respectively. Lsy-6 is expressed only in ASEL neurons and is required for left side neuronal identity (Johnston and Hobert, 2003).

1.1.1.5 Control of developmental timing

The first miRNA identified is encoded by the *lin-4* gene that regulates developmental timing in *C. elegans* (Chalfie et al., 1981; Lee et al., 1993; Wightman et al., 1993).

Lin-4 miRNA targets the *lin-14* mRNA which encodes a transcription factor that is required for completion of the first larval stage (Flynt and Lai, 2008). In *lin-4* loss-of-function mutants, cells reiterate early-stage specific fates, a phenotype that is also observed in *lin-14* gain-of-function mutants that lack the *lin-4* miRNA target sites (Ambros and Horvitz, 1984, 1987; Chalfie et al., 1981; Wightman et al., 1991).

1.1.2 Biological functions in pathological conditions

Generally based on animal models, miRNAs have also many reported roles in pathological conditions such as cardiac hypertrophy (Callis et al., 2009; Care et al., 2007), amyotrophic lateral sclerosis (Williams et al., 2009), (potentially) Alzheimer`s disease (Boissonneault et al., 2009; Hebert et al., 2008; Wang et al., 2008b) and schizophrenia (Begemann et al., 2010; Stark et al., 2008; Xu et al., 2010). Last but not least, miRNAs play a role in cancer (Bou Kheir et al., 2011; Calin et al., 2008; Chan et al., 2005; Cimmino et al., 2005; Lee et al., 2007; Linsley et al., 2007; Liu et al., 2010; Ma et al., 2010; Papagiannakopoulos et al., 2008; Segura et al., 2009; Shi et al., 2011; Tan et al., 2011; Tian et al., 2010; Zhang et al., 2010) acting both as oncogenes (Bjork et al., 2010; He et al., 2005; Jiang et al., 2010) or tumor suppressor genes (Friedman et al., 2009a; Gandellini et al., 2009; Kano et al., 2010; Nohata et al., 2011).

1.2 miRNA biogenesis

How are miRNAs generated in cells? Primary miRNA (pri-miRNA) transcripts are generally produced by RNA polymerase II and contain a cap structure and a poly(A) tail (Figure 1.1, panel A) (Cai et al., 2004; Lee et al., 2004). Most miRNA hairpins are found in non-coding transcripts or in intronic regions of protein-coding pre-mRNAs (Kim et al., 2009). Often multiple miRNA hairpins are clustered in the same transcript (Lee et al., 2002). In a first step of maturation, the miRNA hairpin contained in the pri-miRNA transcript is cleaved by the Drosha/Dgcr8 heterodimer (Han et al., 2006; Lee et al., 2003; Lee et al., 2002). DGCR8 recognizes the dsRNA-ssRNA junction at the base of the miRNA hairpin and directs cleavage near the base of the hairpin by the RNase III-type protein Drosha (Han et al., 2006). This process takes place in the nucleus and releases a small hairpin (typically ~55-70 nucleotides in length) termed

pre-miRNA (Lee et al., 2002). Pre-miRNAs are then exported to the cytoplasm by Exportin-5 and its partner Ran-GTP (Kim, 2004; Lund et al., 2004). In the cytoplasm the RNase III enzyme Dicer cleaves the pre-miRNA hairpin near the terminal loop yielding ~22 nucleotides small RNA duplexes (Bernstein et al., 2001; Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001; Knight and Bass, 2001).

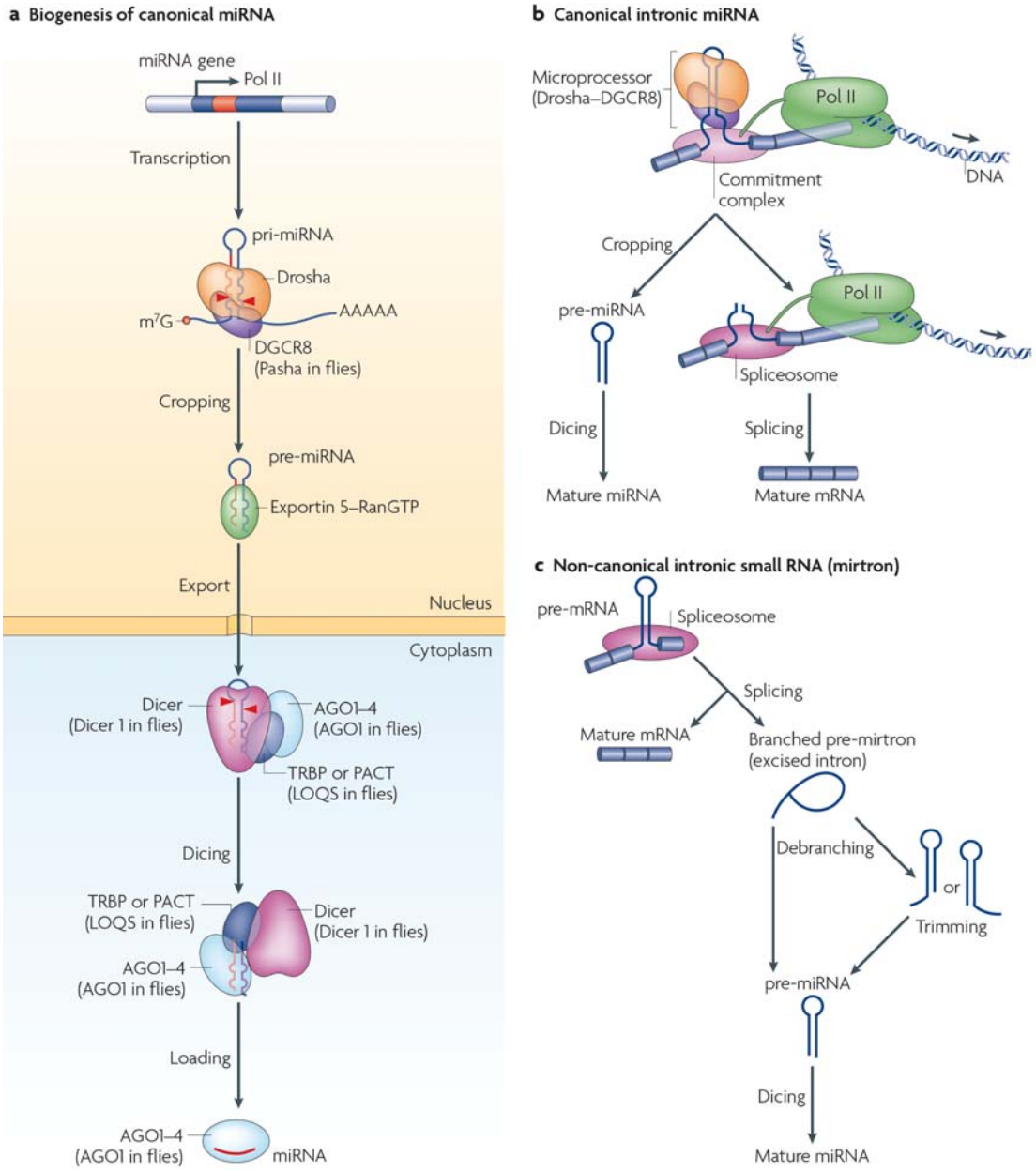


Figure 1.1: miRNA biogenesis pathway. (**A**) Primary miRNA (pri-miRNA) transcripts are generally produced by RNA polymerase II and processed (cropping) in the nucleus by the Drosha-DGCR8 complex (Microprocessor complex) that generates ~65 nucleotide pre-miRNAs. The pre-miRNA is exported from the nucleus by the nuclear export factor exportin 5. In the cytoplasm, the RNaseIII Dicer catalyses the second processing step (dicing) and the resulting miRNA duplex is loaded onto an AGO protein, a process that also seems to involve TAR RNA-binding protein (TRBP). (**B**) A miRNA hairpin located in an intronic region can be

processed in a canonical manner co-transcriptionally before splicing. (**C**) In an alternative biogenesis pathway (mirtron pathway) the pre-miRNA is generated by splicing, lariat debranching and eventually trimming of the 5`- or 3` end. Figure from Kim et al., (2009).

The resulting 22nt RNA dupex is transferred from Dicer to an Argonaute (AGO) protein on a way to form the effector complex called miRNA-induced silencing complex (miRISC). The final step in RISC formation involves the selection of one of the two strands of the small RNA duplex to remain in the AGO protein (miRNA guide strand) whereas the other strand (miRNA* or passenger strand) is degraded (Kim et al., 2009). Usually the strand with a thermodynamically less stable 5` end is selected to remain in the AGO complex, although this rule may not apply to all miRNAs (Han et al., 2006; Khvorova et al., 2003). Removal of the passenger strand may be facilitated by its endonucleolytic cleavage when the AGO protein (e. g., AGO2) contains nuclease activity. When the miRNA duplex is loaded into cleavage incompetent AGO1, AGO3 or AGO4, or when the duplex forms mismatches at the cleavage site removal of the passenger strand may require RNA unwinding activity (Forstemann et al., 2007; Kawamata et al., 2009; Kim et al., 2009; Yoda et al., 2010).

MiRNAs may also be generated by alternative biogenesis pathways that function independently of Drosha/DGCR8 or Dicer. In a biogenesis process that is called mirtron pathway, the pre-miRNA is generated by splicing instead of the Drosha/DGCR8 cleavage (Figure 1.1, panel C) (Okamura et al., 2007; Ruby et al., 2007). After splicing and lariat debranching, the intron (mirtron) is recognized as pre-miRNA and is further processed by the canonical miRNA-biogenesis pathway. Whereas the 5` and 3` ends of conventional mirtrons are determined by splicing, the 5` or 3` ends of alternative mirtrons require trimming by exonucleases (reviewed in Yang and Lai, (2011)).

The biogenesis of miR-451 involves Drosha/DGCR8-mediated cleavage but is independent of Dicer (Cheloufi et al., 2010; Cifuentes et al., 2010; Yang et al., 2010). Instead, the pre-mir-451 consisting of a hairpin with a stem that is too short to be processed by Dicer, is loaded into AGO2 protein and the 5` arm of the hairpin guides cleavage of the 3` arm of the hairpin by AGO2. The resulting 30 nucleotide product is trimmed at its 3` end yielding the mature 23 nt miR-451.

1.3 Principles of target recognition by miRNAs

To exert their repressive effect on gene expression, miRNAs base-pair with their target mRNAs. Extensive pairing complementarity leads to the AGO2-catalyzed cleavage of the target mRNA (Hutvagner and Zamore, 2002; Song et al., 2004; Yekta et al., 2004). More commonly, miRNAs interact with their targets via partial base-pairing complementarity which characteristically involves perfect Watson-Crick pairing of the nucleotides 2-7 from the 5` end of the miRNA, the so called seed region (Bartel, 2009; Doench and Sharp, 2004; Lewis et al., 2005; Lewis et al., 2003). Extended contiguous Watson-Crick base-pairing of positions 2-8 of the miRNA as well as an adenosine residue across position 1 of the miRNA improve its activity (Baek et al., 2008; Bartel, 2009; Lewis et al., 2005; Nielsen et al., 2007). Supplemetary pairing of the 3` portion of the miRNA has been suggested to usually play a modest role in target recognition (Grimson et al., 2007) but there are examples where pairing of the 3` portion of the miRNA compensates for a single nucleotide bulge or mismatch in the seed region (Reinhart et al., 2000; Yekta et al., 2004). Several studies reported examples of miRNA target sites that are functional despite imperfect seed pairing, suggesting that more potential miRNA regulatory sites exist than those predicted when applying only conventional “seed pairing rules” (Chi et al., 2012; Didiano and Hobert, 2006; Ha et al., 1996; Tay et al., 2008; Vella et al., 2004).

The efficacy of miRNA target sites is also influenced by features of the 3`UTR (Bartel, 2009). Target sites within the 3`UTR positioned at least 15 nucleotides from the stop codon are more effective, likely because miRNAs bound to these sites are not displaced by the translating ribosome (Grimson et al., 2007). Further, AU-richness near the target site and other measures of site accessibility positively correlate with site efficacy (Grimson et al., 2007). Sites positioned away from the center of long UTRs are generally more efficient, likely because of favorable site accessibility (Grimson et al., 2007). Finally, sites that are close together tend to act cooperatively (Grimson et al., 2007; Saetrom et al., 2007). Although target sites in the 3`UTR appear to induce more robust repression, miRNAs can also target the 5`UTR and the coding regions of mRNAs (Easow et al., 2007; Gu et al., 2009; Hafner et al., 2010; Kloosterman et al., 2004; Lytle et al., 2007).

Computational studies revealed that highly conserved miRNAs have very many conserved targets (Bartel, 2009; Brennecke et al., 2005; Lewis et al., 2005; Xie et al., 2005), a finding that was supported by experimental approaches which demonstrated that a miRNA can affect the mRNA levels of hundreds of targets (Baek et al., 2008;

Guo et al., 2010; Lim et al., 2005; Selbach et al., 2008). In sum, more than 60% of human protein coding genes appear to have been under selective pressure to maintain pairing to miRNAs (Friedman et al., 2009b).

1.4 Protein components of the miRNA ribonucleoprotein complex

MiRNAs interact directly with proteins of the AGO family which form the core of the miRISC (Peters and Meister, 2007) and are therefore thought to be the key components of the miRNA-mediated silencing pathway (Fabian et al., 2010). There are two subclasses of Argonaute proteins, the Ago subfamily which resembles Arabidopsis AGO1 and the Piwi subfamily which has sequence homology to Drosophila PIWI protein (Peters and Meister, 2007). Proteins of the Piwi subfamily are mainly expressed in the germline where they interact with piRNAs and are involved in germ cell development and silencing of mobile genetic elements (reviewed in Juliano et al., (2011)).

The AGO subfamily is expressed ubiquitously, interacts with miRNAs and siRNAs, and consists of four members in humans, AGO1, AGO2, AGO3, and AGO4 (Peters and Meister, 2007). All four human AGO proteins repress a reporter mRNA when artificially tethered to its 3`UTR and also associate with similar sets of miRNAs, proteins and target mRNAs (Azuma-Mukai et al., 2008; Landthaler et al., 2008; Liu et al., 2004; Meister et al., 2005; Pillai et al., 2004; Pillai et al., 2005; Wu et al., 2008). These observations indicate that all four human AGO proteins are involved in miRNA mediated silencing. Experiments showing that AGO2 is essential for embryogenesis (Liu et al., 2004) and hematopoiesis in mice indicate a paralog-specific function of AGO2 that cannot be complemented by other Argonautes (O'Carroll et al., 2007). The paralog-specific function of AGO2 may indeed involve miRNA regulation, since the endonucleolytic activity of AGO2 is not required for its role in hematopoiesis (O'Carroll et al., 2007).

AGO proteins contain three evolutionarily conserved domains, a Piwi-Argonaute-Zwille (PAZ) domain, a MID domain and a PIWI domain (Fabian et al., 2010). The crystal structures of archaeal and eubacterial AGO proteins and more recent structures of eukaryotic AGO proteins or their fragments revealed that the 3` end of small RNAs binds to a specific binding pocket in the PAZ domain and the 5` terminal nucleotide binds to a pocket in the junction of the MID and PIWI domains (Jinek and

Doudna, 2009; Parker et al., 2005; Schirle and MacRae, 2012; Wang et al., 2008d). Among the four human AGO proteins, only AGO2 can enzymatically cleave the mRNA at the center of the siRNA-mRNA duplex (Liu et al., 2004). The catalytically active site is located in the PIWI domain which structurally resembles the bacterial RNase H that was shown to cleave the RNA strand of RNA-DNA duplexes (Jinek and Doudna, 2009). A ternary complex structure including *Thermus thermophilus* Argonaute, a small RNA mimic and a target RNA revealed that the nucleotides at positions 2-6 of the miRNA contact AGO through the phosphate-ribose backbone and are oriented in a way that the bases are available for hydrogen bonding with the target (Wang et al., 2008d). These observations explain well why perfect complementarity in the seed region of miRNAs is crucial for target mRNA recognition and why the nucleotide at position 1 of the miRNA is not needed for base-pairing (Fabian et al., 2010).

The GW182 family of proteins is another group of factors that is crucial for miRNA-mediated repression. Originally, GW182 was identified in human cells as component of discrete cytoplasmic domains which later turned out to coincide with mRNA-processing bodies (P-bodies) (Eystathioy et al., 2002; Eystathioy et al., 2003). Independent studies identified GW182 as a factor interacting with AGO proteins and required for miRNA-mediated repression (Ding et al., 2005; Jakymiw et al., 2005; Liu et al., 2005a; Meister et al., 2005; Rehwinkel et al., 2005). There are three mammalian GW182 paralogs named TNRC6A (Trinucleotide repeat containing gene 6A protein), TNRC6B and TNRC6C and one homologous protein in *Drosophila*, dGW182. GW182 proteins contain two structured regions, a central ubiquitin associated (UBA)-like domain (only present in some GW182s) and a C-terminal RNA recognition motif (RRM) (Figure 1.2) (Ding and Han, 2007; Eulalio et al., 2007a). The dGW182 RRM adopts a canonical RRM fold but it lacks features that enable canonical RRMs to interact with RNA (Eulalio et al., 2009c). Other regions of GW182 proteins including an N-terminal glycine and tryptophan (GW) repeat-rich region (N-GW-rich), two GW-repeat-containing regions in the C-terminal part of the protein (termed: middle- and C-terminal GW-repeat region (M-GW and C-GW)), and a glutamine-rich region are predicted to be unstructured (Eulalio et al., 2009d). Another conserved region in the C-terminal part of GW182 proteins is called DUF (domain of unknown function) or PAM2 because a sequence within DUF shows similarity with the PAM2 motif of PABP-interacting proteins (Fabian et al., 2010).

GW182 proteins directly interact with AGO proteins and this interaction involves some of the GW-repeats in the N-terminal GW-repeat region (Eulalio et al., 2009a;

Till et al., 2007). Interference with the AGO-GW182 interaction or depletion of GW182 protein by RNAi abrogates miRNA-mediated repression (Behm-Ansmant et al., 2006; Eulalio et al., 2008b; Rehwinkel et al., 2005; Till et al., 2007). Furthermore, direct tethering of GW182 proteins to an mRNA represses the mRNA even in the absence of AGO protein, suggesting that GW182 is the effector protein acting downstream of AGO (Behm-Ansmant et al., 2006).

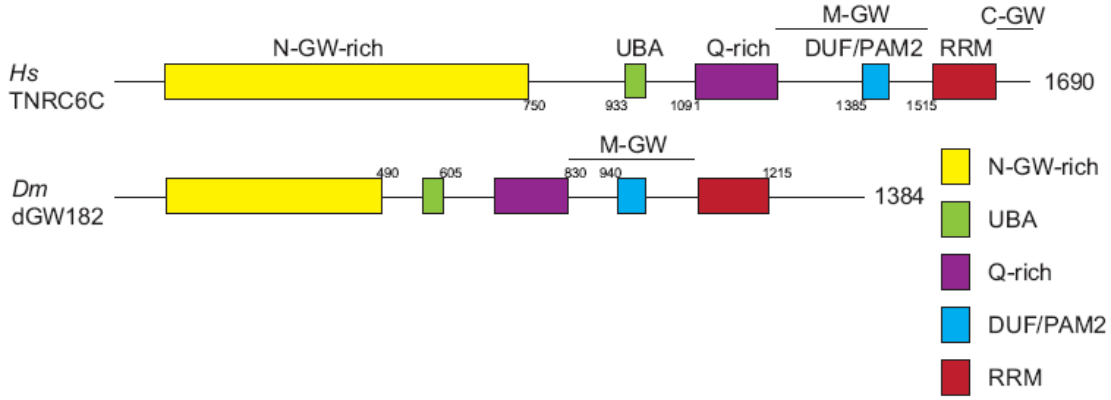


Figure 1.2: Domain structure of selected GW182 proteins. Schematic representation of human (Hs) TNRC6C and *Drosophila* (Dm) GW182 proteins. Positions of N-GW-rich, Q-rich, UBA, DUF/PAM2, M-GW, RRM, and C-GW domains are indicated. Figure modified from Zipprich et al., (2009).

Mammalian mRNA deadenylation involves two cytoplasmic deadenylase complexes, PAN2-PAN3 and CCR4-CAF1-NOT (Yamashita et al., 2005). These two cytoplasmic deadenylase complexes are also involved in miRNA-mediated deadenylation. First, depletion of components of the CCR4-CAF1-NOT complex inhibits deadenylation and decay of mRNAs targeted by miRNAs (Behm-Ansmant et al., 2006; Piao et al., 2010). Second, transcriptome analysis of Drsosophila S2 cells depleted of CCR4-CAF1-NOT complex components revealed that approximately 60% of AGO1 targets are regulated by CAF1 and/or NOT1 (Eulalio et al., 2009b). Third, overexpression of catalytically inactive mutants of CCR4, CAF1, CNOT8 (a CAF1 homolog) or PAN2 interferes with miRNA-mediated deadenylation (Chen et al., 2009; Piao et al., 2010).

The CCR4-CAF1-NOT complex is a large (approximately 1 MDa in yeast) multi-subunit complex that is highly conserved in eukaryotes (Collart and Panasenko, 2012). In humans the complex consists of 7 core subunits. CNOT1, a large scaffold of the complex, interacts with CNOT2, CNOT3, CNOT6 or CNOT6L, CNOT7 or CNOT8, CNOT9, and CNOT10 (Figure 1.3) (Bartlam and Yamamoto, 2010). CNOT6 and CNOT6L as well as the pair CNOT7 and CNOT8 are thought to be present in mutually exclusive manner in CCR4-CAF1-NOT complexes (Lau et al., 2009).

CNOT6 and CNOT6L are the human orthologs of yeast Ccr4p and belong to the exonuclease-endonuclease-phosphatase (EEP) family (Bartlam and Yamamoto, 2010). The two human orthologs of yeast Caf1/Pop2p, a member of the family of DEDD-type deadenylases, are CNOT7 and CNOT8 (Bartlam and Yamamoto, 2010). CNOT6, CNOT6L, CNOT7 and CNOT8 have all been demonstrated to possess deadenylase activity in vitro (Bianchin et al., 2005; Wang et al., 2010).

The function of the CCR4-CAF1-NOT complex has been mainly studied in yeast and the complex or components thereof have been connected to a multitude of cellular processes such as transcription, deadenylation, translational repression, mRNA export, and nuclear surveillance (Collart and Panasenko, 2012). Further, physical or functional links between the complex and the proteasome, ubiquitination, and DNA damage response have been reported (Collart and Panasenko, 2012).

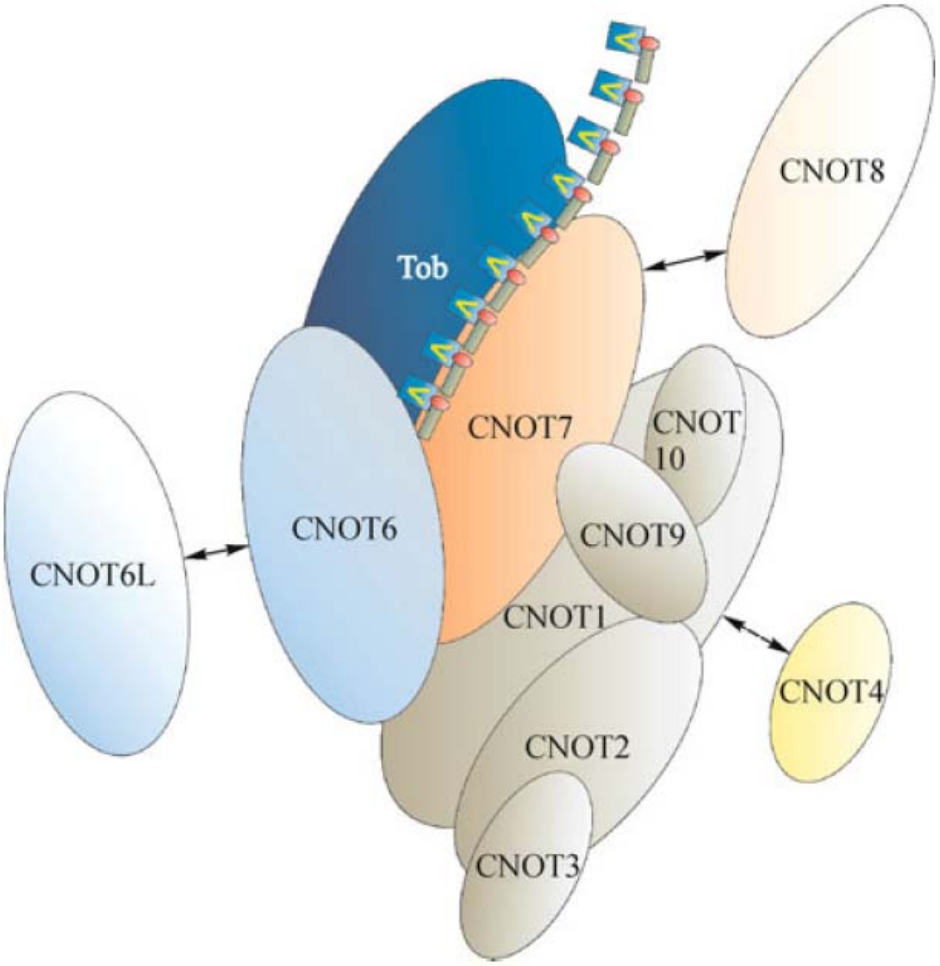


Figure 1.3: A model of the human CCR4-CAF1-NOT complex. CNOT1 acts as a scaffold of the complex which is thought to contain only two deadenylases: one CCR4-type (either CNOT6 or CNOT6L) and one CAF1-type (either CNOT7 or CNOT8). CNOT4 is not stably associated with the complex. Figure from Bartlam and Yamamoto, (2010).

Recruitment of the CCR4-CAF1-NOT complex to the 3`UTR of mRNAs via RNA binding proteins to regulate gene expression post-transcriptionally seems to be a general strategy used in eukaryotes. For example, human CAF1 is recruited by the zinc finger RNA binding protein Tristetraprolin (TTP) to AU-rich elements in the 3`UTR of target mRNAs (Clement et al., 2011; Sandler et al., 2011) and the CCR4-CAF1-NOT complex is recruited to the nanos 3`UTR by Smaug in *Drosophila* embryos (Zaessinger et al., 2006). In yeast, Pop2p is recruited to the 3`UTR of HO mRNA by Mpt5p, a member of the Pumilio family of RNA binding proteins (Goldstrohm et al., 2006; Goldstrohm et al., 2007). In the examples mentioned above, recruitment of the CCR4-CAF1-NOT complex induces deadenylation and decay of the target mRNA. However, the CCR4-CAF1-NOT complex has also been implicated in translational repression. In *Xenopus laevis* oocytes, Caf1 was found to repress translation of a tethered mRNA in a m^7G cap dependent and poly(A) tail independent manner (Cooke et al., 2010). Furthermore, Mpt5p-mediated repression in yeast seems to involve a deadenylation independent mechanism, as in cells lacking Ccr4p, Mpt5p-mediated repression is only modestly affected whereas deadenylation is severely inhibited (Goldstrohm et al., 2006; Goldstrohm et al., 2007). Interestingly, in contrast to Ccr4p, Pop2p is required for this Mpt5p-mediated repression, suggesting that Pop2p plays a role in the deadenylation independent mechanism of repression (Goldstrohm et al., 2007).

miRISCs interact with additional factors that have been shown to play a role in miRNA-mediated repression or may act as modulators of miRNA function (Fabian et al., 2010). One of these factors is the RNA helicase RCK/p54, a P-body component that is essential for inducing repression (Chu and Rana, 2006; Eulalio et al., 2007c). Other factors are the mammalian hyperplastic discs protein EDD, which has a critical function in miRNA-mediated siencing in mouse embryonic stem (ES) cells (Su et al., 2011) and Ataxin-2 (Atx2) that is required for miRNA-mediated repression in *Drosophila* (McCann et al.). Further, Importin 8 (Imp8) functions in miRNA-mediated repression by targeting AGO2 complexes to distinct target mRNAs (Weinmann et al., 2009) and the TRIM-NHL family proteins NHL-2 and TRIM32 were reported to enhance the activity of selected miRNAs in *C. elegans* (Hammell et al., 2009) and mouse (Schwamborn et al., 2009), respectively.

1.5 Cellular compartmentalization of miRNA repression

Where does miRNA-mediated repression take place in a cell? Although clearly more investigation is needed to fully answer this question, some connections between the miRNA machinery and cellular organelles and structures have already emerged.

1.5.1 The role of P-bodies and stress granules

P-bodies are cytoplasmic foci that consist of aggregates of translationally repressed mRNAs associated with a set of proteins of the translation repression and mRNA decay machinery (Parker and Sheth, 2007). Among the proteins that were found in P-bodies are components of the CCR4-NOT deadenylase complex, the decapping enzyme DCP2, the decapping activators DCP1, RCK/p54, HPat1, RAP55, EDC3, Ge-1/Hedls, LSm1-7, and the 5`-3` exonuclease XRN1. mRNAs accumulating in P-bodies are thought not to be engaged in translation as P-bodies lack ribosomes and eIF4E is the only translation initiation factor present (Andrei et al., 2005; Brengues et al., 2005; Ferraiuolo et al., 2005; Teixeira et al., 2005). mRNAs in P-bodies can be stored for later reutilization in translation but on the other hand, P-bodies are also believed to be a site where the final steps of mRNA decay occur (Eulalio et al., 2007a; Parker and Sheth, 2007).

One of the founding components of P-bodies is the miRISC component GW182 (Eystathioy et al., 2002), and miRNAs, AGO proteins and mRNAs targeted by miRNAs were all found to accumulate in P-bodies (Jakymiw et al., 2005; Liu et al., 2005b; Parker and Sheth, 2007; Pillai et al., 2005; Sen and Blau, 2005). Moreover, a correlation between miRNA-mediated repression and P-body localization of repressed mRNAs was observed (Bhattacharyya et al., 2006; Huang et al., 2007; Liu et al., 2005b; Pillai et al., 2005). A connection between P-bodies and miRNA-mediated silencing is further corroborated by the finding that knockdown of P-body components interferes with miRNA-mediated repression (Bagga et al., 2005; Behm-Ansmant et al., 2006; Chu and Rana, 2006; Eulalio et al., 2007c; Rehwinkel et al., 2005). Conversely, a functional miRNA pathway is required for P-body formation as the knock-down of miRNA-biogenesis components (Drosha, DGCR8, Dicer-1) or

factors required for miRNA-mediated repression (AGO1, GW182) resulted in a decline of P-bodies (Eulalio et al., 2007b; Pauley et al., 2006).

Although some P-body components seem to be involved in miRNA-mediated silencing, depletion of other P-body components that results in the dispersion of P-bodies did not affect silencing (Eulalio et al., 2007b). These findings indicate that microscopically visible P-bodies are not essential for miRNA-induced silencing but do not excluded the possibility that submicroscopic P-body-like structures contribute to silencing.

Stress granules are another type of RNA granules that contain untranslated mRNAs and form upon global repression of translation initiation (Fabian et al., 2010; Parker and Sheth, 2007). Like P-bodies, stress granules may play a role in miRNA-mediated repression as AGO proteins and miRNA mimics were observed to accumulate in stress granules (Fabian et al., 2010; Leung et al., 2006). However, this accumulation can just represent passive dragging of mRNA-associated proteins into stress granules upon translational repression.

1.5.2 Role of Multivesicular bodies and endosomes

Evidence for a role of multivesicular bodies (MVBs) in miRNA-mediated silencing was found in two studies using *Drosophila melanogaster* and human cell lines (Gibbings et al., 2009; Lee et al., 2009). Lee et al., (2009) linked miRNA-mediated silencing to endosomal trafficking. Blocking multivesicular body maturation and fusion with lysosomes enhanced miRNA-mediated repression and lead to an accumulation of GW-bodies which were found to be associated with MVBs. Conversely, blocking MVB formation impaired miRNA-mediated silencing. The authors proposed a model in which MVBs promote the turnover of RNA-induced silencing complexes (RISCs) which results in more effective engagement of RISCs with small RNAs and possibly target RNAs. Gibbings et al., (2009) reached similar conclusions after showing that GW-bodies congregate with endomsomes and MVBs and that the depletion of ESCRT (endosomal sorting complex required for transport) complex components required for protein sorting to MVBs compromised miRNA-mediated silencing.

1.6 The mechanism of miRNA-mediated repression

miRNAs regulate gene expression post-transcriptionally by inhibiting translation of target mRNAs and/or by inducing their deadenylation and subsequent degradation (Fabian et al., 2010). First I will provide a brief overview of the mechanism of translation and then summarize the results of studies addressing the mechanism of miRNA-mediated silencing.

1.6.1 Introduction to eukaryotic translation

The process of translation can be divided in three steps: initiation, elongation, and termination (Sonenberg and Hinnebusch, 2009). In the process of translation initiation, the small (40S) ribosomal subunit is recruited to the 5` end of the mRNA and then scans in the 5`-3` direction until it encounters the first AUG start codon which leads to formation of the 80S ribosome by joining of the large (60S) ribosomal subunit. Next, the 80S ribosome moves along the mRNA while catalyzing the formation of an elongating peptide chain. Finally, during translation termination the newly synthesized protein is released and the ribosome dissociates from the mRNA. Efficient translation initiation of an mRNA depends on the m^7GpppN group (termed 5` cap) at the 5` end of the mRNA and on the poly(A) tail at the 3` end. This is explained by the fact that the 5` cap and the poly(A) tail facilitate the recruitment of the 43S pre-initiation complex consisting of translation initiation factors and the small (40S) ribosomal subunit loaded with methionly-tRNA to the 5` end of the mRNA (Figure 1.4, panel A) (Sonenberg and Hinnebusch, 2009). The 5` cap interacts with the translation initiation factor eIF4E which is part of the eIF4F complex. The eIF4F complex contains two more components: eIF4G, which directly interacts with and recruits the 43S pre-initiation complex and eIF4A, an RNA helicase which unwinds secondary structures in the 5`UTR and thereby facilitates scanning of the small ribosomal subunit. The poly(A) tail is bound by the poly(A)-binding protein (PABP) which directly interacts with eIF4G. The PABP-eIF4G interaction stabilizes the interaction of eIF4E with the 5` cap which ultimately enhances translation initiation (Kahvejian et al., 2005).

Translation initiation is not always a cap-dependent process. Originally discovered as part of a viral RNA (Pelletier and Sonenberg, 1988), internal ribosomal entry sites (IRESs) can directly recruit the ribosome and thereby circumvent the requirement of a 5` cap structure for translation initiation. IRESs are also found in many cellular mRNAs (Hellen and Sarnow, 2001). IRESs function independently of the cap but some utilize certain cellular translation initiation factors. For example, the encephalomyocarditis virus (EMCV) IRES recruits the ribosome by directly binding to eIF4G, functions independently of eIF4E but requires otherwise the same set of translation initiation factors as the canonical initiation mechanism (Figure 1.4, panel B) (Hellen, 2009). The hepatitis C virus (HCV) IRES directly assembles the initiation complex containing the 40S ribosomal subunit and eIF3 at the start codon and functions independently of eIFs 4A, 4B, and 4F (Hellen, 2009). In contrast, the cricket paralysis virus (CrPV) IRES recruits the ribosome via a mechanism that does not require any canonical initiation factor (Fabian et al., 2010).

