MicroRNAs (miRNAs) are ~21-nucleotide-long RNAs involved in posttranscriptional regulation in metazoans and plants (5). miRNAs are first synthesized by RNA polymerase II as long primary transcripts in the nucleus (26). The majority of these transcripts undergo a first step of maturation where RNase III Drosha, with the help of the double-stranded RNA-binding domain protein DGCR8, excises the precursor miRNA hairpin of ~70 nucleotides (9, 19, 20). These hairpin precursors are later exported to the cytoplasm by exportin-5 (48), where they are further processed by a second RNase III protein, known as Dicer (22, 25). This endonuclease converts these transcripts into ~21-bp double-stranded RNA and with the help of the double-stranded RNA-binding domain protein TRBP loads one of the strands into the active site of an Argonaute (Ago) family protein (Ago1 to Ago4 in mammals) (7, 18).

Ago family proteins are the central effectors of the posttranscriptional gene silencing (36). Ago proteins contain two main structural domains, the PAZ domain, an oligonucleotide-binding motif responsible for the miRNA association through its 3′-end (29), and a Piwi domain whose fold is related to that of RNase III (42). In the case of full complementary base pairing between the miRNA and its mRNA target, the Piwi domain is able to cleave the target between the residues paired to nucleotides 10 and 11 of the miRNA (27, 33). This property called “slicing” is routinely used for silencing the expression of a particular gene in the cell by transfecting short interfering 21-nucleotide-long RNAs (siRNAs). However, the only mammalian Ago to possess an endonuclease activity is Ago2, and most miRNAs do not have full complementarity with their mRNA targets, resulting in a primarily slicing-independent mechanism of action in mammals (36, 45).

miRNA-loaded Ago proteins are part of larger complexes referred to as miRNA ribonucleoparticles (miRNPs) or miRNA-induced silencing complexes (miRISCs) (6, 8, 34). These complexes repress expression of their target mRNAs through two mechanisms, translation inhibition and mRNA degradation. Depending on the experimental context, miRNAs can inhibit translation at the level of initiation or elongation (21, 37, 38). Independently or in conjunction with translational inhibition, it was also demonstrated that miRNAs were able to induce poly(A) tail deadenylation by the Ccr4-Not complex followed by decapping of the mRNA 5′-end through Dcp1/Dcp2 and further degradation by the 5′-3′ exonuclease Xrn1 (1, 4, 47).

Interestingly, miRNPs and their mRNA targets localize to cytoplasmic foci called P-bodies (28, 41). These structures result from the aggregation of translationally repressed mRNAs with proteins involved in mRNA turnover, such as Dcp1, Dcp2, Xrn1, Ccr4-Not, Lsm1 to Lsm7, or Rck/p54 (2, 15, 23, 46). Moreover, recent experiments have pointed to GW182 (also known as TNRC6A), an Ago-interacting protein, as an important component of miRNPs not only for localization to the P-bodies but also for translation silencing and degradation of the miRNA-targeted mRNAs (4, 13, 28, 39). GW182 belongs to a conserved protein family restricted to metazoans. Caenorhabditis elegans homologs AIN-1 and AIN-2 and Drosophila melanogaster homolog Gawky were also shown to play a major role in miRNA silencing and P-body localization of miRISC (4, 10, 39, 49). In vertebrates, three paralogs were identified, GW182 (or TNRC6A), KIAA1093 (or TNRC6B), and TNRC6C. They all contain an abnormally high content of GW/WG repeats, one or more Q/P-rich motifs, and a C-terminal RNA recognition motif (RRM) domain. The only exception is the worm protein, which does not display a recognizable RRM domain.

In this study, we focused our analysis on the TNRC6B and TNRC6C proteins to better understand their role in transla-
tional inhibition. We employed biochemical purification of TNRC6B- and TNRC6C-containing complexes to characterize their association with mature miRNAs and their RNA-induced silencing activity. Finally, we performed a detailed structure/function analysis to reveal the precise role played by the different domains of TNRC6B in Ago protein binding as well as P-body localization and translational inhibition. Our results point to the RRM domain of TNRC6B as an essential domain for translational inhibition.

