*8692–8705* *Nucleic Acids Research, 2013, Vol. 41, No. 18* *Published online 17 July 2013*
*doi:10.1093/nar/gkt619*

# miRISC recruits decapping factors to miRNA targets to enhance their degradation

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Received April 7, 2013; Revised June 22, 2013; Accepted June 24, 2013

## ABSTRACT

**MicroRNA (miRNA)-induced silencing complexes (miRISCs) repress translation and promote degradation of miRNA targets. Target degradation occurs through the 5′-to-3′ messenger RNA (mRNA) decay pathway, wherein, after shortening of the mRNA poly(A) tail, the removal of the 5′ cap structure by decapping triggers irreversible decay of the mRNA body. Here, we demonstrate that miRISC enhances the association of the decapping activators DCP1, Me31B and HPat with deadenylated miRNA targets that accumulate when decapping is blocked. DCP1 and Me31B recruitment by miRISC occurs before the completion of deadenylation. Remarkably, miRISC recruits DCP1, Me31B and HPat to engineered miRNA targets transcribed by RNA polymerase III, which lack a cap structure, a protein-coding region and a poly(A) tail. Furthermore, miRISC can trigger decapping and the subsequent degradation of mRNA targets independently of ongoing deadenylation. Thus, miRISC increases the local concentration of the decapping machinery on miRNA targets to facilitate decapping and irreversibly shut down their translation.**

## INTRODUCTION

MicroRNAs (miRNAs) are a large family of endogenous non-coding RNAs that post-transcriptionally silence the expression of messenger RNA (mRNA) targets containing complementary sequences and are implicated in nearly all developmental and cellular processes that have been investigated thus far (1). To exert their regulatory functions, miRNAs associate with Argonaute (AGO) proteins in effector complexes known as miRNA-induced silencing complexes (miRISCs). These complexes induce endonucleolytic cleavage of fully complementary targets or translational repression, mRNA deadenylation and 5′-to-3′ exonucleolytic decay of targets with partially complementary binding sites (1–3).

Silencing of mRNA targets containing partially complementary miRNA-binding sites requires the association of AGOs with a protein of the GW182 family, which mediates the translational repression and degradation of these targets (2,4). The mechanism of translational repression has yet to be elucidated, although increasing evidence points to an inhibition of translation initiation (3). In contrast, the mechanism of miRNA target degradation is relatively well understood. It is known that miRNAs accelerate target degradation through the 5′-to-3′ mRNA decay pathway (2). In this pathway, mRNAs are first deadenylated, then decapped and finally degraded by the major cytoplasmic 5′-to-3′ exonuclease XRN1 (5,6). mRNA deadenylation is catalyzed by the sequential action of two cytoplasmic deadenylase complexes (the PAN2-PAN3 and the CCR4-NOT complexes) (6). These complexes are recruited to miRNA targets through interactions with GW182 proteins (7–9). Depending on the cell type and/or specific target involved, the deadenylated mRNA target can be stored in a translationally repressed state, as observed, for example, in *Caenorhabditis elegans* embryos (10). However, in diverse organisms and cell types, deadenylated miRNA targets are rapidly decapped and degraded by XRN1 (11–19).

Decapping is catalyzed by the decapping enzyme DCP2, which requires additional co-factors for full activity/stability (5). These include DCP1, HPat, EDC4 and the DEAD-box protein Me31B (also known as DDX6 or RCK/p54). A role for decapping activators in miRNA-mediated mRNA destabilization is supported by the observation that the abundance of predicted and validated miRNA targets increases when decapping activators are depleted or when dominant-negative forms of decapping factors are overexpressed (12–20).

A question that remains open is whether decapping of miRNA targets occurs exclusively as a consequence of deadenylation or whether miRISCs can also recruit components of the decapping machinery independently of ongoing deadenylation. Evidence for the existence of a specific interaction between decapping factors and miRISC stems from the following observations. First, AGO proteins co-immunoprecipitate with the catalytic

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subunit of the decapping complex, DCP2 and other decapping factors including DCP1, RCK and EDC4 (also known as Ge-1 or Hedls) in human cells (20–24). Second, GW182 co-immunoprecipitates with HPat in *Drosophila melanogaster* (*Dm*) Schneider cells (*Dm* S2 cells) (25). Third, EDC4 was identified as a suppressor of miRNA-mediated gene silencing in *Dm* cells and in *Arabidopsis thaliana* (15,26), and it co-localizes with miRNA targets in human cells (23). Fourth, RCK associates with HIV-1 mRNA in the presence of miR-29a (27). Finally, miRNAs and their targets localize to P-bodies wherein decapping factors, AGOs and GW182 proteins accumulate (21,22,28–30). However, it is unknown whether the interactions between decapping factors and miRISC components are direct and at which step of miRNA-mediated repression decapping activators are recruited to the mRNA target.

In this study, we demonstrate that miRISCs promote the association of DCP1, HPat and Me31B (the *D. melanogaster* RCK ortholog) with miRNA targets. This association was recapitulated on RNA polymerase III (Pol III)-transcribed targets lacking a 5′ cap structure, an open reading frame (ORF) and a poly(A) tail, suggesting that decapping factors are recruited by miRISC onto the target mRNA independently of ongoing deadenylation and decapping. We further show that miRNA targets lacking a poly(A) tail are degraded through decapping. Together with previous studies (1–3), our results indicate that miRISCs accelerate the irreversible degradation of miRNA targets by promoting decapping independently of their effects on deadenylation.

## MATERIALS AND METHODS

### DNA constructs

Luciferase reporters and plasmids for the expression of miRNAs and epitope-tagged proteins in *D. melanogaster* cells have been described elsewhere (17,31–35). Plasmids encoding the Alu and hammerhead ribozyme (HhR) reporters are described in the Supplementary Figure S1. To generate plasmids expressing V5-MBP and V5-DCP1, the corresponding cDNA sequences were amplified by PCR using pAc5.1B-λN-HA-MBP and pAc5.1B- λN-HA-DCP1 as templates (34) and cloned between the *Kpn*I and *Xba*I sites of vector pAc5.1B (Invitrogen). To obtain plasmids for the expression of HA-glutathione S-transferase (GST)-tagged proteins, the corresponding cDNAs were cloned into pAc5.1B- λN-HA-GST, and the region encoding the λN peptide was deleted (31).