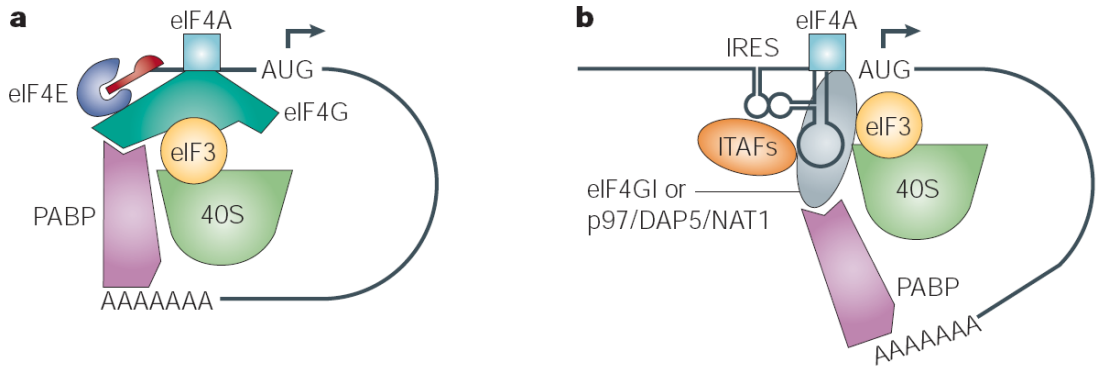


Figure 1.4: Cap-dependent versus internal ribosome-entry site-dependent translation initiation. (**A**) In cap-dependent translation initiation, eIF4E binds to the 5` $m^7$GpppN cap structure (red). The 40S ribosomal subunit is recruited to the 5` end of the mRNA via eIF4E, eIF4G and eIF3. The RNA helicase eIF4A is thought to be involved in the unwinding of secondary structure in the 5` untranslated region. Poly(A)-binding protein (PABP) binds to the poly(A) tail and eIF4G which leads to mRNA circularization. (**B**) A 5` m^7GpppN cap structure and eIF4E are not required for internal ribosome-entry site-dependent translation initiation. Translation initiation mediated by some IRESes is stimulated by internal ribosome-entry site (IRES) trans-acting factors (ITAFs) and eIF4G or a distant homologue thereof (p97). Figure from Holcik and Sonenberg, (2005).

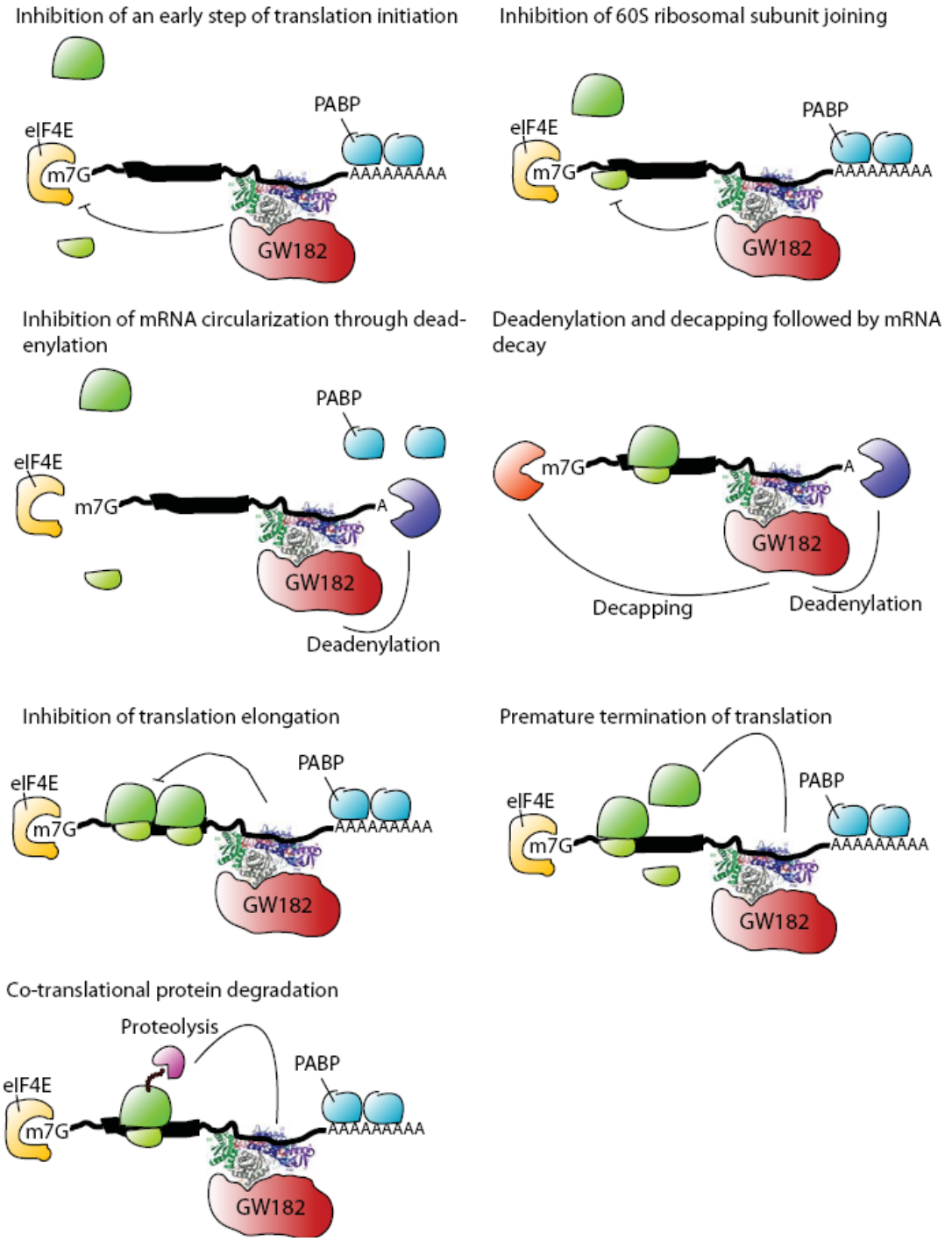


Figure 1.5: Possible mechanisms of miRNA-mediated gene silencing. miRNAs have been suggested to silence gene expression by inhibiting translation at different steps of translation initiation by interfering with cap recognition, interfering with function of translation initiation factors or inducing deadenylation. miRNA-mediated deadenylation may be followed by decapping and mRNA decay. Also, miRNAs have been suggested to affect translation at a step after initiation by inhibiting translation elongation, promoting premature termination of translation or by inducing nascent polypeptide degradation. Figure based on Eulalio et al., (2008a) and Chekulaeva and Filipowicz, (2009).

1.6.2 miRNA-mediated repression of translation

1.6.2.1 miRNA-mediated repression of translation at postinitiation steps

The first miRNA discovered, lin-4, was found to repress *lin-14* post-transcriptionally at the level of translation without causing degradation of *lin-14* mRNA (Lee et al., 1993; Olsen and Ambros, 1999; Wightman et al., 1993). Because lin-4 also did not change the polysomal sedimentation profile of *lin-14* mRNA, the authors concluded that the miRNA does not affect translation initiation but inhibits protein accumulation at a step after initiation (Figure 1.5) (Olsen and Ambros, 1999). Three lines of evidence have been used as arguments supporting the idea that miRNAs mediate repression of translation at a post-initiation step.

First, as observed for lin-4 and lin-14, also a number of subsequent studies found that miRNAs repress protein synthesis without affecting the polysome association of target mRNAs (Gu et al., 2009; Nottrott et al., 2006; Olsen and Ambros, 1999; Petersen et al., 2006; Seggerson et al., 2002).

Second, miRNAs, target mRNAs and AGO proteins were found to be associated with polysomal fractions (Kim et al., 2004; Maroney et al., 2006; Nelson et al., 2004; Nottrott et al., 2006). Although this observation was interpreted as an argument in favor of miRNA-mediated repression taking place at a post-initiation step (Maroney et al., 2006), association of miRISC components with polysomes could also be due to incomplete repression of translation initiation.

Third, miRNAs (or miRNA mimics) were found to repress HCV and CrPV IRES-driven translation (Lytle et al., 2007; Petersen et al., 2006). As IRES-driven translation initiation requires fewer or no (CrPV IRES) canonical translation initiation factors, these results are consistent with repression affecting a step other than canonical translation initiation (Fabian et al., 2010). In the study of Petersen et al., (2006) repression could also involve miRNA-mediated mRNA decay rather than translational repression as the authors did not measure the reporter mRNA level.

What could be the mechanism of translational repression at a post-initiation step? Petersen et al., (2006) observed that after a block of translation initiation, miRNA mimics caused the target mRNA containing polysomes to dissociate more rapidly than in the absence of miRNA mimics. Therefore, the authors proposed that miRNAs promote ribosomes to dissociate prematurely from the mRNA (Figure 1.5). Conflicting with this model, Guo et al., (2010) observed that the number of ribosomes

associated with repressed mRNAs was reduced, but the reduction was constant along the open reading frame.

Nottrott et al., (2006) suggested that miRNAs could cause the degradation of nascent polypeptides co-translationally (Figure 1.5). Arguing against this model, an independent study found that targeting the nascent polypeptide to the endoplasmatic reticulum, which should protect it from proteolysis, did not affect the degree of miRNA-mediated repression (Pillai et al., 2005). Furthermore, mRNAs coding for membrane and ER proteins were found to be overrepresented among translationally repressed miRNA targets (Selbach et al., 2008).

1.6.2.2 miRNA-mediated repression of translation initiation

In contrast to the studies mentioned above, experiments carried out in other laboratories indicated that miRNAs interfere with translation initiation.

First, miRNAs were found to shift target mRNAs from heavy to lighter polysomes in the sedimentation gradient, indicating reduced ribosome association of repressed mRNAs. This was first observed in HeLa cells for reporter mRNAs regulated by endogenous (let-7) or artificial miRNAs (Humphreys et al., 2005; Pillai et al., 2005). Later, similar shifts of target mRNAs were also observed for the endogenous CAT-1 mRNA that is repressed by miR-122 in Huh7 cells (Bhattacharyya et al., 2006), for a miR-16-targeted reporter mRNA in 293T cells (Huang et al., 2007) and for several endogenous mRNAs repressed by let-7 in HeLa cells (Clancy et al., 2011). Importantly, miRNAs were also observed to affect the polysome association of mRNAs in a whole animal, the worm *C. elegans*. Several endogenous targets, among them *lin-14* and *lin-28* mRNAs, were found to be associated with fewer ribosomes when they were repressed by miRNAs (Ding and Grosshans, 2009). Genomic-scale studies using polysome profile analysis or a ribosome profiling approach also support the notion that miRNA-mediated translational repression occurs at the level of initiation (Guo et al., 2010; Hendrickson et al., 2009).

Second, mRNAs whose translation is driven in a m^7G-cap independent manner were found to be refractory to miRNA-mediated repression or to exhibit a reduced extent of repression. In HeLa cells, mRNAs with an ApppG-cap structure were less repressed by miRNA mimics than mRNAs with a normal m^7G-cap structure (Humphreys et al., 2005) and translation driven by the HCV (Pillai et al., 2005), EMCV (Karaa et al., 2009; Pillai et al., 2005) or CrPV (Humphreys et al., 2005) IRESs or by tethered translation initiation factors (eIf4E or eIF4G) (Pillai et al., 2005)

was refractory to miRNA-mediated repression. All these studies indicated that miRNAs target an early step of translation initiation (Figure 1.5).

Subsequent *in vitro* studies using cell free extracts supported the notion that miRNAs inhibit translation at the step of initiation. In extracts prepared from *Drosophila melanogaster* embryos, miRNAs interfered with the formation of 48S and 80S translation initiation complexes (Thermann and Hentze, 2007) and also in mouse Krebs-2 ascites cell-extracts formation of the 80S ribosomal complex was inhibited by miRNAs (Mathonnet et al., 2007). In the cell extracts mentioned above and also in cell extracts from HEK293 cells or in rabbit reticulocyte lysates, the m^7G cap was essential for miRNA-mediated repression as EMCV or HCV IRES-dependent translation and ApppG-capped or uncapped mRNAs were refractory to repression (Mathonnet et al., 2007; Ricci et al., 2011; Thermann and Hentze, 2007; Wakiyama et al., 2007; Wang et al., 2006). In support of the notion that miRNAs target the m^7G-cap-recognition machinery, miRNA mediated repression was found to be affected by modifications of the triphosphate bridge of the cap (Zdanowicz et al., 2009) and by the addition of eIF4F to the cell extract (Mathonnet et al., 2007).

How could miRNAs interfere with the cap-recognition machinery? Iwasaki et al., (2009) provided evidence that in *Drosophila* embryo extracts, dAGO2-RISC inhibits cap-dependent translation by blocking the interaction of eIF4E with eIF4G (Iwasaki et al., 2009). However, generally miRNAs are loaded into dAGO1 and only a subclass of miRNAs is loaded into dAGO2 (Czech and Hannon, 2011; Fabian et al., 2010). Furthermore, the mechanism of dAGO2 action is probably not evolutionarily conserved, since in contrast to dAGO2, human AGO2 has not been obsereved to interact with eIF4E (Fabian et al., 2009; Fabian et al., 2010).

Another model suggests that AGO2 directly binds to the m^7G-cap and inhibits translation initiation by competing with eIF4E for binding to the 5`-cap (Djuranovic et al., 2010; Kiriakidou et al., 2007). Kiriakidou et al., (2007) found two aromatic residues in the AGO2 MID domain to be required for cap interaction and translational repression induced by tethered AGO2 and suggested that these residues bind to the cap structure by stacking interactions similar to those found in eIF4E. Challenging this idea, a homology based structure model of AGO2 revealed that one of the aromatic residues is buried in the hydrophobic core of the domain (Kinch and Grishin, 2009) and mutating the residues also abrogated interaction of AGO with GW182 and miRNAs (Eulalio et al., 2008b). Djuranovic et al., (2010) found that the affinity of Drosophila AGO1 to m^7GTP-Sepharose increased in the presence of miRNAs and suggested that miRNA binding to the 5` phosphate binding site in AGO1 makes an

allosterically regulated cap-binding site available. However, this potential cap binding site was found to be occluded by other parts of the protein in a crystal structure of the MID-PIWI lobe from *Neurospora* Argonaute (Boland et al., 2011). Furthermore, an equivalent potential cap binding site as in the structure of the *Neurospora* Argonaute MID domain was not found in the structure of the human AGO2 MID domain and pull-down experiments with human AGO2 indicated that the interaction with cap analogs is non-specific (Frank et al., 2011).

In contrast to the studies mentioned above suggesting that miRNAs target an early step of translation initiation, other studies indicated that miRNAs interfere with a late step of translation initiation, the joining of the 60S ribosomal subunit (Figure 1.5). In support of this notion Wang et al., (2008a) found 40S but not 60S ribosomal subunits to be associated with mRNAs repressed by miRNA mimics in a rabbit reticulocyte lysate. Another study reported that eIF6, a protein that prevents the 60S ribosomal subunit to join the 40S subunit, is required for miRNA-mediated repression in human cells and in *C. elegans* (Chendrimada et al., 2007). On the other hand, depleting eIF6 had no effect on miRNA-mediated repression in *Drosophila* S2 cells (Eulalio et al., 2008b) and knocking-down eIF6 in *C. elegans* stimulated rather than inhibited let-7-mediated repression (Ding et al., 2008).

1.6.2.2.1 miRNA-mediated repression of translational initiation and the poly(A)-tail

Since the poly(A)-binding protein, which associates with the 3` poly(A) tail of mRNAs, promotes cap-dependent translation initiation, miRNA-mediated deadenylation would be expected to inhibit translation initiation (Figure 1.5) (Fabian et al., 2010). Many studies have therefore addressed the role of a poly(A) tail in miRNA-mediated translational repression. In cell free extracts from HEK293 cells or *Drosophila* embryos, m^7G-capped and poly(A) tail-free mRNAs were found to be refractory to miRNA-mediated repression (Wakiyama et al., 2007; Zdanowicz et al., 2009). Also, miRNA mimics failed to repress m^7G-capped mRNAs lacking a poly(A) tail in nuclease-treated rabbit reticulocyte laysate (Wang et al., 2006). In HeLa cells, blocking deadenylation of a reporter mRNA partially prevented let-7-mediated translational repression, indicating that miRNA-mediated deadenylation contributes to translational repression (Beilharz et al., 2009). In contrast, Pillai et al., (2005) found no difference in the extent of let-7-mediated repression of poly(A)+ and poly(A)- mRNA in transfected HeLa cells. In addition, mRNA reporters in which the poly(A) tail

was replaced by a histone stem-loop or a self-cleavable ribozyme were still repressed by miRNAs (Eulalio et al., 2008b; Eulalio et al., 2009b; Wu et al., 2006). Thus, it seems that miRNAs can inhibit translation in poly(A) tail-dependent and -independent ways (Fabian et al., 2010).

1.6.3 Modulation of miRNA-mediated repression

The repressive effect of miRNAs on their target mRNAs was found to be modulated by various trans-acting factors. HuR, an AU-rich element binding protein, is released from the nucleus upon stress of Huh7 cells and alleviates miR-122-mediated repression of CAT-1 mRNA by binding to its 3`UTR (Bhattacharyya et al., 2006). Similarly, Dead-end 1 (Dnd1), another RNA-binding protein, prevents miRNA-mediated repression in human cells and primordial germ cells of zebrafish by preventing miRNAs to associate with their target mRNAs (Kedde et al., 2007). The protein DAZL (deleted in azoospermia-like) represents another factor that protects some mRNAs in zebrafish primordial germ cells from miRNA activity. DAZL counteracts miR-430-mediated deadenylation by promoting poly(A)-tail elongation of a subset of miR-430 target mRNAs (Takeda et al., 2009).

Trans-acting factors have also been reported to enhance miRNA-mediated repression. Two TRIM-NHL proteins, mammalian TRIM32 and *C. elegans* NHL-2 enhance miRNA activity without changing miRNA levels (Hammell et al., 2009; Krol et al., 2010; Schwamborn et al., 2009). NHL-2 interacts genetically and physically with AGO, GW182, and RCK/p54 proteins and is required for full activity of let-7 and lys-6 miRNAs (Hammell et al., 2009). Interestingly, both TRIM32 and NHL-2 seem to stimulate the activity of only a subset of miRNAs (Hammell et al., 2009; Schwamborn et al., 2009).

1.6.4 miRNA-mediated translational activation

Under certain conditions miRNAs were also found to stimulate the expression of their targets rather than repressing them (Henke et al., 2008; Jopling et al., 2008; Jopling et al., 2005; Orom et al., 2008; Vasudevan and Steitz, 2007; Vasudevan et al., 2007). Vasudevan et al., (2007) found that miRNAs switch from a repression to an activation mode upon G1/G0 growth arrest. However, upregulation of translation in quiescent

cells is probably not a general mechanism as in a different study miRNA-mediated repression was also observed in G1-arrested cells (Li and Carthew, 2005).

Orom et al., (2008) reported that in response to stress or nutrient shortage, miR-10a stimulates translation of 5`-TOP (5`-terminal oligopyrimidine tract) motif containing mRNAs which encode proteins involved in translation. To stimulate translation, miR-10a binds downstream of the 5`-TOP motif in the 5`UTR by seemingly non-canonical miRNA-mRNA base-pairing. Whether AGO proteins or other miRNP components are involved in this type of regulation was not investigated.

The liver specific miR-122 was observed to positively affect expression of the Hepatitis C Virus (HCV) RNA (Henke et al., 2008; Jopling et al., 2008; Jopling et al., 2005). Binding of miR-122 to the 5`UTR of HCV RNA stimulated HCV RNA replication (Jopling et al., 2005) and also its translation (Henke et al., 2008) in an AGO2-dependent manner (Wilson et al., 2011).

1.6.5 miRNA-mediated mRNA deadenylation and decay

Originally, miRNAs were thought to inhibit translation without affecting the level of target mRNAs. However, it is now well established that miRNAs can down regulate target mRNA levels (Bagga et al., 2005; Krutzfeldt et al., 2005; Lim et al., 2005; Wu and Belasco, 2005). Down-regulation of hundreds of miRNA targets was observed in genomic scale studies that measured the abundance of mRNAs after introducing a miRNA into cells (Baek et al., 2008; Guo et al., 2010; Hendrickson et al., 2009; Lim et al., 2005; Linsley et al., 2007; Selbach et al., 2008; Webster et al., 2009) and hundreds of miRNA targets were found to be up-regulated after depleting or inhibiting a miRNA (Baek et al., 2008; Krutzfeldt et al., 2005; Selbach et al., 2008). Similarly, depleting cells of factors essential for miRNA-mediated repression (such as Drosha, Dicer, AGO or GW182) increased the mRNA level of miRNA targets (Behm-Ansmant et al., 2006; Eulalio et al., 2009b; Eulalio et al., 2007c; Giraldez et al., 2006; Rehwinkel et al., 2005; Rehwinkel et al., 2006; Schmitter et al., 2006) and anti-correlated expression changes of miRNAs and their targets were observed in differentiating cells (Farh et al., 2005). Furthermore, there is much evidence that miRNAs cause target mRNA degradation by inducing deadenylation followed by decapping and 5`-3` exonucleolytic digestion (Figure 1.5) (Behm-Ansmant et al., 2006; Beilharz et al., 2009; Chen et al., 2009; Eulalio et al., 2009b; Eulalio et al.,

2007c; Giraldez et al., 2006; Mishima et al., 2006; Piao et al., 2010; Rehwinkel et al., 2005; Wu et al., 2006). Finally, miRNA-mediated deadenylation was also observed in cell free extracts (Fabian et al., 2009; Iwasaki et al., 2009; Wakiyama et al., 2007; Zdanowicz et al., 2009).

1.6.5.1 GW182 and miRNA-mediated deadenylation and decay

The two core components of the miRISC complex, AGO and GW182 proteins, are essential for miRNA-mediated deadenylation and subsequent mRNA decay (Behm-Ansmant et al., 2006; Fabian et al., 2010). Knocking down *Drosophila* AGO1 abrogates miRNA-mediated mRNA decay and immunodepleting AGO2 from Krebs-2 ascites extracts prevents miRNA-mediated deadenylation (Behm-Ansmant et al., 2006; Fabian et al., 2009). Knocking down GW182 also abrogates miRNA-mediated mRNA decay (Behm-Ansmant et al., 2006). Furthermore, interfering with the AGO-GW182 interaction with point mutations or by expressing a competing GW182 fragment or adding a competing peptide (or a GW182 fragment) to cell-free extracts, prevents miRNA-mediated repression and blocks miRNA-mediated deadenylation (Eulalio et al., 2008b; Fabian et al., 2009; Iwasaki et al., 2009; Till et al., 2007). Finally, direct tethering of GW182 to the 3`UTR induces deadenylation and decay of a reporter mRNA (Behm-Ansmant et al., 2006; Chekulaeva et al., 2009; Lazzaretti et al., 2009; Zipprich et al., 2009).

Importantly, besides inducing mRNA decay GW182 proteins are also implicated to be involved in translational repression in human cells, *Drosophila* S2 cells and *C. elegans* (Behm-Ansmant et al., 2006; Chekulaeva et al., 2009; Ding and Grosshans, 2009; Iwasaki et al., 2009; Zipprich et al., 2009).

1.6.5.2 mRNA decay factors and miRNA-mediated deadenylation and decay

miRNAs direct their target mRNAs to the canonical mRNA decay pathway where the mRNA is deadenylated and subsequently decapped and degraded (Behm-Ansmant et al., 2006; Eulalio et al., 2009b; Eulalio et al., 2007c; Giraldez et al., 2006; Piao et al., 2010; Rehwinkel et al., 2005; Wu et al., 2006). Two deadenylase complexes are involved in miRNA-mediated deadenylation and mRNA decay: CCR4-CAF1-NOT and PAN2-PAN3. This is supported by the findings that depleting components of the two deadenylase complexes or overexpressing dominant negative forms interferes

with miRNA-mediated deadenylation and leads to an increase of the mRNA level of miRNA-targets (Behm-Ansmant et al., 2006; Chen et al., 2009; Eulalio et al., 2009b; Fabian et al., 2009; Piao et al., 2010). miRNA-mediated deadenylation is followed by decapping by the DCP1:DCP2 decapping complex and 5`-3` exonucleolytic degradation by XRN1 (Bagga et al., 2005; Behm-Ansmant et al., 2006; Eulalio et al., 2009b; Eulalio et al., 2007c; Rehwinkel et al., 2005). DCP2-mediated decapping is stimulated by decapping activators such as DCP1, GE1, PAT1 and RCK and depletion of these factors was shown to increase the mRNA level of miRNA targets or to interfere with miRNA-mediated repression (Behm-Ansmant et al., 2006; Chu and Rana, 2006; Eulalio et al., 2007c; Rehwinkel et al., 2005).

1.6.5.3 Poly(A)-binding protein and miRNA-mediated deadenylation and decay

The poly(A)-binding protein is another factor that is required for miRNA-mediated deadenylation. Immunodepletion of PABP from Krebs ascite extracts prevented miRNA-mediated deadenylation which could be rescued by the addition of recombinant PABP to the extract (Fabian et al., 2009). Supporting the idea that PABP plays a role in miRNA-mediated silencing two additional studies showed that overexpressing PABP in *Drosophila* S2 cells or in human cells interferes with miRNA-mediated repression (Walters et al., 2010; Zekri et al., 2009). On the other hand, Fukaya and Tomari, (2011) concluded that PABP function is dispensable for AGO1-RISC-mediated deadenylation and translational repression in a *Drosophila* S2 cell lysate, as blocking PABP function by the addition of PABP-interacting protein 2 (Paip2) to the lysate did not affect silencing. Similarly, Mishima et al., (2012) found that miRNA-mediated translational inhibition and target mRNA degradation can occur in a PABP-independent manner in zebrafish embryos.

1.6.6 Translational repression versus mRNA decay

What emerges from the studies mentioned above is that there is compelling evidence for both miRNA-mediated translational repression and miRNA-mediated mRNA decay. Therefore, many studies attempted to elucidate which of the two mechanisms dominates and what is the mechanistic relation between the two processes. Four recent genomic scale studies provide evidence that target degradation is the

predominant mode of regulation by miRNAs in mammalian cell cultures (reviewed by Huntzinger and Izaurralde, (2011)). Baek et al., (2008) and Selbach et al., (2008) used a quantitative mass spectrometry approach to measure the effect of adding or depleting a miRNA from cultured cells on protein and mRNA levels. Both studies found that at later time points, changes in mRNA and protein levels of miRNA targets showed good correlation. However at an early time point after transfection of a miRNA, Selbach et al., (2008) found many miRNA targets that were regulated only at the protein level. Hendrickson et al., (2009) transfected human embryonic kidney cells with miR-124 and analyzed mRNA abundance and translation rate of miR-124 targets. Their data revealed that 75% of the changes observed in protein synthesis are due to target mRNA degradation. Similarly, using a ribosome profiling approach Guo et al., (2010) reported that 84% of the change in protein production can be explained by a decrease of target mRNA steady-state levels.

What is the relation between the different events (translational repression, deadenylation and mRNA decay) of miRNA-mediated silencing? Are these events obligatorily connected or do they occur independently (Djuranovic et al., 2011)? Many studies addressed the question whether target mRNA degradation is simply a consequence of an initial block of translation initiation. Reporter mRNAs whose translation was inhibited by a strong RNA secondary structure in the 5`UTR or by an antisense oligonucleotide blocking the start codon were still deadenylated and degraded by miRNAs (Eulalio et al., 2009b; Giraldez et al., 2006; Wu et al., 2006). Similarly, A-capped reporter mRNAs were found to undergo miRNA-mediated deadenylation (Fabian et al., 2009; Iwasaki et al., 2009; Mishima et al., 2006; Wakiyama et al., 2007; Zdanowicz et al., 2009) and miRNA-mediated deadenylation was also observed in the presence of translation inhibitors such as cycloheximide or hippuristanol (Eulalio et al., 2007c; Fabian et al., 2009; Iwasaki et al., 2009; Wakiyama et al., 2007). Thus, these studies suggested that deadenylation and subsequent degradation are not obligatorily coupled to active translation (Huntzinger and Izaurralde, 2011). Another interesting question that remains to be addressed is to what extent a miRNA-mediated block in translation influences deadenylation of a targeted mRNA.

Conversely, other studies suggested that miRNA-mediated silencing can occur independently of target mRNA deadenylation indicating that besides miRNA-mediated deadenylation there are additional mechanisms that lead to translational inhibition. This is supported by the finding that mRNAs lacking a poly(A)-tail are still repressed by miRNAs and by tethered GW182 protein (Eulalio et al., 2008b; Eulalio

et al., 2009b; Pillai et al., 2005; Wu et al., 2006). miRNAs were also observed to repress mRNA reporters the deadenylation of which was blocked by a non-poly(A) sequence following the poly(A)-tail (Fukaya and Tomari, 2011; Mishima et al., 2012). Furthermore, miRNAs and tethered GW182 protein still silenced reporter mRNAs in cells depleted of the deadenylase complex component NOT1 (Behm-Ansmant et al., 2006; Eulalio et al., 2008b). And finally, in cell free extracts from mouse Krebs ascites cells it was found that miRNA-mediated inhibition of translation preceded deadenylation (Fabian et al., 2009).

Based on these observations the following model of miRNA-mediated silencing was suggested: miRNA-mediated silencing begins with repression of translation initiation that may be enhanced by deadenylation (Djuranovic et al., 2011). The ensuing deadenylation would contribute to silencing and ultimately lead to mRNA decay to consolidate the more transient translational repression (Djuranovic et al., 2011; Fabian et al., 2009; Fabian et al., 2010; Huntzinger and Izaurralde, 2011).

1.7 Aim of this study

The aim of this study was to understand the mechanism of miRNA-mediated gene silencing. Despite an extensive research effort, the molecular events leading to miRNA-mediated repression are not entirely understood. Studies aiming to shed light on this process came to different and sometimes even contradictory conclusions. As a consequence of these discrepant results, a plethora of different models for the mechanism of miRNA-mediated repression has been proposed.

Our idea was to study the protein factors that are responsible for executing the miRNA-mediated repression in order to get a clearer picture of the mode of silencing. As the proteins of the GW182 family emerged as key factors of the silencing mechanism, we decided to focus on these proteins. Deletion and mutational analysis should reveal what parts of the protein and more specifically what sequence elements mediate the repression. Further, we aimed to identify the proteins interacting with GW182 to learn about the molecular events leading to repression. Finally, because the relation and relative contribution of translational inhibition and mRNA deadenylation/decay to silencing were matters of intensive debate, we planed to investigate the role of the poly(A) tail in GW182-mediated repression.

2. Results

The aim of this study was to uncover the mechanism of miRNA-mediated gene silencing. Work performed by others demonstrated that the family of GW182 proteins plays a crucial role in this process. GW182 proteins are recruited to miRNA targets via direct interaction with AGO proteins (Eulalio et al., 2009a; Till et al., 2007). The crucial role of GW182 proteins in miRNA-mediated silencing is evidenced by the facts that interference with the AGO-GW182 interaction and depletion of the GW182 protein alleviates miRNA mediated repression (Behm-Ansmant et al., 2006; Eulalio et al., 2008b; Rehwinkel et al., 2005; Till et al., 2007). Furthermore, direct tethering of GW182 proteins to an mRNA represses the mRNA even in the absence of AGO protein, suggesting that GW182 is the effector protein acting downstream of AGO (Behm-Ansmant et al., 2006). Therefore we reasoned that understanding the function of GW182 proteins would give us insight about the mechanism of miRNA-mediated silencing.

Consequently, we decided to study the role of GW182 in miRNA-mediated gene silencing. The results obtained in the course of this study are described in three published papers that are attached in this thesis. Appendices A and B contain the papers “Importance of the C-terminal domain of the human GW182 protein TNRC6C for translational repression” and “Mammalian miRNA RISC Recruits CAF1 and PABP to affect PABP-Dependent Deadenylation” to which I contributed as a co-author. Here in the results section I briefly summarize the results of these two papers and describe the experiments that were performed by myself in more detail. The third paper “miRNA repression involves GW182-mediated recruitment of CCR4-NOT through conserved W-containing motifs” to which I contributed as a co-first author, is attached in the results section. Finally, in the last section of Results I describe preliminary results about the analysis of the CNOT1 protein that were obtained as part of a follow up study of the third paper.

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

Jakob T. Zipprich, Sankar Bhattacharyya, Hansruedi Mathys, and Witold Filipowicz
(The full paper is attached in Appendix A)

Mammalian genomes encode three GW182 paralogs, TNRC6A, TNRC6B and TNRC6C. A role for TNRC6A and TNRC6B in miRNA-mediated repression had already been demonstrated but it was not known whether this is also the case for TNRC6C. Sankar Bhattacharyya found that RNAi-mediated knockdown of each individual TNRC6 protein partially alleviates repression of a reporter RNA that is repressed by miRNA let-7b, thus demonstrating that all three TNRC6 proteins are important for efficient miRNA-mediated repression (Appendix A, Figure 2c). Sankar Bhattacharyya also tested the function of individual TNRC6 proteins using a tethering assay. In this assay the protein of interest is expressed as a fusion with the phage λN peptide that specifically binds to box B RNA hairpins inserted into the 3`UTR of a Renilla Luciferase (RL) reporter RNA (RL-5BoxB). Artificial tethering of each TNRC6 protein to the 3`UTR of a reporter RNA strongly repressed the expression of the reporter RNA as a result of a combination of effects on the mRNA level and mRNA translation (Appendix A, Figure 3a and 3b).

To gain insight into the mechanism of repression exerted by TNRC6 proteins, Jakob Zipprich generated a collection of deletion mutants of TNRC6C and tested their repressive function in the tethering assay. This analysis revealed the C-terminal part of TNRC6C (CED, also referred to as ΔN1370), encompassing a conserved domain of unknown function (DUF/PAM2) and a RNA recognition motif (RRM), as a key effector domain mediating repression (Appendix A, Figure 4). Sankar Bhattacharyya found that similarly as observed for the full length TNRC6C protein, tethering of the CED fragment (NHA-ΔN1370) affects both mRNA translation and stability (Appendix A, Figure 6a).

2.1.1 The inhibitory effect of tethering TNRC6C or CED on translation is not due to deadenylation

To learn whether translational repression induced by tethering TNRC6C or the CED fragment could be explained by the deadenylation of the reporter mRNA, we investigated the poly(A)-tail status of reporter mRNAs using an RNase H assay. RNase H cleaves DNA-RNA hybrids and therefore in the presence of oligo(dT) removes the poly(A)-tail of reporter mRNAs. Thus, reporter mRNAs bearing a poly(A)-tail are expected to be shortened by the treatment with RNaseH in the presence of oligo(dT), whereas reporter mRNAs that had already been deadenylated in the cell, cannot be shortened any further by the RNase H treatment. We found that

RL-5BoxB reporter mRNAs isolated from cells expressing TNRC6C or the CED fragment fused to the phage λN peptide (NHA-TNRC6C or NHA-ΔN1370) showed an increased mobility in an agarose gel when treated with RNaseH in the presence of oligo(dT) (Figure 2.2, for a schematic representation of TNRC6C and CED see Figure 2.1). These data indicate that the inhibitory effect on translation induced by tethering TNRC6C or the CED fragment is not due to deadenylation of the reporter mRNA.

