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An mRNA m^{7}G Cap Binding-like Motif within Human Ago2 Represses Translation

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SUMMARY

microRNAs (miRNAs) bind to Argonaute (Ago) proteins and inhibit translation or promote degradation of mRNA targets. Human let-7 miRNA inhibits translation initiation of mRNA targets in an m^{7}G cap-dependent manner and also appears to block protein production, but the molecular mechanism(s) involved is unknown and the role of Ago proteins in translational regulation remains elusive. Here we identify a motif (MC) within the Mid domain of Ago proteins, which bears significant similarity to the m^{7}G cap-binding domain of eIF4E, an essential translation initiation factor. We identify conserved aromatic residues within the MC motif of human Ago2 that are required for binding to the m^{7}G cap and for translational repression but do not affect the assembly of Ago2 with miRNA or its catalytic activity. We propose that Ago2 represses the initiation of mRNA translation by binding to the m^{7}G cap of mRNA targets, thus likely precluding the recruitment of eIF4E.

INTRODUCTION

microRNAs (miRNAs) and short interfering RNAs (siRNAs) are ~22 nucleotide (nt) noncoding RNAs that regulate gene expression by binding to recognition elements (such as miRNA recognition elements—MREs) in their RNA targets (reviewed in Ambros [2004], Bartel [2004], and Mello and Conte [2004]). miRNAs and siRNAs assemble with Argonaute proteins in effector complexes known as RNA-induced silencing complexes (RISCs) or miRNPs (Hammond et al., 2001; Mourelatos et al., 2002; Martinez et al., 2002; Hutvagner and Zamore, 2002; Tomari and Zamore, 2005). Argonaute proteins are a large family of ~95 Kda proteins that contain two signature motifs known as PAZ and PIWI and are found in most organisms (Carmell et al., 2002). Further division of Argonaute proteins in two subfamilies termed Ago and PIWI families is based on sequence similarities between the founding members of each subclass, the AGO1 protein from *Arabidopsis* and the PIWI protein from *Drosophila melanogaster*, respectively (Carmell et al., 2002). Ago proteins bind directly to miRNAs and to siRNAs and are the core protein components of RISCs/miRNPs (Hammond et al., 2001; Mourelatos et al., 2002; Martinez et al., 2002; Hutvagner and Zamore, 2002; Tomari and Zamore, 2005; Murchison and Hannon, 2004; Meister and Tuschl, 2004).

The molecular function of miRNAs and siRNAs depends on which Ago protein they bind to and on the degree of complementarity with their cognate MREs. If the complementarity of a miRNA or siRNA with its target mRNA is extensive and the miRNA assembles with catalytically active Ago proteins, the target mRNA is cleaved at a position across from the middle of the guide miRNA or siRNA (Elbashir et al., 2001; Hutvagner and Zamore, 2002; Liu et al., 2004; Meister et al., 2004). Biochemical, genetic, and crystallographic studies of Ago proteins have shown that the PIWI domain of Ago proteins adopts an RNase H fold (Song et al., 2004; Parker et al., 2004; Yuan et al., 2005) and in certain Ago proteins such as mammalian Ago2 provides the endonuclease activity that catalyzes miRNA- and siRNA-directed mRNA cleavage (Liu et al., 2004; Meister et al., 2004).

Partial complementarity between miRNA and MREs leads to translational repression and/or accelerated degradation of the targeted mRNA, but the mechanism of this regulation is not well understood. In human HeLa cells, the let-7 miRNA inhibits the initiation of translation of a reporter construct bearing MREs for let-7 in its 3′-UTR and leads to sequestration of the repressed mRNA in processing bodies (PBs) in a cap-dependent manner (Pillai et al., 2005). PBs are cytoplasmic bodies enriched in mRNA decay enzymes, where translationally repressed mRNAs are localized (Sheth and Parker, 2003). In PBs mRNAs can undergo decapping and degradation (Sheth and Parker, 2003). There is evidence that some miRNA or siRNA-targeted mRNAs can also be sequestered in PBs (Liu et al., 2005b; Sen and Blau, 2005; Pillai et al., 2005). Under certain conditions, this sequestration can be reversible, as in

the case of human hepatoma Huh7 cells, where miR-122 inhibits translation initiation of its endogenous CAT-1 mRNA target and sequesters it to PBs (Bhattacharyya et al., 2006). Interestingly, this inhibition is reversed by stress, which leads to release of the CAT-1 mRNA from PBs and its recruitment to polysomes (Bhattacharyya et al., 2006). In these instances, the targeted mRNA is not destabilized or undergoes a secondary, usually limited degradation (Bhattacharyya et al., 2006; Pillai et al., 2005). Human let-7 miRNA also appears to block production of a reporter protein that is expressed from a construct containing the 3′-UTR of lin-41 (which contains two MREs for let-7), on actively translating ribosomes (Nottrott et al., 2006). Interestingly, the stability of the targeted mRNA may be influenced by the structure of the miRNA:MRE heteroduplex. In HeLa cells, MREs for let-7 in the 3′-UTR of reporter constructs that contain a single bulge when base-paired with let-7, are potent translational repressors but do not destabilize the targeted mRNA (Kiriakidou et al., 2004; Schmitter et al., 2006). In contrast, MREs that contain two opposing loops when base-paired with let-7, in addition to translational repression, lead to degradation of the targeted mRNA. This is proposed to be a secondary consequence of translational repression (Schmitter et al., 2006).

In *C. elegans*, the *lin-4* miRNA cosediments with its mRNA target *lin-14* in polysomes during repression, suggesting that inhibition of translation may occur at a step after initiation (Olsen and Ambros, 1999), although more recent reports showed significant degradation of *lin-14* mRNA during *lin-4*-mediated repression (Bagga et al., 2005). Inhibition of translation after initiation by transfected siRNAs has also been demonstrated in human cells (Petersen et al., 2006). However, another group using the same siRNAs and reporter constructs found that the inhibition was at the level of translation initiation (Humphreys et al., 2005). Finally, independently of translational repression, destabilization of mRNAs targeted by miRNAs may also occur (Rehwinkel et al., 2005; Wu et al., 2006; Giraldez et al., 2006).