### Antibodies and western blotting

The protein co-immunoprecipitations shown in Supplementary Figure S5 were performed as described previously (19). Polyclonal anti-eIF4E and PABPC1 antibodies were generated by immunizing rabbits with purified recombinant *Dm* eIF4E (full-length) and poly(A)-binding protein 1 (PABP) (amino acids 501–634). For western blotting, these antibodies were used at the following dilutions: eIF4E (1:3000) and PABPC1 (1:10 000). HA-tagged proteins were immunoprecipitated using anti-HA mouse monoclonal antibody (HA.11 Clone 16B12, Covance). GFP-tagged proteins were immunoprecipitated using polyclonal antibodies raised in rabbits. HA-tagged and GFP-tagged proteins were detected in western blots using horseradish peroxidase (HRP)-conjugated monoclonal anti-HA (Roche 3F10; 1:5000) and anti-GFP (Roche 11814460001; 1:2000) antibodies, respectively. V5-tagged proteins were immunoprecipitated and detected using anti-V5 antibodies (Invitrogen, dilution for western blot 1:5000). All western blotting experiments were developed with the enhanced chemiluminescence western blotting detection system (GE Healthcare) as recommended by the manufacturer.

### RNA interference, transfections, luciferase assays and RNA analysis

Protein depletions were performed as described previously (15). S2 cells were depleted on days 0 and 4 and transfected on day 6. Transfections were performed in 6-well plates using Effectene transfection reagent (Qiagen). The transfection mixtures contained the following amounts of expression plasmids: miRNA target (600 ng), R-Luc control (500 ng), miRNA (200 ng), GW182 (200 ng), DCP2 (wild-type or mutant 300 ng), HA-GST-tagged AGO1, Me31B and HPat (25 ng), HA-GST-MBP (12.5 ng), HA-eIF4E (10 ng), HA-DCP1 or V5-DCP1 (100 ng), HA-MBP (300 ng), POP2* (500 ng). When necessary, transfection mixtures were supplemented with pAc5.1B (Invitrogen). Firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase reporter assay system (Promega). Northern blotting was performed as described previously (15).

### RNA co-immunoprecipitations and GST pull downs

S2 cells were collected two days after transfection, washed with ice-cold phosphate buffered saline and resuspended in 50 µl of lysis buffer [10 mM Hepes (pH 7.8), 10 mM potassium acetate, 0.2 mM EDTA and 0.5 mM dithiothreitol] containing 1% NP-40, 0.4 unit/µl riboLock (Fermentas) and 1 unit/µl protease inhibitors (EDTA-free, Roche). The lysates were immediately diluted by adding 450 µl of lysis buffer containing 0.1% NP-40 and 1 unit/µl protease inhibitors, incubated on ice for 15 min with occasional sonication (30 s, three times), and then centrifuged at 16 000 *g* for 15 min at 4°C. The cleared lysates were diluted with 350 µl of lysis buffer containing 0.1% NP-40 and 350 mM KCl. To immunoprecipitate epitope-tagged or endogenous proteins, the lysates were supplemented with the corresponding antibodies and incubated for 30 min at 4°C. Then, 10 µl of Protein G-agarose (GE Healthcare) was added, and the incubation was continued for 2 h at 4°C. For GST pull downs, the diluted lysates were incubated with 10 µl of Prothino Glutathione Agarose 4B beads (Macherey-Nagel) for 2 h at 4°C. The beads were washed three times with lysis buffer containing 0.1% NP-40 and 150 mM KCl and the purified mRNPs were eluted with 60 µl of 2× protein sample buffer for 5 min at 25°C. For RNA analysis, 120 µl of peqGOLD TrifastTM, 800 µl of TrifastTM FL (peqlab) and 1.5 µg of a 700-bp

double-stranded RNA was added to the lysates (input) and eluate. RNA samples were purified and analyzed by northern blotting as described previously (15). The glutathione agarose and Protein G-agarose beads were preblocked by incubation with 50 µg of yeast RNA (Boehringer Mannheim GmbH) and 4 µg of BSA in lysis buffer for 1 h. Beads were washed twice with lysis buffer before use.

## RESULTS

### DCP1, Me31B and HPat associate with miRNA targets

To monitor the association of decapping factors with silenced miRNA targets, we performed co-immunoprecipitation assays with epitope-tagged versions of these proteins and determined mRNA levels of an actively translated or silenced miRNA reporter in the immunoprecipitates using northern blotting. We used an miRNA reporter consisting of the firefly luciferase (F-Luc) ORF followed by the 3′ untranslated region of the *Dm* gene *CG5281*, which is silenced by miR-12. The rationale for selecting this reporter is that its silencing is suppressed in *Dm* S2 cells that are depleted of decapping factors (17). Additionally, silencing of this reporter is observed only on co-expression of exogenous miR-12 because miR-12 is expressed at low levels in S2 cells (36).

To validate our approach, we first analyzed the interaction of GST-tagged AGO1 with the F-Luc-CG5281 mRNA. We observed that GST-AGO1 pulled down the F-Luc-CG5281 mRNA on glutathione agarose beads in the presence of miR-12 (Supplementary Figure S2A, lane 4). The interaction of AGO1 with the F-Luc-CG5281 mRNA was slightly enhanced in cells that were overexpressing GW182 (Supplementary Figure S2A, lane 8). As a negative control, GST-tagged MBP did not detectably precipitate the reporter (Supplementary Figure S2A, lanes 1, 2, 5 and 6). To confirm that equal amounts of total RNA were included in the precipitations, we probed for the presence of ribosomal RNA in the input samples. In addition, input and pull-down fractions were spiked with a 700 bp dsRNA to control for recovery of the RNA after precipitation.

Because the mRNA target is partially degraded at the steady state (Supplementary Figure S2A; e.g. lane 2 versus 1), to avoid target degradation and trap mRNA decay intermediates, the pull-down experiments were repeated in S2 cells overexpressing a catalytically inactive DCP2 mutant (DCP2*, carrying the E361A substitution). In these cells, miRNA targets accumulate in a deadenylated form (because deadenylation precedes decapping), but decapping and further degradation are partially inhibited (Figure 1A versus Supplementary Figure S2A) (15–18). We observed that GST-AGO1 pulled down the deadenylated reporter in a miR-12-dependent manner (Figure 1A, lanes 4 and 8). Western blot analyses indicated that GST-AGO1 was expressed and pulled down at comparable levels in the presence or absence of miR-12 (Supplementary Figure S2B).