MATERIALS AND METHODS

Antibodies. Anti-DCP1a rabbit serum was a kind gift from J. Lykke-Andersen (Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, CO). Anti-Flag M2 was purchased from Sigma. Anti-Ago2 polyclonal antibodies were raised against an internal peptide (KLMRSASPNTDPYVRE, amino acids 881 to 906) of the human Ago2 sequence (Open Biosystems). Anti-mouse immunoglobulin G (IgG) antibodies coupled to Alexa Fluor 488 and anti-rabbit IgG antibodies coupled to Alexa Fluor 568 were purchased from Molecular Probes. Phosphatase-conjugated anti-IgG secondary antibodies were purchased from Promega.

Affinity purification of Flag-tagged proteins. Flag-tagged protein-expressing constructs and a selectable marker for puromycin resistance were cotransfected in HEK293T (Ago1) or H1299 (TNRC6B and TNRC6C) cells. Transfected cells were grown in the presence of 2.5 μg/ml puromycin (Sigma) for selection. Individual colonies were isolated and screened for Flag-tagged protein expression. Cytoplasmic extracts (100 μg) of Flag fusion-expressing cells (50 μg) were incubated with 250 μl of anti-Flag M2 affinity gel (Sigma) for 2 h at 4°C. Beads were washed four times with 1 ml of BC500 buffer (20 mM Tris [pH 8], 0.5 M KCl, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol [DTT], 0.1% NP-40) and incubated with 1 μg/ml each of the last three antibodies (1:1,000 dilutions). Bound peptides were eluted with 400 μg/ml Flag peptide (Sigma) in BC100 buffer. The flow rate was fixed at 0.35 ml/min, and 0.5-ml fractions were collected.

Northern blotting for miRNA detection. Flag-tagged TNRC6B and Flag-tagged TNRC6C were immunoprecipitated from total cell extract from approximately 10^7 H1299 cells stably expressing TNRC6 (trinucleotide repeat containing 6) proteins using anti-Flag M2 affinity gel (Sigma). Bound peptides were eluted with 400 μg/ml Flag peptide (Sigma) in BC100 buffer, and RNAs were extracted with TRIzol reagent (Invitrogen) and precipitated with 10 μg of RNA as a carrier. RNAs were resolved on a 15% acrylamide-7 M urea gel and transferred to a Hybond-N+ membrane (GE Healthcare). miRNAs were detected by hybridization with short DNA probe complementary to the miRNA sequences labeled with [α-32P]dATP by the method of Behlke et al. (31).

Plasmid constructs. Full-length TNRC6B and related mutants were amplified by standard PCR techniques from the KIAA1093 cDNA clone (Kazusa DNA Research Institute) using vent polymerase (New England Biolabs) and cloned between the HindIII and BamHI restriction sites of the pFlag-CMV2 (Sigma) vector. Point mutants were generated using the QuikChange site-directed mutagenesis kit (Stratagene). Full-length TNRC6C was amplified by standard PCR techniques from the KIAA1582 cDNA clone (Kazusa DNA Research Institute) using vent polymerase (New England Biolabs) and cloned between the HindIII and SalI restriction sites of the pFlag-CMV2 (Sigma) vector. Full-length Ago1 was amplified by standard PCR techniques using vent polymerase (New England Biolabs) and cloned between the EcoRI and BamHI restriction sites of the pFlag-CMV2 (Sigma) vector.

MS2 fusion proteins were generated by PCR amplification of the MS2-binding domain and cloning into the HindIII restriction site of the different pFlag-CMV2 constructs. The pGL3-MS2 construct was generated by cloning an oligonucleotide containing the sequence of two MS2-binding aptamers (5′-CTA GACGCGTACACGATCACGGTACGCTGAATTAGATCTCTGCGGATAG 3′) into the XbaI restriction site of the pGL3-Ctrl vector (Promega). The EMCV-luciferase-MS2 construct was generated as follows. The encephalomyocarditis virus (EMCV) fragment was excised from the EMCV-LEF plasmid (kind gift of Fatima Gebauer, Centro de Regulación Genómica, Barcelona, Spain) after digestion by SacI restriction enzyme, filling in by T4 DNA polymerase, and further digestion by NcoI restriction enzyme. This fragment was cloned into the pGL3-MS2 plasmid previously digested by HindIII, filled in by T4 DNA polymerase, and further digested by NcoI.