The protein complexes mediating miRNA-dependent translational repression are not well defined. Ago proteins are the leading protein candidates due to their engagement with miRNAs. Artificial tethering of human Ago proteins to the 3′-UTR of reporter constructs leads to inhibition of translation initiation without affecting the mRNA levels of the reporters (Pillai et al., 2004; Pillai et al., 2005). Other proteins, such as GW182, the decapping enzyme complex Dcp1 and Dcp2, and RCK/p54, a general translation inhibitor, can also participate in miRNA-dependent translational repression and mRNA degradation. (Liu et al., 2005a, 2005b ; Rehwinkel et al., 2005 ; Chu and Rana, 2006).

The 5′ end of eukaryotic mRNAs is modified by the addition of a 7-methyl guanosine cap (m^7GpppN; m^7G cap; where N is any nucleotide) in a 5′-5′ triphosphate linkage. Eukaryotic initiation factor 4E (eIF4E) binds the m^7G cap directly, and this interaction is essential for the initiation of translation of most eukaryotic mRNAs (reviewed in Merrick [2004], Gebauer and Hentze [2004], and Richter and Sonenberg [2005]). Regulation of translation initiation is the most common target of translational control, and preventing binding of eIF4E to the m^7G cap is a commonly employed cellular strategy to inhibit translation (Richter and Sonenberg, 2005; Gebauer and Hentze, 2004).

In this study we present evidence that a subdomain within the middle domain of Ago2 demonstrates m^7G cap binding activity and is required for translational repression but not for assembly with miRNA or endonucleolytic activity. We propose that Ago2 represses the initiation of mRNA translation by binding to the m^7G cap of mRNA targets, thus likely precluding the recruitment of eIF4E.

RESULTS

A Cap-Binding-like Domain (MC) within Ago2

We undertook a bioinformatics approach to study the primary structure of Argonaute proteins. BLAST searches of the Swiss-Prot database showed that there was significant similarity between a portion of the middle (Mid) domain of human Argonaute 2 (hAGO2) encompassing amino acids 468–520, which we term the MC domain, with the human eIF4E. The MC domain is present in all four human and mammalian Agos, in Agos from chordates (such as zebrafish), in *Drosophila* AGO1, and in *C. elegans* ALG-1 and ALG-2. This domain is not found in Ago proteins of plants, archaea, or fission yeast, in *Drosophila* AGO2 and in most members of the *C. elegans* Ago protein family, with the exception of ALG-1 and ALG-2. In addition, the MC domain is absent from proteins of the PIWI family (Figure 1 and our unpublished data).

Biochemical and crystallographic analyses of m^7G cap-binding proteins show that the most important determinants of cap recognition are π-π stacking interactions between the methylated base of the cap (m^7G) and the side chains of two protein aromatic residues. In the case of eIF4E the m^7G is sandwiched between the side chains of two tryptophan residues, W56 and W102 (numbering refers to the murine and human eIF4E) (Marcotrigiano et al., 1997). Substitution of these tryptophan residues with phenylalanines still supports cap binding but with reduced affinity (Altmann et al., 1988). In human 4EHP, an eIF4E homologous protein with orthologs in nematodes (IFE-4) and plants (nCBP), the m^7G is sandwiched between the side chains of a tyrosine (Y78) and a tryptophan residue (W124) (Rom et al., 1998). In cap-binding protein 20 (CBP20), the m^7G is sandwiched between the side chains of two tyrosine residues (Y20 and Y43 of human CBP20) (Calero et al., 2002). In VP39, a vaccinia virus protein, the m^7G is sandwiched between the side chains of a tyrosine (Y22) and phenylalanine (F180) residues (Hodel et al., 1997; Hu et al., 1999). It is interesting to note that although CBP20 and VP39 do not show any homology with eIF4E, they bind the cap by using the same strategy: stacking interactions between aromatic residues and the m^7G.

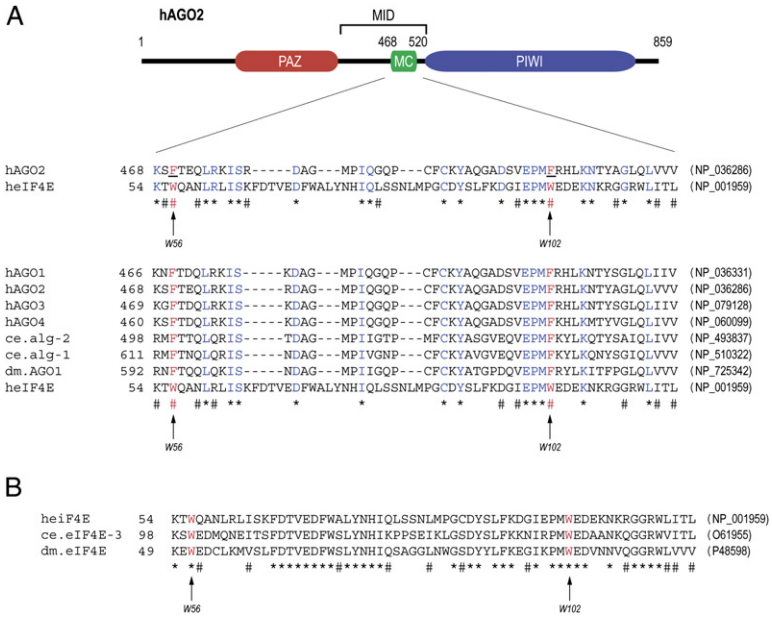

Figure 1. Similarity between the MC Domain of Ago Proteins and the m^{7}G Cap-Binding Domain of eIF4E

(A) Indicated sequences were aligned using CLUSTALW. Arrows indicate the two tryprophan residues (W56 and W102) of eIF4E that bind to the m^{7}G cap and the conserved phenylalanines (F) of Ago proteins. The two phenylalanines of hAgo2 (F470 and F505) that were mutated to valines or tryptophans are underlined. Accession numbers are listed in parentheses; (*) indicates identical and (#) indicates similar.

(B) Alignments of cap-binding domains of eIF4E homologs.

The similarity between the MC domain of Ago proteins and eIF4E encompasses the m^{7}G cap-binding domain of eIF4E and is centered around W56 and W102, the two tryptophan residues of eIF4E that are crucial for m^{7}G binding. In Ago proteins these tryptophans are replaced by two phenylalanines (F470 and F505), conserved aromatic residues (Figure 1). This similarity between the cap-binding domain of eIF4E and Agos prompted us to ask whether Ago proteins might bind to the m^{7}G cap.