Binding of RNA by RRM domains of RNA-binding proteins commonly involves stacking interactions between aromatic amino acid side-chains and nucleotides (Clery et al., 2008). Interestingly, the RRM domain of GW182 proteins contains several conserved aromatic amino acids and Jakob Zipprich found that some of these residues are required for maximal silencing activity of the CED in a tethering assay (Appendix A, Figure 5c). Furthermore, Sankar Bhattacharyya analyzed the effect of tethering the CED or its RRM domain mutants on reporter mRNA translation and stability. He found that compared to the wild-type CED, the RRM domain mutants F1543A and H1537A/Y1556A were specifically impaired in repressing translation (Appendix A, Figure 6a). As we considered the possibility, that the repressive effect on translation seen upon tethering of the CED could be due to mRNA deadenylation, we asked whether the RRM domain mutants were also impaired in inducing mRNA deadenylation compared to the wild-type CED. We found that the mRNA that escaped degradation by tethered CED or its RRM domain mutant (H1537A/Y1556A) was not detectably deadenylated (Figure 2.2). Thus, as mentioned above, the inhibitory effect on translation induced by tethering the CED seems not to be due to deadenylation of the reporter mRNA. Consequently, impaired translational repression activity of the RRM domain mutants compared to the wild-type CED is unlikely due to an impaired ability to induce deadenylation.

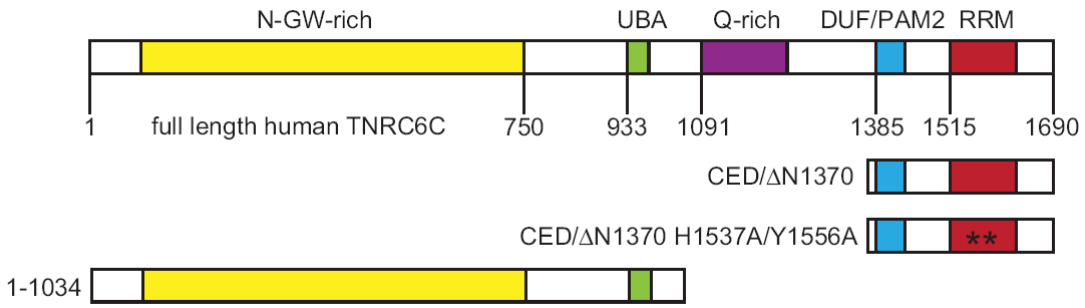


Figure 2.1: Schematic representation of human TNRC6C and its deletion mutants used in the experiments shown in figures 2.2 and 2.3. Positions of N-GW-rich, Q-rich, UBA,

DUF/PAM2, and RRM domains are indicated. Numbers correspond to amino acid positions. The positions of aromatic amino acids mutated to alanines are indicated by asterisks.

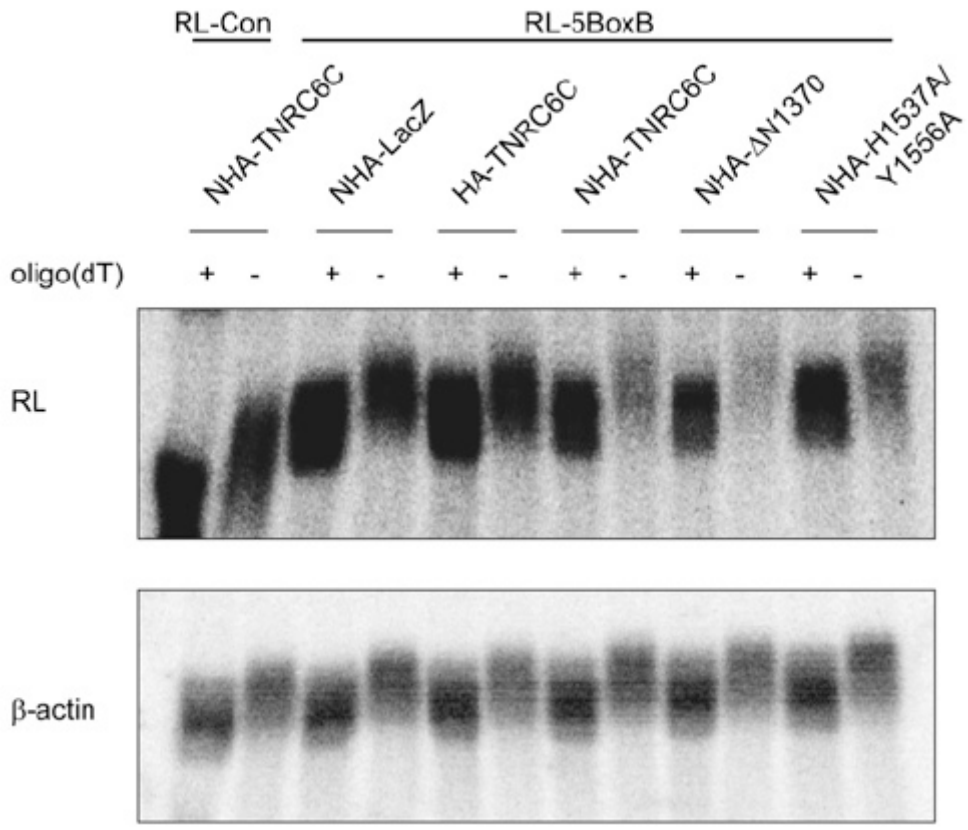


Figure 2.2: Treatment with RNase H in the presence of oligo(dT) results in faster mobility of both control mRNAs and mRNAs repressed by tethering TNRC6C or the C-terminal effector domain of TNRC6C. RL-Con and RL-5BoxB mRNAs were coexpressed in HEK293 cells with proteins indicated above the panels. RNA 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 βactin mRNAs. Figure 6b from Zipprich et al., (2009), see Appendix A.

2.1.2 The CED fragment does not interact with endogenous AGO or TNRC6C proteins

We considered the possibility that the CED fragment (Figure 2.1) induces repression by recruiting the endogenous miRNP complex via interaction with endogenous AGO or TNRC6 proteins. Using immunoprecipitation assays we tested whether the CED fragment (as a fusion with an HA tag and the λN peptide, NHA-CED (NHA-ΔN1370)) interacts with endogenous AGO proteins or TNRC6C. NHA-CED did co-immunoprecipitate detectable amounts of neither AGO proteins nor TNRC6C,

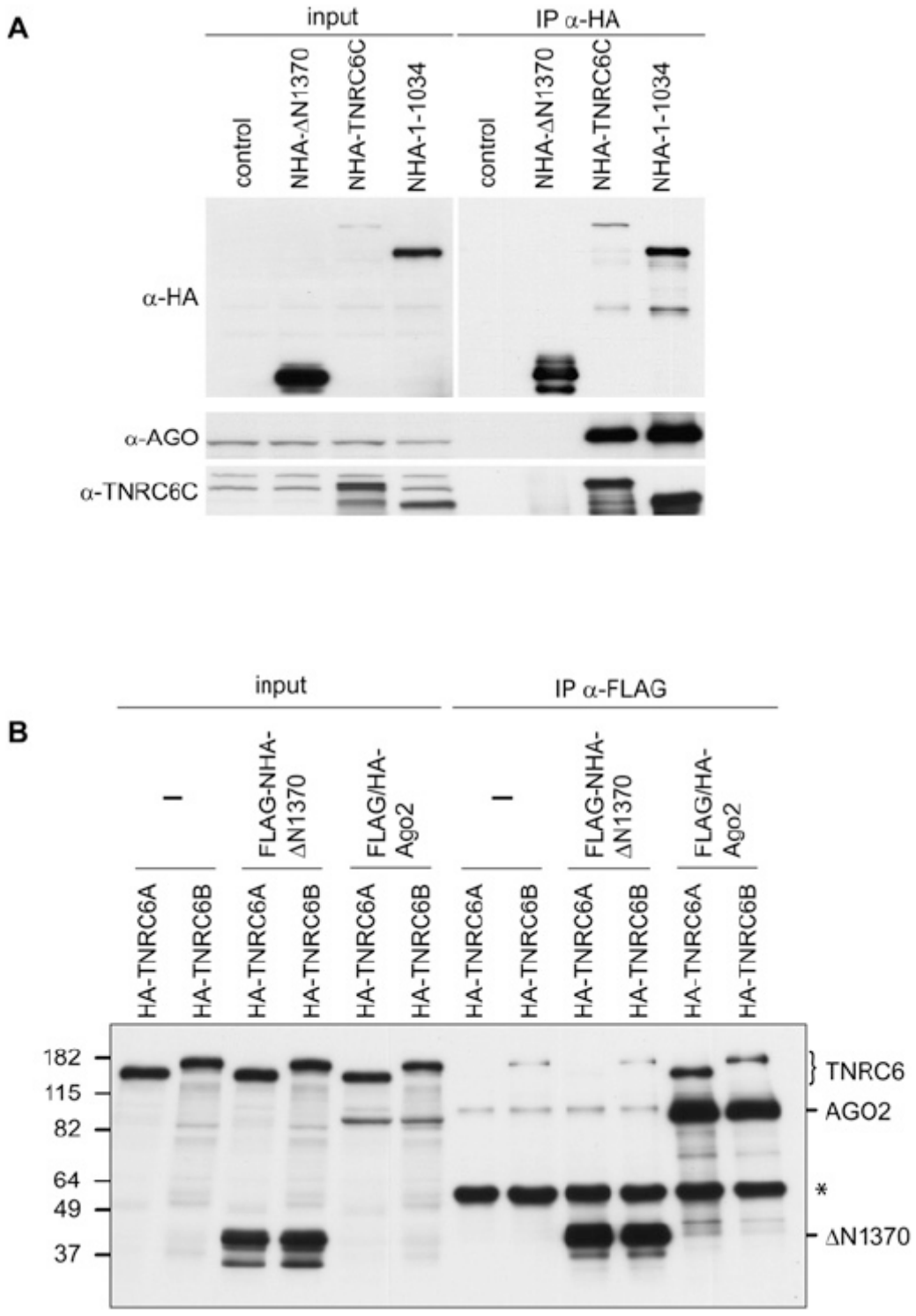


Figure 2.3: The CED (ΔN1370 fragment) of TNRC6C does not interact with endogenous Ago and TNRC6C proteins. (**A**) Cell extracts of HEK293 cells transiently expressing the indicated fusion proteins were incubated with anti-HA Affinity Matrix (Roche), and immunoprecipitated proteins (45% of the total immunoprecipitate) were analyzed by Western blotting using the indicated antibodies. 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**)

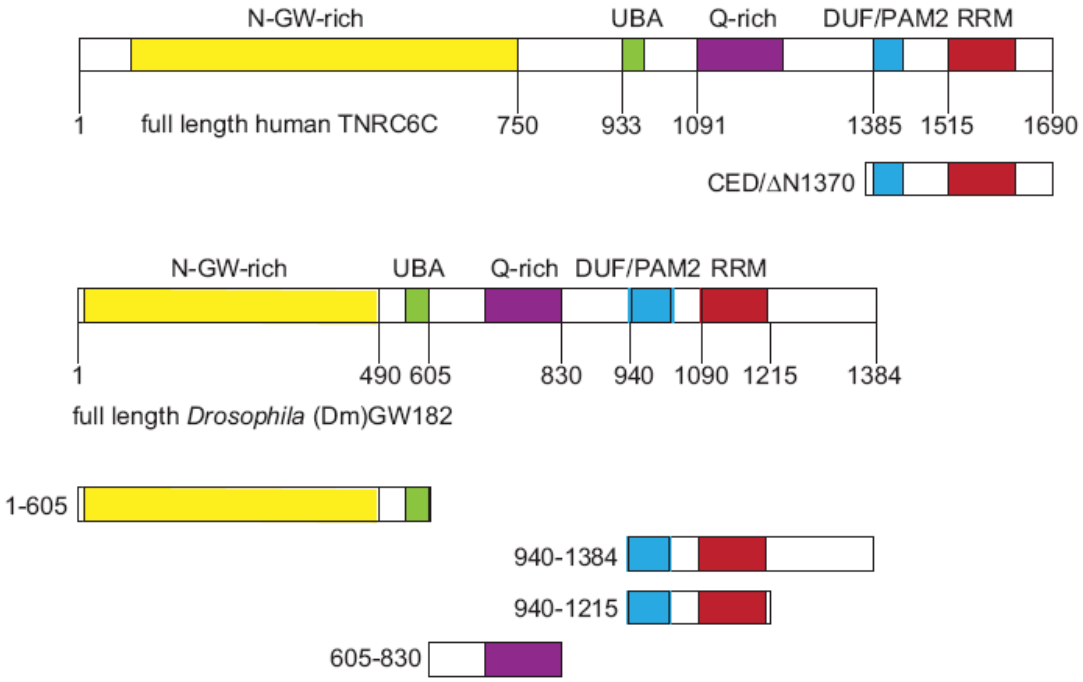
ΔN1370 does not interact with TNRC6A and TNRC6B proteins. Cell extracts of HEK293 cells transiently expressing indicated epitope-tagged proteins were incubated with anti-Flag M2-Agarose Affinity Gel (Sigma), and immunoprecipitated proteins (45% of the total immunoprecipitate) were analyzed by Western 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 7 from Zipprich et al., (2009), see Appendix A.

whereas full length TNRC6C (NHA-TNRC6C) and an N-terminal fragment of TNRC6C (NHA-1-1034) efficiently co-immunoprecipitated endogenous AGO proteins (Figure 2.3, panel A). Since no antibody detecting TNRC6A and TNRC6B was available, we co-expressed HA-tagged TNRC6A (HA-TNRC6A) or TNRC6B (HA-TNRC6B) together with FLAG-HA-tagged CED (FLAG-NHA-CED (FLAG-NHA-ΔN1370)) or AGO2 (FLAG/HA-Ago2) in HEK293 cells and performed co-immunoprecipitation experiments. FLAG-NHA-CED co-immunoprecipitated neither HA-TNRC6A nor HA-TNRC6B, whereas both proteins were co-immunoprecipitated with FLAG/HA-Ago2 (Figure 2.3, panel B). These data indicate that the repression induced by the CED fragment is not due to its interaction with AGO or TNRC6 proteins and suggest that the CED functions as an autonomous repressive domain.

2.1.3 Cross species repressive activity of GW182 proteins and their mutants

Chekulaeva et al., (2009) found that the *Drosophila* GW182 protein contains three distinct regions that induce repression when artificially tethered to a reporter RNA in *Drosophila* S2 cells: the N-terminal GW-rich domain, the Q-rich domain and a C-terminal fragment encompassing the DUF and RRM regions (Figure 2.4, panel A). We tested whether tethering the *Drosophila* GW182 protein and its subfragments can induce repression when tethered to the RL-5BoxB reporter in human HEK293 cells. Tethering the full length *Drosophila* GW182 protein induced repression of the RL-5BoxB reporter in HEK293 cells to a similar extent as tethering of human TNRC6C did (Figure 2.4, panel B). The N-terminal GW-rich region (1-605) of the *Drosophila* GW182 protein also strongly repressed the RL-5BoxB reporter upon tethering whereas the Q-rich domain (605-830) and two C-terminal fragments (940-1385 and 940-1215) of the *Drosophila* GW182 protein induced only mild repression of the RL-5BoxB reporter in HEK293 cells (Figure 2.4, panel B). Hence, whereas the full length

A



B

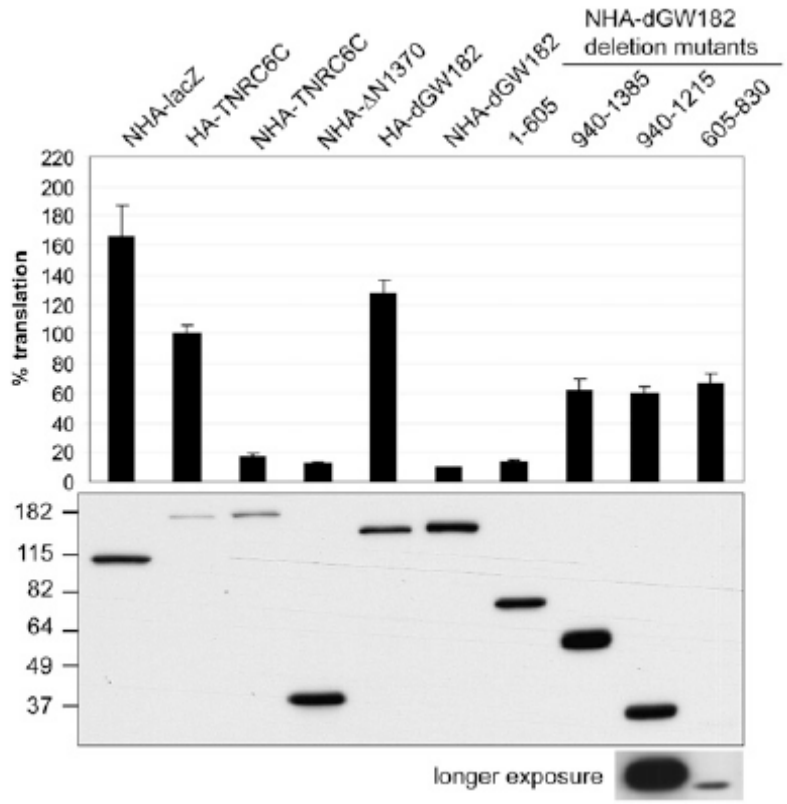


Figure 2.4: Effect of tethering of DmGW182 and its deletion mutants on activity of RL-5BoxB reporter in human cells. (**A**) Schematic representation of TNRC6C, TNRC6C CED, and *Drosophila* GW182 and its deletion mutants used for the experiment shown in B.

Positions of N-GW-rich, Q-rich, UBA, DUF/PAM2, and RRM domains are indicated. Numbers correspond to amino acid positions. (**B**) (Upper panel) Tethering of DmGW182 and its deletion mutants represses activity of RL-5boxB reporter in HEK293 cells. Indicated plasmids expressing human TNRC6C or *Drosophila* DmGW182, 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 antibody. (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. The data represent means from three independent experiments. Figure 8 from Zipprich et al., (2009), see Appendix A.

Drosophila GW182 protein is active in repression in HEK293 cells, not all of its subfragments that are active repressors in S2 cells function efficiently in HEK293 cells.

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

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 III, Gregory J. Hannon, Witold Filipowicz, Thomas F. Duchaine, and Nahum Sonenberg
(The full paper is attached in Appendix B)

Marc Fabian in the laboratory of Nahum Sonenberg found that a let-7 miRNA loaded RNA-induced silencing complex interacts with the poly(A)-binding protein (PABP) and the CAF1 and CCR4 deadenylases (Appendix B, Figure 2). Furthermore Marc Fabian demonstrated that miRNA-mediated deadenylation depends on CAF1 activity and PABP in mouse Krebs-2 ascites extracts (Appendix B, Figure 3f and 4b).

Having identified the CED of TNRC6C as an autonomous repressive domain, we aimed to elucidate the mechanism by which the CED represses its targets. We hypothesized that the CED may function as a repressive domain by interacting with other proteins. To identify those proteins, we expressed the CED as a fusion with GST in HEK293 cells and pulled it down from cell extracts via Glutathione Sepharose beads. Pulled-down proteins were resolved by SDS-PAGE and analyzed by mass

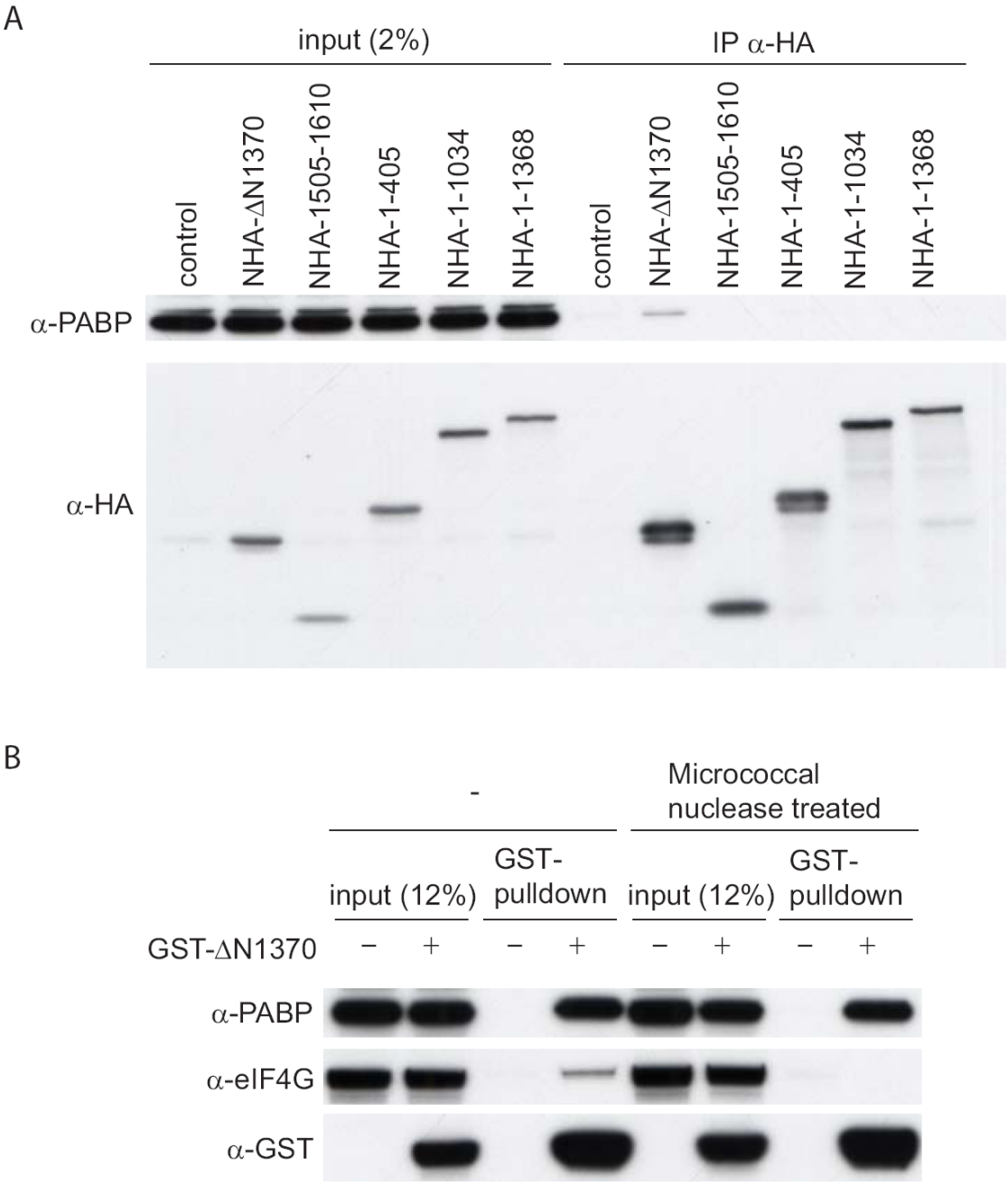


Figure 2.5: The C-terminal effector domain of TNRC6C interacts with PABP. (**A**) Cell extracts of HEK293 cells, transiently expressing the indicated fusion proteins, were incubated with Anti-HA Affinity Matrix (Roche), and immunoprecipitated proteins were analyzed by western blotting using the indicated antibodies. Inputs represent 1% of the cell extract used for IP. Nontransfected cells served as a control. (**B**) Cell extracts of HEK293 cells transiently expressing GST-CED (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. Figure 6 B and C modified from Fabian et al., (2009), see Appendix B.

spectrometry. This analysis identified the poly(A)-binding protein (PABP) as a factor interacting with the CED. To test whether other regions of TNRC6C also interact with PABP, we expressed HA-tagged fragments spanning different regions of TNRC6C in HEK293 cells and performed immunoprecipitation experiments. Only the CED (NHA-ΔN1370) but not other fragments of TNRC6C co-immunoprecipiated endogenous PABP (Figure 2.5, panel A). GST-CED (GST-ΔN1370) also pulled down endogenous PABP from HEK293 cell extracts that were treated with micrococcal nuclease, indicating that the CED interacts with PABP in an RNA-independent manner (Figure 2.5, panel B). Notably, in the absence of micrococcal nuclease GST-CED pulled down both PABP and eIF4G. In contrast, after micrococcal nuclease treatment GST-CED pulled down only PABP, providing evidence for the effectiveness of the micrococcal nuclease treatment.

Marc Fabian could then demonstrate that GW182 directly interacts with PABP and that this interaction is required for maximal miRNA-mediated deadenylation (Appendix B, Figures 6a and 7d).

2.3 miRNA repression involves GW182-mediated recruitment of CCR4-NOT through conserved 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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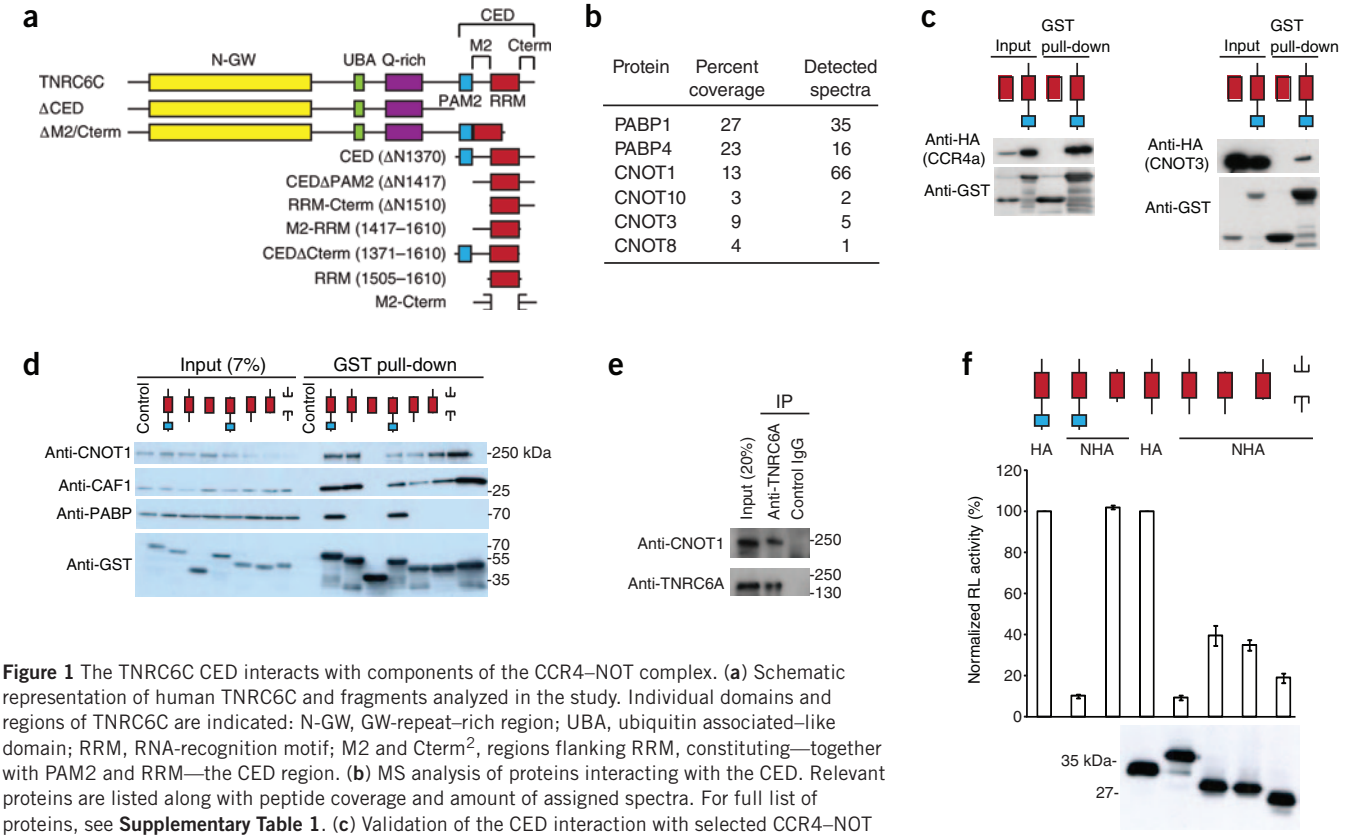

Figure 1 The TNRC6C CED interacts with components of the CCR4–NOT complex. (**a**) Schematic representation of human TNRC6C and fragments analyzed in the study. Individual domains and regions of TNRC6C are indicated: N-GW, GW-repeat–rich region; UBA, ubiquitin associated–like domain; RRM, RNA-recognition 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.

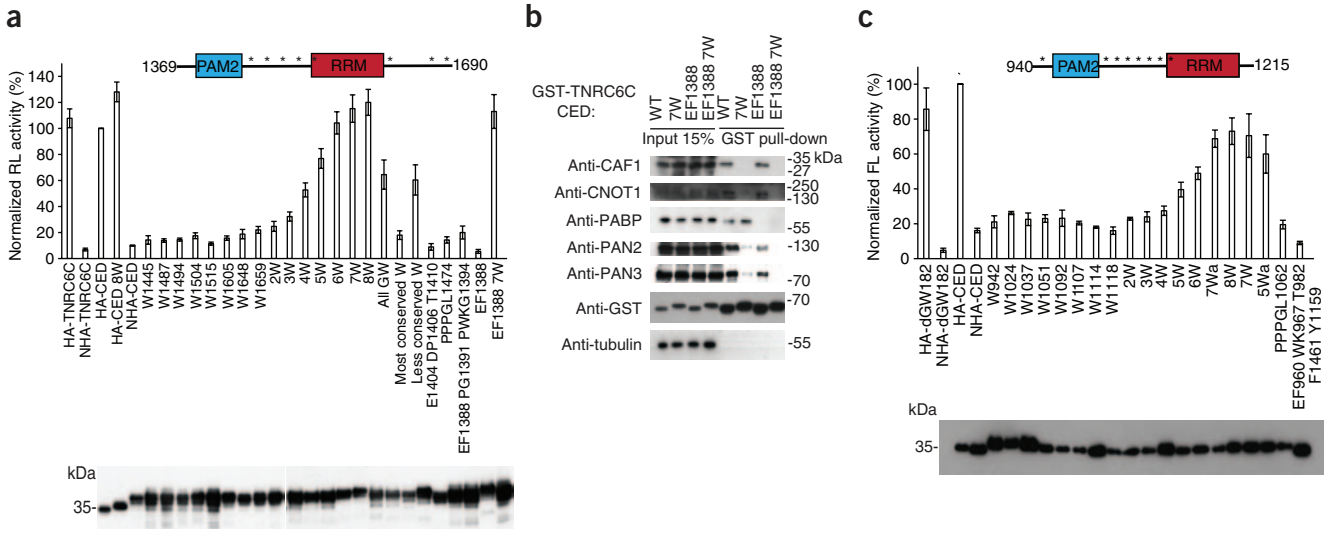

Figure 2 W-motifs in GW182 proteins mediate mRNA repression by recruiting CCR4–NOT and PAN2–PAN3 deadenylation complexes. (**a**) Mutations of tryptophan residues in W-motifs alleviate repression by the TNRC6C CED. Schematic representation of the TNRC6C CED with positions of W-motifs marked with asterisks 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),

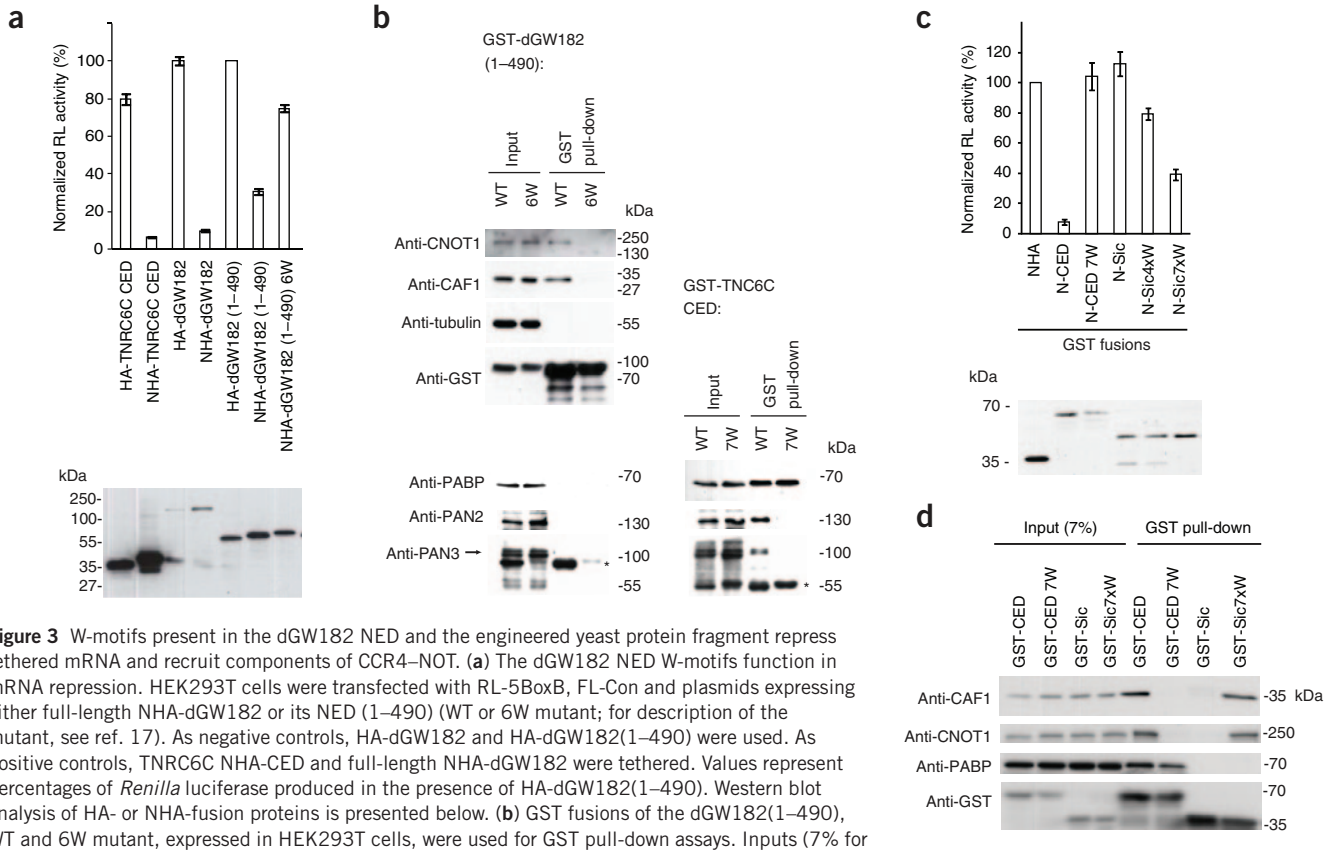

Figure 3 W-motifs present in the dGW182 NED and the engineered yeast protein fragment repress tethered mRNA and recruit components of CCR4–NOT. (**a**) The dGW182 NED W-motifs function in mRNA repression. HEK293T cells were transfected with RL-5BoxB, FL-Con and plasmids expressing either full-length 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].