To further validate our approach, we examined the association of the mRNA target with GW182 in cells expressing the catalytically inactive DCP2 mutant. F-Luc-CG5281 mRNA was co-immunoprecipitated with V5-tagged GW182 in the presence of miR-12 (Figure 1B, lane 4). In contrast, a GW182 mutant that does not interact with AGO1, i.e. in which all 12 N-terminal GW-repeats were mutated to alanines (12xGW mutant) (37) did not co-immunoprecipitate the F-Luc reporter, regardless of the presence of miR-12 (Figure 1B, lanes 5 and 6). The V5-GW182 proteins were expressed at comparable levels and were precipitated with similar efficiencies, regardless of miR-12 (Supplementary Figure S2C). Together, these results validate the specificity of our approach and further indicate that AGO1 and GW182 remain bound to the target mRNA when mRNA degradation is inhibited, as shown previously (31,35).

Having established that the association of AGO1 and GW182 with F-Luc-CG5281 mRNA is enhanced in the presence of miR-12, we next used a similar approach to investigate the association of the decapping factors Me31B, HPat, DCP1, DCP2 and EDC4. We selected these decapping factors because their depletion suppresses the degradation of a large number of miRNA targets in S2 cells (15), whereas depletion of EDC3 or subunits of the LSm1–7 complex stabilized only 5% of AGO1 targets (15). Therefore, EDC3 and the subunits of the LSm1–7 complex were not included in our analysis.

In contrast to the association of AGO1 and GW182 with the reporter, which was strongly stimulated by miR-12, DCP1, HPat and Me31B associated with the reporter in the absence of miR-12, but their association was enhanced in the presence of miR-12 (Figure 1C–E, lanes 4 versus 3). Western blot analyses indicated that the precipitation efficiency of these decapping factors was comparable in the absence or presence of miR-12 (Supplementary Figure S2D–F). EDC4 did not give reliable results in this assay because the protein was expressed at low levels. Unexpectedly, binding of the DCP2 catalytic mutant to the target was barely detectable (data not shown), despite the fact that its overexpression inhibited target degradation. This suggests that DCP2 may bind to the target only transiently or that its binding may require the prior dissociation of eIF4E, which (as shown below) remained bound to the target under our experimental conditions. We conclude that miRISC promotes the association of a subset of decapping factors with deadenylated miRNA targets.

### Decapping factors associate with miRNA targets in the presence of eIF4E

It has been suggested that AGOs compete with the cap-binding protein eIF4E for binding to the cap structure (38,39). Although subsequent functional and structural studies indicate that AGOs do not bind to the cap as proposed by these earlier studies (40,41), the possibility that miRISCs promote the dissociation of eIF4E from the cap by alternative mechanisms cannot be ruled out. An increased accessibility of the cap by displacement of eIF4E could explain the observed miRNA-mediated association of decapping factors with F-Luc-CG5281 mRNA. To test this possibility, we analyzed the association of