Specific Interactions between Ago2 and a Cap-Analog Resin

To test whether human Ago2 associates with the m^{7}G cap, we transfected human 293T cells with vectors expressing HA-tagged, wild-type Ago2 or mutant Ago2$^{F470V;F505V}$ (Ago2-F2V2) where the two phenylalanines (F470 and F505) of the Ago2 MC domain were substituted with valines. The overexpressed, wild-type, and mutant Ago2 proteins were purified with immunoaffinity chromatography using agarose beads with covalently bound anti-HA antibody, followed by acidic elution of the HA-tagged proteins, neutralization, and dialysis (Figure 2A). The eluted proteins were then tested for binding to m^{7}GTP sepharose or GTP-sepharose. The binding buffer included heparin to eliminate nonspecific interactions between the basic Ago2 protein and the negatively charged m^{7}GTP or GTP. After extensive washes, retained proteins were analyzed by NuPAGE electrophoresis and western blotting. As shown in Figure 2B, Ago2 (lane 2), but not Ago2-F2V2 (lane 7), bound to the m^{7}GTP sepharose. To further characterize the specificity of this interaction, we carried out m^{7}GTP sepharose-binding reactions including m^{7}GpppG cap analog as a competitive inhibitor or nonmethylated GpppG. As shown in Figure 2B, the presence of the methylated cap analog competed with binding of Ago2 to the m^{7}GTP sepharose (lane 3), whereas the presence of nonmethylated GpppG did not affect retention of the Ago2 to the m^{7}GTP sepharose (lane 4); wild-type Ago2 protein did not

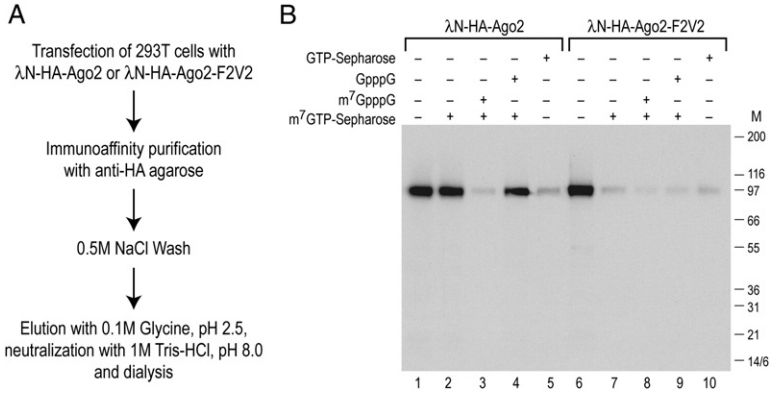

Figure 2. Human Ago2 Binds to m^{7}GTP Sepharose, and This Interaction Is Abolished by Mutations of the Two Conserved Phenylalanines of the MC Domain in Ago2-F2V2 Mutant

(A) Wild-type human λN-HA-Ago2 or point mutant λN-HA-Ago2$^{F470V;F505V}$ (λN-HA-Ago2-F2V2) was immunoaffinity purified and eluted.

(B) Purified proteins were incubated with m^{7}GTP sepharose, a cap analog resin, in the presence or absence of m^{7}GpppG competitive inhibitor, or nonmethylated cap analog (GpppG), or they were incubated with GTP sepharose. Lanes 1 and 6 show 5% of input fraction used for binding. Bound proteins were analyzed by Western blot with 16B12 anti-HA monoclonal antibody.

bind to nonmethylated GTP sepharose (lane 5). Ago2-F2V2 mutant did not bind to m^{7}GTP sepharose or GTP sepharose under any conditions (Figure 1B, lanes 7–10). Taken together these results demonstrate that Ago2 associates specifically with m^{7}G and that the two conserved phenylalanines of the MC domain are required for binding.

The MC Domain of Ago2 Is Required for Translational Repression but Not for Endonucleolytic Activity

The Filipowicz lab has previously shown that tethering of all human Ago proteins (Ago1–4) to the 3′-UTR of a Renilla Luciferase reporter represses translation without affecting the mRNA levels of the reporter, and this is similar to the inhibition of translation initiation by let-7 miRNA (Pillai et al., 2004, 2005). We employed the same strategy (shown in Figure 4A) to test whether mutations of the two phenylalanines (F470 and F505) of the MC domain of human Ago2 also abolish the inhibitory effect of Ago2 in translation. Briefly, the tethering assay involves the creation of a fusion protein between Ago2 or Ago2-F2V2 and the λN peptide, a 22 amino acid peptide derived from the λ phage, which binds with high affinity to its cognate RNA-binding site, known as BoxB (Legault et al., 1998). Five BoxB sites are placed in the 3′-UTR of an RL reporter construct (RL-5BoxB), and the effect of the fusion proteins to the reporter is assessed in cotransfection experiments (De Gregorio et al., 1999; Pillai et al., 2004, 2005). An Ago2 construct that does not contain the λN peptide serves as negative control, and an HA epitope placed at the amino terminus of Ago2 or Ago-F2V2 serves as a tag to detect the proteins by western blot and for immunoprecipitation experiments.

First, we wanted to test whether the λN and HA tags and more importantly the mutations of the two phenylalanine residues in Ago-F2V2 affected the activity of the fusion proteins. Pillai et al. have previously shown that λN-HA-Ago2 was catalytically active (Pillai et al., 2004). We transfected HeLa cells with HA-Ago2, λN-HA-Ago2, and λN-HA-Ago2-F2V2 constructs and performed immunoprecipitations with anti-HA antibody beads. We then tested whether the immunoprecipitated proteins were able to cleave a radiolabeled RNA target complementary to the endogenous let-7 miRNA (let7-TP target, shown in Figure 3A). As positive control, we used beads containing endogenous human Ago2 protein obtained after immunoprecipitation with the 2A8 anti-Ago monoclonal antibody. We used anti-HA immunoprecipitates from mock-transfected HeLa cells as negative control. As shown in Figure 3B, HA-Ago2, λN-HA-Ago2, and λN-HA-Ago2-F2V2 were able to cleave the let7-TP RNA target. These findings indicate that the transfected proteins, including the Ago2-F2V2 mutant, are able to assemble with endogenous let-7 in HeLa cells into catalytically active Ago2/miRNA ribonucleoprotein complexes. Thus, the two point mutations in Ago2-F2V2 did not perturb the folding or structural properties of Ago2.