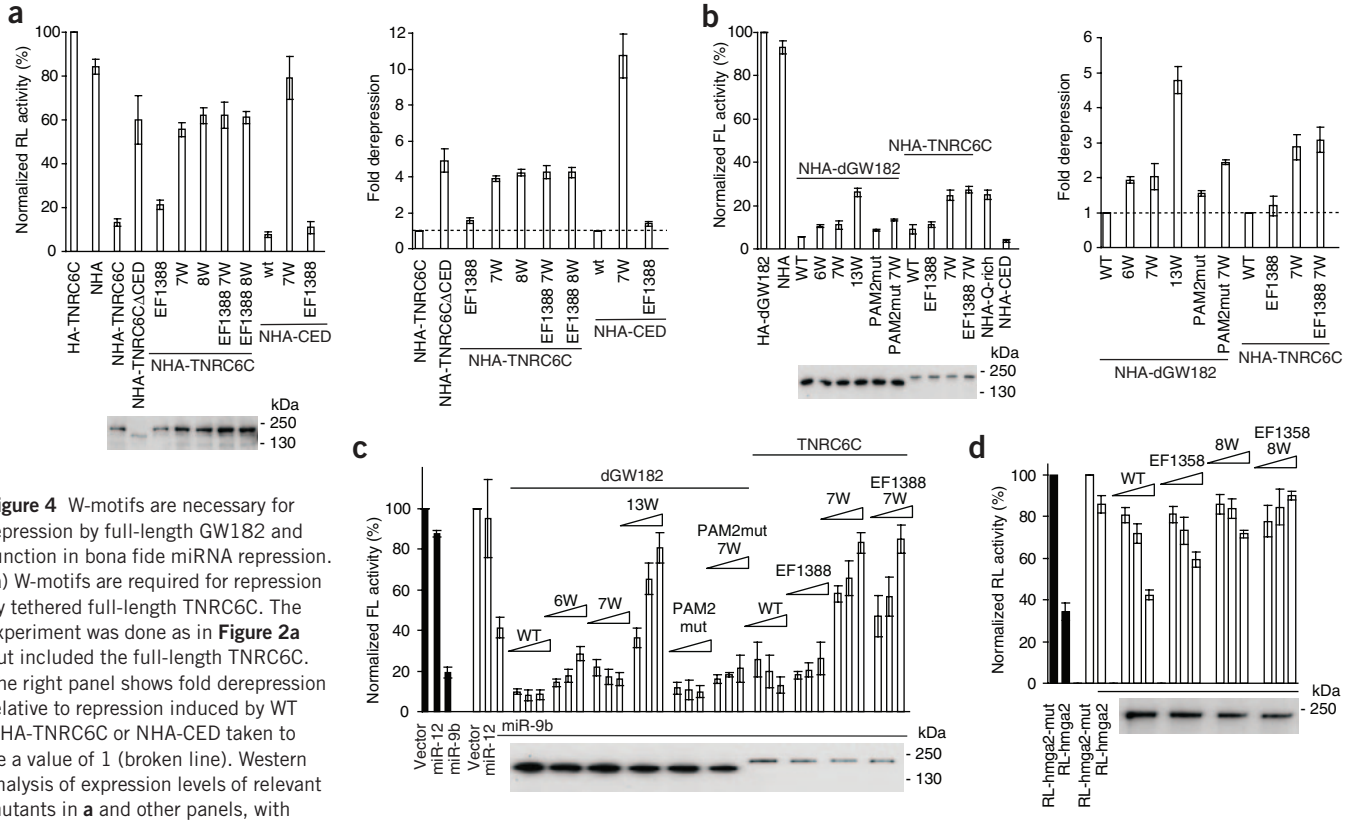

Figure 4 W-motifs are necessary for repression by full-length GW182 and function in bona fide miRNA repression. (**a**) W-motifs are required for repression by tethered full-length TNRC6C. The experiment was done as in **Figure 2a** but included the full-length TNRC6C. The right panel shows fold derepression 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

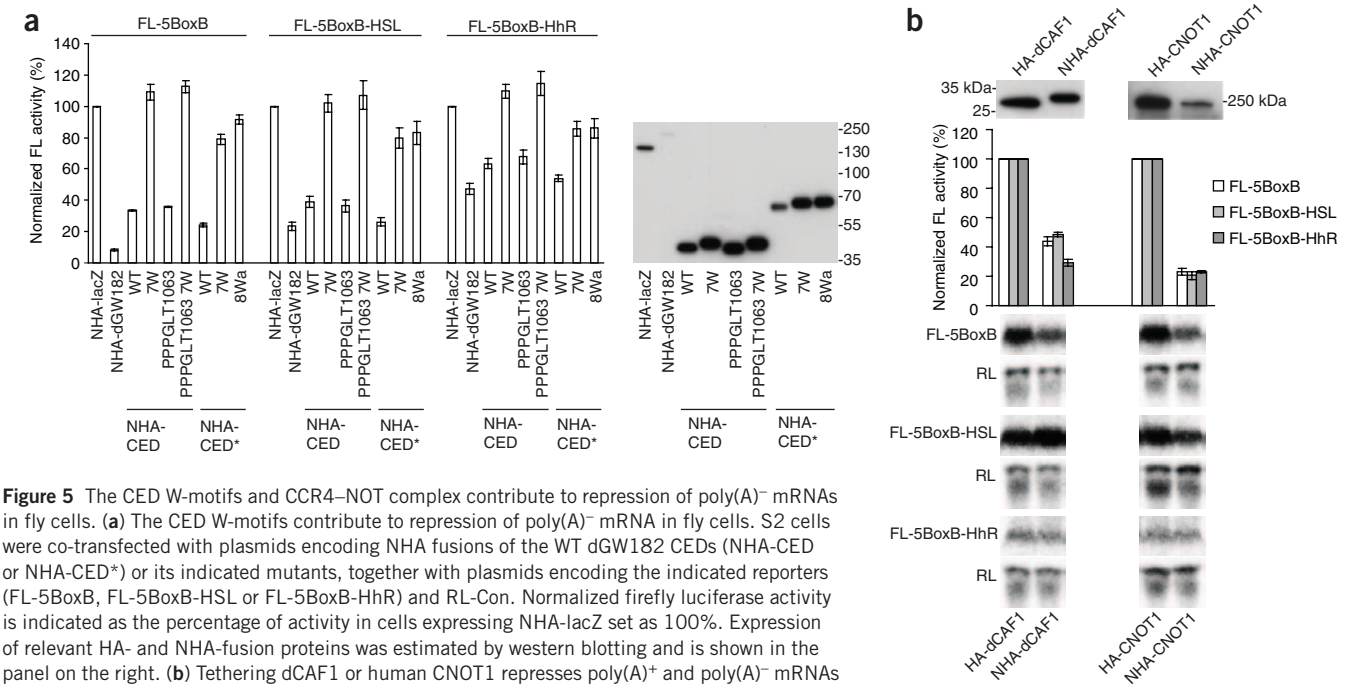

Figure 5 The CED W-motifs and CCR4–NOT complex contribute to repression of poly(A)⁻ mRNAs in fly cells. (**a**) The CED W-motifs contribute to repression of poly(A)⁻ mRNA in fly cells. S2 cells were co-transfected with plasmids encoding NHA fusions of the WT dGW182 CEDs (NHA-CED or NHA-CED*) or its indicated 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].

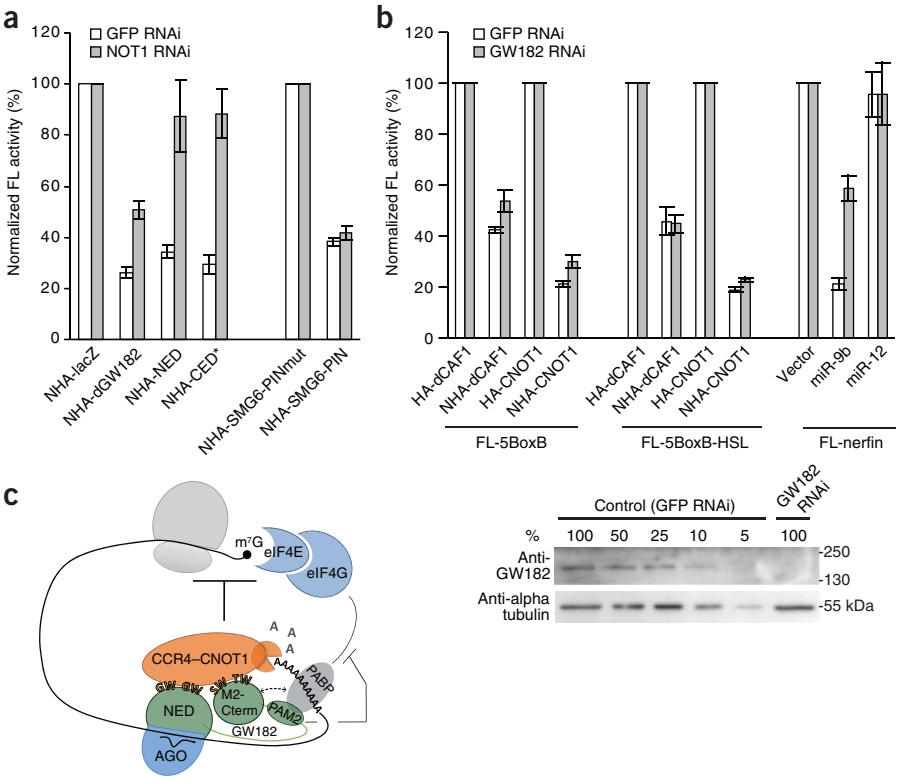

Figure 6 Repression of poly(A)⁻ RNA by tethering dGW182 or its fragments depends on NOT1, but repression by tethered CCR4–NOT components is dGW182-independent. (**a**) Repression of FL-5BoxB-HSL reporter by tethering dGW182 or its fragments is alleviated in S2 cells depleted of NOT1. S2 cells treated 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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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.

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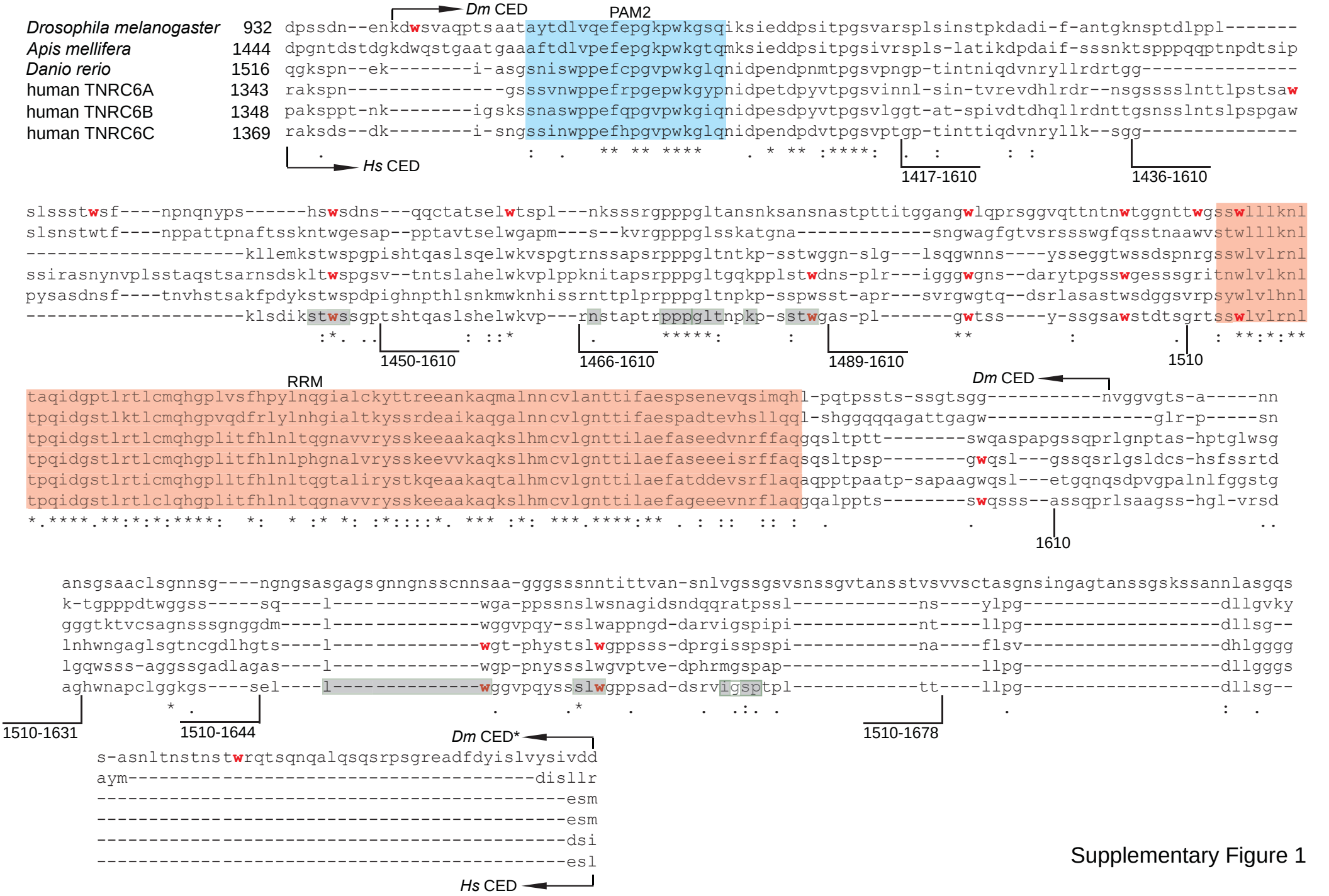

Supplementary Figure 1

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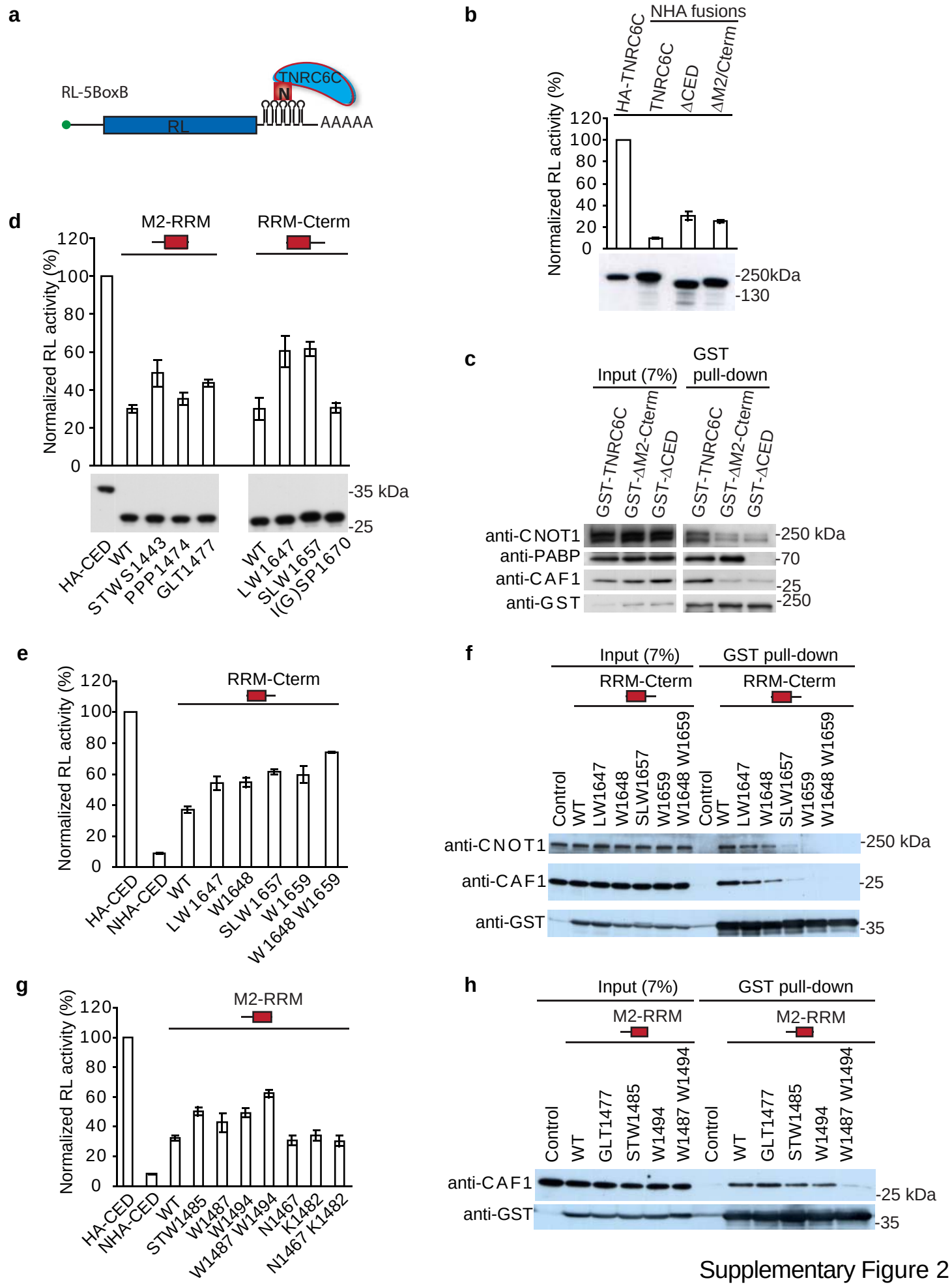
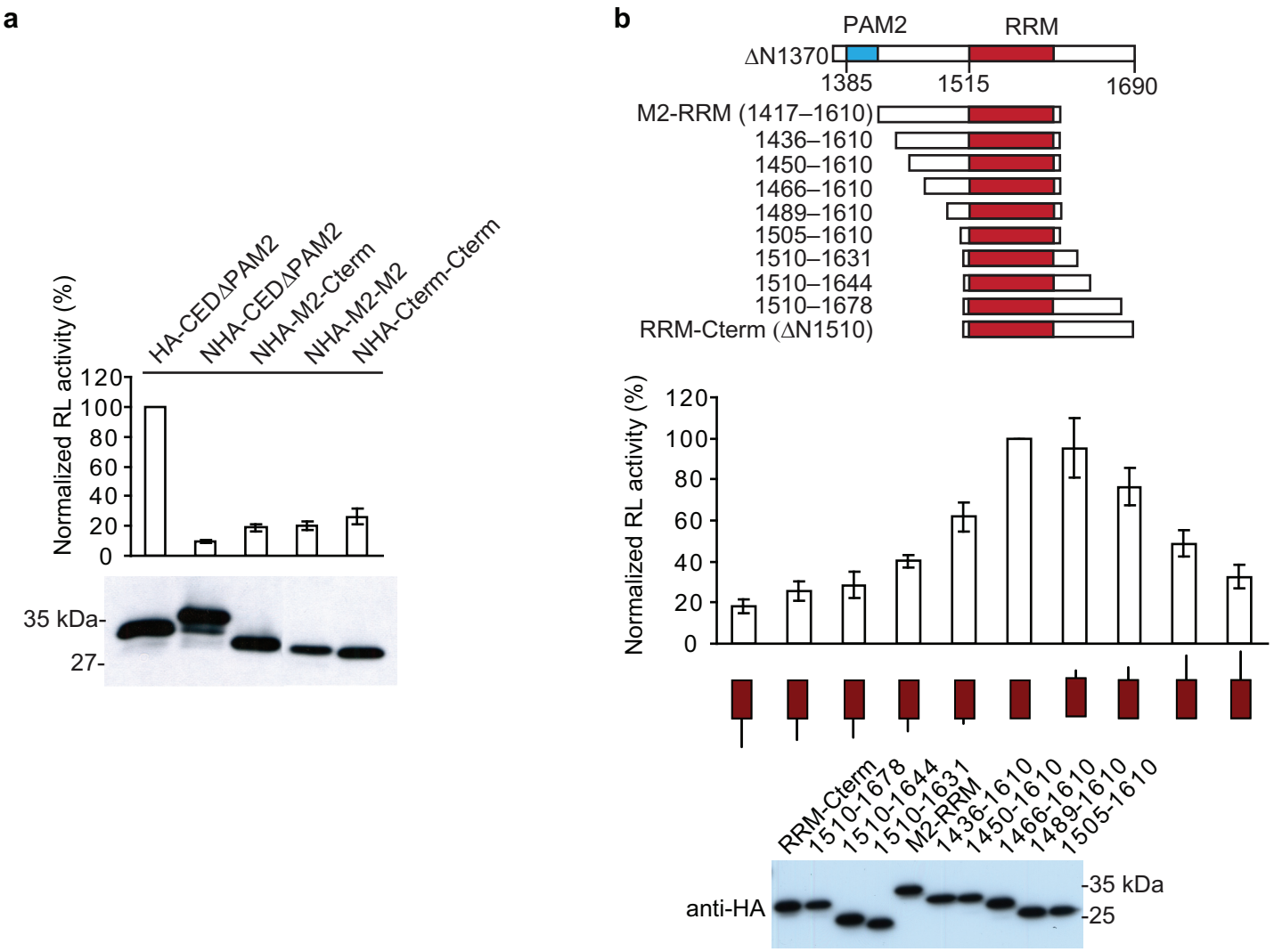
Supplementary Figure 1. Alignment of CED regions of GW182 proteins from different species. Alignment was performed using the T-Coffee tool. The CED regions used throughout the work are delineated with bent arrows. Note that in the case of *D. melanogaster* GW182, two different variant of the region, CED and CED*, were used. PAM2 and RRM motifs are highlighted in blue and pink, respectively. Mutated tryptophan residues are shown in red, other mutations are highlighted in grey. Amino acids were always mutated to alanines. G residue in the sequence LGSP has not been mutated what is indicated by putting it in brackets in Supplementary Figure 2d. Starts and ends of deletion mutants analyzed in Supplementary Figure 3b are marked with vertical and bent lines. The numbers correspond to amino acid positions in full-length GW182 proteins. Numbers of TNRC6A correspond to the protein described in[3]. Asterisks mark residues identical in all sequences, colons mark conservative substitutions, and dots mark semi-conservative substitutions.



Supplementary Figure 2

Nature Structural & Molecular Biology: doi:10.1038/nsmb.2166

Supplementary Figure 2. Repressive potential of the TNRC6C CED correlates with its association with the CCR4–NOT complex. (**a**) Schematic representation of the RL-5BoxB reporter construct used in mRNA tethering assays. In this assay, protein fusions with the phage λ N-peptide are co-expressed in HEK293T cells, together with *Renilla* luciferase (RL) mRNA reporter containing BoxB hairpins in the 3' UTR (RL-5BoxB). (**b**) Repression of RNA function by NHA-TNRC6C and its deletion mutants (schematically shown in Figure 1a). Tethering constructs were co-transfected with RL-5BoxB and FL-Con. Values represent percentages of RL activity measured in the presence HA-TNRC6C. (**c**) TNRC6C and its mutants tested in panel (b) were analyzed for their ability to bind the CCR4–NOT complex and PABP, using GST pull-downs followed by western blotting analysis with the antibodies indicated. (**d**) NHA-M2-RRM and NHA-RRM-Cterm-encoding constructs or indicated mutants were co-transfected with RL-5BoxB and FL-Con in HEK293T cells. Values represent percentages of RL produced in the presence of non-tethered HA-CED control. Expression levels of HA- or NHA-fusion proteins were analyzed by western blotting. (**e**) Mutations of W residues in Cterm lead to alleviation of repression by RRM-Cterm. Tethering assay with indicated RRM-Cterm mutants was performed as in (d). (**f**) Point mutants of RRM-Cterm that showed alleviation of repression in (e) were tested by GST pull-downs and western blotting for interaction with components of the CCR4–NOT complex. (**g**) Mutations of W residues in M2-RRM lead to alleviation of repression induced by its tethering to mRNA. Tethering assay with the indicated M2-RRM mutants was performed as in panel (d). Values represent percentages of RL activity seen in the presence of HA-CED. (**h**) M2-RRM mutants that showed alleviation of repression in panel (g) were analyzed for interaction with CAF1 protein using GST pull-downs and western blotting. The assay was done as described in Figure 1d.



Supplementary Figure 3. Redundancy of the CED sequences responsible for mediating repression of mRNA function. (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 HACEDΔPAM2 which is taken as 100%. (b) Activity of M2-RRM and RRM-Cterm fragments bearing step-wise deletions of M2 and Cterm regions. Deletion mutants of NHA-M2-RRM and NHA-RRM-Cterm fragments are schematically shown on the top. Numbers correspond to amino acid positions (for more details, see Supplementary Fig. 1). Activity of mutants was analyzed in the mRNA tethering assay (shown in the middle). The tethering assay was performed as described in Figure 1f. Values represent percentages of RL activity seen in the presence of NHA-RRM, which is known not to repress the tethered mRNA (ref. 3; Fig. 1f). Expression of HA- and NHA-fusions was estimated by western blotting with anti-HA antibody (bottom).

a

GST-TNRC6C CED:	Detected spectra WT	7W
TNRC6C	195	145
PABP4	135	148
PABP1	67	69
CNOT1	92	1
CNOT10	21	0
CNOT3	17	0
PAN2	10	0
PAN3	4	0

b

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Sic          MAPSAPPRSRGTRYLAQPSGNTSSSALMQGQKAPQKPSQNLVPVAPSTTK
Sic4xW       MAPSAPPRSWGTRYLAQPSGWTSSSALMQGQKAPQKPSQNLVPVAPSTTK
Sic7xW       MAPSAPPRSWGTRYLAQPSGWTSSSALMWGQKAPQKPSWNLVPVAPSTTW

Sic          SFKNAPLLAPPNSNMGMTAPFNGLTAPQRAPFPKSSVKRT
Sic4xW       SFKNAPLLAPPNSNMGWTAPFNGWTAPQRAPFPKSSVKRT
Sic7xW       SFKNAPLLAPPNSNMGWTAPFNGWTAPQWAPFPKSSVKRT
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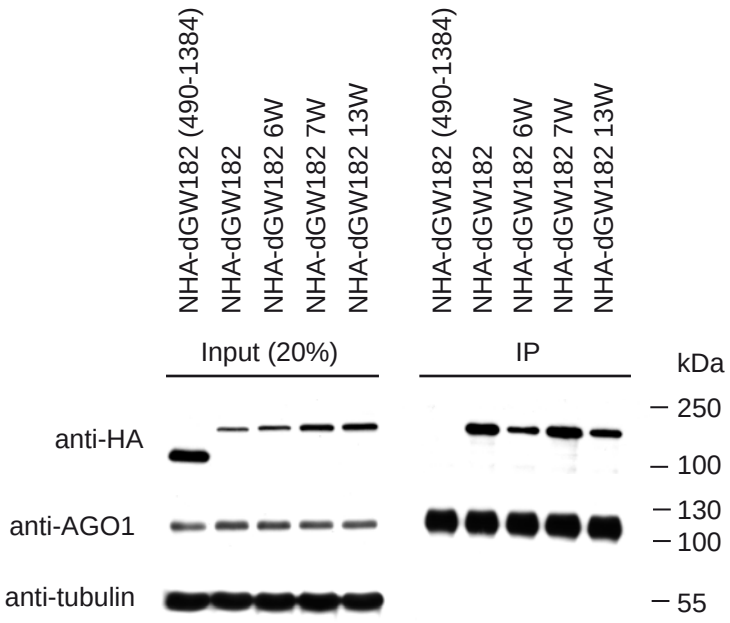
c



Supplementary Figure 4

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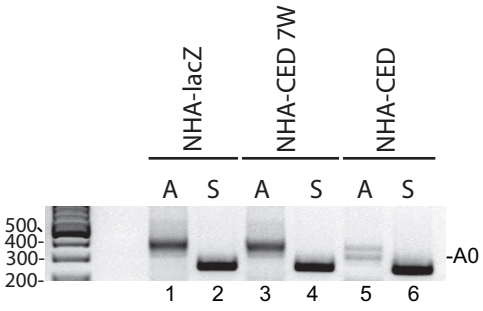
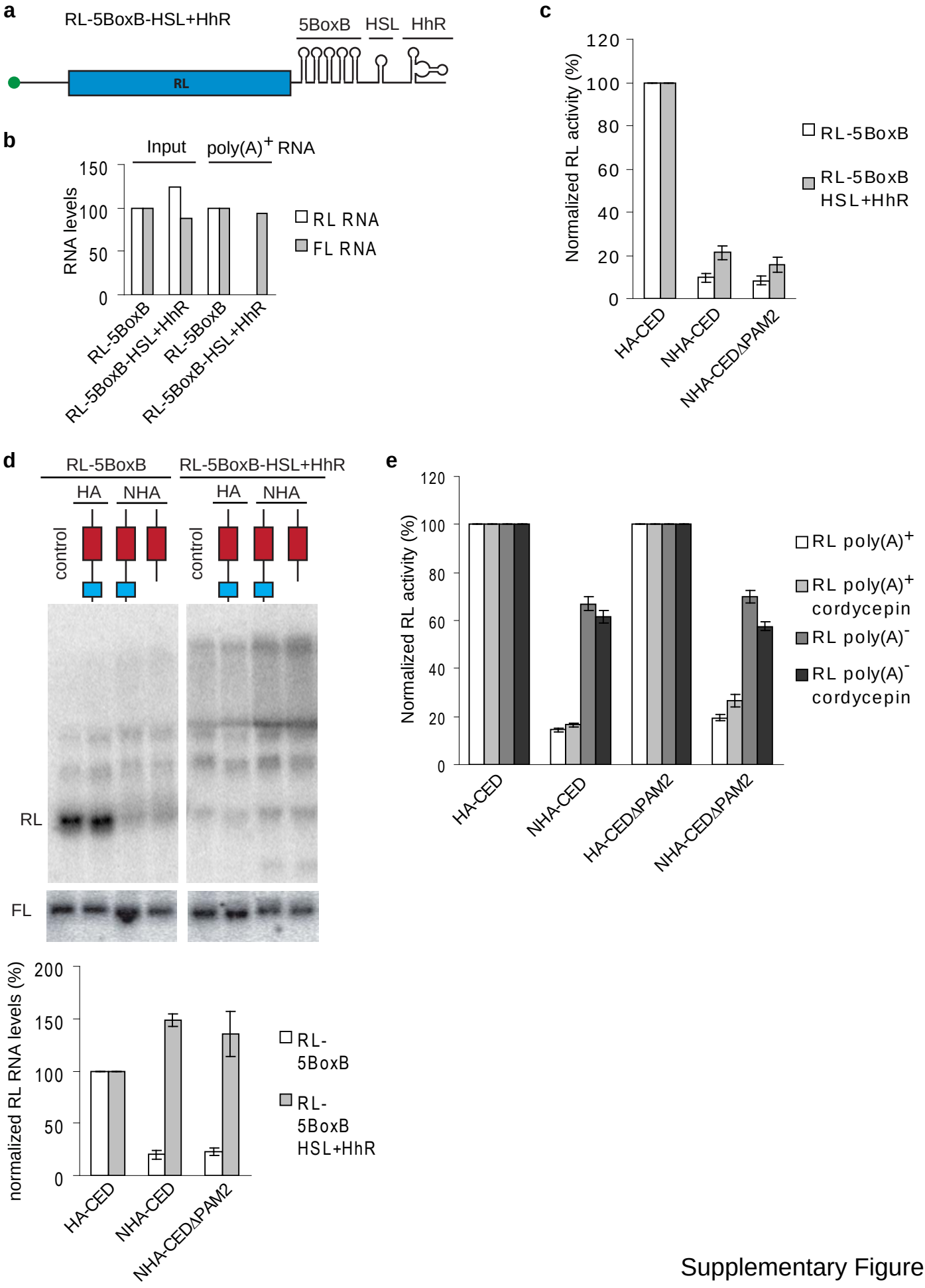
Supplementary Figure 4. MS analysis of proteins associating with the TNRC6C CED in a W-dependent manner and estimation of the role of W-motifs in mRNA repression. (**a**) MS analysis of proteins associating with the TNRC6C CED in a W-dependent manner. The GST-TNRC6C CED, either wild-type or 7W mutant, was expressed in HEK293T cells and protein content of GST pull-downs was analyzed by MS. Relevant identified proteins are listed, along with numbers of detected spectra. The PAN2–PAN3 complex was selected as a specific CED interactor since it was not pulled down by the 7W mutant. PAN2 (but not PAN3) was also identified in MS analysis shown in Figure 1b (1 assigned spectrum) but its role was originally not pursued since the 7W mutant providing specificity control was not available at that time. Of note, MS analysis presented in Figure 1b was performed with the less sensitive MS equipment (see Supplementary Methods) than the one shown in this figure. (**b**) Sequences of engineered Sic, Sic4xW, and Sic7xW protein fragments tested in the tethering assay in Figure 3c. An N-terminal fragment of the *S. cerevisiae* Sic1p protein (positions 1-90), in which several phosphorylation sites are mutated (Ser/Thr->Ala; shown in italics) served as a control (Sic). Introducing either four or seven X->W amino acid mutations (see the alignment, mutations are in red), always next to G, S, or T residues, resulted in Sic4xW and Sic7xW constructs. (**c**) Contribution of W-motifs within NED and CED to the dGW182-dAGO1 interaction. *D. melanogaster* S2 cells were transfected with plasmids encoding NHA-dGW182 or its mutants described in Figure 4b and c: 6W has six tryptophanes mutated in W-motifs in the NED[1], 7W contains mutations of W-motifs within the CED (Fig. 2c), and 13W represents a combination of both. The dGW182 (490-1384) fragment was analyzed as a negative control. Cell lysates were used for immunoprecipitations with an anti-AGO1 antibody, according to[1]. Inputs and immunoprecipitates were analyzed by western blotting using anti-HA or anti-AGO1 antibodies, as indicated on the left. Expression of anti-tubulin was estimated as a loading control. Positions of protein size markers are indicated.