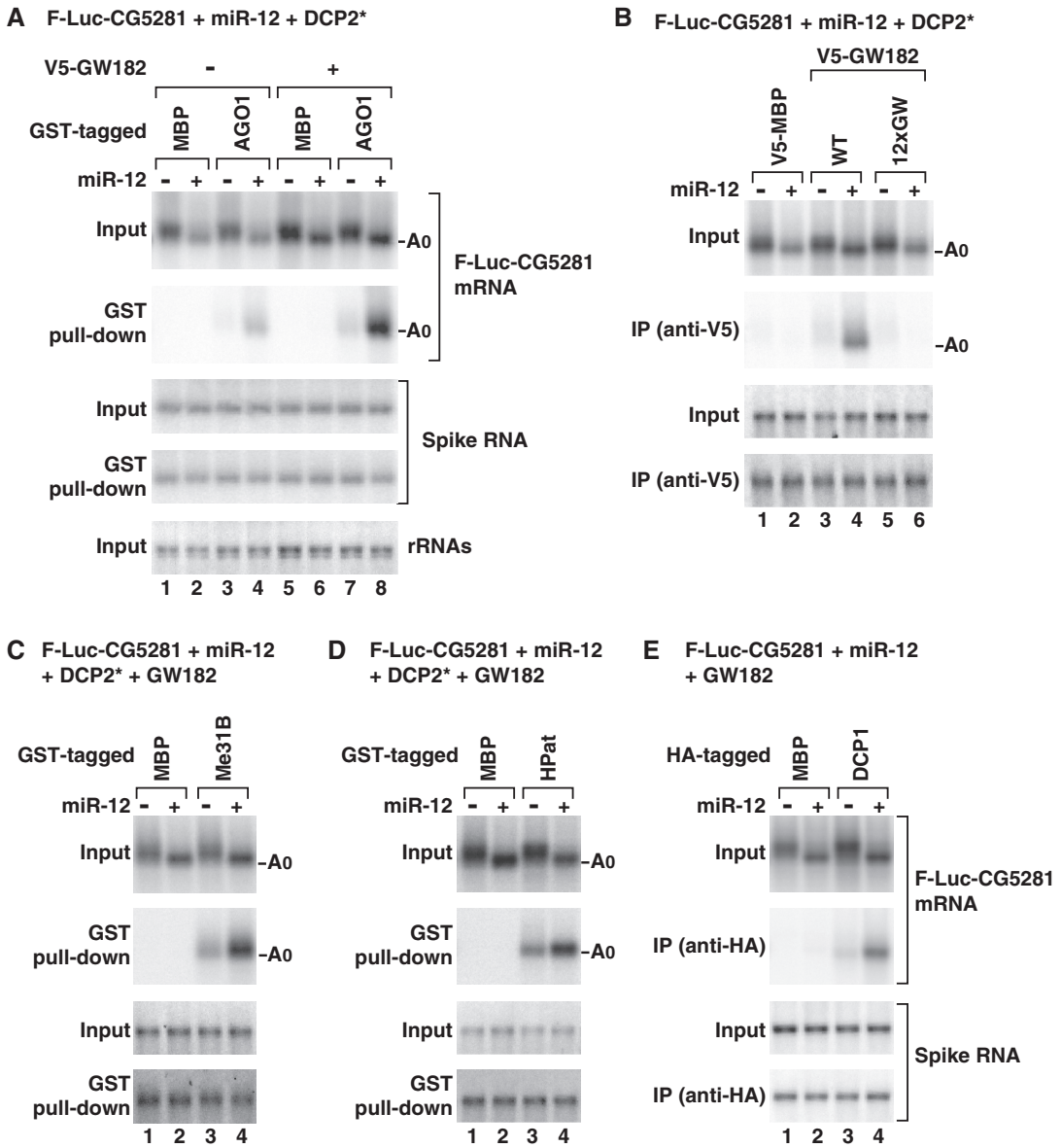


**Figure 1.** miRNAs enhance the association of Me31B, HPat and DCP1 with miRNA targets. (**A**) The association of GST-tagged AGO1 with the F-Luc-CG5281 mRNA reporter was analyzed in the absence or presence of miR-12 in cells expressing the DCP2* catalytic mutant. GST-tagged MBP (maltose-binding protein) served as negative control. The pull downs were performed in cells expressing V5-GW182 or the corresponding empty vector (−). RNAs in the input and pull-down fractions were analyzed by northern blotting. Ribosomal RNA (rRNA) and a spike RNA were visualized by ethidium bromide staining. (**B**) The association of V5-tagged GW182 [wild-type (WT) or the 12xGW mutant] with the F-Luc-CG5281 mRNA reporter was analyzed in the absence or presence of miR-12. V5-MBP served as a negative control. RNAs in the inputs and immunoprecipitates (IP) were analyzed as described in panel (A). (**C–E**) The association of GST-tagged Me31B and HPat or of HA-tagged DCP1 with the F-Luc-CG5281 reporter in the absence (−) or presence (+) of miR-12 was analyzed as described in panel (A). HA-DCP1 was immunoprecipitated using anti-HA antibodies. HA-MBP served as a negative control. The DCP2* mutant was included in panels (B–D) but not in panel (E) because DCP1 overexpression inhibits decapping of the miRNA target as is evident by the accumulation of the deadenylated F-Luc-CG5281 mRNA in the presence of miR-12 (Figure 1E, input, lanes 2 and 4 versus 1 and 3). In this panel, untagged DCP1 was included in cells expressing HA-MBP to prevent degradation of the reporter. The position of the deadenylated ($A_0$) mRNA reporter is indicated.

endogenous eIF4E with F-Luc-CG5281 mRNA. We observed that eIF4E co-immunoprecipitated with F-Luc-CG5281 mRNA even in the presence of miR-12 in cells expressing the DCP2* mutant (Supplementary Figure S3A). eIF4E also co-immunoprecipitated with R-Luc mRNA (which served as a transfection control) as expected. Thus, in cells in which decapping is inhibited, eIF4E remains bound to deadenylated miRNA targets, suggesting that in the absence of decapping, miRISCs do not fully release eIF4E from the mRNA cap structure, in agreement with our previous study (35). The results also show that DCP1, HPat and Me31B can be recruited to an mRNA, even though the cap may still be protected by eIF4E. However, we cannot rule out that eIF4E and decapping factors are bound to different mRNA pools.

### Decapping factors associate with miRNA targets lacking a 5′ cap and a poly(A) tail

The observed association of decapping activators with deadenylated miRNA targets could simply be an indirect consequence of deadenylation because decapping normally follows deadenylation (5,6). To discriminate between deadenylation-dependent or -independent recruitment of decapping factors, we constructed an artificial miRNA reporter that is transcribed by Pol III and therefore lacks a 5′ cap structure, an ORF and a poly(A) tail. This Pol III reporter is derived from the *Dm* 7SL RNA gene locus and includes the Pol III enhancer/promoter and transcriptional terminator regions. The 7SL RNA consists of an Alu and S domains and is a component of the signal recognition particle, which is involved in targeting secretory or membrane proteins to the endoplasmic reticulum membrane (Figure 2A) (42). Our 7SL-derived miRNA reporter contains only the Alu domain but lacks the S domain, which is required for the recognition of a signal peptide on a newly synthesized protein and for binding to the signal recognition particle receptor on the endoplasmic reticulum membrane (Figure 2A) (42). The Alu domain is an independent folding unit (43) that is sufficient for nuclear export (44). The Alu domain was extended by adding two miR-12 or miR-1-binding sites (Alu-miR-12 and Alu-miR-1 RNAs; Figure 2A).

The Alu-miR-12 RNA did not co-immunoprecipitate with HA-tagged eIF4E or endogenous cytoplasmic PABP as was expected for an RNA lacking a 5′ cap structure and a poly(A) tail (Figure 2B and C). As a control, the Pol II transcribed *Renilla* luciferase mRNA associated with both eIF4E and PABP (Figure 2B and C). Both proteins were immunoprecipitated at comparable levels independently of GW182 (Supplementary Figure S3B and C). Because Alu-miR-12 RNA is not a substrate for degradation through deadenylation or decapping, the pull-down experiments described below could be conducted without overexpressing the catalytically inactive DCP2* mutant. First, we confirmed that Alu-miR-12 RNA associated with AGO1 only in the presence of miR-12 (Figure 2D, lanes 4 versus 3), although AGO1 was pulled down with similar efficiencies, regardless of the presence of miR-12 (Supplementary Figure S3D). The association of AGO1 and Alu-miR-12 RNA was enhanced in cells overexpressing GW182 (Figure 2D, lane 8). Furthermore, in the presence of miR-12, Alu-miR-12 RNA associated with wild-type GW182 but not with the GW182 mutant that does not bind to AGO1 (12xGW mutant; Figure 2E, lanes 4 and 6), although both proteins were expressed and precipitated at comparable levels (Supplementary Figure S3E).

Next, we investigated whether Alu-miR-12 RNA associates with DCP1, Me31B and HPat in a miRNA-dependent manner. We observed that DCP1 co-immunoprecipitated with Alu-miR-12 RNA. Efficient association required both the presence of miR-12 and GW182 co-expression (Figure 3A, lane 4), indicating that the recruitment of decapping factors to this RNA is mediated by miRISC. Furthermore, DCP1 binding occurred only with the relevant combinations of reporters and miRNAs. Indeed, miR-12 caused the association of DCP1 with Alu-miR-12 RNA, but not with Alu-miR-1 RNA (Figure 3B). Conversely, miR-1 caused the association of DCP1 with Alu-miR-1 RNA, but not with Alu-miR-12 RNA (Figure 3B), although DCP1 was expressed and immunoprecipitated at similar levels in all conditions (Supplementary Figure S4A and B).