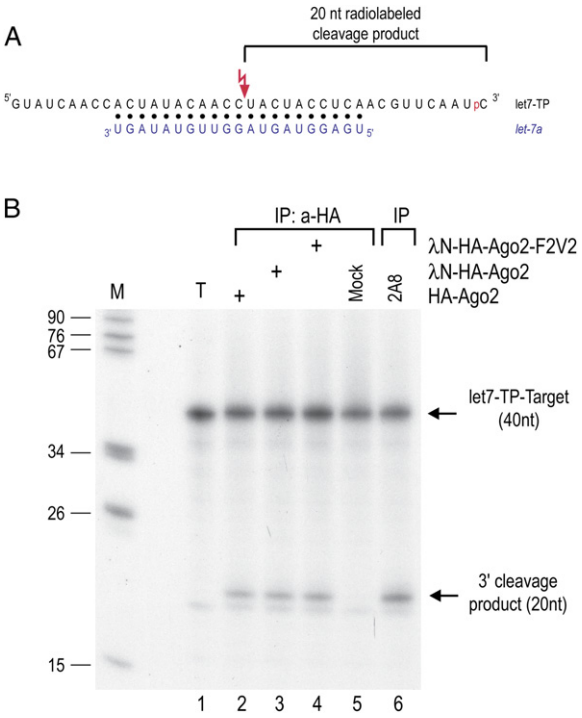

Figure 3. λN-HA-Ago2-F2V2 Assembles with Endogenous let-7 miRNA and Contains an Active Endonuclease Domain

(A) Schematic of the RNA target (let7-TP) and the base-pairing with let-7a (blue); the [5′-^{32}P] of pCp is shown in red. Cleavage site is indicated with red lightning bolt.

(B) The 3′-end radiolabeled let7-TP RNA target was incubated with beads containing indicated immunoprecipitated proteins; mock refers to mock-transfected cells (negative control); 2A8 is an anti-Ago monoclonal antibody and served as positive control. The products of the reactions were analyzed on 20% denaturing polyacrylamide gel. Nucleotide sizes of the radiolabeled marker (M) are shown on the left.

Next, we transfected HeLa cells with HA-Ago2, λN-HA-Ago2, and λN-HA-Ago2-F2V2 along with RL-5BoxB and firefly luciferase (FL; as normalization control) and quantitated the levels of normalized RL/FL using standard luminometric assays. As shown in Figure 4B, λN-HA-Ago2 led to a ~4-fold repression of RL-5BoxB, as previously reported (Pillai et al., 2004, 2005). In contrast, λN-HA-Ago2-F2V2 was unable to repress the expression of RL-5BoxB. We next tested the protein levels of the transfected Ago2 constructs by probing HeLa cell lysates with anti-HA antibody and also with anti-β-tubulin antibody, as loading control. As shown in Figure 4C, all transfected Ago2 proteins were expressed in similar levels. Finally, we assayed the mRNA levels of RL-5BoxB by Northern blotting using radiolabeled riboprobes against RL and against FL (as normalization control). As shown in Figure 4D, the mRNA levels of RL-5BoxB were similar between the samples that were transfected with HA-Ago2, λN-HA-Ago2, or λN-HA-Ago2-F2V2. We also tested whether translation initiated at an internal ribosome entry site (IRES), and thus bypassing the cap, was subjected to repression by

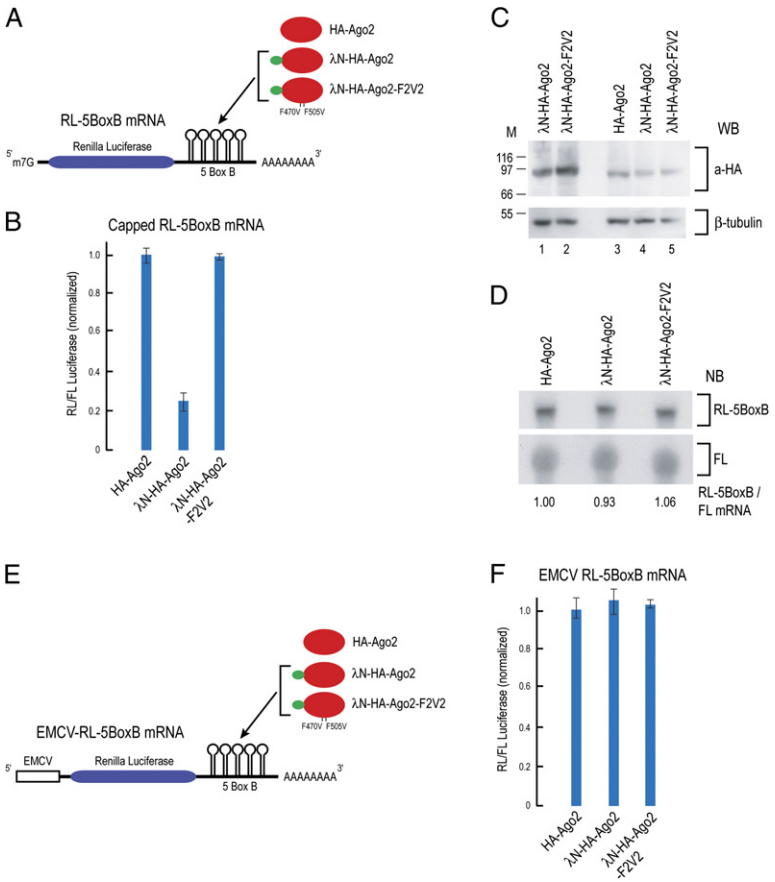

Figure 4. λN-HA-Ago2 Represses Translation in an m^{7}G Cap-Dependent Manner, but λN-HA-Ago2 F2V2 Is Unable to Repress Translation

(A) Schematic of the λN-tethering assay, the capped RL-5BoxB reporter, and the Ago2 constructs used.

(B) HeLa human cells were cotransfected with Renilla Luciferase RL-5BoxB bearing 5BoxB-binding sites for λN in the 3′-UTR, along with firefly luciferase (FL; as a normalization control) and the indicated Ago constructs. Results shown are average values (with standard deviations) of normalized RL/FL activities obtained from six separate experiments.

(C) Protein levels of indicated HA-tagged Ago proteins were analyzed by western blots (WB) of lysates from transfected cells; β-tubulin served as normalization control.