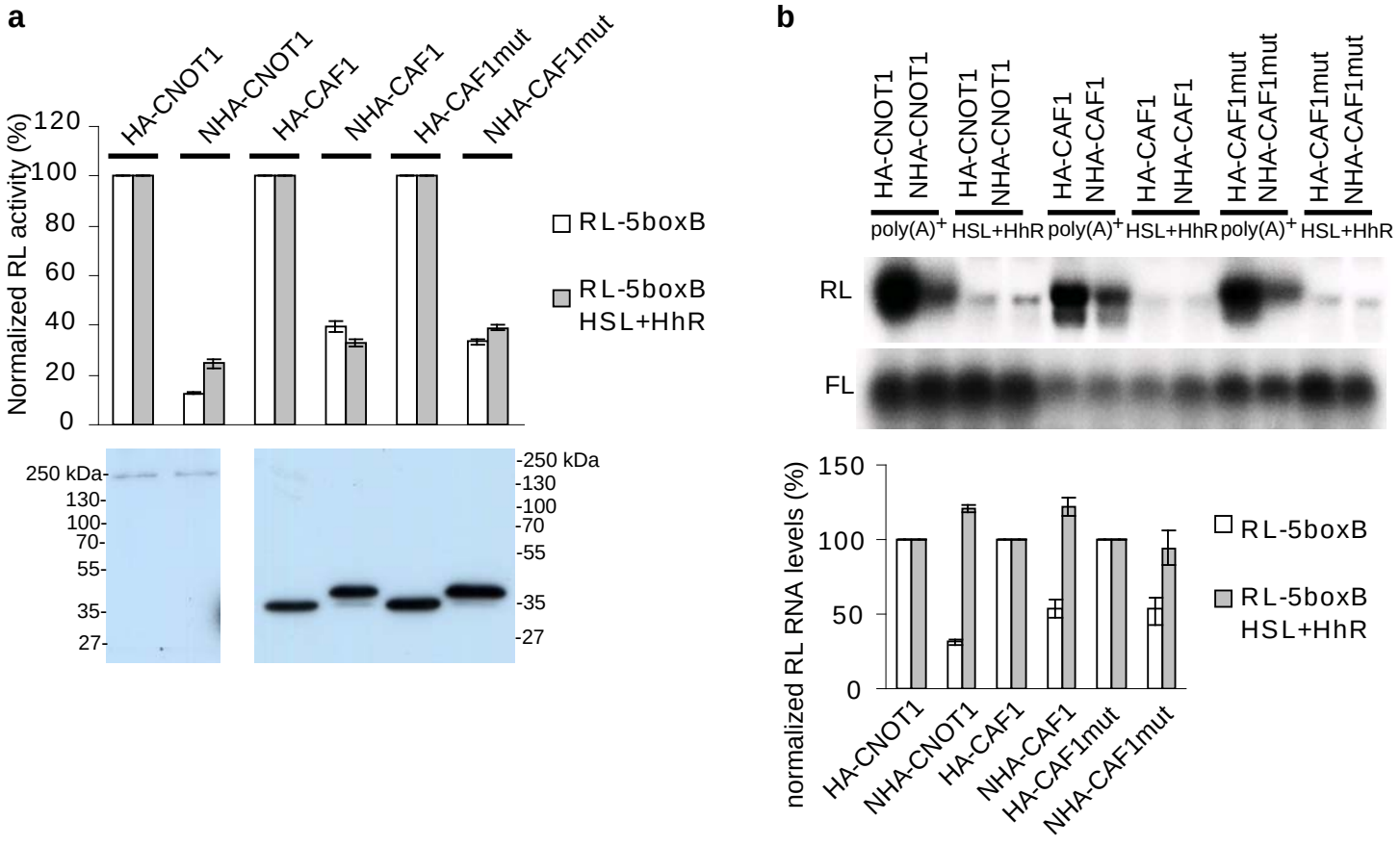
Supplementary Figure 5. W-motifs of dGW182 are involved in mRNA deadenylation. S2 cells were transfected with FL-5BoxB and either NHACED, NHA-CED 7W or NHA-lacZ expressing plasmids. Poly(A) tails of FL-5BoxB reporter were estimated using G-tailing/PAT [poly(A) test] assay[19]. To prevent decapping and degradation of deadenylated mRNAs, S2 cells were depleted of decapping activators Ge1 and Me31b[22]. Lanes 1, 3, and 5 show PCR products corresponding to the fragment of the reporter 3'UTR including poly(A) tails (A). PCR products in lanes 2, 4, and 6 correspond to the 263 base-pairs product originating from the 3'-UTR region starting ~30-nt upstream of the polyadenylation site. These products (S) do not include poly(A) tails. Position corresponding to fully deadenylated mRNA (A0) is indicated. DNA size markers are shown on the left.

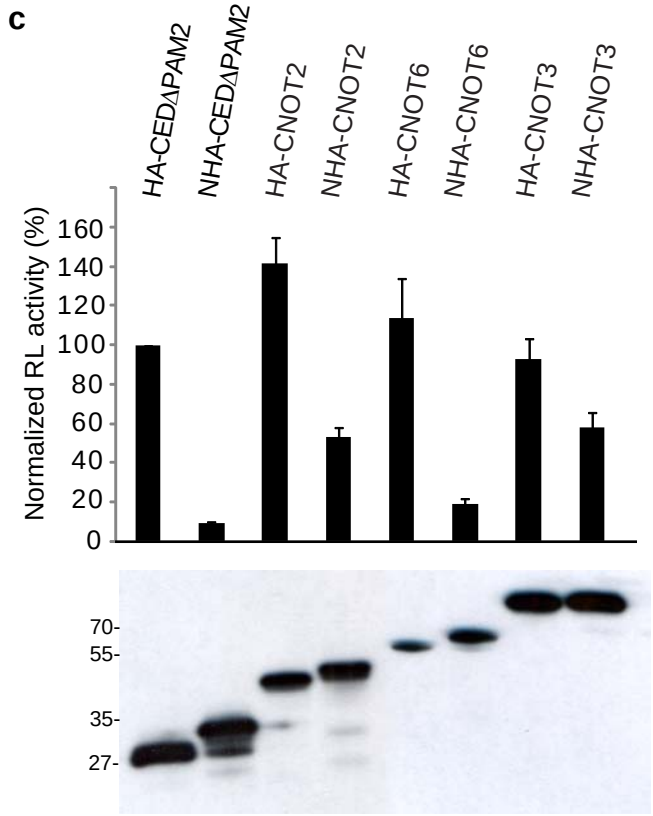


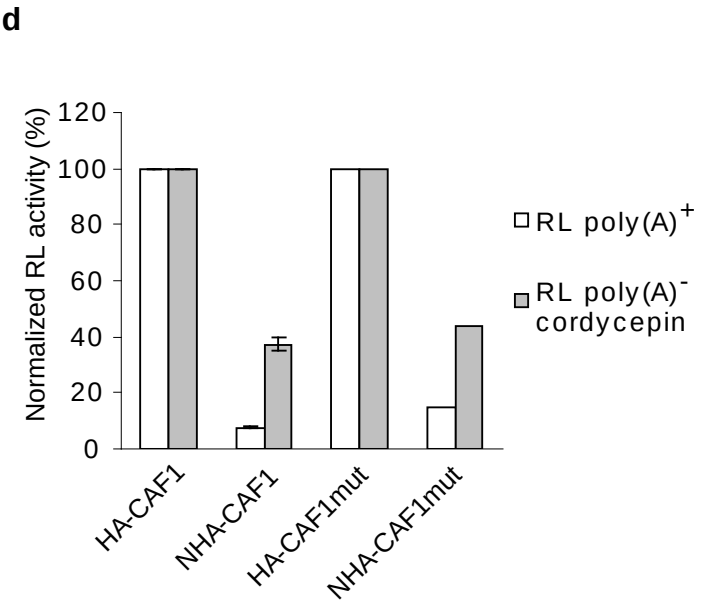
Supplementary Figure 6

Nature Structural & Molecular Biology: doi:10.1038/nsmb.2166

Supplementary Figure 6. Tethering of the TNRC6C CED represses function of both poly(A)$^+$ and poly(A)$^-$ mRNAs. (**a**) Schematic representation of the RL-5BoxB-HSL+HhR reporter construct. (**b**) The poly(A) status of mRNA expressed from the transfected RL-5BoxB-HSL+HhR reporter was assessed by fractionation of total cellular RNA on oligo(dT)$_{25}$ beads. HEK293T cells were co-transfected with plasmids expressing HA-CED and plasmids encoding either RL-5BoxB or RL-5BoxB-HSL+HhR, and also with FL-Con. Total cellular RNA was isolated and fractionated on oligo(dT)$_{25}$ beads. The reporter RNA levels in the total cellular RNA and in the fraction retained on oligo(dT)$_{25}$ beads [corresponding to poly(A)$^+$ RNA] were determined by quantitative real-time PCR. Average RNA levels of *Renilla* luciferase and firefly luciferase reporters from two independent experiments are shown as percentage of the levels in cells expressing RL-5BoxB reporter, which are set as 100%. (**c**) Effect of tethering the CED or CED∆PAM2 on activity of RL-5BoxB and RL-5BoxB-HSL+HhR reporters. HEK293T cells were co-transfected with plasmids expressing NHA-CED or NHA-CED∆PAM2, 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%. (**d**) Analysis of RL-5BoxB and RL-5BoxB-HSL+HhR RNA levels by Northern blotting in an experiment performed as described in panel (c). Total RNA isolated from cells transfected with indicated plasmids was analyzed using RL- or FL-specific probes. Control, cells transfected only with RL and FL reporters. Positions of RL and FL mRNAs are indicated. Quantification of Northern blots, shown in the lower panel, was performed using a PhosphorImager and the Image-Quant software. Normalized values represent means ± SE (n = 3). (**e**) Effect of tethering CED or CED∆PAM2 on activity of the in vitro transcribed RL-5BoxB mRNAs, either poly(A)$^+$ or poly(A)$^-$, and either containing or not the cordycepin residue at the 3' end, which were transfected into cells. HEK293T cells were also co-transfected with the in vitro transcribed RNAs encoding HA or NHA fusions of CED or CED∆PAM2 (as indicated), as well as with the in vitro transcribed RNA encoding FL. Normalized RL activity is indicated as the percentage of activity in cells expressing HA-CED or HA-CED∆PAM2 set as 100%.







Supplementary Figure 7

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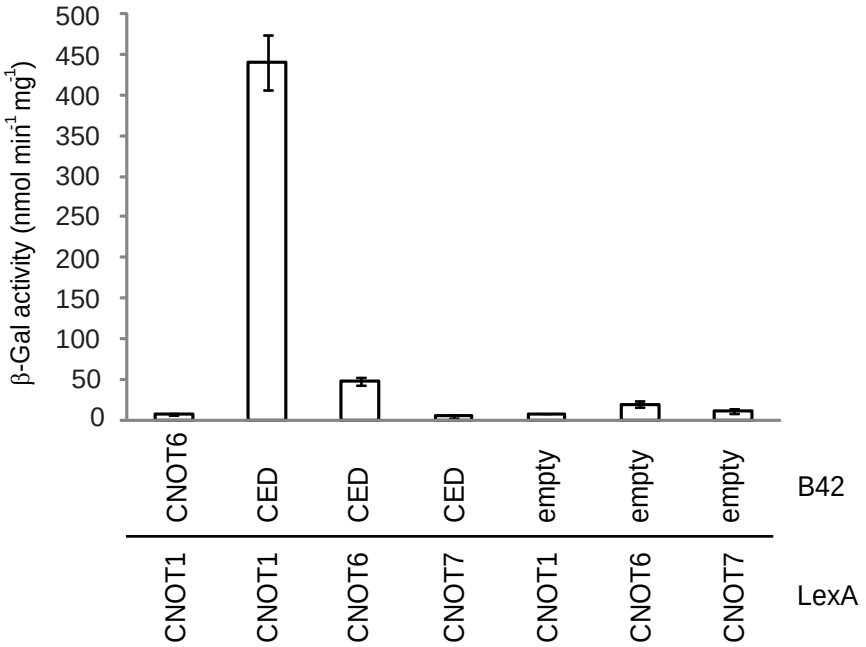
Supplementary Figure 7. Tethering of the CCR4–NOT complex components represses activity of both poly(A)$^+$ and poly(A)$^-$ mRNAs in HEK293 cells. (**a**) Effect of tethering CNOT1 or CAF1 (or its catalytic mutant, CAF1mut) on activity of RL-5BoxB and RL-5BoxB-HSL+HhR reporters. HEK293T cells were co-transfected with plasmids expressing HA or NHA fusions of the indicated proteins and plasmids encoding RL-5BoxB or RL 5boxB HSL+HhR, and FL-Con. Normalized *Renilla* luciferase activity is indicated as percentages of activity in cells expressing indicated HA-fusion protein, which are set as 100%. The expression of fusion proteins was analyzed by Western blotting using αHA antibody (shown in the lower panel). (**b**) Northern blot analysis of the mRNA levels of the RL-5BoxB and RL-5boxB-HSL+HhR reporters in an experiment performed as described in Supplementary Figure 7a. RNA isolated from transfected cells was subjected to Northern blotting analysis to estimate changes in the reporter mRNA levels upon tethering of indicated proteins. Quantification of Northern blots, shown in the lower panel, was performed using a PhosphorImager and the Image-Quant software. The normalized RL mRNA levels are indicated as percentages of the levels in cells expressing indicated HA-fusion proteins, which are set as 100%. Values represent means ± SE (n = 3). (**c**) Repression of RL-5BoxB reporter by tethering the indicated CCR4–NOT complex components. HEK293T cells were co-transfected with plasmids expressing HA and NHA fusions of the indicated CCR4–NOT complex components and plasmids encoding RL-5BoxB and FL-Con reporters. As a control, the effect of plasmids expressing either HA-CEDΔPAM2 or NHA-CEDΔPAM2 was measured. Normalized RL activity is indicated as the percentage of activity in cells expressing HA-CEDΔPAM2 set as 100%. (**d**) Effect of tethering CAF1 or CAF1mut on activity of the in vitro transcribed poly(A)$^+$ or poly(A)$^-$ RL-5BoxB reporter RNAs transfected into cells. HEK293T cells were co-transfected with the in vitro transcribed RNAs encoding HA or NHA fusions of CAF1 or CAF1mut and the in vitro transcribed RNA reporters, both RL-5BoxB and FL-Con. The poly(A)$^-$ RL-5BoxB RNA contained cordycepin residue at the 3' end. Normalized RL activity is indicated as the percentage of activities in cells expressing HA-CAF1 or HA-CAF1mut, which are set to 100%. Bars represent means ± SE (n=4) for CAF1 and averages from two independent experiments for CAF1mut.



Supplementary Figure 8. The TNRC6C CED interacts with CNOT1 in yeast two-hybrid system. Yeast strain W303-1B was transformed with the indicated LexA- and B42-fusion expression plasmids (or an empty pJG4-5 vector) together with a LacZ reporter gene containing eight LexA operator sites. CNOT1, CNOT6 and CNOT7 were also transformed together with empty pJG4-5 vector as control for lack of self-activation. The CNOT1-CNOT6 combination served as another negative control. The graph shows a representative experiment (of four experiments performed). Bars show β-Gal activity [nmol min^{-1} mg^{-1}]. Values represent mean +/- SEM.

Supplementary Table 1. Mass spectrometry analysis of GST-CED pull-down.

Accession	Description	Number of Assigned Spectra
GST26_SCHJA	Glutathione S-transferase class-mu 26 kDa isozyme	382
CLH1_HUMAN	Clathrin heavy chain 1	283
TNR6C_HUMAN	Trinucleotide repeat-containing gene 6C protein	257
CNOT1_HUMAN	CCR4-NOT transcription complex subunit 1	66
GSTP1_HUMAN	Glutathione S-transferase P	50
GRP78_HUMAN	78 kDa glucose-regulated protein	42
PABP1_HUMAN	Polyadenylate-binding protein 1	35
AP2A1_HUMAN	AP-2 complex subunit alpha-1	31
RENT1_HUMAN	Regulator of nonsense transcripts 1	28
A8K916_HUMAN	cDNA FLJ78481, highly similar to Homo sapiens adaptor-related protein complex 2	27
SND1_HUMAN	Staphylococcal nuclease domain-containing protein 1	25
PK1L2_HUMAN	Polycystic kidney disease protein 1-like 2	23
CBR1_HUMAN	Carbonyl reductase [NADPH] 1	21
HSP7C_HUMAN	Heat shock cognate 71 kDa protein	19
AP2A2_HUMAN	AP-2 complex subunit alpha-2	19
A4D1U3_HUMAN	Single-stranded DNA binding protein 1	18
GSTM3_HUMAN	Glutathione S-transferase Mu 3	17
PABP4_HUMAN	Polyadenylate-binding protein 4	16
A8K795_HUMAN	cDNA FLJ75751, highly similar to Homo sapiens eukaryotic translation elongation factor 1 b	13
A8K5I0_HUMAN	cDNA FLJ75127, highly similar to Homo sapiens heat shock 70kDa protein 1A	13
OGT1_HUMAN	UDP-N-acetylglucosamine--peptide N-acetylglucosaminyltransferase 110 kDa subunit	13
DHX30_HUMAN	Putative ATP-dependent RNA helicase DHX30	12
P3H1_HUMAN	Prolyl 3-hydroxylase 1	11
GSTM2_HUMAN	Glutathione S-transferase Mu 2	11
A6NNH4_HUMAN	Fragile X mental retardation 1, isoform CRA_e	10
TR150_HUMAN	Thyroid hormone receptor-associated protein 3	10
ROA2_HUMAN	Heterogeneous nuclear ribonucleoproteins A2/B1	9
DHX9_HUMAN	ATP-dependent RNA helicase A	9
KIF7_HUMAN	Kinesin-like protein KIF7	9
AP1B1_HUMAN	AP-1 complex subunit beta-1	8
DESP_HUMAN	Desmoplakin	8
TIM13_HUMAN	Mitochondrial import inner membrane translocase subunit Tim13	8
ADT2_HUMAN	ADP/ATP translocase 2	7
FXR1_HUMAN	Fragile X mental retardation syndrome-related protein 1	7
HNRPU_HUMAN	Heterogeneous nuclear ribonucleoprotein U	7
UHMK1_HUMAN	Serine/threonine-protein kinase Kist	6
MATR3_HUMAN	Matrin-3	6
PPIB_HUMAN	Peptidyl-prolyl cis-trans isomerase B	6
DDX1_HUMAN	ATP-dependent RNA helicase DDX1	6
HNRPC_HUMAN	Heterogeneous nuclear ribonucleoproteins C1/C2	6
B2R7N5_HUMAN	cDNA, FLJ93526, Homo sapiens ribosomal protein S3 (RPS3)	6
Q5SUJ3_HUMAN	Ribosomal protein S18	6

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MCM7_HUMAN	DNA replication licensing factor MCM7	5
SFPQ_HUMAN	Splicing factor, proline- and glutamine-rich	5
DDX21_HUMAN	Nucleolar RNA helicase 2	5
CNOT3_HUMAN	CCR4-NOT transcription complex subunit 3	5
BCLF1_HUMAN	Bcl-2-associated transcription factor 1	5
DJC10_HUMAN	DnaJ homolog subfamily C member 10	5
ROA3_HUMAN	Heterogeneous nuclear ribonucleoprotein A3	5
ZBT37_HUMAN	Zinc finger and BTB domain-containing protein 37	4
AP2S1_HUMAN	AP-2 complex subunit sigma	4
F120A_HUMAN	Constitutive coactivator of PPAR-gamma-like protein 1	4
A8K4Z8_HUMAN	cDNA FLJ75550, highly similar to Homo sapiens heterogeneous nuclear ribonucleoprotein A1	4
A8K7H3_HUMAN	cDNA FLJ77670, highly similar to Homo sapiens ribosomal protein S15a (RPS15A)	4
Q0IIN1_HUMAN	Q0IIN1_HUMAN	4
RCD1_HUMAN	Cell differentiation protein RCD1 homolog	4
B2R6F3_HUMAN	cDNA, FLJ92926, Homo sapiens splicing factor, arginine/serine-rich 3 (SFRS3)	4
B4DPV7_HUMAN	cDNA FLJ54534, highly similar to Homo sapiens cysteinyl-tRNA synthetase (CARS)	4
A2A3R6_HUMAN	A2A3R6_HUMAN	4
HSP74_HUMAN	Heat shock 70 kDa protein 4	4
USP9X_HUMAN	Probable ubiquitin carboxyl-terminal hydrolase FAF-X	4
B2R549_HUMAN	cDNA, FLJ92341, Homo sapiens ribosomal protein S13 (RPS13)	4
HNRPM_HUMAN	Heterogeneous nuclear ribonucleoprotein M	4
WDR68_HUMAN	WD repeat-containing protein 68	4
A8MUS3_HUMAN	Putative uncharacterized protein RPL23A	4
HS90B_HUMAN	Heat shock protein HSP 90-beta	3
LARP1_HUMAN	La-related protein 1	3
NUCL_HUMAN	Nucleolin	3
B0ZBD0_HUMAN	40S ribosomal protein S19	3
B4DTG2_HUMAN	cDNA FLJ56389, highly similar to Elongation factor 1-gamma	3
RL22_HUMAN	60S ribosomal protein L22	3
B2RDD5_HUMAN	cDNA, FLJ96562, Homo sapiens ribosomal protein S16 (RPS16)	3
B3KMN4_HUMAN	cDNA FLJ11706 fis, clone HEMBA1005101, highly similar to RNA-binding protein 14	3
ELAV1_HUMAN	ELAV-like protein 1	3
B2RMP5_HUMAN	EEF2 protein (Eukaryotic translation elongation factor 2, isoform CRA_a)	3
SC23B_HUMAN	Protein transport protein Sec23B	3
Q5JVH5_HUMAN	Ribosomal protein 26 (RPS26) pseudogene	3
FXR2_HUMAN	Fragile X mental retardation syndrome-related protein 2	2
CNOTA_HUMAN	CCR4-NOT transcription complex subunit 10	2
RS14_HUMAN	40S ribosomal protein S14	2
F136A_HUMAN	Protein FAM136A	2
PERQ2_HUMAN	PERQ amino acid-rich with GYF domain-containing protein 2	2
PPIA_HUMAN	Peptidyl-prolyl cis-trans isomerase A	2
IF2B3_HUMAN	Insulin-like growth factor 2 mRNA-binding protein 3	2
Q05D43_HUMAN	YBX1 protein (Fragment)	2
CLCB_HUMAN	Clathrin light chain B	2

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A9X7H1_HUMAN	Eukaryotic translation elongation factor 1 alpha (Fragment)	2
B2R4T2_HUMAN	cDNA, FLJ92205, Homo sapiens ribosomal protein S5 (RPS5)	2
A8K4W3_HUMAN	cDNA FLJ77116, highly similar to Homo sapiens clathrin, light polypeptide	2
CBR3_HUMAN	Carbonyl reductase [NADPH] 3	2
B4E3B6_HUMAN	cDNA FLJ54408, highly similar to Heat shock 70 kDa protein 1	2
A3R0T7_HUMAN	Liver histone H1e	2
B0YJA5_HUMAN	Mitochondrial import inner membrane translocase subunit Tim8 B	2
B2R4M7_HUMAN	cDNA, FLJ92149, Homo sapiens ribosomal protein S25	2
H2A1B_HUMAN	Histone H2A type 1-B/E	2
P5CR3_HUMAN	Pyrroline-5-carboxylate reductase 3	2
TIM8A_HUMAN	Mitochondrial import inner membrane translocase subunit Tim8 A	2
TTC19_HUMAN	Tetratricopeptide repeat protein 19, mitochondrial	2
HNRPR_HUMAN	Heterogeneous nuclear ribonucleoprotein R	2
B2R4C1_HUMAN	cDNA, FLJ92036, highly similar to Homo sapiens ribosomal protein L31 (RPL31)	2
Q5T0P7_HUMAN	40S ribosomal protein S24	2
CA163_HUMAN	Hcp beta-lactamase-like protein C1orf163	2
A4D1Q6_HUMAN	Similar to dJ753D5.2 (Novel protein similar to RPS17 (40S ribosomal protein S17))	2
Q76M58_HUMAN	40S ribosomal protein S12	2
PAN2_HUMAN	PAB-dependent poly(A)-specific ribonuclease subunit 2	1
B2R4H3_HUMAN	cDNA, FLJ92089, Homo sapiens ribosomal protein L17	1
DHX36_HUMAN	Probable ATP-dependent RNA helicase DHX36	1
NUP93_HUMAN	Nuclear pore complex protein Nup93	1
WDR47_HUMAN	WD repeat-containing protein 47	1
A9C4C1_HUMAN	Ribosomal protein S9 (Ribosomal protein S9)	1
TPC10_HUMAN	Trafficking protein particle complex subunit 10	1
Q5HY50_HUMAN	Ribosomal protein L10 (Fragment)	1
A8K674_HUMAN	cDNA FLJ75516, highly similar to Xenopus tropicalis ubiquitin C	1
A6ND19_HUMAN	Putative uncharacterized protein ENSP00000352315	1
B2R4E3_HUMAN	cDNA, FLJ92058, Homo sapiens ribosomal protein S10	1
RL26L_HUMAN	60S ribosomal protein L26-like 1	1
B2REA7_HUMAN	Ribosomal protein L36a	1
A6NEC0_HUMAN	Putative uncharacterized protein MAGOHB	1
A8K517_HUMAN	cDNA FLJ77921, highly similar to Homo sapiens ribosomal protein S23 (RPS23)	1
AP1S1_HUMAN	AP-1 complex subunit sigma-1A	1
RL35_HUMAN	60S ribosomal protein L35	1
YOT5_CAEEL	Uncharacterized protein ZK632.5	1
HNRPG_HUMAN	Heterogeneous nuclear ribonucleoprotein G	1
HNRH3_HUMAN	Heterogeneous nuclear ribonucleoprotein H3	1
B2RD15_HUMAN	B2RD15_HUMAN	1
SYTL2_HUMAN	Synaptotagmin-like protein 2	1
RL21_HUMAN	60S ribosomal protein L21	1
A4D1G5_HUMAN	Ribosomal protein S27	1
B2R5H2_HUMAN	cDNA, FLJ92473, Homo sapiens enhancer of rudimentary	1

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	homolog (Drosophila) (ERH)	
RS30_HUMAN	40S ribosomal protein S30	1
ZN184_HUMAN	Zinc finger protein 184	1
B3KSK8_HUMAN	cDNA FLJ36521 fis, clone TRACH2002138, highly similar to Adenylate cyclase type 5	1
DCR2_YEAST	Phosphatase DCR2	1
PCBP1_HUMAN	Poly(rC)-binding protein 1	1
A8K094_HUMAN	cDNA FLJ76205, highly similar to Homo sapiens ribosomal protein L8 (RPL8)	1
B2RE88_HUMAN	cDNA, FLJ96465, highly similar to Homo sapiens solute carrier family 25	1
CNOT8_HUMAN	CCR4-NOT transcription complex subunit 8	1

Mass spectrometry analysis of GST-CED pull-down. Identified proteins are listed with a short protein description and the number of assigned spectra.

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SUPPLEMENTARY RESULTS

Mutational analysis of the TNRC6C CED identifies functional elements containing W residues

To determine the features of M2 and Cterm that repress mRNA function, we identified conserved regions of 2–4 amino acids by alignment of vertebrate and insect GW182 proteins (Supplementary Fig. 1). Mutagenesis of the selected sequences in the context of full-length CED or CEDΔPAM2 had a very limited effect on their ability to repress mRNA function (data not shown). Hence, the effect of mutations was tested in a context of fragments containing only the M2 or Cterm region adjacent to RRM, referred to as M2-RRM or RRM-Cterm, respectively (see Fig. 1a).

Two of the three mutations in RRM-Cterm, LW1647 and SLW1657 (all mutations throughout the paper are to alanines; if several consecuitive amino acids are mutated, numbers always correspond to the first mutated residue) reduced repression by approximately two times (Supplementary Fig. 2d). We noted that amino acids mutated in LW1647 and SLW1657 include W residues in a context of GW or WG dipeptides (see Supplementary Fig. 1), shown previously to be important not only for the interaction of GW182 proteins with Argonautes but also for the AGO-independent repression of protein synthesis by the N-terminal domain of both *D. melanogaster* and human GW182s[1,2]. Hence, we generated mutants in which only W residues of LW1647 and SLW1657 amino acids are mutated, either singly or both. We found that mutation of W residues alone (mutants W1648 and W1659) had an effect similar to that of mutants LW1647 and SLW1657. Mutation of both W residues (mutant W1648 W1659) resulted in a stronger reduction in repression than for the single W mutants (Supplementary Fig. 2e). We also observed that the mutants LW1647, SLW1657, W1648, W1659, and W1648 W1659, which were all less inhibitory than the wild-type RRM-Cterm, were also strongly affected in their activity to pull down CNOT1 and CAF1, with the strongest effect seen with W1659 and the W1648 W1659 double mutant (Supplementary Fig. 2f). These results indicated that the Ws in Cterm are important both for mRNA repression and interaction with the CCR4–NOT complex.

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A similar conclusion emerged from the analysis of the M2 domain. This analysis revealed that mutations of sequences bearing W residues, or of W residues alone (mutants STW1485, W1487, W1495) generally reduced repression more than mutations of amino acids not including tryptophanes (mutants PPP1474 and GLT1477) or individual non-W amino acids or combinations thereof (mutants N1467, K1482, and N1467 K1482) (Supplementary Fig. 2d and 2g). Notably, simultaneous mutation of two W residues, both present in the GW (or WG) context, produced the strongest effect among the M2-RRM mutants tested (Supplementary Fig. 2g). We also observed that the ability of these mutants to interact with CAF1 correlated with their repressive activity. For example, mutant GLT1477 interacted with CAF1 as much as wild-type M2-RRM did. In contrast, the interaction was partially affected in mutants STW1485 and W1495, and was nearly eliminated for the mutant W1487 W1495 (Supplementary Fig. 2h).

The data presented above pointed towards a considerable redundancy of sequences in the CED responsible for mediating both its interaction with CCR4–NOT and its repressive function in protein synthesis. Such redundancy is additionally indicated by step-wise deletion of the M2 and Cterm regions in a context of M2-RRM and RRM-Cterm fragments (Supplementary Fig. 3b). Moreover, we found that fragments containing duplications of either M2 (mutant M2-M2) or Cterm (mutant Cterm-Cterm) induced the repression to the level similar of that of the M2-Cterm fusion (Supplementary Fig. 3a). Hence, the M2 region does not seem to carry any unique functional elements which differ from those present in Cterm, and vice versa.

Together, the data presented above suggested redundancy in the features of the CED domain that repress mRNA function, and indicated that W residues may be important for that repression activity in a manner that involves recruitment of the CCR4–NOT complex.

Tethering of the TRNC6C CED represses protein synthesis from both poly(A)$^+$ and poly(A)$^-$ mRNAs in HEK293 cells

We investigated whether the TNRC6C CED can repress tethered mRNA independently of poly(A) tail. To generate a poly(A) tail-free reporter, we initially replaced the cleavage and polyadenylation signal in the RL-5BoxB

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reporter with sequences comprising a histone stem-loop and a downstream element (referred to as HSL) 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 to produce RL-5BoxB-HSL+HhR reporter (Supplementary Fig. 6a). We have verified, by fractionating the total cellular RNA on oligo$(dT)_{25}$ beads and performing quantitative real-time PCR (RT-qPCR), that more than 99% of transcripts generated from transfected RL-5BoxB-HSL+HhR is poly(A)-free (Supplementary Fig. 6b).

Tethering of CED or CEDΔPAM2 repressed expression of the RL-5BoxB poly$(A)^+$ reporter by approximately ten times when compared to the CED lacking the N-peptide (Supplementary Fig. 6c; see also Zipprich et al.[3]). Interestingly, expression being driven by RL-5BoxB-HSL+HhR, yielding poly$(A)^-$ RNA, was also strongly repressed (approximately five times; Supplementary Fig. 6c). Northern analysis showed that whereas tethering of TNRC6C fragments induced marked degradation of the polyadenylated RNA, the level of RNA transcribed from RL-5BoxB-HSL+HhR plasmid remained unaffected (Supplementary Fig. 6d; quantification of multiple notherns is shown in a lower panel).

Northern analysis revealed that, in addition to RNA of the size expected for RL-5BoxB HSL+HhR RNA, larger RNA species cross-reacting with the RL probe also accumulated in transfected cells (Supplementary Fig. 6d). This raised a possibility that RL activity measured in cells expressing RL-5BoxB-HSL+HhR reporter might originate, at least partially, from transcripts other than those processed at HSL and HhR sites. We therefore sought for additional evidence that the TNRC6C CED and CEDΔPAM2 fragments can induce repression independently of a poly(A) tail. Towards this end, we transfected HEK293 cells with *in vitro* transcribed poly(A)-tail free RL-5BoxB RNA. To minimize a possibility that the poly$(A)^-$ *in vitro* transcript gets polyadenylated upon its accumulation inside the cell, we also used a form of poly$(A)^-$ transcript which contains a residue of cordycepin, an AMP analog, incorporated at the 3' end of RNA; cordycepin lacks the ribose 3'-OH group which would be required for extension of RNA with additional As or other

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nucleotide residues[4]. Tethering of either CED or CEDΔPAM2 repressed expression of RL from the in vitro synthesized poly(A)$^+$ RL-5BoxB RNA by 70 to 80%. Importantly, it also repressed, by approximately 30 to 40%, the expression from transfected non-polyadenylated RL-5BoxB RNA, irrespective whether it bore the cordycepin end or not (Supplementary Fig. 6e). Of note, expression of RL from cordycepin-bearing RL-5BoxB RNA was approximately three times higher then that from non-modified poly(A)$^-$ 5boxB RNA (data not shown), consistent with a reported protective effect of cordycepin against 3'→5 exonucleolytic degradation[5]. In further experiments, cordycepin-modified transcripts were used as a poly(A)$^-$ RNA.

Tethering of CCR4–NOT complex components represses protein synthesis from both poly(A)$^+$ and poly(A)$^-$ mRNAs in HEK293 cells

We tested whether direct tethering of the CCR4–NOT complex components to mRNA is sufficient to induce its silencing in HEK293 cells. Cooke et al.[6] reported previously that tethering of CAF1 or its catalytically inactive mutant to the microinjected reporter mRNA, either poly(A)$^+$ or poly(A)$^-$, can repress translation at the initiation step in *Xenopus leavis* oocytes. We found that also in HEK293 cells, tethering of CAF1 or its catalytic mutant represses RL expression from the RL-5BoxB reporter (Supplementary Fig. 7a). Interestingly, tethering of CNOT1, CNOT2, CNOT6 and, to lesser extend, CNOT3 (all expressed as NHA fusions), also repressed the RL-5BoxB reporter function (Supplementary Fig. 7a,c). Comparison of the effect on activity of RL-5BoxB and RL-5BoxB-HSL+HhR reporters, expressing poly(A)$^+$ or poly(A)$^-$ RNAs respectively, revealed that also poly(A)$^-$ RNA was repressed by three to four times by tethering of CNOT1, CAF1 or CAF1 catalytic mutant (Supplementary Fig. 7a). Notably, similarly as observed with tethering of the TNRC6C CED and CEDΔPAM2 fragments (see Supplementary Fig. 6), tethering of CAF1 or CNOT1 proteins resulted in a marked (two to three times) decrease of the level of polyadenylated RL-5BoxB RNA but not RNA transcribed from the RL-5BoxB-HSL+HhR reporter, which should be poly(A)-free (Supplementary Fig. 7b).

We also tested if tethering of CAF1 or its catalytic mutant will repress function of the *in vitro* transcribed RL-5BoxB RNA, either polyadenylated or

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poly(A)-free, transfected to HEK293 cells. We found that tethering of either CAF1 protein repressed expression of RL from the poly(A)$^+$ RL-5BoxB RNA by 80–90%. Importantly, it also repressed, by approximately 60%, the expression from transfected poly(A)-free (cordycepin-modified) RL-5BoxB RNA (Supplementary Fig. 7d).