RESULTS

TNRC6B and TNRC6C form distinct protein complexes with the four human Argonaute proteins. To identify stable partners of Ago proteins, we generated HEK293T-derived stable cell lines expressing Flag epitope-tagged human Ago1, Ago2, and Ago3. Following anti-Flag immunoaffinity purification, the associated proteins were eluted from the affinity matrix using Flag peptide. The affinity eluate was then fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to colloidal blue staining (Fig. 1a and data not shown). Specific bands were excised, digested with trypsin, and analyzed using mass spectrometric sequencing. The predomin-
FIG. 1. The TNRC6 family of proteins interacts with Argonaute proteins. (a) Purification of Ago1-associated protein complexes. (Left) Purification scheme. Flag-Ago1, Flag-tagged Ago1. (Right) Ago1-associated polypeptides. HEK293 cell lines stably expressing a Flag-tagged Ago1 protein were established. Polypeptides associated with Ago1 were immunopurified from S100 fractions from these cells using agarose beads conjugated with anti-Flag M2 monoclonal antibodies. After the polypeptides were washed and eluted, they were resolved by SDS-PAGE and silver stained. The parental HEK293 cell line was used as a control (mock) for the immunopurification. The positions of molecular mass markers are shown to the left of the gels. (b) Schematic alignment of TNRC6 family proteins and their \textit{D. melanogaster} (dm) and \textit{C. elegans} (ce) homologs. Gray blocks represent domains of higher amino acid sequence identity. The positions of domains I to IV, Q/P-rich domain (Q/P), RNA recognition motif (RRM), and ubiquitin-associated motif (UBA) are shown. The percent identity (percent similarity in parentheses) between the conserved domains of TNRC6C and Gawky is indicated. aa, amino acids. (c) Purification of TNRC6B- and TNRC6C-associated protein complexes. H1299 cell lines stably expressing a Flag-tagged TNRC6C or TNRC6B protein were established. Polypeptides associated with TNRC6C or TNRC6B were immunopurified from S100 fractions from these cells using agarose beads conjugated to anti-Flag M2 monoclonal antibodies. After the polypeptides were washed and eluted, they were resolved by SDS-PAGE and silver stained. The positions of major degradation fragments of Flag-tagged TNRC6B (Flag-TNRC6B) and TNRC6C are indicated by asterisks besides the gels.
nant sequences obtained with all three Agos corresponded to the three human GW182-related proteins (also known as TNRC6 [trinucleotide repeat containing 6]; see Fig. 1b for a schematic alignment of the proteins). We focused on TNRC6B and TNRC6C, since a number of studies had characterized GW182 (TNRC6A) previously (4, 10, 28, 39). To confirm the association of TNRC6B and TNRC6C with the Agos, we generated stable cell lines expressing Flag epitope-tagged TNRC6B and TNRC6C. Mass spectrometric sequencing of affinity eluates confirmed the presence of all four human Ago proteins in TNRC6B and TNRC6C affinity eluates (Fig. 1c). We next subjected the affinity eluates to size exclusion chromatography using a Superose 6 column. As shown in Fig. 2a and b, TNRC6B and the four Agos coelute as a complex of about 700 kDa. Our results are consistent with a previous report of association of TNRC6B with Agos (34). Importantly, we did not find Agos in association with other Agos. Moreover, TNRC6 proteins did not associate with other TNRC6 proteins. Therefore, it is likely that each TNRC6 protein forms a complex with an individual Ago, resulting in the existence of 12 distinct TNRC6-Ago complexes.

The TNRC6 proteins are associated with Agos loaded with mature miRNAs. As TNRC6 complexes contain Ago2, the RNase H fold endonuclease responsible for siRNA-directed cleavage of mRNAs (40, 42), we assessed the enzymatic activity of the TNRC6 complexes. We measured their activity in an Ago2-mediated targeted RNA cleavage assay. Ago2 activity was triggered by a siRNA complementary to let-7 and directed against a small 22-nucleotide radiolabeled probe fully complementary to its sequence (18). As shown in Fig. 2c, the cleavage activity nicely coeluted with the TNRC6B/Ago2-containing fractions. Interestingly, while Dicer-containing complexes displayed RISC activity following the addition of exogenously added siRNAs in vitro (10), the cleavage activity obtained from TNRC6-containing complexes did not require additional siRNA trigger (Fig. 3a). This result suggested that Agos in association with TNRC6 proteins are already loaded with miRNAs (30). Indeed, Northern blot analysis confirmed the presence of different miRNAs in association with the TNRC6B and TNRC6C complexes (Fig. 3b). Similar to the TNRC6B complex and in accordance with its association with mature