As observed for DCP1, recruitment of HPat and Me31B to the Alu-miR-12 RNA was stimulated in the presence of miR-12 and wild-type GW182 (Figure 3C). The association of Me31B with Alu-miR-12 or Alu-miR-1 RNA reporters was enhanced in the presence of the corresponding miRNA (Figure 3C and D). The pull-down efficiency of the GST-tagged proteins was not altered in the presence of the miRNAs (Supplementary Figure S4C and D). We conclude that miRISC recruits a subset of decapping activators to RNA targets in the absence of a 5′ cap structure, an ORF and a poly(A) tail and, therefore, to miRNA targets that do not undergo deadenylation and decapping.

The miRNA-binding sites on the Alu reporter can be replaced by any other binding site for sequence-specific RNA-binding proteins providing a new and adaptable tool that has the potential for use in investigating the assembly of protein complexes on RNA *in vivo*, independently of the contributions of the cap structure or the poly(A) tail. We have termed this RNA reporter Ev*Alu*ator.

### Interaction of decapping factors with AGO1 and GW182

The association of decapping factors with the Ev*Alu*ator reporters described earlier in the text indicates that the recruitment of these factors is miRNA-dependent and enhanced by GW182 but does not require direct recognition of the cap structure or any Polymerase II-specific mRNP component, such as the eIF4F complex (consisting of the cap-binding protein eIF4E, the scaffolding protein eIF4G and the helicase eIF4A) or PABP. Thus, decapping factors most likely interact with miRISC components bound to the mRNA target. Therefore, we searched for potential interactions between decapping factors and AGO1 or GW182. GFP-tagged AGO1 or GW182 were co-expressed with HA- or V5-tagged decapping factors in S2 cells and immunoprecipitated using anti-GFP antibodies.

We observed that HPat co-immunoprecipitated with GW182 (Supplementary Figure S5A) as reported previously (25). Interestingly, HPat also interacted with AGO1 (Supplementary Figure S5B). The interaction of HPat with AGO1 was independent of GW182 because an AGO1 mutant that no longer binds GW182 (the F2V2 mutant) (32) interacted with HPat as well as wild-type AGO1 (Supplementary Figure S5C). In contrast, a GW182 mutant that does not interact with AGO1 (the 12xGW mutant) was impaired in its binding to HPat (Supplementary Figure S5D). This suggests that the interaction of HPat with miRISC is mediated primarily by AGO1, although it is possible that the 12xGW mutations affect HPat binding directly because the GW182