Taken together, the results presented in the last two sections indicate that recruitment of the TNRC6C CED or different components of the deadenylase CCR4–NOT complex to the 3' UTR of mRNAs induces their silencing even if the transcripts have no poly(A) tail. Since the repression of poly(A)$^-$ RNA was not associated with deacrease of mRNA levels, the data also suggest that the CCR4–NOT complex not only mediates deadenylation but also translational repression of mRNA. These findings extend recent observations of Cooke et al.[6] showing that tethering of CAF1 can induce translational repression in microinjected *X. laevis* oocytes. They also indicate that the repression may not result from a direct effect of CAF1 but rather is due to the recruitment of a whole CCR4–NOT complex to mRNA.

SUPPLEMENTARY METHODS

DNA constructs and protein mutants

Reporter plasmids RL-5BoxB and FL-Con[7], and FL-5BoxB, RL-Con and FL-nerfin[8], as well as FL-5BoxB-HSL and FL-5BoxB-HhR[9] have been described previously. Plasmids encoding NHA, NHA-lacZ, dGW182 and its deletion fragments[10], as well as miR-9b and miR-12[8] have also been described. Plasmids encoding TNRC6A, TNRC6C and its deletion fragments[3], the pEBG-Δ1370 plasmid encoding TNRC6C GST-CED[11], and pSR-HA-CCR4[12] have been reported. Point mutations in TNRC6C, TNRC6A and dGW182 constructs were introduced by site-directed mutagenesis according to[13]. The TNRC6A clone lack the first 312 amino acids[14]. Plasmids encoding the dGW182 N-terminal effector domain (1-490) and its point mutants used for tethering assays in human cells, as well as sub-fragments of TNRC6C, were generated by PCR-amplification of the corresponding fragments of the NHA- or HA-

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dGW182 regions (wild-type or its indicated mutants[1]) or TNRC6C, and cloning into either pCI-neo vector (Promega) or pCI-neo bearing an HA- or NHA-tag[7]. 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[7]. A plasmid expressing TNRC6C lacking the M2 and Cterm regions was generated by cloning a PCR amplified fragment encoding the RRM into a construct encoding amino acids 1-1413 of TNRC6C in pCI-NHA vector[7].

To generate N-Sic-GST and its W-containing versions, the Sic-, Sic4xW-, and Sic7xW-encoding fragments were chemically synthesized (Genscript; for protein sequence information, see Supplementary Figure 4B) and cloned into a pCI-neo vector containing NHA-GST, by removing the HA-tag and inserting Sic sequences between the N and GST regions. The TNRC6C N-CED-GST construct and its 7W mutant were generated similarly, by replacing the HA-tag in pCI-NHA-GST with PCR-amplified CED fragments. Plasmids expressing GST-tagged dGW182 (1-490), TNRC6C, and their deletions and point mutants, all used for GST pull-downs, were generated by cloning the corresponding PCR-amplified fragments into the pEBG vector (Addgene). Plasmids encoding GST-tagged Sic and Sic7xW, used for GST pull-downs, were obtained by cloning the chemically synthesized fragments mentioned above into the pEBG vector.

To obtain plasmids expressing HA-CNOT1 and NHA-CNOT1, the CNOT1 coding region from the plasmid pME-FLAG-CNOT1[15] (a kind gift of Dr. T. Yamamoto, University of Tokyo) was PCR amplified and cloned into pCI-HA or pCI-NHA vector[7] using the In-Fusion 2.0 Dry-Down PCR Cloning Kit (Clontech) or cloned into a pAC5.1A vector containing NHA- or HA-tag. For generating plasmids expressing HA- or NHA-tagged CNOT2, CNOT3L or CNOT6, the corresponding coding sequences were amplified by PCR using B42 vectors described in Lau et al.[16] (kindly provided by Dr. H.T.M. Timmers, University of Utrecht) as template and cloning the PCR product into pCI-HA or pCI-NHA vector[7]. Plasmids expressing HA-CAF1, NHA-CAF1, HA-CAF1 D40A (CAF1 D40A is referred to as CAF1mut in the text), and NHA-CAF1mut were obtained by PCR amplification of the CAF1-encoding sequence from plasmids pSR-HA-Caf1 or pSR-HA-Caf1 D40A[17] (kind gifts of Dr. A-B. Shyu,

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The University of Texas Medical School, Houston) and cloning into pCI-HA or pCI-NHA vector[7].

The plasmid RL-5BoxB-poly(A) that was used for generating *in vitro* transcribed reporter RNA was obtained by cloning an Xba1-Stu1 fragment of the plasmid RL-5BoxB-pA[18] (a kind gift of Dr. M. Fabian, McGill University, Montreal), which encodes 5BoxB sites and a 98-nt-long poly(A) tail, into the Xba1-Stu1 digested RL-5BoxB reporter plasmid. The sequence between the T7 promoter and ATG codon was modified in all plasmids (except FL-Con) used for the *in vitro* transcription to obtain transcripts bearing identical 5` UTR. Correctness of all plasmids was verified by sequencing.

Immunoprecipitations and Western blotting

For western blotting analysis, inputs and the 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 or rescue 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).

Mass spectrometry analysis

Protein content from GST pull-downs shown in Supplementary Fig. 4b was analyzed by MS, using an LTQ/Orbitrap VELOS mass spectrometer (Thermo Fischer Scientific) equipped with Agilent 1100/1200 Series Nanoflow LC system (Agilent Technologies). For MS analysis shown in Figure 1b, a 4000 Q TRAP mass spectrometer (Applied Biosystems, Foster City, CA) was used.

Northern blotting and poly(A) tail length measurement

10–20 µg of total RNA isolated from HEK293T or S2 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 were hybridized to the RNA on the membrane in ULTRAhyb Ultrasensitive

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Hybridization Buffer (Ambion) at 68°C. After washing the membrane with 0.2XSSC containing 0.1% (v/v) SDS at 68°C, the signal was detected using a PhosphorImager screen and a GE TyphoonTM 9400 scanner.

For poly(A) tail length measurement, total RNA was isolated from S2 cells expressing FL-5BoxB and either NHA-CED, NHA-CED 7W or NHA-lacZ with Trizol LS reagent (Invitrogen). Poly(A) tails of FL-5BoxB mRNA were estimated using the poly(A) tail length assay kit (Affymetrix) based on G-tailing/PAT (poly(A) test) assay[19]. FL-5BoxB-specific primers were: TTATCTCGAGGTCACCCATT (forward); and GCAATAGCATCACAAATTTCA (reverse). The expected PCR amplification product is 263 base-pairs long and corresponds to the region starting at 30 nucleotides upstream of the polyadenylation site. The same forward primer was also used for estimation of poly(A) length.

Fractionation of total cellular RNA and RT-qPCR

Total cellular RNA, isolated from HEK293T cells 24 h post-transfection with Trizol, was used for poly(A)$^+$ RNA selection using Dynabeads® Oligo $(dT)_{25}$ (Invitrogen). 7 μg of RNA dissolved in 100 μl of water was mixed with 100 μl of Binding Buffer (20 mM Tris-HCl, pH 7.5, 1M LiCl) and heated at 65°C for 2 min. After cooling down on ice the RNA was mixed with 0.1 mg Dynabeads® Oligo $(dT)_{25}$ in 100 μl of Binding Buffer. After incubation for 5 min at room temperature the beads were washed twice with 200 μl of Washing Buffer (10 mM Tris-HCl, pH 7.5, 0.15 M LiCl) and the selected RNA was eluted with 100 μl of 10 mM Tris-HCl, pH 7.5 at 75°C for 2 min. The fractionation procedure was repeated two more times, the input and selected RNA fractions were treated with RQ1 RNase-free DNase (Promega) according to the manufacturers protocol, and used for RT-qPCR.

300 ng of each RNA was reverse-transcribed using SuperScript™ III First-Strand Synthesis SuperMix (Invitrogen) and the resulting cDNA was used as a template for qPCR with the ABI 7500 Real-Time PCR system and Platinum SYBR Green qPCR SuperMix, using reporter-specific primers amplifying the RL or FL coding regions.

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In vitro transcription and transfection of RNA

Plasmids used as templates for the *in vitro* transcription were linearized using the following restriction enzymes: plasmids encoding HA- or NHA-fusion proteins with Mfe1, FL-Con with Hpa1, and plasmids encoding RL-5BoxB reporters with Not1 [5' of the plasmid-encoded poly(A) stretch] to yield a template for poly(A)$^-$ RNA synthesis or with Age1 [3` of the poly(A) stretch]. Before *in vitro* transcription, the Age1-linearized plasmid was treated with mung bean nuclease to remove the overhang produced by Age1 to ensure the DNA template ends with a stretch of 98 A residues. The in vitro transcription was performed using the MEGAscript T7 kit (Ambion), following the manufacturers instructions, and RNA was purified using the RNeasy mini kit (Qiagen). 3` ends of some of the in vitro transcribed RNAs were modified by incorporating a cordycepin nucleotide (3'-deoxyadenosine 5'-triphosphate) using yeast poly(A) polymerase[4]. Briefly, purified in vitro transcripts (0.2 μM) were incubated for 10 min at 37°C with 20 μM cordycepin triphosphate (Jena Bioscience) and yeast poly(A) polymerase (Affymetrix). Transcripts were then repurified as described above.

RNA transfections were performed in six-well plates with ~90% confluent HEK293T cells, using Lipofectamine 2000 transfection reagent (Invitrogen). 50 ng of the RL reporter RNA, 150 ng of the FL-Con RNA and 600 ng of RNA encoding HA- or NHA-fusion protein were transfected per well. Cells were harvested 16 h post-transfection in Passive Lysis Buffer (PLB; Promega) and activity of RL and FL was analyzed as described above.

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[16]). 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, also provided by

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H.T.M. Timmers. The reporter plasmid pSH18-34 was used to measure β-Gal activity[20]. Transformations with different plasmids were carried out according to the protocol described in[21].

For measurements of β-Gal activity, 100 µl SC [-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 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[21].

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2.4 Deletion analysis of the human CNOT1 protein

As our experiments suggested that GW182 interacts directly with CNOT1, we were interested in characterizing the molecular interaction between these two proteins. Furthermore, we were interested to identify the parts of CNOT1 that mediate repression. To answer these questions, we generated a collection of deletion mutants of CNOT1 (Figure 2.6, panel A) and tested their repressive potential in the tethering assay in HEK293 cells and also probed their interaction with the C-terminal region of TNRC6C in GST pull-down assays.

Tethering NHA-tagged CNOT1 strongly repressed (7-fold) activity of the RL-5BoxB reporter when compared to HA-tagged CNOT1 protein lacking the λN peptide. Progressive deletion from the N-terminus revealed that a central part of the CNOT1 protein is required for inducing maximal repression of reporter mRNA in the tethering assay. Whereas deletion of the N-terminal 243 amino acids did not affect repression, deleting the N-terminal 1068- and 1602 amino acids reduced repression from 7-fold to 3.5-fold and 1.5-fold, respectively (Figure 2.6, panel B and Figure 2.11).

Conversely, progressive deletions carried out from the C-terminus revealed that amino acids 1827-2376 are required for maximal repression but a fragment lacking this part was still efficiently repressing (5-fold repression) the reporter mRNA (Figure 2.6, panel B and Figure 2.11). Whereas a CNOT1 fragment containing amino acids 1-1601 efficiently repressed (5.5-fold) mRNA function, a fragment containing amino acids 1-1069 was almost inactive, suggesting that the region between amino acids 1069 and 1601 is particularly important for inducing repression (Figure 2.6, panel B and Figure 2.11).

As the aforementioned results indicated that a central region of CNOT1 is important for inducing repression, we tested whether a central fragment encompassing amino acids 727-1601 would be active in repression. Indeed, tethering this fragment reduced activity of the RL-5BoxB reporter 5-fold, which is comparable to the extent of repression induced by the fragments 1-1601 and 840-2376 (Figure 2.6, panel B and Figure 2.11). Further deletion analysis of the central region did not reveal a shorter fragment with the same repressive activity as the fragment 727-1601, suggesting that maximal repressive activity is only achieved when this region of the protein is intact (Figure 2.6, panels B and C and Figure 2.11). Interestingly, the central region of CNOT1 that seems to be important for repression also encompasses the minimal fragment able to interact with CAF1 as identified by

Sandler et al., (2011), suggesting that CAF1 may play a role in the repression induced by tethered CNOT1 (Figure 2.11).

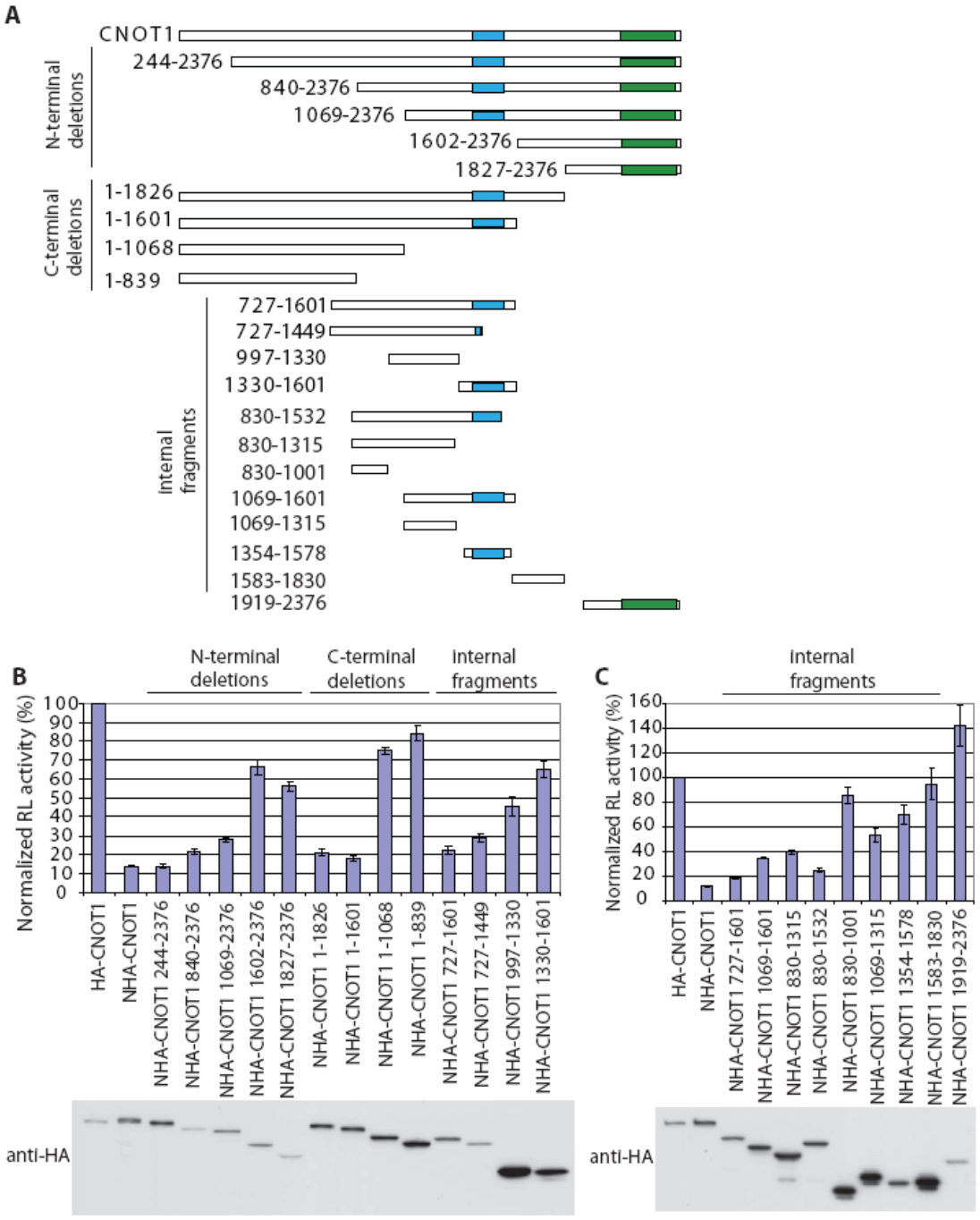

Figure 2.6: Tethering a central fragment of CNOT1 efficiently represses the expression of Renilla Luciferase (RL) reporter mRNA. (**A**) Schematic representation of CNOT1 and its deletion mutants. Conserved domains according to the NCBI Protein Database are indicated (blue: DUF3819 super family, green: Not1 super family). (**B** and **C**) Plasmids encoding indicated HA- or NHA-tagged CNOT1 or fragments thereof were co-transfected into HEK293T cells with RL-5BoxB and FL-Con plasmids. RL expression was normalized to the activity of FL and is shown as percentage of activity in cells expressing HA-CNOT1. The values are means (±standard error of the mean (SEM)) from three experiments. (Lower panels) Expression

levels of HA- or NHA-tagged proteins was assessed by Western blotting using anti-HA antibody.

To test which regions of CNOT1 interact with the CED, we expressed the CED as a glutathione S-transferase (GST) fusion together with NHA-tagged CNOT1 and fragments thereof in HEK293 cells and performed pull-down experiments. All the N-terminal deletion fragments of CNOT1 were efficiently pulled down by the CED, suggesting that the NHA tagged C-terminal region 1827-2376 of CNOT1 is sufficient to interact with the CED (Figure 2.7, panel A). Deletions from the C-terminus of CNOT1 revealed however that the C-terminus is not the only fragment that contributes to the interaction with the CED, as the NHA-tagged CNOT1 fragment 1-1826 was still efficiently pulled down by the CED (Figure 2.7, panel B). Whereas the fragment NHA-CNOT1 1-1601 was efficiently pulled down by the CED, a fragment encompassing amino acids 1-1068 interacted very poorly with the CED, suggesting that the region 1069-1601 is important for the interaction with the CED (Figure 2.7, panel B). Indeed, further pull-down experiments revealed that the NHA-tagged CNOT1 fragments encompassing amino acids 727-1601, 1069-1601 and 1354-1578 all interacted with the CED or CED∆PAM2 indicating that the region 1354-1578 is sufficient for the interaction (Figure 2.8 and Figure 2.9). Importantly, interaction of these fragments with the CED depended on the tryptophan residues present in the CED (Figure 2.10).

In summary, the analysis of CNOT1 fragments revealed that at least two different regions of CNOT1 are capable of interacting with the CED of TNRC6C in HEK293 cell extracts. Hence, the CED is able to contact both the middle- and the C-terminal part of CNOT1. The identification of CNOT1 fragments interacting with the CED in HEK293 cell extract should serve as a basis for further biophysical and structural characterization of the interaction between GW182 and CNOT1 proteins. It will be interesting to learn how multiple W-motifs of the CED contribute to the interaction with the two regions of CNOT1.

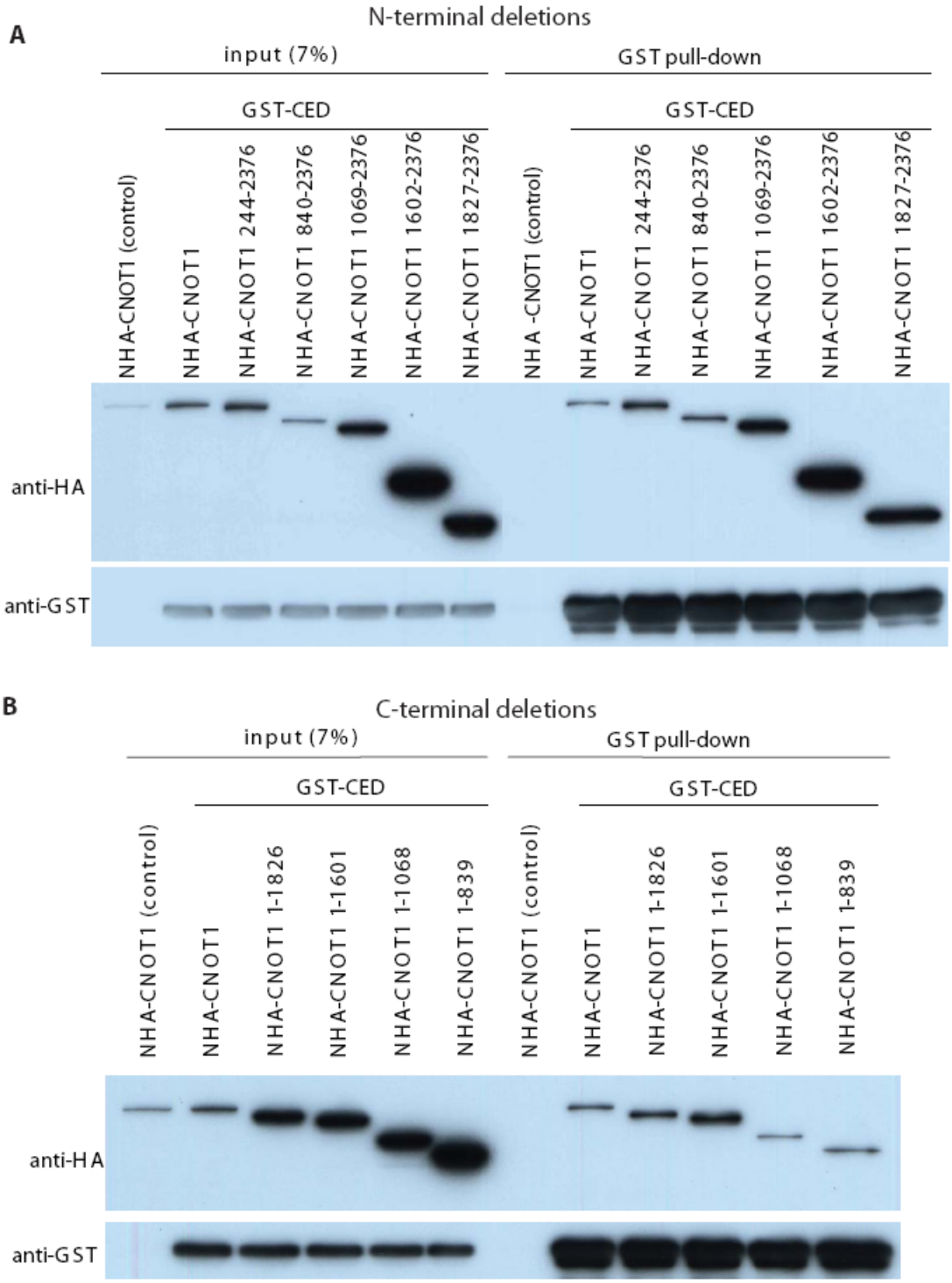

Figure 2.7: A C-terminal fragment of CNOT1 is sufficient to interact with the TNRC6C CED in HEK293 cell extracts. (**A**) GST pull-down assay with GST-tagged CED and HA-tagged human CNOT1 and fragments thereof. HEK293T cells were co-transfected with plasmids encoding NHA-CNOT1 or indicated fragments and GST-tagged CED. Inputs (7%) and pull-down assays were analyzed by western blotting. Extracts from cells not transfected with GST-CED were used as a control. (**B**) GST pull-down assay was performed as described in A but with different fragments of CNOT1.

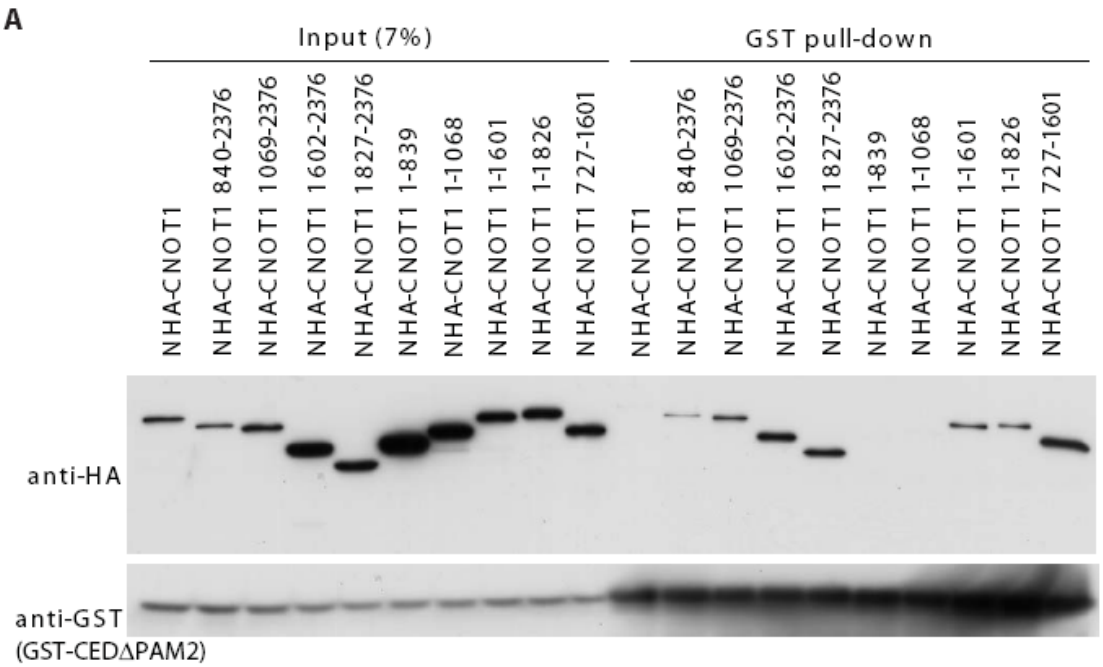

Figure 2.8: A central NHA-tagged fragment of CNOT1 is sufficient to interact with the TNRC6C CEDΔPAM2 in HEK293 cell extracts. GST pull-down assay was performed as described in Figure 2.7A using GST-tagged CEDΔPAM2 instead of GST-tagged CED.

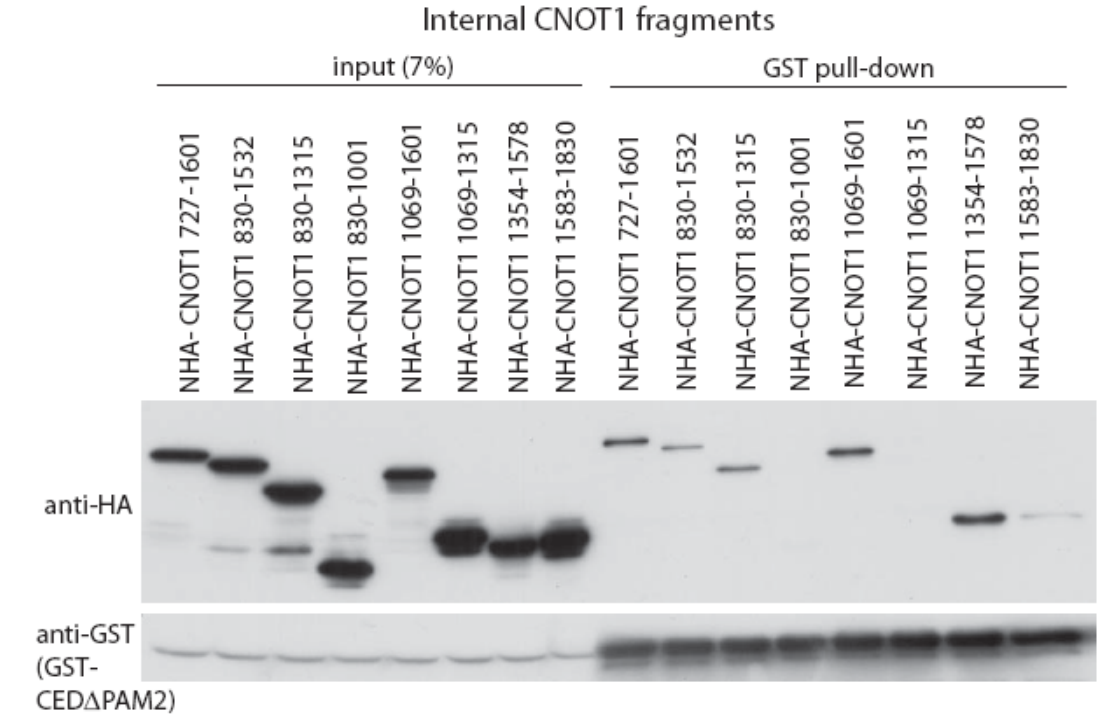

Figure 2.9: The NHA-tagged fragments of CNOT1 encompassing amino acids 1069-1601 and 1354-1578 interact with GST-CEDΔPAM2 in HEK293 cell extracts. GST pull-down assay was performed as described in Figure 2.8.

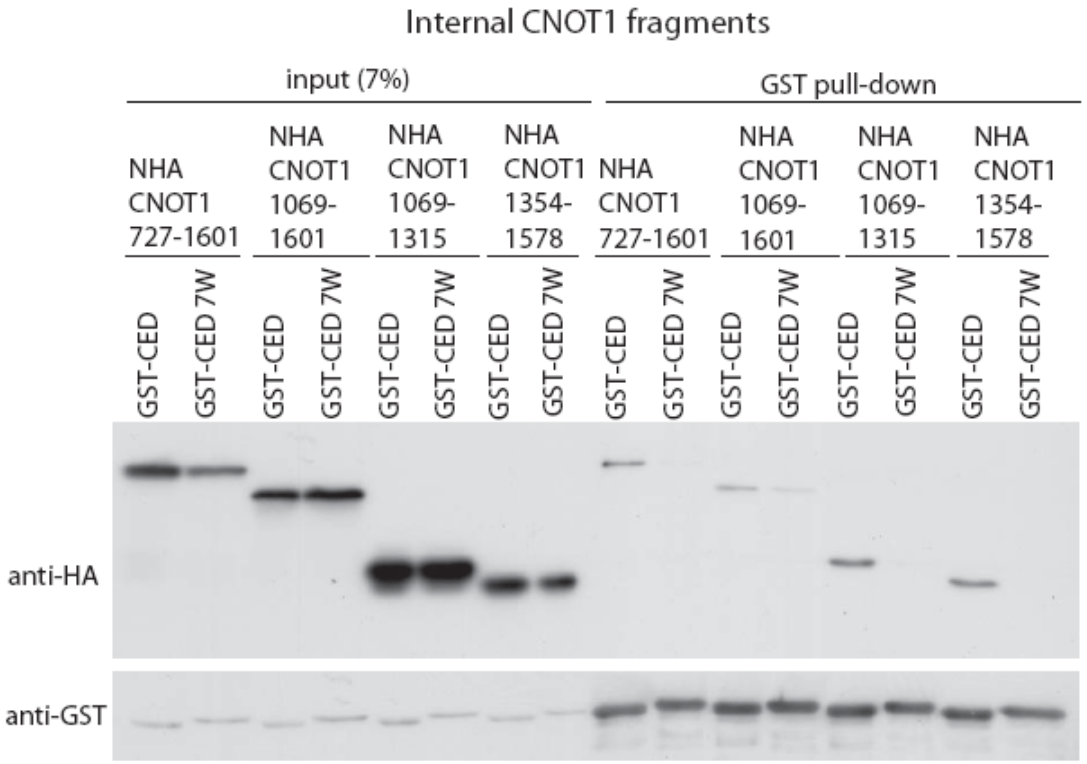

Figure 2.10: The interaction between the CED and NHA-tagged fragments of CNOT1 depends on the tryptophan residues in the CED. GST pull-down assay was performed as described in Figure 2.7A using GST-tagged CED or a mutant version thereof containing seven tryptophan to alanine mutations (GST-CED 7W).

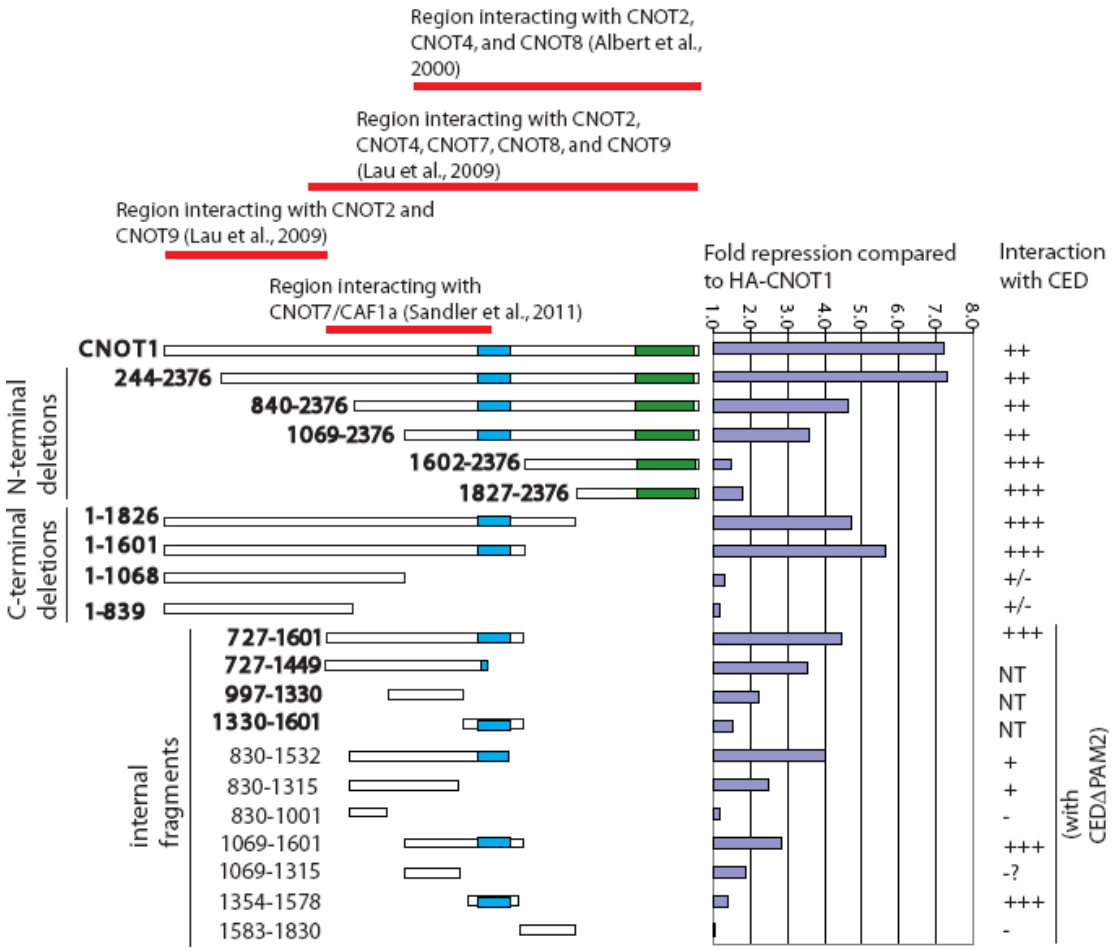

Figure 2.11: Summary of the results from tethering assays and GST pull-down assays. The collection of deletion mutants of CNOT1 analyzed is represented schematically. Conserved domains according to the NCBI Protein Database are indicated (blue: DUF3819 super family, green: Not1 super family). The region of CNOT1 that was found by Sandler et al., (2011) to be sufficient for the interaction with CAF1 is marked with a red bar. Other regions of CNOT1 found to interact with the indicated proteins in yeast two-hybrid assays by Lau et al., (2009) and Albert et al., (2000) are also marked with a red bar. The strength of repression induced by each fragment in the tethering assay is indicated as fold repression. We estimated the enrichment of each of the fragments in the GST-CED pull-down assay and indicate our estimations on the right using the following scale: +++, ++, +/-, - (+++ meaning strongest enrichment, - meaning weakest enrichment). The fragment 1069-1315 is labeled with "-?" because it did not interact with CEDΔPAM2 in two experiments but interacted with the full CED in one performed experiment. The fragments labeled with NT were not tested in GST pull-down assays.