![FIG. 2. Analysis of the TNRC6B-associated complexes. (a) SDS-PAGE analysis of the TNRC6B-associated complex fractionated on a Superose 6 gel filtration column after silver staining. The Superose 6 fractions (fractions 14 to 38) are shown above the gel. The positions of molecular mass markers are indicated to the left of the gel. The positions of major contaminant polypeptides are indicated by asterisks. (b) The same fractions were analyzed by Western blotting with anti-Flag M2 and anti-Ago2 antibodies. (c) RISC activity after preincubation of the Superose 6 fractions with an siRNA trigger corresponding to the let-7 sequence.](https://example.com)
miRNAs, the TNRC6C complex displayed targeted cleavage in the absence of any exogenously added trigger (Fig. 3c).

The presence of the target mRNA does not influence the loading of TNRC6-containing complexes. We next assessed the mechanism by which TNRC6B protein is loaded with miRNAs in vivo. Cells stably expressing Flag-tagged TNRC6B were transfected with either a synthetic construct mimicking mature miRNA or a precursor RNA hairpin. We first confirmed the

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FIG. 3. Ago proteins interacting with TNRC6B and TNRC6C are already loaded with mature miRNAs and active. (a) Comparison of RISC activity between the TNRC6B- and Dicer-associated complexes with (+) or without (−) preincubation with a siRNA trigger (let-7). Flag-TNRC6B, Flag-tagged TNRC6B. (b) Northern blot analysis of TNRC6B- and TNRC6C-associated complexes. RNAs were extracted from the TNRC6B- and TNRC6C-associated complexes, precipitated, and resolved on a 15% acrylamide–7 M urea gel. After the RNAs were transferred from the gel to a membrane, the membranes were hybridized with 32P-labeled probes corresponding to the indicated miRNAs. The parental HEK293 cell line was used as a control (mock) for the immunopurification. Flag-IP, Flag immunoprecipitation. (c) Comparison of RISC activity corresponding to the let-7 miRNA between TNRC6B- and TNRC6C-associated complexes in the absence of any exogenous siRNA trigger. (d) Pre-miRNA processing assay. Dicer- and TNRC6B-associated complexes were immunopurified from stably expressing cells. Increasing amounts (in microliters) were tested for processing of pre-miR30a into mature miR30a. (e) Cells stably expressing TNRC6B were transfected with either 70-bp hairpins or siRNA corresponding to the luciferase sequence. After 24 h, the TNRC6B-associated complexes were purified by immunoaffinity, and their RISC activity was assayed against a luciferase probe (Luc probe) and a let7 probe. For controls, untransfected cells were used and the specificity was assessed by adding 2′-O-methyl (2′OMe) oligonucleotides complementary to the luciferase siRNA sequence. GL3 hp, luciferase hairpin. (f) Same as panel e except the luciferase hairpin was cotransfected with increasing amounts of a plasmid expressing the target sequence (pGL3-ctrl; Promega).
absence of Dicer and its pre-miRNA processing activity in the TNRC6B-immunoprecipitated complexes (Fig. 3d). Next, we measured the RISC activity of the eluted fractions, which indicated that TNRC6B-containing complexes could be efficiently triggered by either an siRNA or a RNA hairpin corresponding to precursor miRNA (Fig. 3e). Moreover, either mode of triggering the RISC activity could be efficiently inhibited using a 2′-O-methyl construct (Fig. 3e). Consistent with our previous analysis of Dicer—TRBP-Ago complexes (7, 18), the present results indicated that a double-stranded siRNA could be efficiently loaded into a TNRC6B-containing complex. To further assess whether the presence of increasing amounts of target influences the loading of the siRNA into a TNRC6B complex, we titrated in various amounts of synthetic target with perfect complementarity to the siRNA. These experiments revealed that the siRNA loading of the TNRC6B complex is not influenced by the presence of the target mRNA (Fig. 3f).