(D) mRNA levels of RL-5BoxB and FL (as a normalization control) were analyzed by Northern blot (NB) of lysates from transfected cells using radiolabeled riboprobes and quantitated with storage phosphor autoradiography. The ratio of RL-5BoxB mRNA to FL mRNA is shown under each lane.

(E) Schematic of the uncapped, IRES-driven, reporter mRNA (EMCV-RL-5BoxB), and the Ago2 constructs used.

(F) 293 human cells were first transfected with the indicated Ago constructs and then transfected with uncapped EMCV-RL-5BoxB mRNA and FL mRNA (as a normalization control). Results shown are average values (with standard deviations) of normalized RL/FL activities obtained from four separate experiments.

tethered Ago2. As shown in Figures 4E and 4F, the translation of transfected reporter RNA containing the IRES of the encephalomyocarditis virus (EMCV RL-5BoxB) was not repressed by tethered Ago2.

These results demonstrate that translational repression by Ago2 requires an m^{7}G cap and cancellation of the cap binding activity of Ago2 by mutations of the two conserved phenylalanines (F470 and F505) of the MC domain abolishes the effect of Ago2 on translation. The mRNA levels of the reporter are not affected. Furthermore, these mutations do not affect the ability of Ago2-F2V2 to assemble with miRNAs or its catalytic activity. Thus, we have effectively dissected the inhibitory activity of Ago2 in translation from its endonucleolytic activity.

The MC Domain of Ago2 Mediates Inhibition of Translation Initiation

We next analyzed the polysome profiles of RL-5BoxB mRNA in HeLa cells transfected with HA-Ago2, λN-HA-Ago2, or λN-HA-Ago2-F2V2. Cytoplasmic lysates from transfected HeLa cells were subjected to sedimentation on 10%–50% sucrose gradients in the presence of cycloheximide (to preserve the association of translating ribosomes with mRNAs). The gradients were divided in 12 equal-volume fractions, and the position of soluble, light particles, 40S subunits, monoribosomes, and heavier sedimenting polyribosomes was monitored with continuous A_{254} measurements during fractionation. Total RNA was isolated and equal-volume aliquots were fractionated by electrophoresis (Figure S1 shows the ethidium bromide-stained gels) and analyzed by Northern blots using radiolabeled riboprobes against RL-5BoxB mRNA or DNA probes against endogenous β-actin mRNA. The sedimentation of β-actin mRNA is an accurate indicator for the position and integrity of polysomes, since almost all β-actin mRNA is actively translated. As shown in Figure 5A, a significant fraction of RL-5BoxB mRNA was shifted to the light fractions in the presence of λN-HA-Ago2, as previously reported Pillai et al. (2005), indicating a translation initiation block. In contrast, in the presence of HA-Ago2 or λN-HA-Ago2-F2V2, most of RL-5BoxB mRNA is found toward the bottom of the gradient, cosedimenting with actively translating polysomes (Figures 5B and 5C). These results (quantitation shown in Figure 5D) indicate that mutations abolishing the cap-binding activity of Ago2 are also unable to negatively regulate translation of its mRNA target.

Specific Aromatic Amino Acids in the MC Domain of Ago2 Mediate Translational Repression

We next asked whether a single-valine mutation of the MC domain of Ago2 (F470) would be sufficient to cancel the

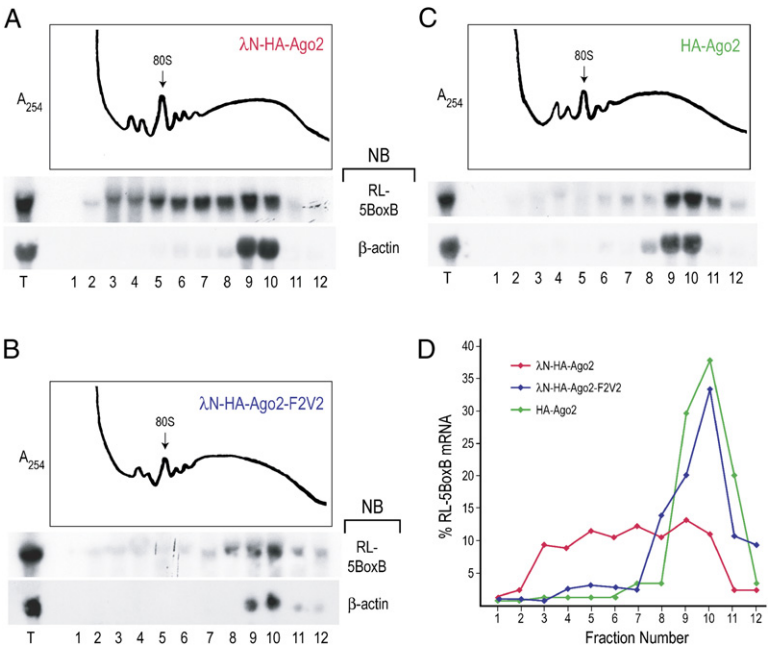

Figure 5. λN-HA-Ago2-F2V2, Unlike Wild-Type λN-HA-Ago2, Cannot Inhibit Translation Initiation

(A)–(C) Polysomal profiles from HeLa cells that had been cotransfected with Renilla Luciferase RL-5BoxB (bearing 5BoxB-binding sites for λN in the 3′-UTR), along with *λN-HA-Ago2* (A), *λN-HA-Ago2-F2V2* (B), or *HA-Ago2* (C) were analyzed by sucrose gradient sedimentation. RNA was extracted from each fraction and analyzed with probes for RL-5BoxB and endogenous β-actin on Northern blots (NB).

(D) Quantitation of mRNA distribution plotted as percent of total radioactivity present in each lane.

translational regulatory activity of Ago2 and whether the translational repressive activity of Ago2 would be affected by substitution of one or both phenylalanines of the MC domain (F470 alone or F470 and F505) by tryptophans (mimicking the cap-binding domain of eIF4E, which utilizes tryptophans to engage the cap). Using the tethering assay described above, we transfected HeLa cells with λN-HA-Ago2, λN-HA-Ago2-F1V1(Ago2^{F470V}), λN-HA-Ago2-F2V2 (Ago2$^{F470V;F505V}$), λN-HA-Ago2-F1W1(Ago2^{F470W}), or λN-HA-Ago2-F2W2(Ago2$^{F470W;F505W}$) along with RL-5BoxB and FL plasmids. As shown in Figure 6A, both λN-HA-Ago2-F1V1 and λN-HA-Ago2-F2V2 were unable to repress the translation of RL-5BoxB, whereas λN-HA-Ago2-F1W1 and λN-HA-Ago2-F2W2 restored translational regulatory activity, as compared to the wild-type λN-HA-Ago2. The λN-HA-Ago2-F2V2 and λN-HA-Ago2-F2W2 mutants were also tested in human 293T cells with similar results (Figure 6B). These findings highlight the importance of the two aromatic residues of the MC domain of Ago2 in translational repression and support a model (Figure 7) in which the MC domain of Ago2 protein exerts translational repression via interaction with the m^{7}G cap.