3. Discussion

Owing to the important role of miRNAs as post-transcriptional regulators of gene expression, the molecular mechanism of miRNA-mediated gene silencing has been subject to intensive studies by many research groups worldwide. However, as studies aiming to shed light on this process came to different and sometimes even contradictory conclusions, it has been difficult to draw a comprehensive picture of the molecular events leading to miRNA-mediated repression. In order to extend our understanding of the mechanism of miRNA-mediated gene silencing, we studied the function of GW182 proteins that have emerged as key components executing miRNA-mediated repression.

In this work we identify the C-terminal fragment of TNRC6C (CED) as a key region mediating miRNA-induced repression by interacting with PABP via its PAM2 motif and by recruiting the PAN2-PAN3 and CCR4-CAF1-NOT deadenylase complexes via conserved tryptophan-containing motifs.

In the following part of the discussion I do not indicate references for our own results presented in this thesis. These results are described in chapter two (2. Results) and appendices A and B of the thesis. The results obtained by others are however specifically referenced in the text.

3.1 The role of different regions of GW182 proteins in miRNA-mediated silencing

The following evidence supports the conclusion that the C-terminal region of TNRC6 proteins represents a key region mediating miRNA-induced repression. First, in the tethering assay the CED represses a reporter mRNA to the same extent as full length TNRC6C and it appears to do so in an autonomous manner as we do not detect it to interact with AGO or TNRC6 family proteins. In contrast to the CED which reduces the reporter protein level 10 times, the GW-rich and Q-rich domains of TNRC6C only repress reporter protein levels 2-3 times. Second, whereas the expression of wild-type TNRC6A rescues miRNA mediated repression in HeLa cells depleted of

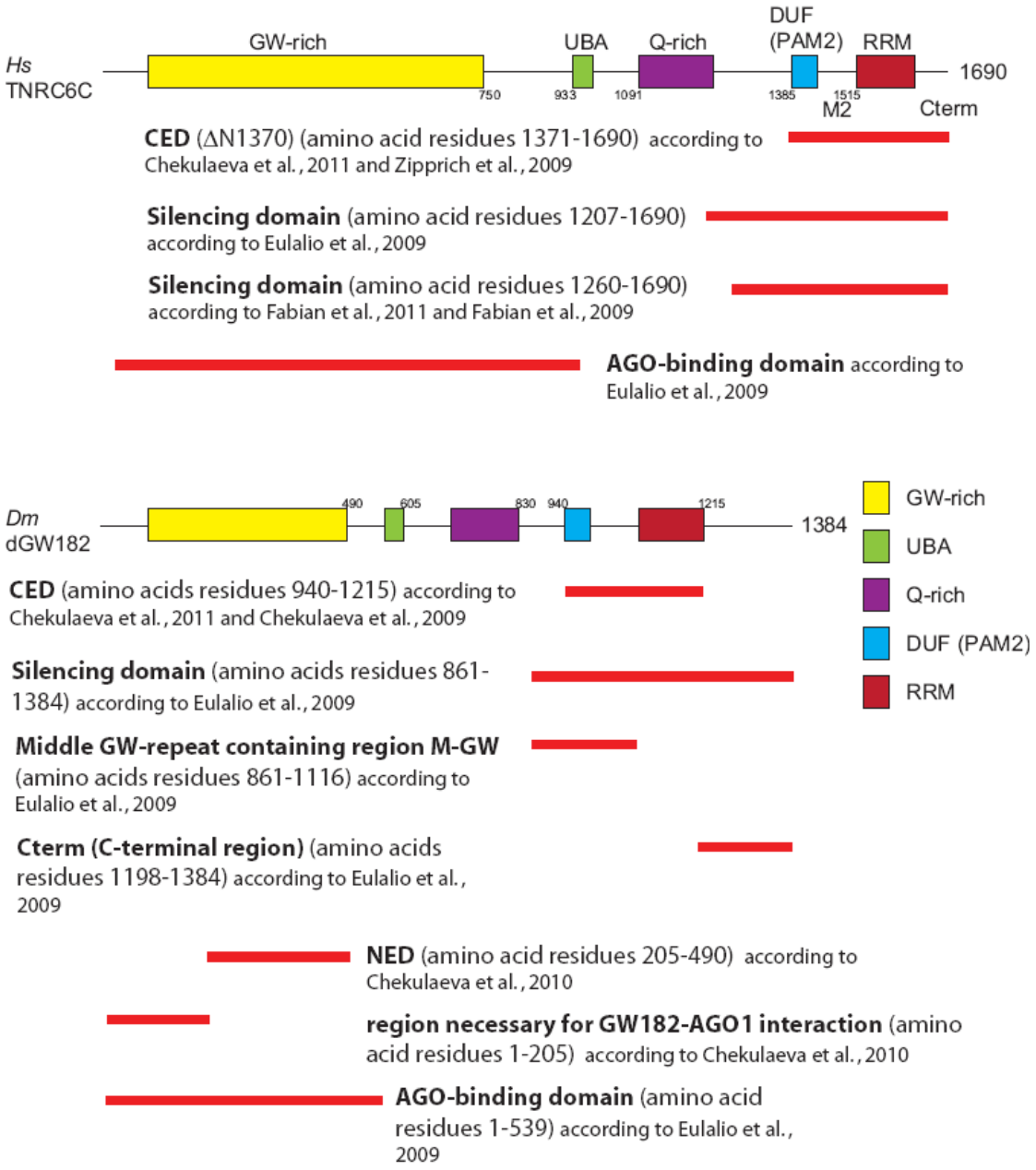

Figure 3.1: Domains and important regions of selected GW182 proteins. Schematic representation of human (Hs) TNRC6C and *Drosophila* (Dm) GW182 proteins. Positions of GW-rich, Q-rich, UBA, DUF (PAM2), and RRM domains are indicated. Important regions of the proteins discussed in the text are marked with red. The regions indicated are based on the following references: (Chekulaeva et al., 2009; Chekulaeva et al., 2011; Chekulaeva et al., 2010; Eulalio et al., 2009a; Eulalio et al., 2009d; Fabian, 2011; Fabian et al., 2009; Zipprich et al., 2009). Figure modified from Zipprich et al., (2009).

TNRC6 proteins, a version of TNRC6A containing mutations in residues critical for CED function, fails to do so completely. Third, in contrast to wild-type TNRC6C, TNRC6C containing mutations critical for CED function fails to rescue miRNA-mediated repression in Drosophila S2 cells depleted of DmGW182.

These observations are in agreement with findings by Lazzaretti et al., (2009) showing that the N-terminal parts of TNRC6A, TNRC6B and TNRC6C proteins containing the sequence upstream of the UBA domain were inactive in the tethering assay in HEK293 cells whereas the C-terminal fragments of the proteins strongly reduced reporter RNA activity. Furthermore, Huntzinger et al., (2010) found that in contrast to wild-type TNRC6A, a mutant TNRC6A lacking the silencing domain was unable to rescue miRNA-mediated silencing in HeLa cells depleted of TNRC6A and TNRC6B. Similarly, the silencing activity of zebrafish TNRC6A in a tethering assay in zebrafish embryos was found to depend largely on a C-terminal part encompassing amino acids 1310-1567 and this part of the protein was also sufficient to induce silencing to the same extent as the full length protein (Mishima et al., 2012).

A key role in miRNA-mediated silencing was also demonstrated for the C-terminal region of *Drosophila* GW182. Deleting the middle (M-GW) and the Cterm regions of *Drosophila* GW182 completely abolished its silencing activity in a complementation assay in *Drosophila* S2 cells (Eulalio et al., 2009a) and in a tethering assay a C-terminal region of DmGW182 was sufficient to silence bound reporter transcripts (Chekulaeva et al., 2009).

What do we know about the contribution to miRNA-mediated silencing of regions of GW182 other than its C-terminal part? It is well established, and our data confirm it, that the N-terminal part of GW182 mediates the interaction with AGO proteins (Behm-Ansmant et al., 2006; Lazzaretti et al., 2009; Till et al., 2007) - but does it have additional (more direct) functions in silencing? The following evidence indicates a contribution of the N-terminal region of GW182 proteins to silencing. First, we observed mild repression of transcripts bound to fragments containing the N-terminal GW-rich region or the Q-rich region of TNRC6C in a tethering assay. Similarly, a fragment of the N-terminal part of TNRC6A (amino acids 896-1045) induced repression of bound mRNA in HEK293 cells (Yao et al., 2011). Second, N-terminal fragments of DmGW182 strongly repressed bound transcripts in *Drosophila* S2 cells (Chekulaeva et al., 2009) as well as in HEK293 cells in a cross-species experiment and induced their deadenylation in a tethering assay in S2 cell lysates (Fukaya and Tomari, 2011). Third, N-terminal fragments of DmGW182 encompassing the N-terminal GW-rich region or both the N-terminal GW-rich region and the Q-rich region, partially rescued miRNA-mediated silencing in S2 cells depleted of endogenous GW182 (Chekulaeva et al., 2009).

It is not clear why the N-terminal part of human TNRC6C can induce silencing in the tethering assay whereas in a complementation assay its paralog, TNRC6A, with

mutations only in the C-terminal region is inactive in silencing. Also, it needs to be explained why the N-terminal region of DmGW182 can rescue miRNA-mediated silencing of one (Chekulaeva et al., 2009) but not other (Eulalio et al., 2009a) reporters.

More detailed analysis of the C-terminal region of the human TNRC6C protein revealed that the M2 and Cterm regions are required and sufficient for inducing efficient repression in a tethering assay and for the interaction with the CCR4-CAF1-NOT complex components.

In support of these data, the M2 and Cterm regions of DmGW182 and human TNRC6B were found to contribute to miRNA-mediated silencing in complementation assays in S2 cells (Huntzinger et al., 2010) and a function in miRNA-mediated silencing was also demonstrated for the M2 and Cterm regions of TNRC6A in a complementation assay in HeLa cells (Braun et al., 2011). Further, in a mammalian cell-free extract from mouse Krebs-2 ascites cells, deadenylation induced by a tethered C-terminal fragment of TNRC6C was severely reduced when the tethered fragment was lacking the Cterm region (Fabian, 2011). Also, the M2 and Cterm regions of the silencing domain of TNRC6B were shown to contribute to the interaction with *in vitro* translated CNOT1 (Braun et al., 2011) and the interaction between recombinant TNRC6C silencing domain and CCR4-CAF1-NOT complex components from HeLa cell extracts largely depended on the Cterm region (Fabian, 2011).

What is the role of the RNA recognition motif (RRM) which represents the most conserved part of vertebrate and insect GW182 proteins? Tethering the RRM of TNRC6C did not induce repression of bound RNA which is in line with the observation that the RRM also was not observed to interact with the CCR4-CAF1-NOT complex. However, introducing point mutations into the RRM of the CED fragment and deleting the RRM from the CEDΔPAM2 fragment modestly but significantly reduced silencing, indicating that the RRM is required for maximal silencing efficiency. A modest contribution of the RRM domain of DmGW182 to miRNA-mediated silencing was also observed by (Eulalio et al., 2009c). Taken together these data indicate a modest contribution of the RRM domain to GW182-mediated silencing but the mode of this contribution is not clear.

Our findings together with data of others indicate that GW182 proteins act as poly(A)-binding protein (PABP)-interacting proteins. Mass spectrometry analysis

identified PABP as an interaction partner of the C-terminal domain of TNRC6C and subsequent deletion and mutational analysis of the CED revealed that the PAM2 region is essential for the interaction between the CED and PABP. The PAM2 region (previously termed domain of unknown function (DUF)) shows sequence similarity with the PABP-interacting motif 2 (PAM2) which is present in PAIP1 and PAIP2 and other proteins involved in translation and mRNA degradation. The PAM2 motif of PAIP1 and PAIP2 binds to the C-terminal MLLE domain of PABP (Kozlov et al., 2001). Indeed, structural and biochemical analyses demonstrated that the PAM2 motif of TNRC6C binds to the MLLE domain of PABP in a similar way as the PAM2 of PAIP1 and PAIP2 (Fabian et al., 2009; Jinek et al., 2010; Kozlov et al., 2010).

Several lines of evidence indicate that the interaction between GW182 proteins and PABP has a functional role in miRNA-mediated silencing. First, we observed that mutating residues of the PAM2 region characterized to be critical for PABP interaction, interfered with the activity of TNRC6A in miRNA-mediated silencing in a complementation assay. The PAM2 region of TNRC6A was also found to contribute to miRNA-mediated silencing by Huntzinger et al., (2010) and Braun et al., (2011). Second, an excess of PAIP2-derived PAM2 peptide that competes with the TNRC6C-PABP interaction, interfered with miRNA-mediated deadenylation in mouse Krebs-2 ascites cell extract (Fabian et al., 2009). In the same *in vitro* system, mutation of residues of the PAM2 region critical for the interaction with PABP reduced the rate of deadenylation by the C-terminal domain of TNRC6C in a tethering assay (Fabian, 2011; Jinek et al., 2010). Finally, a mutation introduced into the PAM2 motif of a fragment of zebrafish TNRC6A that completely abolished interaction with PABP, slightly reduced repression activity of the fragment in a tethering assay in zebrafish embryos (Mishima et al., 2012).

It is not clear why we did not observe any significant influence of deleting or mutating the PAM2 region on the silencing activity of tethered CED in HEK293 cells. Possibly, a modestly reduced rate of deadenylation would not influence the protein steady state level. Further, the function mediated by the PAM2 region may be dispensable when the CED is tethered to a target mRNA, consistent with the observation that PABP promotes association of miRISC with miRNA-regulated mRNAs (Moretti et al., 2012).

3.2 The role of tryptophan-containing motifs in GW182 proteins

In this work we provide evidence that the human and *Drosophila* GW182 proteins recruit the CCR4-CAF1-NOT deadenylase complex in a PABP-independent manner via tryptophan-containing motifs (W-motifs). The following evidence supports the idea that W-containing motifs in GW182 proteins are crucial elements for recruiting the deadenylase complex and inducing repression. First, mutational analysis of sub-fragments of the TNRC6C CED identified W-containing motifs in the M2 and Cterm regions required for silencing activity and interaction with the CCR4-CAF1-NOT deadenylase complex. The analysis demonstrated a strong correlation between repression and interaction with the CCR4-CAF1-NOT complex. Second, introducing an increasing number of tryptophan to alanine mutations in the CEDs of TNRC6C and DmGW182 caused a gradual alleviation of repression mediated by these domains in tethering assays. Third, tryptophan-containing motifs in the N-terminal effector domain (NED) of DmGW182 that were shown to be essential for the silencing activity of this domain (Chekulaeva et al., 2010), are also crucial for the interaction with the deadenylase complex. Fourth, tryptophan-containing motifs proved to be essential for maximal miRNA-mediated silencing activity of DmGW182, TNRC6C and TNRC6A in complementation assays. Fifth, introducing tryptophan residues into an unstructured fragment of the yeast Sic1p enabled the engineered protein to mediate repression in tethering assays and to interact with the CCR4-CAF1-NOT complex.

The crucial role of W-motifs in miRNA-mediated silencing is further supported by the finding of Fabian et al., (2011) who demonstrated that the interaction between TNRC6C and the CCR4-CAF1-NOT complex is mediated via two conserved tryptophan-containing motifs that were also found to be important for promoting deadenylation of targeted mRNA. Furthermore, substitution of all tryptophan residues in the *C. elegans* GW182 protein AIN-1 abrogated its interaction with CeALG-1, CeNOT1, CeNOT2, and CePAN3 (Kuzuoglu-Ozturk et al., 2012).

How do the W-motifs promote the interaction with the CCR4-CAF1-NOT complex? The involvement of multiple hydrophobic tryptophan residues is reminiscent of previously reported examples of protein-protein interactions. For example, the interaction between the spliceosomal component SF3b155 with the large subunit of

the U2 small nuclear RNA auxiliary factor (U2AF65) is mediated via multiple motifs containing an essential tryptophan. Similarly to GW182 proteins, the tryptophan containing motifs are located in an unstructured region of SF3b155 (Thickman et al., 2006). Another example are the (FG)-nucleoporines which are characterized by unfolded domains with extensive repeats of phenylalanine-glycine (FG) repeats and act as a selectively permeable barrier in nuclear pore complexes (Walde and Kehlenbach, 2010). Importantly, structural studies revealed that the FG repeats directly can bind to multiple hydrophobic FG-binding sites formed by α-helices of HEAT repeats in nuclear transport receptors (Bayliss et al., 2000; Terry and Wente, 2009). It will be interesting to determine whether a similar structural principle applies to the interaction between GW182 and deadenylase complexes.

Based on the observations that first, GW182 proteins interact with the poly(A)-binding protein (Fabian et al., 2009; Zekri et al., 2009) and second, that CCR4-CAF1-NOT complex components are co-precipitated with the poly(A)-binding protein (Zekri et al., 2009), it has been suggested that PABP might act as an adaptor protein allowing GW182 to recruit the CCR4-CAF1-NOT deadenylase complex to the mRNA to induce miRNA-mediated deadenylation (Zekri et al., 2009). Our data indicate that GW182 proteins can recruit the CCR4-CAF1-NOT deadenylase complex in a PABP-independent manner. First, deletion or mutation of the PAM2 motif which abrogates the interaction of the C-terminal domain of human TNRC6C with PABP, did not affect the interaction of the CED with the deadenylase complex components. Second, the CED containing 7 tryptophan to alanine mutations still interacted with PABP but not with the CCR4-CAF1-NOT complex components. Similarly, the interaction between TNRC6C and PABP was unaffected by the deletion of the M2 and Cterm regions of TNRC6C, whereas these deletions significantly reduced the interaction between TNRC6C and the CCR4-CAF1-NOT complex components. Third, an N-terminal fragment of DmGW182 (amino acids 1-490) and the Sic1p fragment containing tryptophan residues interacted with CCR4-CAF1-NOT complex components but not with PABP.

It is likely that the interaction between GW182 proteins and the CCR4-CAF1-NOT complex is mediated via the CNOT1 protein, as a fragment of human CNOT1 but not CNOT7/CAF1 or CNOT6/CCR4 interacted with the CED in a yeast two-hybrid assay. Furthermore, depletion of CNOT1 in HEK293 cells strongly reduced the interaction between TNRC6C and CNOT2, CNOT6/CCR4, or CNOT7/CAF1 (Braun et al., 2011). In addition, in HeLa cells depleted of CNOT1, the C-terminal region of TNRC6C failed to interact with components of the CCR4-CAF1-NOT complex (Fabian, 2011).

Finally, in a pull-down assay with recombinant proteins expressed in E. coli, a GST fusion of the C-terminal domain of TNRC6B interacted with CNOT1 *in vitro* (Braun et al., 2011) and the recombinant silencing domain of TNRC6C expressed in E. coli interacted with human CNOT1 produced in insect SF9 cells (Fabian, 2011).

3.3 The role of the poly(A) tail in silencing mediated by the CED and components of the CCR4-CAF1-NOT complex

MiRNAs regulate gene expression through translational repression and mRNA deadenylation and degradation. However, the relative contribution of these effects is unclear. Whereas some studies reported mRNA decay as a predominant effect (Baek et al., 2008; Bagga et al., 2005; Guo et al., 2010; Lim et al., 2005) other studies show translational repression without accompanying mRNA decay (Lee et al., 1993; Mathonnet et al., 2007; Olsen and Ambros, 1999; Pillai et al., 2005; Thermann and Hentze, 2007).

Our data suggest, that the C-terminal region of TNRC6C can mediate all the effects attributed to miRNAs: translational repression, deadenylation, and mRNA decay. In tethering assays the CED reduced the reporter mRNA level maximally five times but reduced the reporter protein level 10 times, indicating that part of the repression is due to inhibition of translation. Examination of the poly(A) tail status revealed that the reporter mRNA that escaped degradation by tethered TNRC6C or tethered CED was not deadenylated, indicating that translational repression of the reporter is not due to removal of the poly(A) tail but rather results from a more direct interference with the translation process. In line with this idea, the following evidence indicates that the C-terminal regions of GW182 proteins are able to repress not only polyadenylated but also poly(A)-free mRNAs. First, the CEDs of human TNRC6C and DmGW182 were able to repress mRNAs in which the polyadenylation signal is substituted by a histone stem-loop (HSL) or a hammerhead ribozyme (HhR). Second, the CED of human TNRC6C repressed *in vitro* transcribed poly(A) tail-free mRNAs that were transfected into HEK293 cells. It is unlikely that these *in vitro* transcripts were repressed only upon their polyadenylation in cells, as incorporation of a cordycepin residue at the 3` end of the *in vitro* transcripts (cordycepin lacks the

ribose 3` OH group that would be required for the extension of the RNA with additional nucleotides) did not reduce repression induced by the tethered CED.

Further, our data indicate that the repression of poly(A)-free mRNAs by GW182 proteins is mediated via the CCR4-CAF1-NOT deadenylase complex. First, we observed that the W-motifs, which are essential for the CED - CCR4-CAF1-NOT complex interaction, are also required for the repression of poly(A)-free mRNAs by the tethered CEDs of human TNRC6C and DmGW182 (data for human TNRC6C are not shown). Second, tethering of CCR4-CAF1-NOT complex components in HEK293 or S2 cells repressed not only polyadenylated but also poly(A)-free mRNAs and the RNA levels of poly(A)-free mRNAs were either not affected at all or only slightly reduced, indicating that the major part of the repression was due to inhibition of translation. Third, repression of poly(A)-free mRNAs in *Drosophila* S2 cells by tethered DmGW182 or its CED depended on NOT1 but repression by tethered CAF1 or CNOT1 was not affected by the depletion of GW182, indicating that NOT1 acts downstream of GW182 in the repression of poly(A)-free mRNAs.

The ability of the CCR4-CAF1-NOT deadenylase complex to mediate translational repression is corroborated by the findings of Cooke et al., (2010) who demonstrated that tethered CAF1 can repress translation of microinjected mRNAs at the step of initiation in *Xenopus laevis* oocytes. Confirming our observations, an involvement of NOT1 in DmGW182-mediated repression of a poly(A)-free reporter mRNA, was also observed by Braun et al., (2011).

Collectively, these data indicate that recruitment of the CCR4-CAF1-NOT complex via tryptophan-containing motifs in GW182 proteins can induce both translational repression and deadenylation of mRNAs targeted by miRNAs.

3.4 Discussion of our data and the current literature addressing the mechanism of miRNA-mediated gene silencing

Our findings revealing the GW182-mediated recruitment of PABP and deadenylase complexes are in accordance with previous studies indicating that mRNA degradation is a widespread effect of miRNA regulation as well as studies demonstrating a role of mRNA decay factors in miRNA-mediated repression (reviewed in Fabian et al., (2010) and Huntzinger and Izaurralde, (2011)). Importantly,

our findings indicate that miRNA-mediated deadenylation may be at least partially a direct effect of miRNA regulation (due to the recruitment of deadenylase complexes by GW182) and not merely a consequence of a translational block. This idea is consistent with data showing that even mRNAs with an artificial Appp-cap structure that impairs translation undergo miRNA-mediated deadenylation (Fabian et al., 2009; Mishima et al., 2006; Wakiyama et al., 2007). Collectively, these data support the notion that mRNA deadenylation and decay are an important component of miRNA-mediated gene silencing (Djuranovic et al., 2011; Huntzinger and Izaurralde, 2011). The evidence that deadenylation is a widespread effect of miRNA regulation raises the question whether deadenylation alone could account for translational repression of target mRNAs. This scenario is unlikely as we observed that tethering GW182 or its C-terminal domain as well as tethering of the deadenylase complex components can also repress reporter mRNAs lacking a poly(A) tail. Our observations are consistent with data of others demonstrating the ability of GW182 and miRNAs to repress poly(A) tail-free mRNAs (Braun et al., 2011; Eulalio et al., 2008b; Eulalio et al., 2007c; Wu et al., 2006). Furthermore, reporter mRNAs refractory to deadenylation were still found to be repressed by miRNAs (Fukaya and Tomari, 2011; Mishima et al., 2012) and kinetic analyses of miRNA-mediated silencing revealed that translational repression preceds detectable deadenylation (Bazzini et al., 2012; Djuranovic et al., 2012; Fabian et al., 2009; Zdanowicz et al., 2009)(Béthune et al., 2012). Collectively, these data indicate that besides deadenylation an additional mechanism of translational repression is operating during miRNA-mediated repression and that the CCR4-CAF1-NOT complex is involved in this process.

What do we know about this mechanism of translational repression? Although we have not performed more detailed experiments addressing this question ourselves, Cooke et al., (2010) reported that CAF1, a component of the CCR4-CAF1-NOT complex, represses translation in a m^7G cap dependent manner in *Xenopus laevis* oocytes. This finding is reminiscent of studies suggesting that miRNAs inhibit translation initiation and studies that reported a importance of the m^7G cap structure in repression (Bhattacharyya et al., 2006; Ding and Grosshans, 2009; Mathonnet et al., 2007; Pillai et al., 2005; Thermann and Hentze, 2007; Zdanowicz et al., 2009). Therefore, it is intriguing to speculate that the CCR4-CAF1-NOT deadenylase complex contributes to the repression of translation initiation in a m^7G cap dependent manner during miRNA-mediated gene silencing. Interestingly, the CCR4-CAF1-NOT complex is known to interact with the decapping activator and translational repressor Dhh1/Me31b in yeast and *Drosophila* (Coller et al., 2001; Temme et al., 2010) and indeed, orthologs of Dhh1 have been reported to be required for miRNA-mediated

repression in other organisms (Chu and Rana, 2006; Eulalio et al., 2007c). Thus, these data suggest a possible mechanism by which the CCR4-CAF1-NOT complex may repress translation.

What could be the role of the interaction between GW182 and PABP in miRNA-mediated gene silencing? Interestingly, in a tethering assay in zebrafish embryos it was observed that the PAM2 motif contributed to translational repression of a reporter that is refractory to deadenylation. Because the contribution was no longer observed in the presence of excess PAIP2, a protein that was shown to displace PABP from the poly(A) tail and eIF4G (Sonenberg and Dever, 2003), the authors concluded that the PAM2 motif contributes to translational repression by counteracting the function of PABP in translation (Mishima et al., 2012). PABP is thought to function in translation by binding to eIF4G and thereby promoting the formation of an mRNA closed loop. Indeed, one attractive model of miRNA action states that GW182 competes with eIF4G for binding to PABP and thereby GW182 can interfere with the formation of an mRNA closed loop (which is thought to stimulate mRNA translation). Supporting this model, cell extract containing overexpressed GW182 silencing domain can compete away eIF4G from PABP (Zekri et al., 2009). Further evidence which may support this model is provided by the observations that adding a fragment of eIF4G that binds PABP blocks miRNA-mediated deadenylation *in vitro* (Fabian et al., 2009) and that overexpression of PABP in HEK293T cells reduced miRNA-mediated repression (Walters et al., 2010). It is not known whether and how the binding of the PAM2 region to the C-terminal MLLE domain of PABP can influence the interaction of eIF4G with the N-terminal RRMs of PABP. An alternative but not mutually exclusive model states that, similarly as described for PAIP2 (Derry et al., 2006), the interaction between GW182 and PABP could reduce the affinity of PABP for the poly(A) tail which would expose the poly(A) tail to deadenylases and interfere with mRNA circularization (Huntzinger and Izaurralde, 2011). Indeed, a GW182 silencing domain fragment unable to bind PABP failed to induce processive deadenylation (Fabian, 2011). Rather, the deadenylation process was blocked along the poly(A) tail at increments of approximately 27 nucleotides, which is the number of adenosines bound by a single PABP molecule, arguing that the GW182 silencing domain displaces PABP from the poly(A) tail to allow for processive deadenylation (Fabian, 2011). Another idea about the role of the GW182-PABP interaction states that this interaction may juxtapose the PABP-associated poly(A) tail with the miRISC associated deadenylase complex to facilitate initiation of the deadenylation reaction (Fabian et al., 2009; Fabian et al., 2010).

In summary, our data and evidence published by others put forth the following model of miRNA-mediated gene silencing. GW182 acts as key repressor mediating miRNA-induced silencing by interacting with PABP via its PAM2 motif and by recruiting the PAN2-PAN3 and CCR4-CAF1-NOT deadenylase complexes via tryptophan-containing motifs. Both, the contact to PABP and the recruitment of the CCR4-CAF1-NOT complex mediate translational repression and facilitate deadenylation of target mRNAs.

Although this model is in accordance with the majority of published data about the mechanism of miRNA-mediated gene silencing - not surprisingly - it cannot explain all reported observations. For example, this model does not predict any regulatory events of translation at post-initiation steps.

It is important to note that our own data described in this thesis are the result of experiments with only two cell types: cultured mammalian HEK293 cells and *Drosophila* S2 cells. Similarly, the current view of miRNA regulation is based on studies in a limited set of cell types typically cultured *in vitro* (Fabian et al., 2010; Huntzinger and Izaurralde, 2011). Therefore, it will be interesting to investigate the mechanism of miRNA-mediated silencing in a more diverse set of cell types in their physiological context to learn whether miRNAs function in a cell- and/or development-dependent manner. For example, it has been suggested that in oocytes and embryonic or neuronal cells in which deadenylated mRNAs are often stable, miRNA targets may accumulate in a deadenylated, translationally silent form and eventually be translated again after being readenylated (Huntzinger and Izaurralde, 2011).

It will also be interesting to more comprehensively learn whether and how the mechanism of miRNA-mediated silencing is influenced in a target specific manner. It is well established, that the extent of miRNA-mediated repression can be influenced by miRISC- or mRNA-associating proteins (Bhattacharyya et al., 2006; Hammell et al., 2009; Kedde et al., 2007; Takeda et al., 2009). Moreover, evidence suggests that the mode of miRNA-mediated repression is influenced in a target specific manner (Eulalio et al., 2008a). Repression of some reporter miRNA targets in *Drosophila* S2 cells could be entirely accounted for by mRNA degradation whereas others were mainly silenced by translational repression or by a more equal contribution of both processes (Behm-Ansmant et al., 2006; Eulalio et al., 2009b; Eulalio et al., 2007c). To explain these differences it has been suggested that the mode of miRNA-mediated repression depends on specific features of the miRNA binding site and on

the specific complement of proteins associated with a given target mRNA (Eulalio et al., 2008a).

Investigation of these and many more aspects will define a more comprehensive mechanistic picture of miRNA-mediated gene silencing.

4. Materials and Methods

Most methodologies and materials used in this study have been described in the corresponding sections in the attached paper published in Nature Structural & Molecular Biology (Chekulaeva et al., 2011) and in the published papers attached as Appendices A (Zipprich et al., 2009) and B (Fabian et al., 2009).

In the following section I describe materials and methods used in experiments that are part of this study but have not yet been published.

4.1 DNA constructs

Plasmids encoding the fragments of CNOT1 used for tethering and pull-down assays were generated by PCR-amplification of the corresponding fragments of the plasmid NHA-CNOT1 (Chekulaeva et al., 2011) and cloning into pCI-neo vector bearing a NHA-tag (Pillai et al., 2004).

4.2 Tethering assays with NHA-tagged CNOT1 and fragments thereof

Transfection of human HEK293T cells were done in 6-well plates with Nanofectin (PAA Laboratories) according to the manufacturers instructions. In tethering experiments, cells were transfected with 50 ng RL-5BoxB (Pillai et al., 2004), 300 ng FL-Con (Pillai et al., 2004) and 400 ng HA- or NHA-fusion constructs per well. Cells were lysed 24 hours post-transfection and luciferase activities were measured with the Dual-Luciferase Reporter Assay System (Promega).

4.3 Pull-down assays

Pull-down assays with GST-tagged CED or CEDΔPAM2 and NHA-tagged CNOT1 and fragments thereof were performed as described previously (Chekulaeva et al., 2011).