The conserved glutamine/proline-rich domain (domain III) of TNRC6B is responsible for P-body localization. Since TNRC6A was shown to be an integral component of P-bodies (14), we assessed the cytoplasmic localization of TNRC6B and TNRC6C. TNRC6B and TNRC6C proteins were expressed in HeLa cells and examined for their cytoplasmic localization compared to that of the decapping enzyme subunit DCP1a. DCP1a has previously been shown to be enriched in P-bodies and is used extensively as a P-body marker (16). Similar to previously reported localization of GW182 to P-bodies, we found that both TNRC6B and TNRC6C proteins were enriched in P-bodies (see Fig. S1a in the supplemental material). Interestingly, increased expression of TNRC6B and TNRC6C resulted in enhanced P-body size and number (data not shown), consistent with previous reports examining the ectopic expression of other P-body protein components (12). We next examined the domain(s) in TNRC6B responsible for localization to P-bodies. Previous studies had pointed to the C-terminal part of GW182/Gawky as being critical for P-body localization (4). Using N- and C-terminal truncations of TNRC6B (Fig. 4a), we narrowed down the region necessary for P-body localization to the conserved C-terminal glutamine/proline-rich (Q/P-rich) domain (Fig. 4b). This glutamine/proline-rich domain (domain III) is reminiscent of prion-related or polyglutamine domains involved in the formation of physiological or pathological protein aggregates (35). Our results suggest that such domains may contribute to accumulation of some proteins in P-body-like cytoplasmic structures.

Distinct domains in TNRC6B are responsible for Ago2 binding and P-body localization. To analyze whether there is a distinct domain in the TNRC6B protein that mediates the association with Agos, we used our multiple C- and N-terminal truncations of TNRC6B to map for Ago binding (Fig. 4a and 5a). We found that in contrast to P-body localization in which domain III played the critical role, domain I is necessary and sufficient for the association of TNRC6B with Ago2 (Fig. 5a). Removal of this domain from the full-length protein abrogated Ago2 binding but had no effect on TNRC6B localization to the P-bodies (Fig. 5b). Moreover, ectopic expression of domain I could be communoprecipitated with endogenous Ago2 but failed to localize to the P-body (Fig. 5a and b). We also confirmed that similar to TNRC6A (4), TNRC6B is interacting with the Piwi domain of Ago2 (see Fig. S1b in the supplemental material). Taken together, these results indicate that Ago2 binding to TNRC6B is functionally uncoupled from P-body localization.

TNRC6B domain I corresponds to the WG/GW-rich region of GW182 (TNRC6A) that was recently described as an evolutionarily conserved platform for Ago protein recruitment (11, 44). However, after alignment of domain I sequences corresponding to TNRC6 proteins from different organisms (Fig. 5c), it appeared that only a few WG/GW pairs are conserved, suggesting that these conserved amino acids might have a specific role in the interaction between TNRC6 and Ago proteins. To test this contention, we mutated the highly conserved tryptophan 623 to alanine (W623A) in full-length TNRC6B as well as in the isolated domain I from TNRC6B and repeated the Ago-binding experiments. Interestingly, the W623A mutation completely abrogated the interaction between TNRC6B and Ago2 (Fig. 5c, gels). These results suggest a more specific mechanism of molecular recognition between TNRC6B and Ago2 rather than the existence of a large nonspecific interaction platform.

Inhibition of translation by TNRC6B does not require either association with Agos or localization to P-bodies. D. melanogaster Gawky (GW182-like) protein has been reported to strongly repress translation when tethered to a luciferase reporter construct, leading to a decrease in the mRNA transcript (4). We used a similar tethering methodology to investigate the translation-inhibitory properties of TNRC6B and TNRC6C. Similar to GW182, MS2 fusions of TNRC6B and TNRC6C showed a strong translational inhibitory activity (Fig. 6a) when brought to a reporter construct containing MS2-binding sites. However, other proteins shown to play a role in miRNA-mediated silencing displayed a weaker inhibitory potential in such an assay (Fig. 6a). Interestingly, while luciferase expression was inhibited by nearly 80%, we did not detect any change in the mRNA levels (see Fig. S2a in the supplemental material).

We next investigated whether the domains shown to be involved in Ago binding (domain I) and P-body localization (domain III) contributed to translation inhibition in such tethering experiments. Multiple MS2 fusion constructs of TNRC6B were developed and tested for their effects on translation (Fig. 6b). Surprisingly, the deletion of neither the Ago binding nor the P-body localization had an impact on translation inhibition (Fig. 6c), suggesting that their associated functions are uncoupled from translation inhibition. Indeed, direct recruitment of Agos through tethering of domain I failed to exert translational repression (Fig. 6c). Consistent with these data, a single point mutation (W623A) in either full-length TNRC6B or domain I, which abrogates its association with Agos, maintains the full extent of translational inhibition (Fig. 6d) (see Fig. S2b in the supplemental material). These results indicate that once tethered to a reporter construct, TNRC6B does not require the association with Agos or localization to P-bodies to bring about translational inhibition.