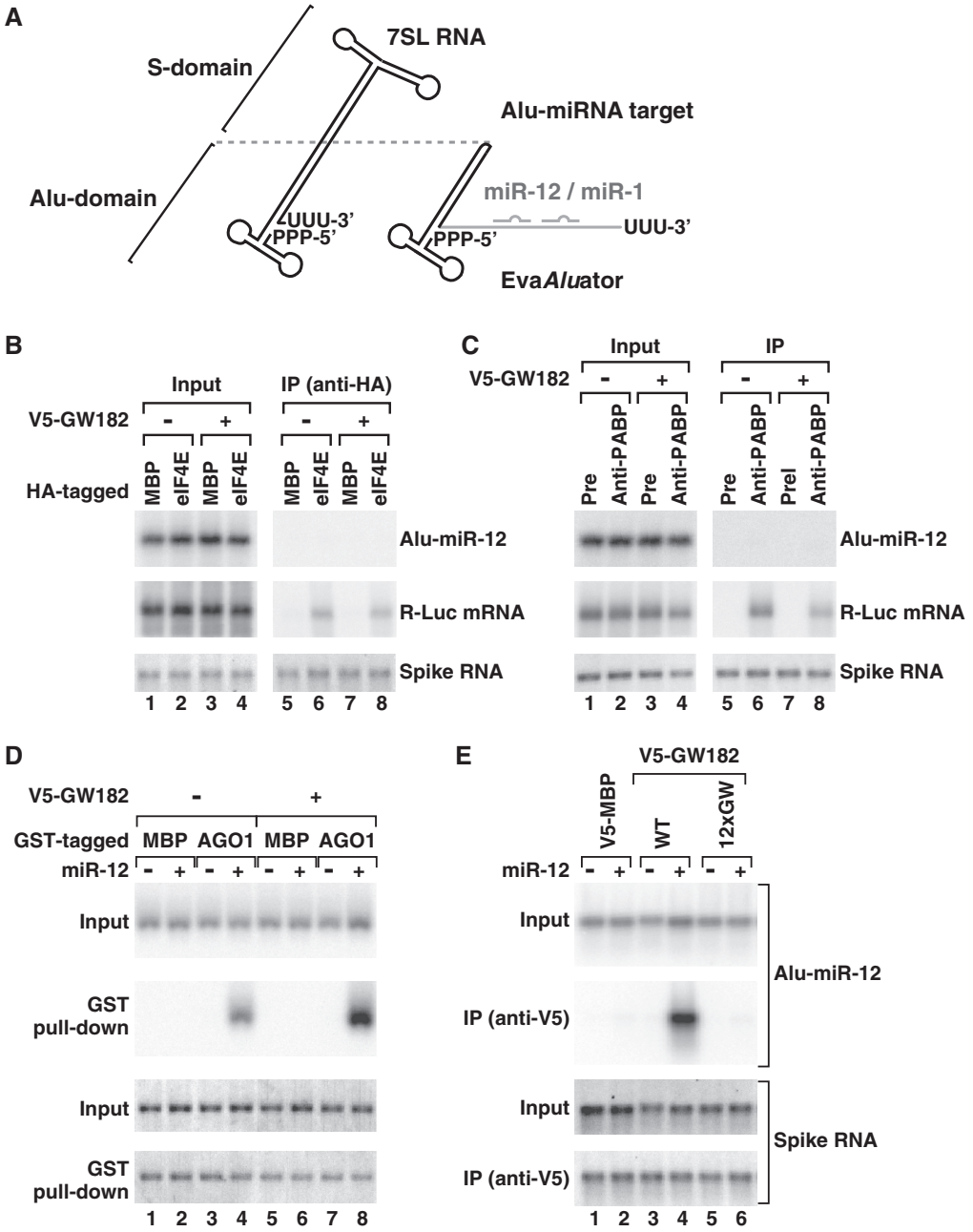


**Figure 2.** AGO1 and GW182 associate with miRNA targets lacking a 5′ cap structure and a poly(A) tail. (**A**) Schematic representation of *Dm* 7SL RNA and the Alu-miRNA targets that we termed Ev*Alu*ators. (**B**) The association of the Alu-miR-12 RNA with HA-tagged MBP or eIF4E in control cells or in cells expressing V5-GW182 was analyzed by co-immunoprecipitation using anti-HA antibodies. RNA samples were analyzed as described in Figure 1A. (**C**) The association of endogenous PABPC1 with the Alu-miR-12 RNA was analyzed using a polyclonal anti-PABPC1 antibody. The corresponding preimmune (Pre) serum served as a negative control. (**D** and **E**) The association of the Alu-miR-12 target with GST-AGO1 and V5-GW182 (wild-type or 12xGW mutant) in the absence or presence of miR-12 was analyzed as described in Figure 1.

N-terminal fragment (ΔSD) exhibits residual HPat binding activity (Supplementary Figure S5D).

We also detected a reproducible but weak binding of EDC4 and DCP2 to AGO1 (Supplementary Figure S5E and F), as reported previously (21,23). In contrast, EDC4 and DCP2 did not interact with GW182 (Supplementary Figure S5G and H). All immunoprecipitations were performed in cell lysates treated with RNase A, suggesting that the observed interactions are not mediated by RNA. Finally, DCP1, EDC3 and Me31B did not significantly co-immunoprecipitate with AGO1 or GW182 at levels above the background (Supplementary Figure S5I–N). Together, the results of the immunoprecipitation assays suggest that recruitment of DCP1 and Me31B to miRNA targets is not mediated directly by AGO1 or GW182. DCP1 and Me31B may interact with AGO1-GW182 complexes or recognize AGO1 or GW182 only in a conformation that is induced by target binding. Alternatively, decapping factors may be

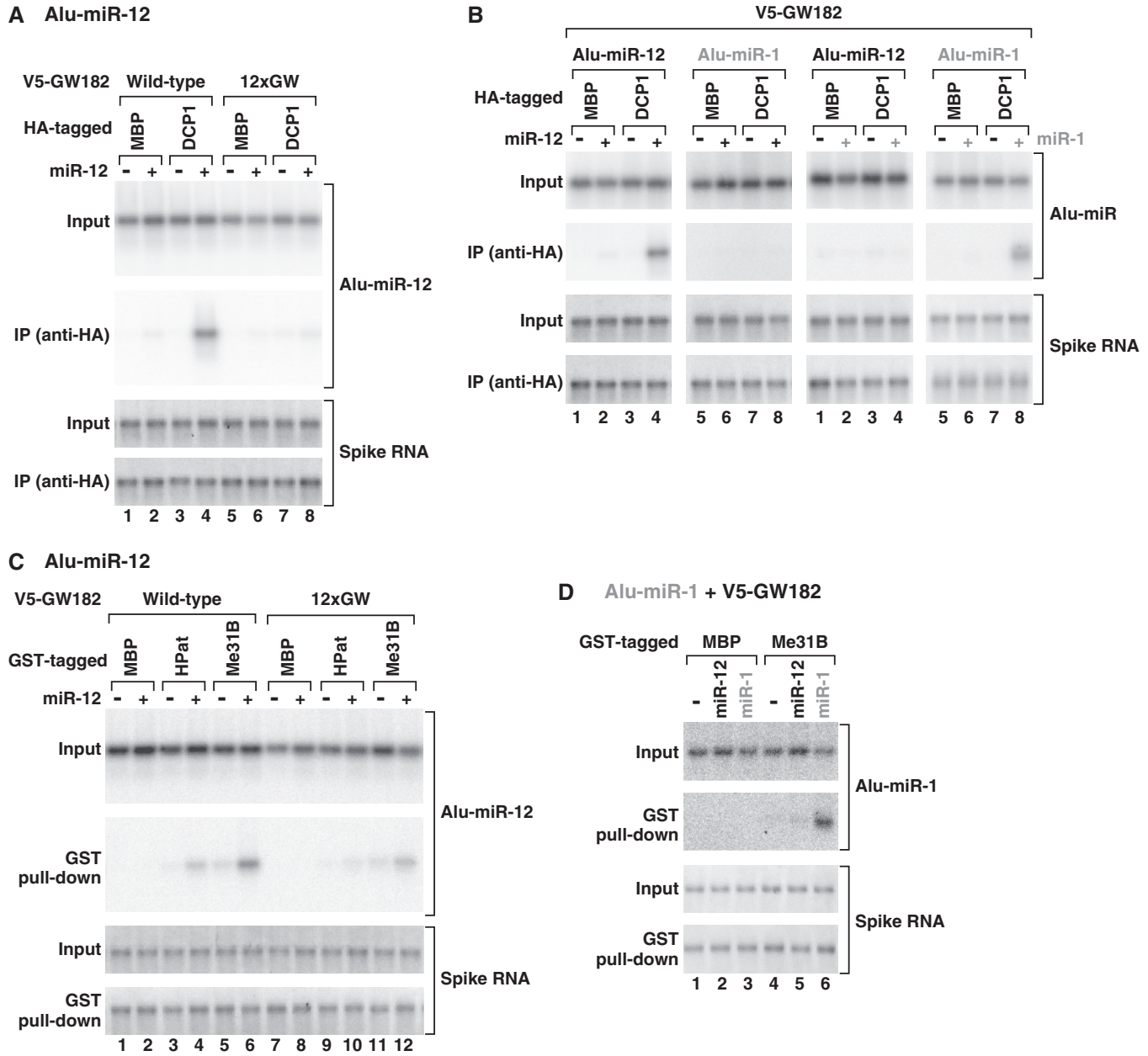


**Figure 3.** DCP1, HPat and Me31B are recruited to the Alu-miRNA targets. (**A**) The association of HA-DCP1 with the Alu-miR-12 target was analyzed as described in Figure 1, except that DCP2* was omitted. (**B**) The specificity of the association of DCP1 with the Alu-miR-12 or Alu-miR-1 target was analyzed in the presence of the corresponding miRNAs. (**C**) The association of GST-HPat and GST-Me31B with the Alu-miR-12 target was analyzed as described in Figure 1. (**D**) Association of GST-Me31B with the Alu-miR-1 target in the presence of miR-1 or miR-12.

recruited through interactions with the PAN2-PAN3 or CCR4-NOT deadenylase complexes, which bind GW182 proteins directly (7–9).