DISCUSSION

Our findings support a model where the MC domain of Ago2 represses mRNA translation by binding to the m^{7}G cap of mRNA targets. We propose a model where binding

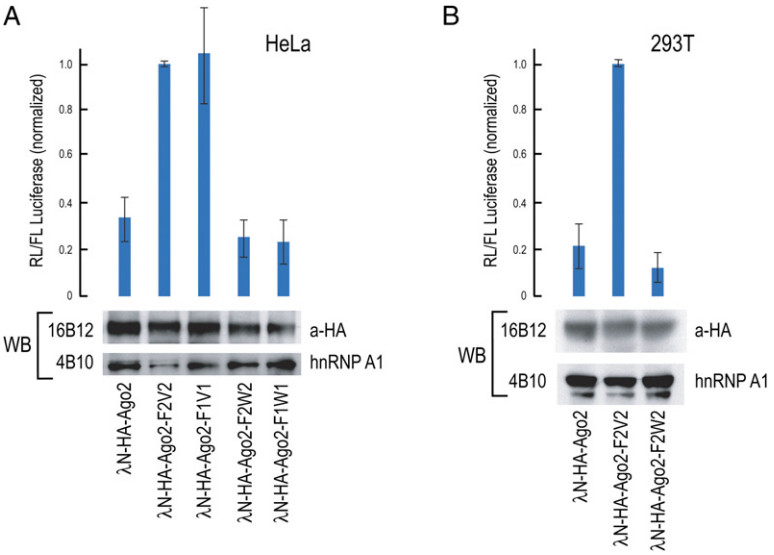

Figure 6. λN-HA-Ago2-F1V1 Single Mutant Is Unable to Repress Translation while Substitution of Phenylalanines by Tryptophans Restores Translational Repressing Activity of Ago2

(A and B) HeLa (A) or 293T (B) human cells were cotransfected with Renilla Luciferase RL-5BoxB bearing 5BoxB-binding sites for λN in the 3′-UTR, along with firefly luciferase (FL; as a normalization control) and the indicated Ago2 constructs. Results shown are average values (with standard deviations) of normalized RL/FL activities obtained from ten (A) or nine (B) separate experiments. Protein levels of indicated HA-tagged Ago proteins were analyzed by western blots (WB) of lysates from transfected cells; hnRNP-A1 served as normalization control.

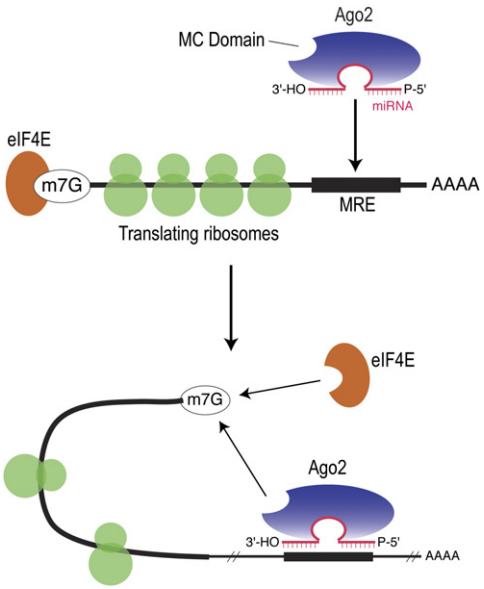

Figure 7. Proposed Mechanism of Translational Repression by Ago2

miRNA-guided deposition of Ago2 to an mRNA target leads to binding of the MC domain of Ago2 to the mRNA cap (m7G) and thus exclusion of eIF4E and inhibition of translation initiation.

of the m^7G cap by Ago2 precludes the recruitment of eIF4E (Figure 7). In this model, the miRNA acts as a specificity determinant to deposit the Ago2 protein onto mRNA targets and provides a simple and direct way to explain inhibition of translation initiation as seen by mammalian miRNAs and first proposed by the Filipowicz lab (Pillai et al., 2005). Additional mechanisms, such as inhibition of protein production on actively translating ribosomes or ribosome dropoff during elongation, and additional factors such as GW182, Dcp1, Dcp2, and RCK/p54, may function with Ago proteins and miRNAs for optimal repression in vivo.

An important feature of miRNA-directed translational repression is its apparent cooperativity: increasing the number of MREs in the 3′-UTR of an mRNA target enhances translational repression (Doench et al., 2003; Bartel, 2004; Pillai et al., 2005). Cooperativity is also seen when multiple MREs for different miRNAs are found in the 3′-UTR of the same mRNA target, arguing that common factors, notably Ago proteins, bound to all miRNAs are responsible for the enhanced translational repression (Krek et al., 2005). Indeed, this cooperativity is accurately recapitulated in experiments with tethered Ago2; increasing the number of BoxB sites in the 3′-UTR of the reporter leads to enhancement of the translational repression by λN-HA-Ago2 (Pillai et al., 2004). We propose that multiple MREs, within the same mRNA target, increase the number of Ago2 molecules bound to the mRNA, thus increasing the probability that they will interact with the m^7G cap and augment translational repression by limiting availability of the m^7G cap to eIF4E. In this model, Ago2 binds to m^7G cap less efficiently than eIF4E. Therefore, optimal repression by Ago2 and thus optimal eIF4E competition would require multiple Ago2 molecules. Weak Ago2 binding to the m^7G cap also makes biological sense, since an Ago2 protein with high affinity to the m^7G cap would lead to generalized and strong translational inhibition. Our model is also consistent with weak translational repression of mRNA targets that bear single MREs. Indeed, the vast majority of mRNA targets contain a single MRE for any given miRNA and the level of translational repression is typically modest (usually 1.5- to 2-fold repression) (Poy et al., 2004; Kiriakidou et al., 2004; Stark et al., 2005; Farh et al., 2005). Such modest and noncomplete repression may also explain why many miRNAs cosediment with actively translating, endogenous, mRNAs in polysomes (Nelson et al., 2004; Kim et al., 2004; Maroney et al., 2006). Lastly, our findings do not exclude additional mechanisms of miRNA and Ago regulation, perhaps in the presence of additional factors such as inhibition of protein production on actively translating ribosomes (Olsen and Ambros, 1999; Petersen et al., 2006; Nottrott et al., 2006) or ribosome dropoff (Petersen et al., 2006) during elongation or degradation of mRNAs (Rehwinkel et al., 2005; Wu et al., 2006; Giraldez et al., 2006).