The C-terminal domain of TNRC6B, comprising an RRM, exerts a strong translation inhibition potential. The above findings led us to look for the domain(s) in the TNRC6B protein responsible for mediating its translational inhibition. MS2 fusion constructs of C-terminal truncations were engi-
neered, and their translational inhibitory activities were assessed (Fig. 6b and 7a). While full-length TNRC6B resulted in a sixfold inhibition, truncation of the RNA recognition motif (ΔC1415) relieved the inhibition to threefold. Further C-terminal deletions resulted in a gradual decrease in translational inhibition (Fig. 7a). Consistent with these findings, the tethering of the RRM-containing C-terminal fragment from positions 1409 to 1722 to the luciferase messenger resulted in an

FIG. 4. The glutamine/proline-rich domain localizes TNRC6B to P-bodies. (a) Schematic representation of the Flag-tagged TNRC6B deletion mutants used. The positions of domains I to IV, the Q/P-rich motif, and RRM are shown. aa, amino acids. (b) Cellular localization of TNRC6B. HeLa cells were transfected with the indicated constructs. After fixation and permeabilization, the cells were probed with anti-Flag M2 monoclonal antibodies (revealed with anti-mouse IgG antibodies coupled to Alexa Fluor 488) and with anti-DCP1a polyclonal antibodies (revealed with anti-rabbit IgG antibodies coupled to Alexa Fluor 568).
FIG. 5. A single point mutation in domain I of TNRC6B abrogates Ago binding. (a) HEK293 cells were transfected with the different TNRC6B constructs described in the legend to Fig. 4a. Total cell extracts were immunoprecipitated with anti-Flag M2 antibodies. Immunoprecipitates were resolved by SDS-PAGE and analyzed by Western blotting with anti-Flag M2 antibodies and anti-Ago2 antibodies. Flag-IP, Flag immunoprecipitation. (b) HeLa cells were transfected with the indicated constructs. After fixation and permeabilization, the cells were probed with anti-Flag M2 monoclonal antibodies (revealed with anti-mouse IgG antibodies coupled to Alexa Fluor 488) and with anti-DCP1a polyclonal antibodies (revealed with anti-rabbit IgG antibodies coupled to Alexa Fluor 568). (c) (Top) Protein alignment for the highly conserved region in the Ago-binding domain of TNRC6B proteins from human (Homo sapiens [Hs]), zebra fish (Danio rerio [Dr]), and fruit fly (Drosophila melanogaster [Dm]) sources. (Bottom) Same as in panel b using the wild-type (WT) and W623A point mutant (mut) of full-length TNRC6B and D-I. ctrl, control.
FIG. 6. TNRC6B exerts a strong Ago-independent translational inhibition. (a) HEK293 cells were transfected with pGL3-MS2, pRL-CMV, and the various MS2 fusion constructs indicated. Twenty-four hours posttransfection, the cells were lysed, and firefly and Renilla luciferase activities were measured and plotted as the firefly luciferase/Renilla luciferase ratio. (b) Schematic representations of the TNRC6B-MS2 fusion constructs and the luciferase constructs used in this figure. The positions of MS2 domain, domains I to IV, Q/P-rich motif, and RRM are shown. aa, amino acids; SV40, simian virus 40; MBS x2, two copies of an MS2 binding site. (c and d) Same as in panel a. CTRL, control.
inhibition comparable to that observed with the full-length TNRC6B (Fig. 7a), suggesting a critical role for this region in TNRC6B-associated translational inhibition.

We envisioned a possible mechanism for translation inhibition through a competition between TNRC6B and eIF4E, the cap-binding protein, for binding to the cap and thus preventing efficient translation initiation. To assess this possibility, we measured the ability of the RRM domain of TNRC6B for binding to the cap in vitro. While we observed the association of CBP20, an RRM-containing subunit of the nuclear cap-binding complex (32), with the cap, we failed to detect the association of TNRC6B RRM domain (ΔN1409) with the 7mG cap (Fig. 7b). As an alternative approach for investigating the cap-dependent mechanism of action, we assessed the extent of translational repression when TNRC6B is tethered to a reporter construct directed for translation by the internal ribosome entry site of the EMCV. Since the translation of this construct does not require eIF4E, TNRC6B effects on translation cannot be mediated through an eIF4E-mediated mechanism. Indeed, the addition of MS2-TNRC6B to this construct resulted in the same extent of translational inhibition as seen from an eIF4E-dependent reporter, indicating that the inhibition does not seem to be directed through TNRC6B inhibition of eIF4E binding (Fig. 7c).