### Decapping factors are recruited before the completion of deadenylation

The observation that miRISC recruits decapping factors to Pol III-transcribed miRNA targets that do not undergo deadenylation prompted us to examine whether decapping factors associate with the target before or after deadenylation. We impaired the deadenylation of miRNA targets by overexpressing a catalytically inactive POP2 mutant (POP2*, carrying a D53A substitution). We used two miRNA reporters, F-Luc-CG5281 (which is targeted by miR-12) and an F-Luc-miR-1 target (which is degraded in the presence of miR-1) (Supplementary Figure S6A, lane 3 versus 1). Overexpression of the POP2* mutant inhibited deadenylation and resulted in the accumulation of polyadenylated mRNAs both in the presence and absence of miRNAs (Figure 4A, input panel lanes 3, 4 versus 1). The association of these polyadenylated mRNA targets with decapping factors was compared with the association observed in cells overexpressing the

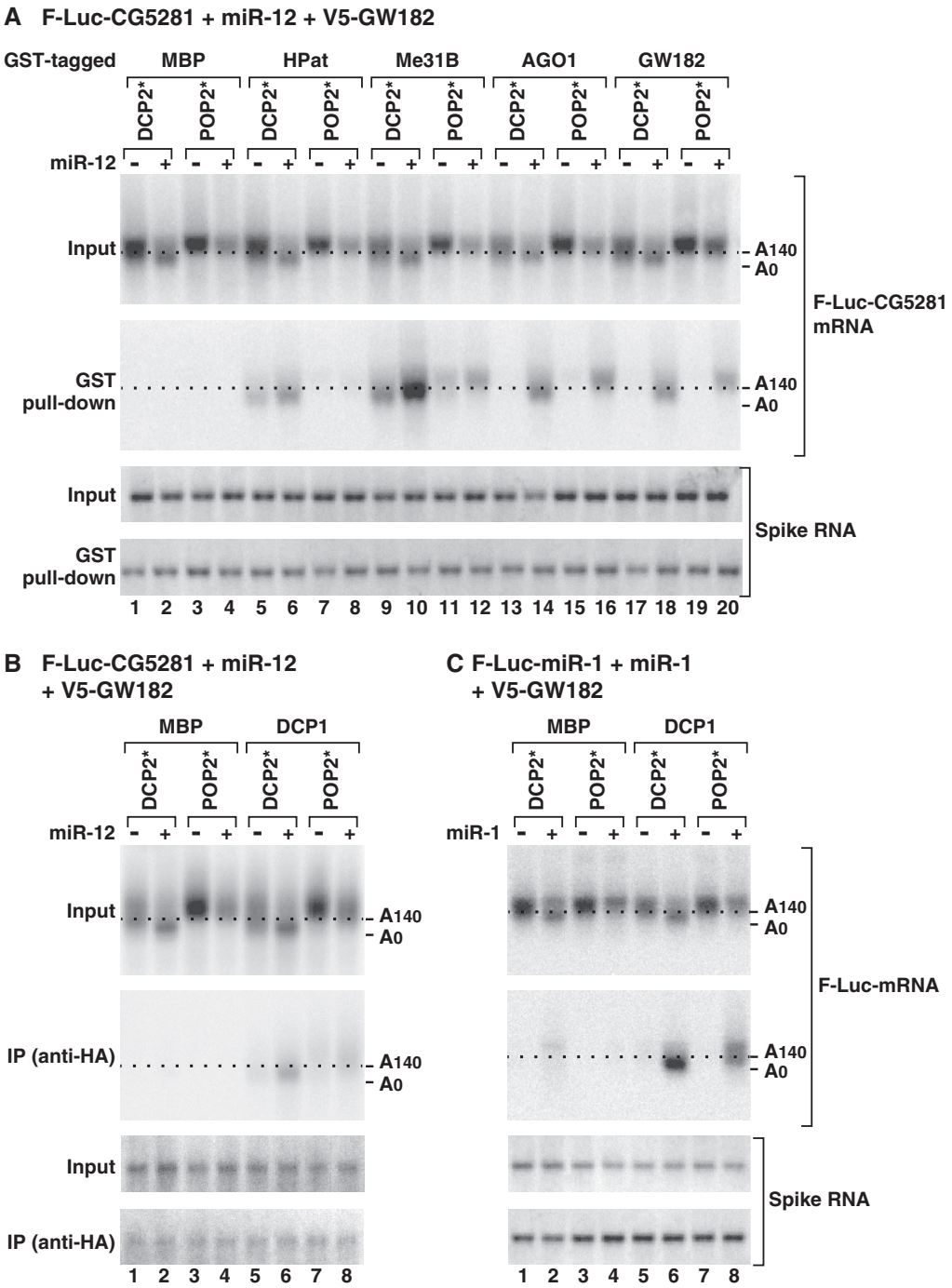


**Figure 4.** DCP1 and Me31B associate with miRNA targets independently of the length of the poly(A) tail. (**A–C**) The association of GST-tagged MBP, HPat, Me31B, AGO1 and GW182 with F-Luc-CG5281 in the absence or presence of miR-12 was analyzed in cells overexpressing the DCP2* or POP2* mutant as described in Figure 1. (B and C) The association of HA-DCP1 with a miR-12 or miR-1 target was analyzed in cells expressing the DCP2* or POP2* mutant. The positions of the polyadenylated ($A_{140}$, dotted line) and deadenylated ($A_0$) mRNA are indicated in all panels.

inactive DCP2* mutant, wherein the targets accumulate in a deadenylated form (Figure 4A, input panel, lanes 2 versus 1).