Appendix A

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

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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

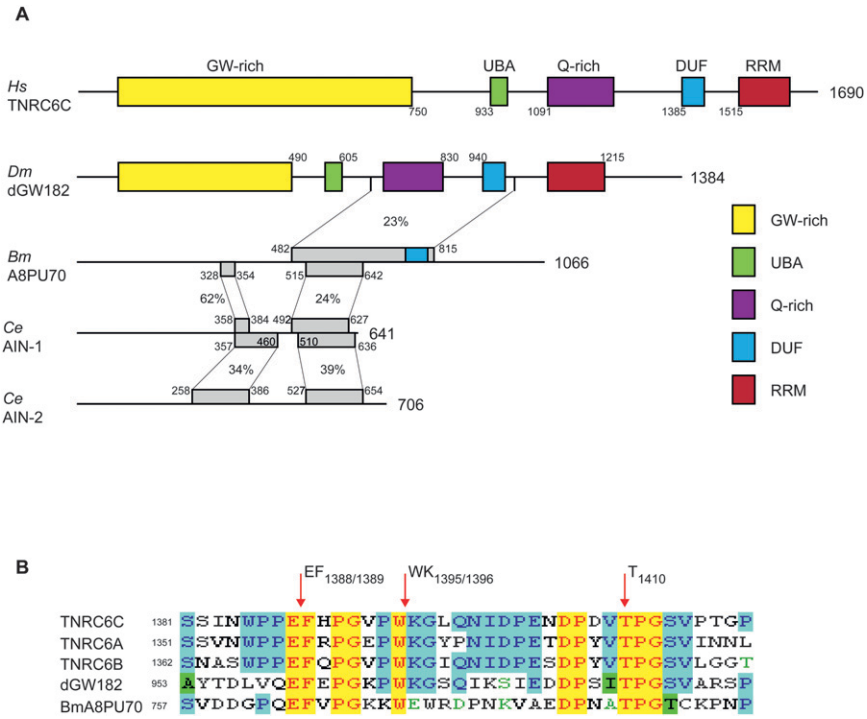

FIGURE 1. Domain structure of selected GW-182-like proteins. (*A*) Schematic representation of human (Hs) TNRC6A, TNRC6B, and TNRC6C proteins, the *Drosophila* (Dm) GW182 (dGW182), *C. elegans* (Ce) AIN-1 and AIN-2, and a candidate GW182 ortholog of *Brugia malayi* (Bm). Positions of GW-rich, Q-rich, 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

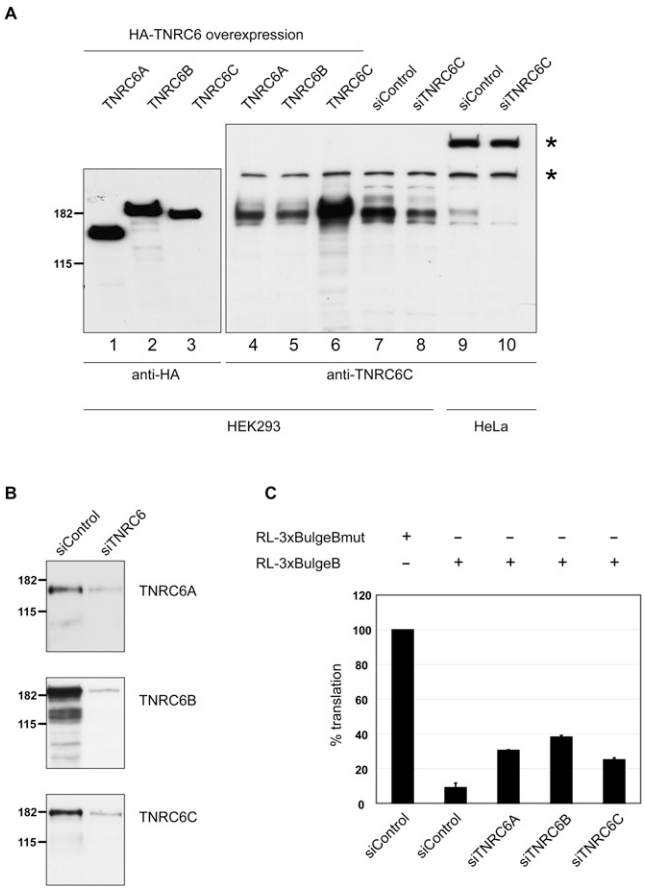

FIGURE 2. Expression of TNRC6C protein in HEK293 and HeLa cells and its importance for effective miRNA-mediated repression. (*A*) Anti-TNRC6C Abs specifically recognize endogenous TNRC6C in HEK293 and HeLa cell extracts and do not cross-react with overexpressed TNRC6A and TNRC6B proteins. (Lanes *1–6*) 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 than 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 the 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

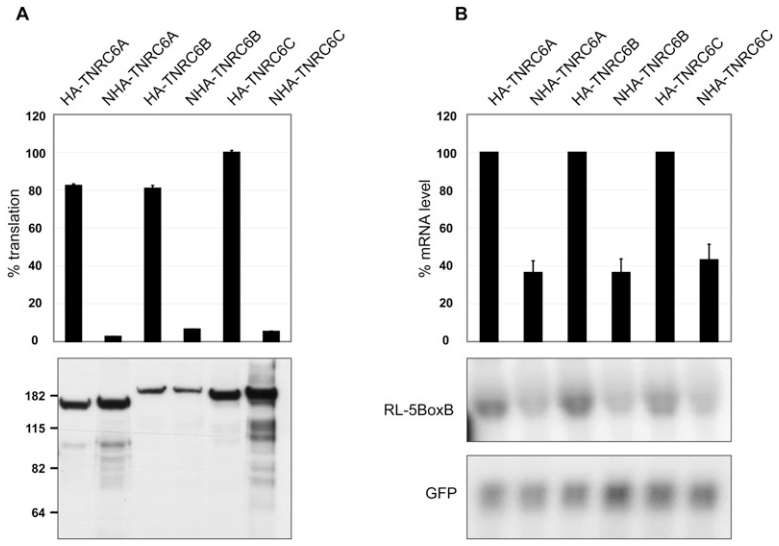

FIGURE 3. Tethering of TNRC6 proteins to mRNA causes strong repression of protein synthesis and partial mRNA degradation. (*A*) Indicated HA- or NHA-tagged TNRC6 proteins were coexpressed into HEK293 cells with RL-5BoxB, FL-Con, and (in some transfections) GFP reporters. (*Upper* panel) RL expression 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

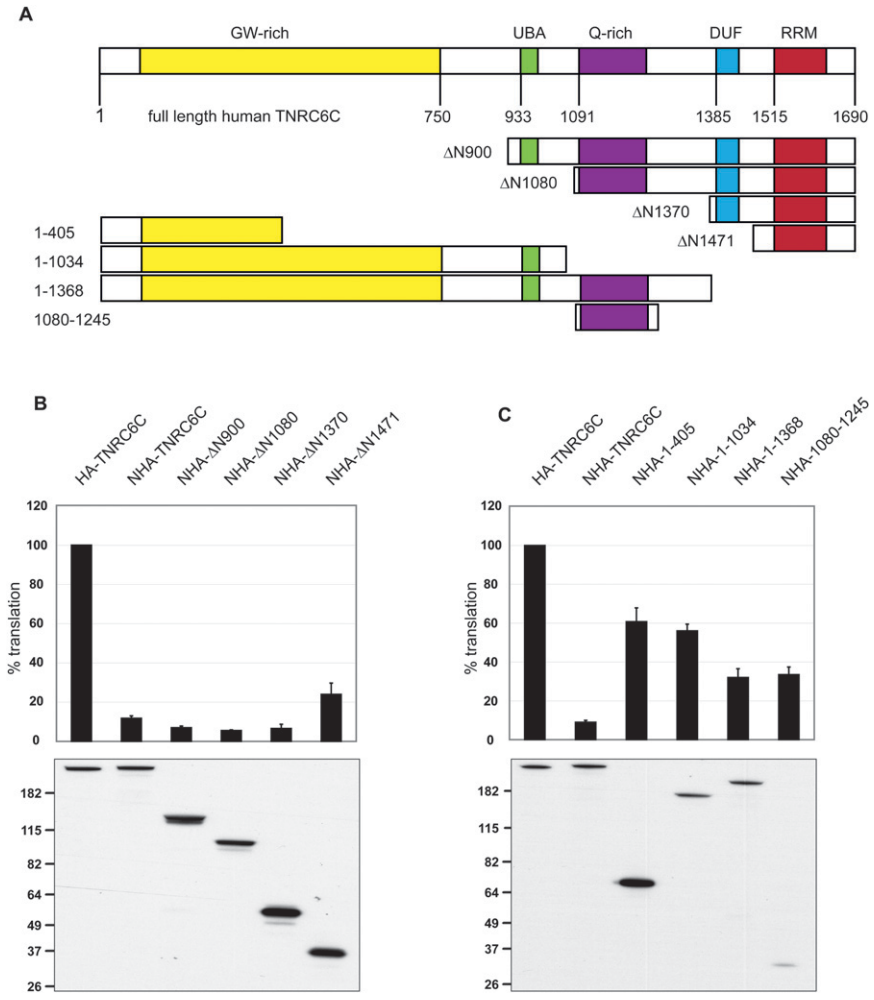

FIGURE 4. Characterization of the TNRC6C deletion mutants. (*A*) Schematic representation of TNRC6C and its deletion mutants. Mutants with progressive deletions from the (*upper* part of scheme) N terminus and (*lower* part of scheme) C terminus. Numbers correspond to amino acid positions. (*B*) The 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

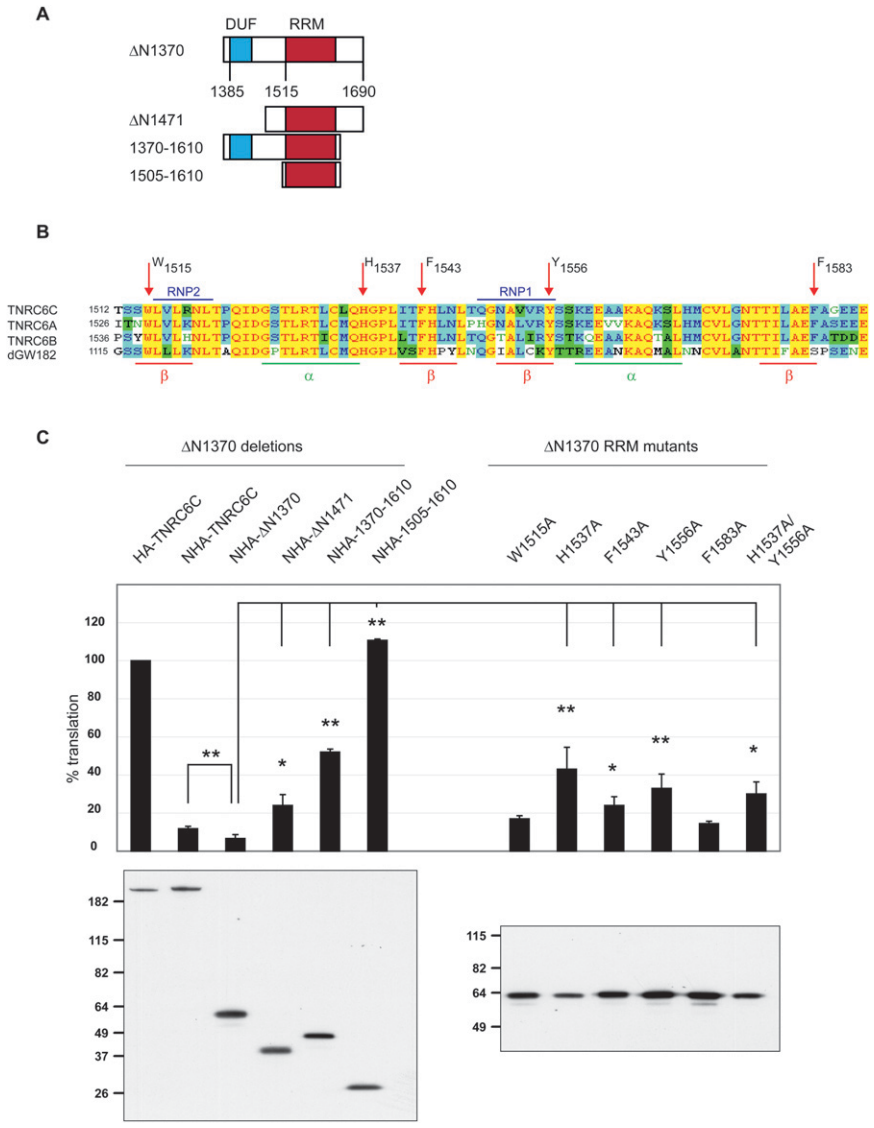

FIGURE 5. Detailed characterization of the C-terminal ΔN1370 fragment of TNRC6C. (*A*) Schematic representation of deletion mutants of the C-terminal ΔN1370 fragment of TNRC6C. (*B*) Sequence alignment of RRM domains of selected GW182 proteins. Aromatic amino acids mutated to alanines, either singly 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

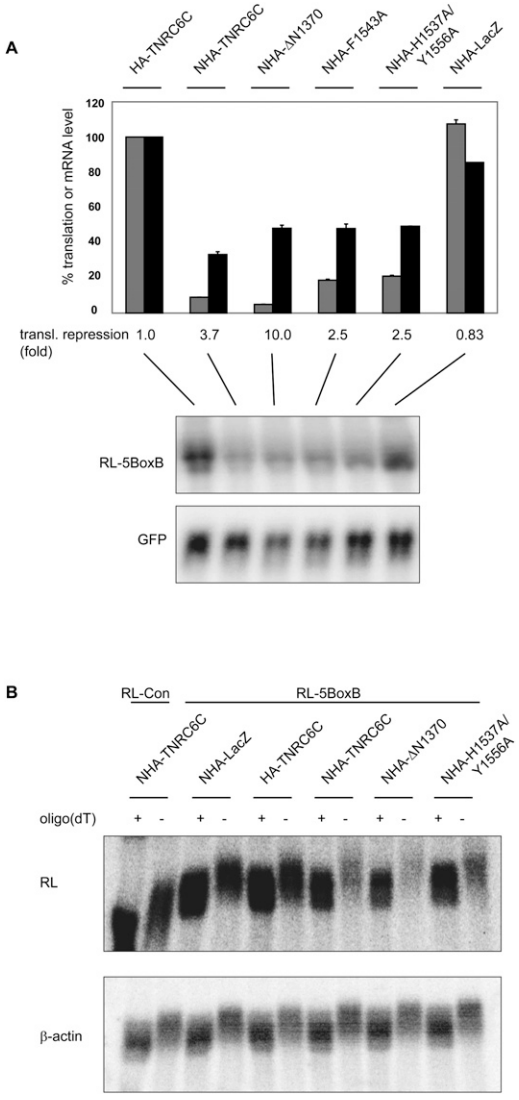

FIGURE 6. (Legend on next page)

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.

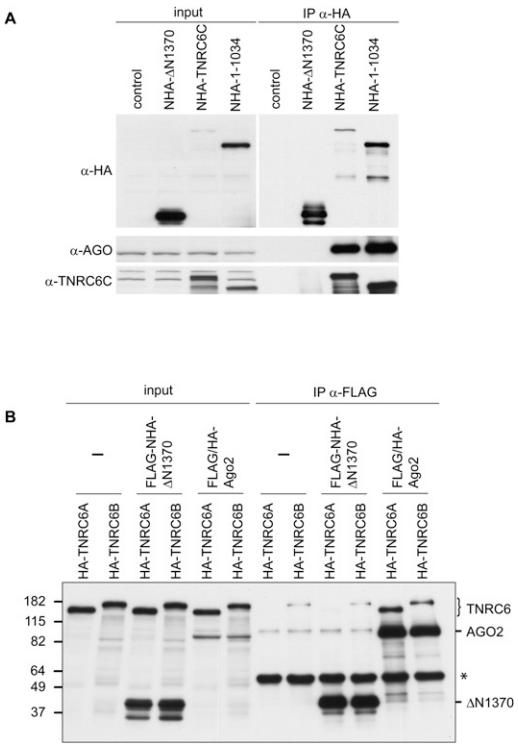

FIGURE 7. ΔN1370 does not interact with endogenous Ago and TNRC6C proteins. Cell extracts of HEK293 cells transiently expressing the indicated fusion proteins were incubated with anti-HA Affinity Matrix (Roche), and immunoprecipitated proteins (45% of the total immunoprecipitate) were analyzed by Western 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* the bars ($n = 3$, with the 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.

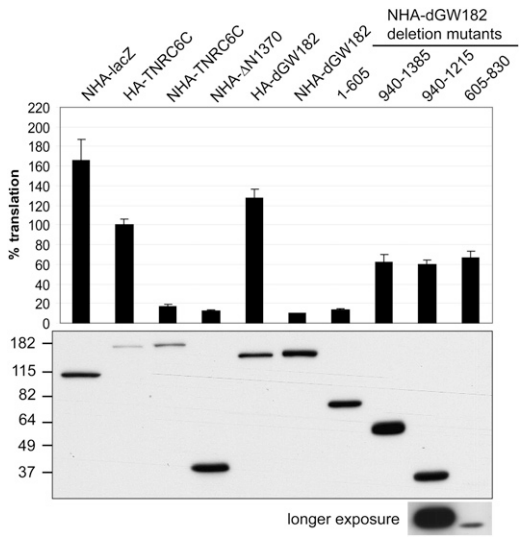

FIGURE 8. Effect of tethering of dGW182 and its deletion mutants on activity of RL-5BoxB reporter in human cells. (*Upper* panel) Tethering of dGW182 and its deletion mutants represses activity of RL-5boxB reporter in HEK293 cells. Indicated plasmids expressing human TNRC6C or *Drosophila* dGW182, or 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 of 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 from 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) were 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 were 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 the 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 were 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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Appendix B

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

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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

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

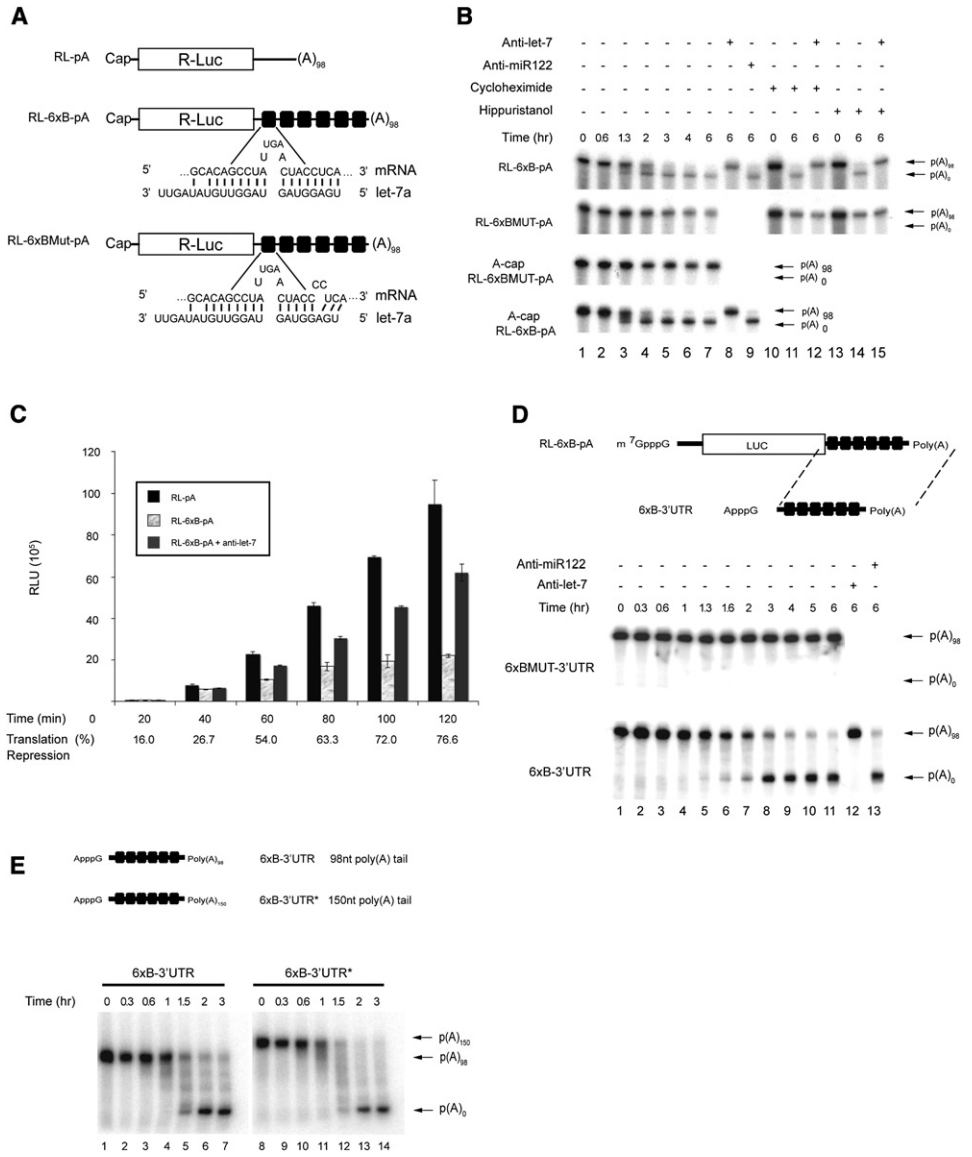

Figure 1. Deadenylation Mediated by let-7 miRNA in a Krebs Extract

(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).

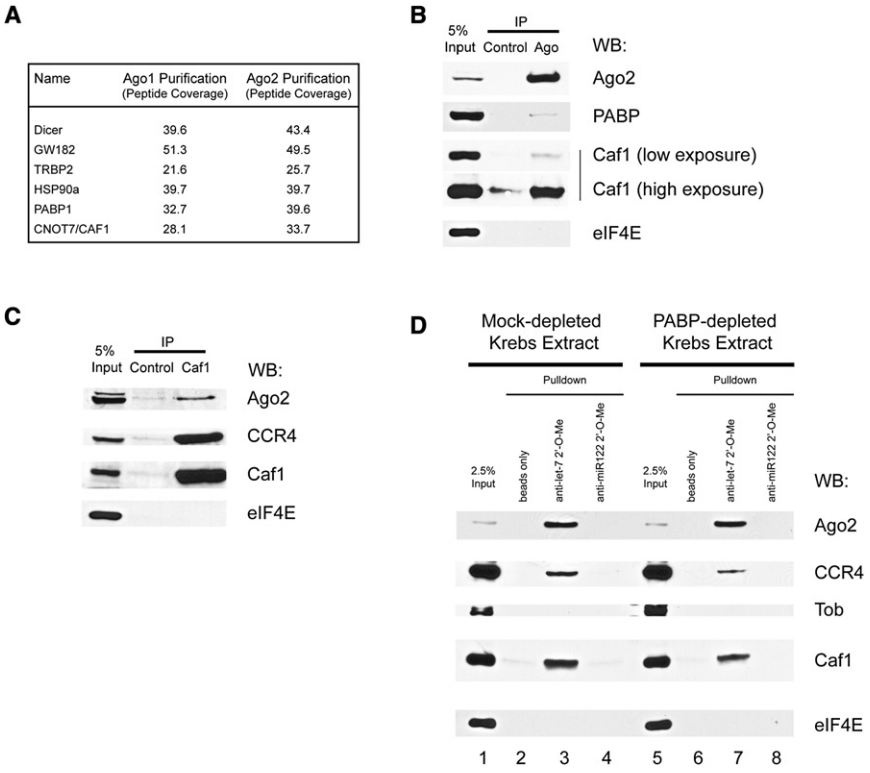

Figure 2. Ago Proteins Interact with PABP and the CAF1/CCR4 Deadenylase Complex

(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

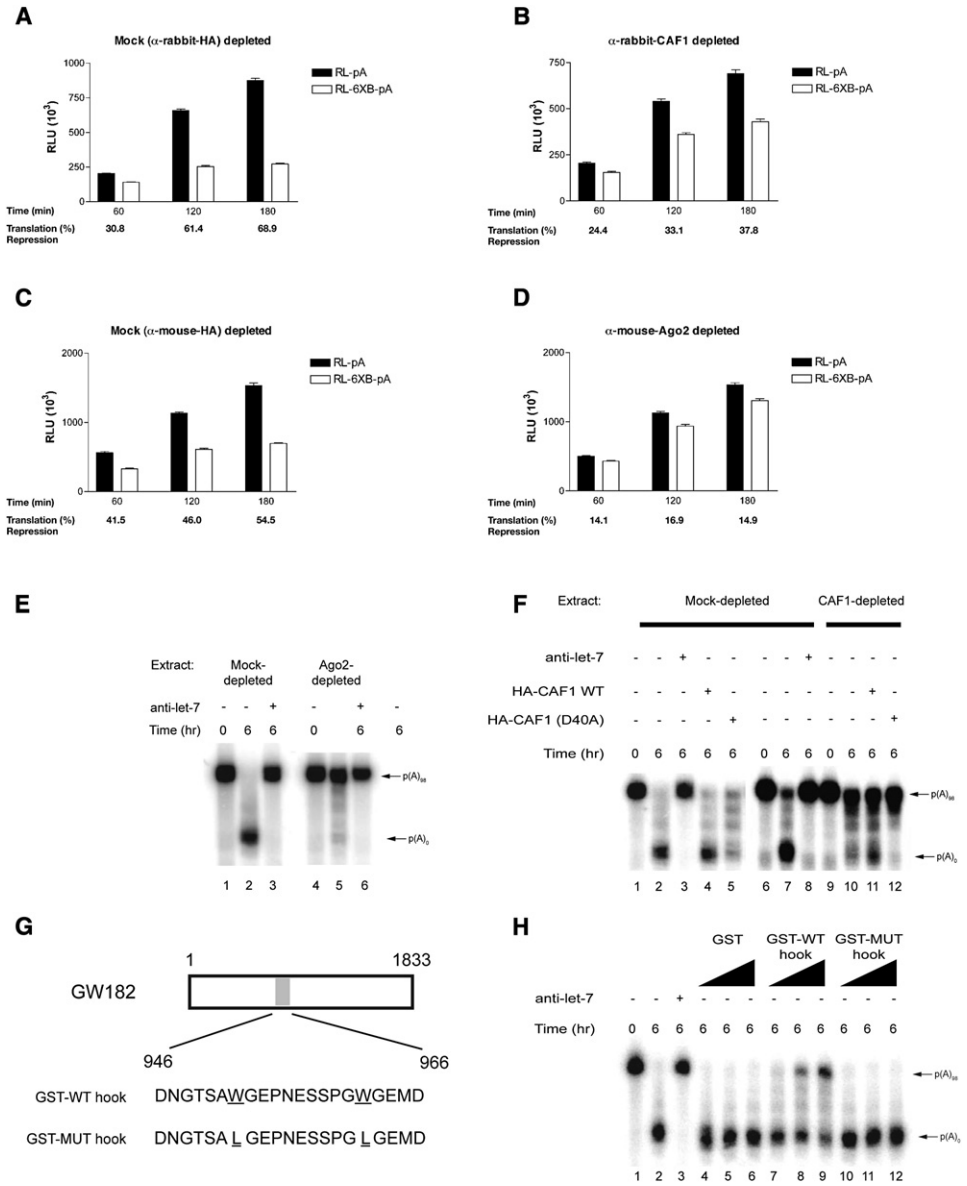

Figure 3. let-7-Mediated Deadenylation Requires CAF1, Ago2, and GW182

(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

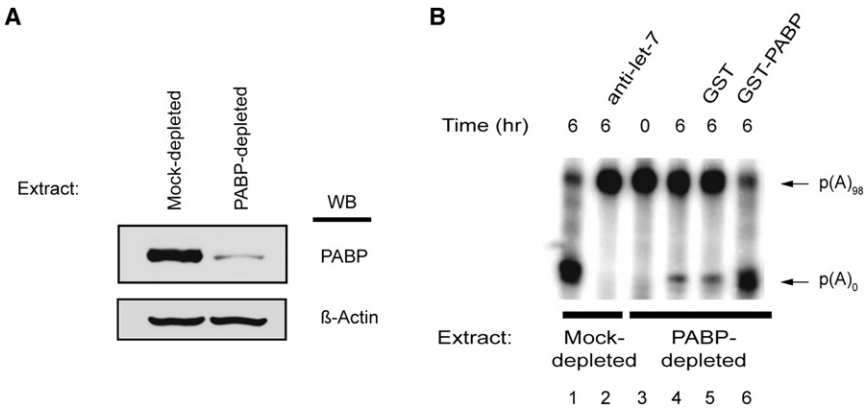

Figure 4. let-7-Dependent Deadenylation Requires PABP

(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

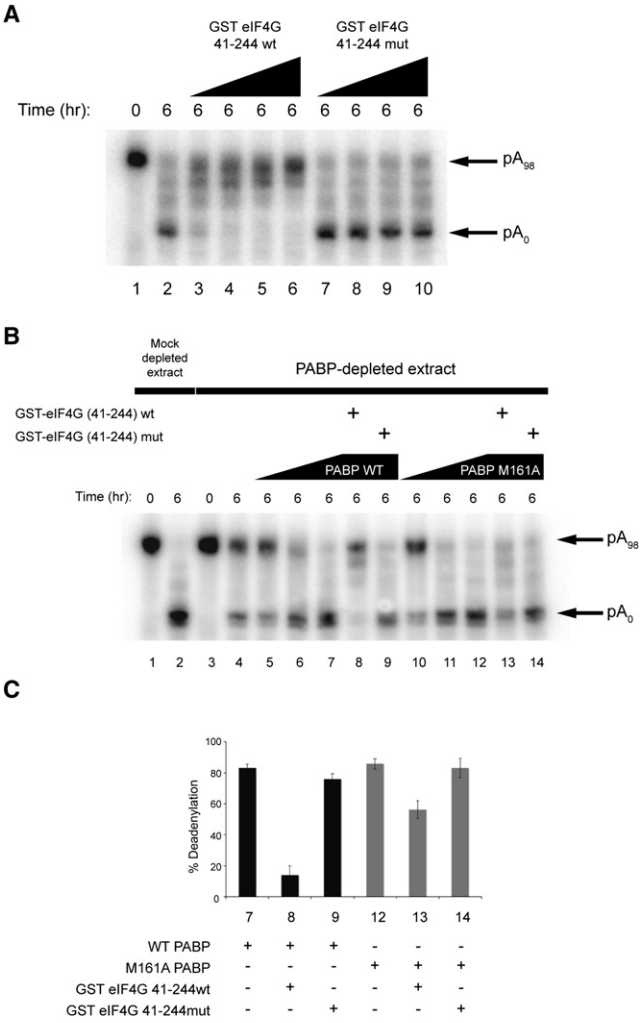

Figure 5. eIF4G Contact with PABP Antagonizes miRNA-Mediated Deadenylation

(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.

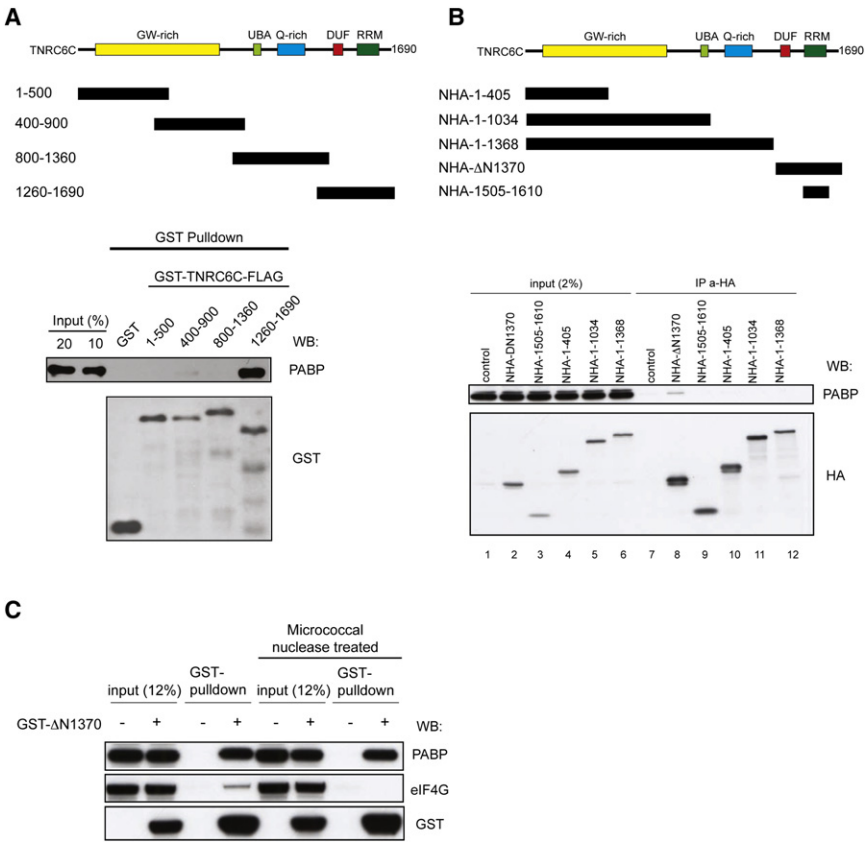

Figure 6. The C Terminus of TNRC6C Directly Binds PABP

(A) Schematic representation of human TNRC6C and GST- and FLAG-tagged recombinant protein fragments. Western blot analysis of GST pull-downs 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. Non-transfected 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.,

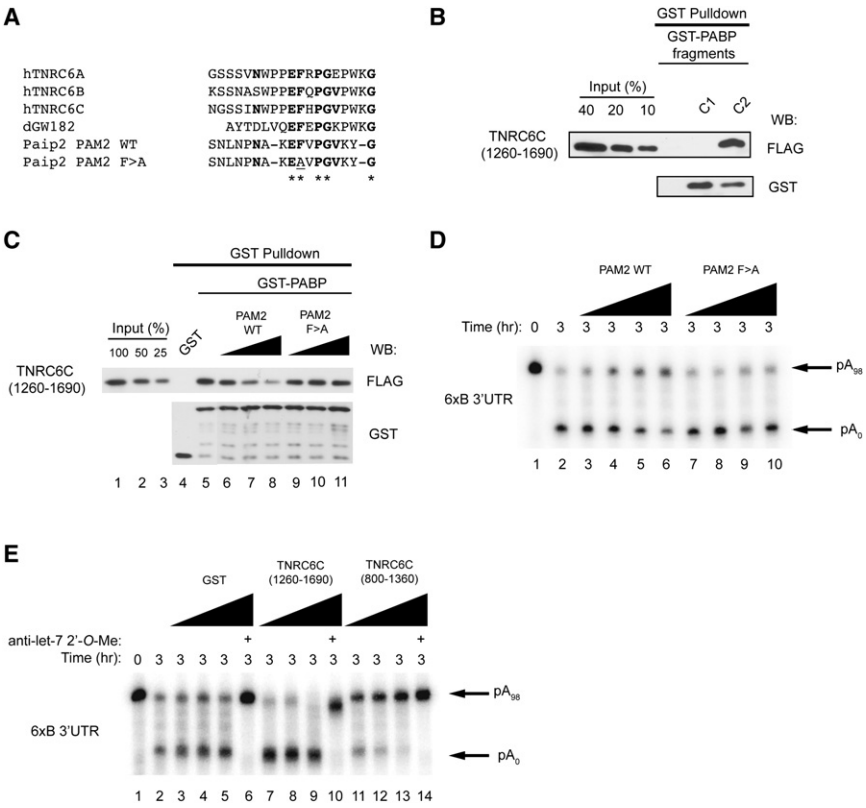

Figure 7. GW182 Binding to PABP Is Required for miRNA-Mediated Deadenylation

(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

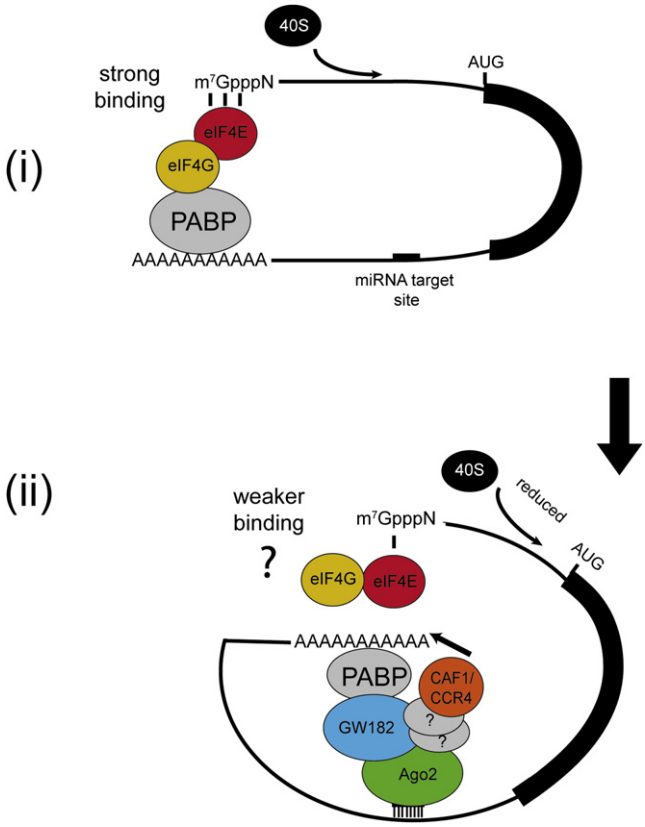

Figure 8. Model for Temporal Stepwise miRNA-Mediated Gene Silencing

(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

(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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1998-2002	Deutsches Gymnasium Biel
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Publications

Chekulaeva, M.*, Mathys, H.*, Zipprich, J.T., Attig, J., Colic, M., Parker, R., and Filipowicz, W. (2011). miRNA repression involves GW182-mediated recruitment of CCR4-NOT through conserved W-containing motifs. *Nature Structural & Molecular Biology*
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