In addition to CBP20, the RRM domain is present in multiple RNA-binding proteins. Indeed, a number of different RRM domains were shown to bind RNA directly (32). To assess whether the repression of translation by TNRC6B RRM domain requires the integrity of the RRM domain, we introduced mutations (H1642P and Y1678P) in aromatic residues that could potentially play a role in protein-RNA or protein-protein interactions according to the known structures of various RRM domains (32). Analysis of these mutants supported a role for the TNRC6B RRM domain in mediating the translational repression (Fig. 7d). Taken together, our results indicate that while the association of the RRM domain with the TNRC6B C-terminal domain of TNRC6B. (a) HEK293 cells were transfected with pGL3-MS2, pRL-CMV, and the various MS2 fusion constructs indicated. Twenty-four hours posttransfection, the cells were lysed, and firefly and Renilla luciferase activities were measured and plotted as the change in repression from the relative luciferase activity measured with the MS2 domain alone. (b) Cap-binding assay. HEK293 cells were transfected with Flag-tagged CBP20 or Flag-tagged ΔN1409. After 48 h, the cells were lysed, and Flag proteins were immunopurified. Eluted proteins were tested for cap binding using 7mGTP beads in the presence of specific (7mGpppG) or nonspecific (GpppG) competitor. Bound proteins were eluted with 7mGpppG and analyzed by Western blotting using anti-Flag antibodies. (c) (Top) Schematic representation of the EMCV internal ribosome entry site (IRES) luciferase construct. luc, luciferase; SV40, simian virus 40; MBS x2, two copies of an MS2 binding site. (Bottom) HEK293 cells were transfected with EMCV-luc-MS2 construct, pRL-CMV, and the MS2 fusion constructs indicated. Twenty-four hours posttransfection, the cells were lysed, and firefly and Renilla luciferase activities were measured and plotted as the firefly luciferase/Renilla luciferase ratio. (d) Same as in panel a but relative luciferase activity (firefly luciferase/Renilla luciferase) is plotted.

FIG. 7. Translation inhibition exerted by the RRM-containing C-terminal domain of TNRC6B. (a) HEK293 cells were transfected with pGL3-MS2, pRL-CMV, and the various MS2 fusion constructs indicated. Twenty-four hours posttransfection, the cells were lysed, and firefly and Renilla luciferase activities were measured and plotted as the change in repression from the relative luciferase activity measured with the MS2 domain alone. (b) Cap-binding assay. HEK293 cells were transfected with Flag-tagged CBP20 or Flag-tagged ΔN1409. After 48 h, the cells were lysed, and Flag proteins were immunopurified. Eluted proteins were tested for cap binding using 7mGTP beads in the presence of specific (7mGpppG) or nonspecific (GpppG) competitor. Bound proteins were eluted with 7mGpppG and analyzed by Western blotting using anti-Flag antibodies. (c) (Top) Schematic representation of the EMCV internal ribosome entry site (IRES) luciferase construct. luc, luciferase; SV40, simian virus 40; MBS x2, two copies of an MS2 binding site. (Bottom) HEK293 cells were transfected with EMCV-luc-MS2 construct, pRL-CMV, and the MS2 fusion constructs indicated. Twenty-four hours posttransfection, the cells were lysed, and firefly and Renilla luciferase activities were measured and plotted as the firefly luciferase/Renilla luciferase ratio. (d) Same as in panel a but relative luciferase activity (firefly luciferase/Renilla luciferase) is plotted.
cap may not be a mechanism for TNRC6B repression of translation, the RRM domain is playing a structural role in such inhibition, perhaps through associating with RNA or a protein partner.

**DISCUSSION**

Our results indicate that each of the TNRC6 family members can form a complex with one of the four Ago proteins. Therefore, our data suggest the presence of 12 different distinct TNRC6-Ago complexes in human cells. Since we do not know the exact temporal localization of TNRC6 and Agos in different tissues, it is tempting to speculate that they may be expressed in a tissue-specific and developmentally regulated fashion. More-detailed analyses of TNRC6 and Ago protein expression patterns are needed to fully address individual complex expression profiles and functions. Recent results in embryonic stem cells (43) suggest that in cells lacking all four Ago proteins, the expression of any of the Agos was sufficient to rescue miRNA-directed silencing. However, a recent study of *Drosophila* suggests that different Ago proteins can exert their action through different mechanisms (24). Taken together, these results suggest that while the system may allow redundant mechanisms of action, different Agos may use different repression pathways. Indeed, there is ample evidence pointing to the abilities of TNRC6 and Agos to induce both translational repression and RNA destabilization through deadenylation.