An important observation is that the MC motif is not detected in Ago proteins from organisms that do not contain miRNAs, or do not use miRNAs for translational repression. Specifically, all mammalian Ago proteins and certain Ago proteins from nematodes and flies, where translational repression by miRNAs has been demonstrated, contain the MC domain, and thus these Ago proteins may be capable of repressing translation. The MC domain is present in *Drosophila* AGO1, which is required for miRNA function, but not in *Drosophila* AGO2, which functions predominantly in siRNA pathways (Kataoka et al., 2001; Okamura et al., 2004), although more recent studies show overlapping functions of *Ago1* and *Ago2* pathways in flies (Meyer et al., 2006). The MC domain is present in *C.elegans* ALG-1 and ALG-2 Ago proteins but absent from the remaining 25 members of the *C.elegans* Argonaute protein family, consistent with the finding that there are distinct RNAi-related pathways in nematodes, with ALG-1 and ALG-2 proteins participating in the microRNA pathway and all other nematode Argonaute proteins being associated with exo- or endo-RNAi pathways (Yigit et al., 2006). Finally, the MC domain is absent from Ago proteins in organisms that do not have miRNAs such as fission yeast and Archaea. Although the MC motif is not found in Archaeal Agos, the structures of the *P. furiosus* and *A. aeolicus* Ago proteins show that a major portion of the Mid domain is accessible and thus may be capable of interacting with other factors (Song et al., 2004; Yuan et al., 2005). The MC domain is also not present in PIWI proteins, which are almost exclusively expressed in the germline (reviewed in Kim [2006]). Notably, tethering of HIWI, a human PIWI protein, in the 3′-UTR of RL-5BoxB, is unable to repress RL translation (Pillai et al., 2004). In contrast, tethering of all human Ago proteins (Ago1–4) in the 3′-UTR of RL-5BoxB results in strong repression of RL translation

(Pillai et al., 2004). These studies along with the finding that translational repression is unaffected in Ago2 null mouse embryonic fibroblasts (Liu et al., 2004) also show that the endonuclease activity of mammalian Ago proteins is not required for translational repression. In flies, PIWI proteins associate with repeat-associated siRNAs (Vagin et al., 2006; Saito et al., 2006). Mammalian PIWI proteins do not assemble with miRNAs or siRNAs but bind to slightly larger RNAs termed piRNAs (Aravin et al., 2006; Girard et al., 2006; Lau et al., 2006; Grivna et al., 2006a; Watanabe et al., 2006). The mouse MIWI protein can associate with m^7GTP sepharose, suggesting that MIWI proteins may also function in translation (Grivna et al., 2006b). However, since the MC domain is absent from the MIWI protein, it is possible that MIWI contains another cap-binding motif or associates with the cap-analog resin indirectly, via interactions with another cap-binding protein. However, the biochemical function of MIWI proteins and of piRNAs is unknown, and it is difficult to ascertain the functional consequences of this interaction at this point. Finally, the absence of the MC motif from plant Agos is intriguing and suggests that plant miRNAs may not be capable of repressing translation through interactions with the cap (but other mechanisms cannot be excluded). So far translational repression by miRNAs in plants has only been implicated for the control of very few mRNA targets (Aukerman and Sakai, 2003; Chen, 2004; Gandikota et al., 2007), while most known plant miRNAs show extensive complementarity with their targets, directing target mRNA cleavage (Bartel, 2004; Schwab et al., 2005; Bonnet et al., 2006).

EXPERIMENTAL PROCEDURES

Constructs and Generation of Point Mutants

The RL-5BoxB, λN-HA-Ago2, and HA-Ago2 constructs were a generous gift from R. Pillai and W. Filipowicz. pGL3 plasmid expressing FL was purchased from Promega. Site-directed mutagenesis was performed using the Quickchange XL kit (Stratagene). EMCV-RL-5BoxB was generated by cloning the 5BoxB sequence in the 3′-UTR of EMCV-RL plasmid (a gift from R. Pillai and W. Filipowicz). Details are provided in the Supplemental Experimental Procedures.

Cell Culture, Transfections, Luciferase Assays, and Western Blots

Details are provided in the Supplemental Experimental Procedures.

Immunopurification of λN-HA-Ago2 and λN-HA-Ago2-F2V2 and m^7GTP Sepharose Bindings

293T cells (2×10^7) were transfected with 16 μg of appropriate Ago2-expressing plasmid (λN-HA-Ago2 or λN-HA-Ago2-F2V2). The next day, the cells from each transfection were split 1:1 (in four 100 mm dishes) and the cells were harvested 48 hr posttransfection. Immunoaffinity purification and elution of proteins was performed as previously described (Mourelatos et al., 2001). Briefly, the cell pellet was resuspended in lysis buffer (20 mM Tris-HCL at pH 7.5, 200 mM NaCl, 2.5 mM $MgCl_2$, and 0.5% Triton X-100 plus protease inhibitors) lysed by sonication and clarified. The supernatant was used for immunoprecipitations with 100 μl of anti-HA agarose resin (16B12 covalently bound to the agarose matrix, Covance). The resin was then washed extensively with the same buffer, except that the NaCl concentration was raised to 500 mM. The final wash was with 20 mM Tris-HCL, 100 mM NaCl, 2.5 mM $MgCl_2$, and 1 mM DTT. Captured HA-tagged Ago2 proteins were eluted with 0.1 M glycine at pH 2.5 and neutralized with 1 M Tris-HCL at pH 8.0 and dialyzed against phosphate-buffered saline (PBS). Eluates (20 μl) were incubated with 10 μl bed volume of m^7GTP sepharose (GE Healthcare) or with 10 μl bed volume of GTP-sepharose (Axxora) in 200 μl of binding buffer (20 mM Tris-HCL, 100 mM NaCl, 2.5 mM $MgCl_2$, 1 mM DTT, and 1 mg/ml heparin) for 1 hr at 4°C using constant rotation. The beads were washed four times with 800 μl of 20 mM Tris-HCL, 100 mM NaCl, 2.5 mM $MgCl_2$, and 1 mM DTT, and the proteins were eluted with NuPAGE sample buffer (Invitrogen). As a competitive inhibitor, 75 μM of cap analog (m^7GpppG; New England Biolabs) was added to the binding reaction were indicated; nonmethylated GpppG (NEB) was also used at 75 μM. Proteins were analyzed by electrophoresis in 4%–12% NuPAGE (Invitrogen) and visualized by western blot using the anti-HA monoclonal antibody 16B12 (Covance).

Cellular Fractionation, Polysome Analysis, and Northern Blots

These were performed as previously described (Pillai et al., 2005; Nelson et al., 2004), with few modifications. Forty-eight hours posttransfection, cycloheximide (final 100 μg/ml) was added to the cells 5 min prior to harvest to arrest translation elongation. For each gradient, the cells from two 100 mm plates were washed with PBS containing 100 μg/ml cycloheximide, trypsinized (again in the presence of 100 μg/ml cycloheximide), combined in one 15 ml Falcon tube, and washed twice with ice-cold PBS containing 100 μg/ml cycloheximide and the cell pellet was lysed with 600 μl of lysis buffer (20 mM Tris-HCL at pH 7.5, 200 mM NaCl, 5 mM $MgCl_2$, 0.5% Triton X-100, 100 μg/ml cycloheximide, 0.1 U/ml Rnasin, complete EDTA-free protease inhibitors; Roche) on ice by passing the cells four times through a 25G needle. The lysate was clarified by centrifugation at 14,000 g for 10 min at 4°C. A 50 μl aliquot was removed for total RNA isolation, and the clarified supernatant was loaded on 10%–50% sucrose gradients (prepared in a buffer containing 20 mM Tris-HCL at pH 7.5, 200 mM NaCl, and 5 mM $MgCl_2$). All ultracentrifugations were carried with a SW41 rotor at 4°C at 36,000 rpm for 2 hr. Twelve fractions were collected with a Biocomp collector, and the A_{254} absorbance was monitored with a UVM-II monitor (GE healthcare) using a Dataq DI-158U USB data acquisition interface (Dataq) connected to a PC and visualized using WinDaq software (Dataq). Total RNA was isolated from each fraction using Trizol-LS (Invitrogen). The RNA pellet from each fraction was digested with three units of RQ1 RNase-free DNase I (Promega) for 15 min at 37°C and was then extracted with phenol/chloroform. The RNA pellet was resuspended in 12 μl of Millipore H_2O, and one-half was used for Northern blots.

To generate riboprobes for hRL and FL, the cDNA inserts from RL-5BoxB plasmids and pGL3 plasmids were amplified with PCR, using reverse primers that contained the T7 RNA polymerase promoter sequence. Primer sequences were the following for hRL: Forward, hRL-F, 5′-ATGGCTTCCAAGGTGTACGACCCCGAG; Reverse, hRL-T7-R, 5′-TAATACGACTCACTATAGGGTTACTGCTCGTTCTTCAGCA CGCGCTC. Primer sequences were the following for FL: Forward, FL-F, 5′- ATGGAAGACGCCAAAAACATAAAGAAA; Reverse, FL-T7-R, 5′-TAATACGACTCACTATAGGGTTACACGGCGATCTTTCCGCCC TTCTT. The PCR products were gel purified, and 200 ng of each was used as template with the Strip-EZ T7 RNA kit (Ambion). In vitro transcription was performed according to the manufacturer's instructions with the following exceptions: 11 μl of [α-^{32}P]UTP at 3000 Ci/mmol and 2 μl of 67 μM cold UTP was used per reaction. To generate radiolabeled DNA probe for β-actin, 30 ng of β-actin cDNA (Ambion) was used in a random priming reaction with 5 μl of [α-^{32}P]dCTP at 3000 Ci/mmol using the Ready-To-Go DNA labeling beads (GE Healthcare). RNA was denatured with glyoxal (Ambion), resolved on 1% glyoxal-agarose gels, and electroblotted onto Hybond-N+ membranes (GE Healthcare) using the Transblot SD apparatus (Biorad); electroblotting was with 0.5× TBE, at 20 V constant for 1 hr per gel transfer. Membranes were blocked with Ultrahyb (Ambion) and hybridized

overnight with probes diluted in Ultrahyb at 68°C (riboprobes) or at 44°C (DNA probes for actin). Membranes were washed twice, 30 min each with 2× SSC, 0.1% SDS at room temperature and twice, 1 hr each, with 0.1X SSC, 0.1% SDS at 68°C (riboprobes) or at 44°C (DNA probes for actin). Quantitation of signal was performed with storage phospor autoradiography using a Storm 860 (GE Healthcare). Stripping of the membranes was performed according to Ambion's instructions.

Immunoprecipitations and Cleavage Assays

These were performed essentially as described in (Kiriakidou et al., 2005). For immunoprecipitation of endogenous Ago2 protein, 2 μl of 2A8 ascites was used with 20 μl of protein-G agarose (Invitrogen) and HeLa cell lysate from one 100 mm dish. For immunoprecipitations of HA-tagged Ago constructs, lysate from two 100 mm HeLa dishes transfected with appropriate Ago2-expressing plasmid as described above was used with 20 μl bed volume of anti-HA (16B12)-conjugated agarose beads (Covance). Cells were lysed in 20 mM Tris-HCL at pH 7.5, 200 mM NaCl, 2.5 mM $MgCl_2$, 0.5% Triton X-100, and 0.1 U/ml RNasin, complete EDTA-free protease inhibitors, as described above. The beads were washed extensively with the same buffer except that the NaCl concentration was raised to 600 mM. The final washes were with 20 mM HEPES-KOH at pH 7.5, 100 mM KCl, and 0.5 mM DTT. Preparation of radiolabeled target complementary to let-7 and cleavage assays were performed as previously described (Kiriakidou et al., 2005). RNA from cleavage assays was resolved using 20% UREA-PAGE and visualized by autoradiography.

Supplemental Data

Supplemental Data include one figure and Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/cgi/content/full/129/6/1141/DC1/.

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