We do not find a stable association of TNRC6 proteins with Dicer. However, we were able to load TNRC6B using synthetic hairpins, indicating that the loading of small siRNAs requires prior Dicer participation, the only enzyme known to convert pre-miRNA hairpins into mature miRNAs. Moreover, since the Agos associated with TNRC6 proteins are already loaded with miRNAs, it is reasonable to surmise that the association of miRNA with Agos may enhance their interaction with the TNRC6 family of proteins. Indeed, we were unable to find TNRC6 complexes that could be loaded in vitro using single-stranded siRNAs (data not shown), which is consistent with their full occupancy with miRNAs. Once loaded, these complexes could associate with the targets of each miRNA. Interestingly, our studies indicate that the system does not incorporate a feedback pathway, as the increased presence of a miRNA target does not affect the loading of a specific miRNA into an Ago complex. It would be interesting to investigate whether and how the miRISC complex associated with its mRNA target is recycled following its localization to P-bodies.

Our detailed analysis of TNRC6B indicated that this protein uses a specific domain, known as domain I, to interact with the Ago proteins. Importantly, a single point mutation in this domain is sufficient to completely abrogate Ago binding. A similar domain in the plant large subunit of RNA polymerase IV and the TAS3 protein in *Schizosaccharomyces pombe* had been previously proposed to contribute to Ago binding (11). These results point to an evolutionarily conserved protein-protein interaction interface in different components of RNA interference machinery across species. Interestingly, this domain which was originally present in nuclear proteins in plants and yeasts evolved to play a role in the cytoplasm in animals.

While we find domain I to be a major determinant in Ago interaction, a different domain in TNRC6B, domain III, was required for the localization of this protein to P-bodies. This is consistent with previous studies mapping GW182 localization to P-bodies (4). The intriguing finding is the resemblance of proline/glutamine-rich domain III to glutamine-rich domain of the prion-like domain-containing proteins (17). While this finding suggests that the proline/glutamine-rich domain in other P-body components may serve as a P-body localization signal, our deletion analysis of number of other proteins localized to P-bodies indicated that such a domain does not serve a universal role in P-body localization (data not shown).

Importantly, beside Agos, we were unable to find other stable partners of TNRC6B and TNRC6C proteins. In addition, we did not find any evidence for the stable association of either the translation or RNA deadenylation machinery with TNRC6B or TNRC6C proteins. However, these results do not rule out the possibility of a transient functional interaction between TNRC6 proteins and the machineries involved in translational regulation and RNA metabolism. Indeed, our functional analysis of TNRC6B which was tethered to the reporter constructs suggests a crucial role for this protein in translational inhibition. Our domain mapping studies indicate that the tethering of the C-terminal domain of TNRC6B consisting of an RRM domain is able to repress translation as efficiently as the full-length protein. Interestingly, the repression of translation by the RRM domain does not require the interaction with Agos or the localization to the P-bodies as was demonstrated by our mapping studies. Since this domain is conserved in all three mammalian TNRC6 proteins as well as the *Drosophila* GW182, it may contribute to an evolutionarily conserved mechanism of repression. The only caveat is the absence of a detectable RRM domain in the worm proteins AIN-1 and AIN-2 (10, 49). However, future structural analysis of the worm proteins may uncover a RRM-like domain in these proteins.

While we do not know the precise mode by which the RRM domain may contribute to silencing, it is likely that it may operate via multiple mechanisms. Other RRM domains have been shown to be versatile modules, capable of RNA binding, 7mG cap binding, and providing a platform for protein-protein interactions (32). We show that TNRC6B was unable to bind the mRNA 5′ cap. Moreover, the presence of a single RRM domain is shown to be more in line with a mechanism evoking protein-protein interaction, as several RRM domains are usually required for RNA binding (32). The characterization of the detailed mechanism by which the RRM domains of TNRC6 proteins are required for translational repression should provide further insight into the action of miRNAs.

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