We observed that AGO1 and GW182 associated with F-Luc-CG5281 mRNA independently of the length of the poly(A) tail and their association was strictly dependent on the presence of miR-12 (Figure 4A, lanes 13–20). Similarly, DCP1 and Me31B associated with both the polyadenylated and deadenylated reporter (Figure 4A, lanes 9–12 and Figure 4B andC), although the association with polyadenylated targets was less efficient compared with the association observed with deadenylated targets (Figure 4A, lanes 12 versus 10; and Figure 4C, lanes 8 versus 6). For both proteins, the association with the mRNA target was enhanced in the presence of the relevant miRNA (Figure 4B and C). These results

suggest that DCP1 and Me31B are recruited before the completion of deadenylation. In contrast, HPat preferentially associate with the deadenylated reporter (Figure 4A, lanes 5–8). Western blot analyses indicate that decapping factors were expressed and immunoprecipitated at comparable levels in cells expressing the DCP2* or POP2* mutants (Supplementary Figure S6B–D). Together, our data indicate that complete deadenylation is not required for the association of DCP1 and Me31B with miRNA targets.

### miRISC induces decapping independently of ongoing deadenylation

We next examined whether the observed recruitment of decapping factors caused degradation of the miRNA target independently of deadenylation. To this end, we used miRNA reporters in which the polyadenylation signal was replaced by a self-cleaving HhR. Additionally, the reporters used in this experiment contained the F-Luc coding region, miR-12 or miR-1-binding sites and five BoxB hairpins located upstream of the HhR cleavage site (Figure 5A, F-Luc-miR-12-5BoxB-HhR or F-Luc-miR-1-5BoxB-HhR). Because these mRNAs are expressed at low levels, we stabilized them by artificially tethering PABP. PABP tethering increased reporter mRNA levels 2.5-fold (Figures 5B and C, gray bars) and translation efficiency 4.5-fold (Figures 5B and C, black bars). Importantly, the designed reporters were silenced in a miRNA-dependent manner. Indeed, the miRNAs caused a 5-fold and 2-fold reduction of F-Luc activity and reporter mRNA levels, respectively (Figure 5D and E and F and G, lanes 2 versus 1).

To investigate whether reporter mRNA degradation occurs through decapping, we depleted endogenous DCP2 and additionally expressed a dsRNA resistant version of the DCP2* catalytic mutant. This combination completely abolished miRNA-mediated degradation of the reporters and partially restored F-Luc protein levels (Figure 5D, E and F, G, lanes 4 versus 3), without affecting the levels of the reporter in the absence of the miRNA. Expression of a dsRNA-resistant version of wild-type DCP2 restored reporter mRNA degradation in cells that have been depleted of endogenous DCP2 (Figure 5D, E and F, G, lanes 6 versus 4). Consistent with the lack of a poly(A) tail, migration of the mRNA reporters did not change on expression of the corresponding miRNAs, even when decapping was blocked (Figure 5F and G, lanes 4 versus 3), whereas the corresponding polyadenylated reporter accumulated in a deadenylated form in DCP2-depleted cells expressing the DCP2* mutant and the corresponding miRNA (Figure 5F, lanes 8 versus 7). These results indicate that DCP2-catalyzed decapping is responsible for the degradation of non-adenylated miRNA reporters.

Next, we sought to obtain an orthogonal validation of the role of decapping factors in miRNA target degradation. To this end, we used a reporter containing three miR-12-binding sites and an internal poly(A) tail of 93 residues [F-Luc-miR-12-$(A)_{93}$-Hhr; Figure 6A]. This reporter is immune to deadenylation because its 3′ end is generated by a self-cleaving HhR and contains a 3′ poly(C) tail of six residues (Figure 6A) (35). The reporter was silenced by miR-12, which caused a 4-fold and 1.7-fold reduction of F-Luc activity and reporter mRNA levels, respectively (Figure 6B and C). The corresponding polyadenylated reporter [F-Luc-miR-12-poly(A)] was silenced more efficiently resulting in a 4-fold reduction of F-Luc activity and mRNA levels (Figures 6B–D).

To investigate whether degradation of the reporter containing an internal poly(A) tail occurs through decapping, we overexpressed XRN1. We recently showed that XRN1 overexpression inhibits decapping and subsequent mRNA degradation in *Dm* cells (19). Accordingly, we observed that the degradation of the F-Luc-miR-12-$(A)_{93}$-Hhr reporter by miR-12 was inhibited and mRNA levels were restored (Figures 6B and D). Consistent with the lack of a poly(A) tail, migration of the reporter did not change on XRN1 overexpression (Figure 6D, lane 4 versus 3), indicating that the reporter is not deadenylated. By contrast, the corresponding polyadenylated reporter accumulated in a deadenylated form in cells expressing XRN1 (wherein decapping is blocked; Figure 6D, lane 8 versus 7) because deadenylation precedes decapping (15). Consequently, although the levels of the F-Luc-miR-12-poly(A) reporter were partially restored, an equivalent increase in F-Luc activity was not observed, as deadenylated mRNAs are not efficiently translated. From these results, we conclude that miRNA targets that are immune to deadenylation can nevertheless be degraded through decapping.

## DISCUSSION

In this study, we demonstrate that miRISCs enhance the association of DCP1, Me31B and HPat with miRNA targets in a miRNA-dependent manner. This association occurred even when the miRNA target lacked a 5′ cap structure, an ORF and a poly(A) tail. Furthermore, mRNA reporters that are immune to deadenylation were degraded through decapping in the presence of the miRNA, indicating that miRISCs can promote decapping independently of deadenylation.

### miRISC enhances the association of decapping factors with mRNA targets

It is known that miRNAs promote the degradation of partially complementary targets through the 5′-to-3′ decay pathway. In this pathway, decapping is coupled to deadenylation and does not occur on polyadenylated and fully functional mRNAs (5,6). Here, we investigated whether the decapping of miRNA targets occurs by default, as a consequence of this coupling, or whether miRISCs can also recruit decapping factors independently of deadenylation. We showed that miRISCs enhanced the association of DCP1, Me31B and HPat with unadenylated 7SL-derived miRNA targets that have been transcribed by Pol III (Figure 3, Ev*Alu*ator reporters), indicating that the cap, a poly(A) tail and ongoing deadenylation are not required for the recruitment of decapping factors to miRNA targets. DCP1 association with the Ev*Alu*ator

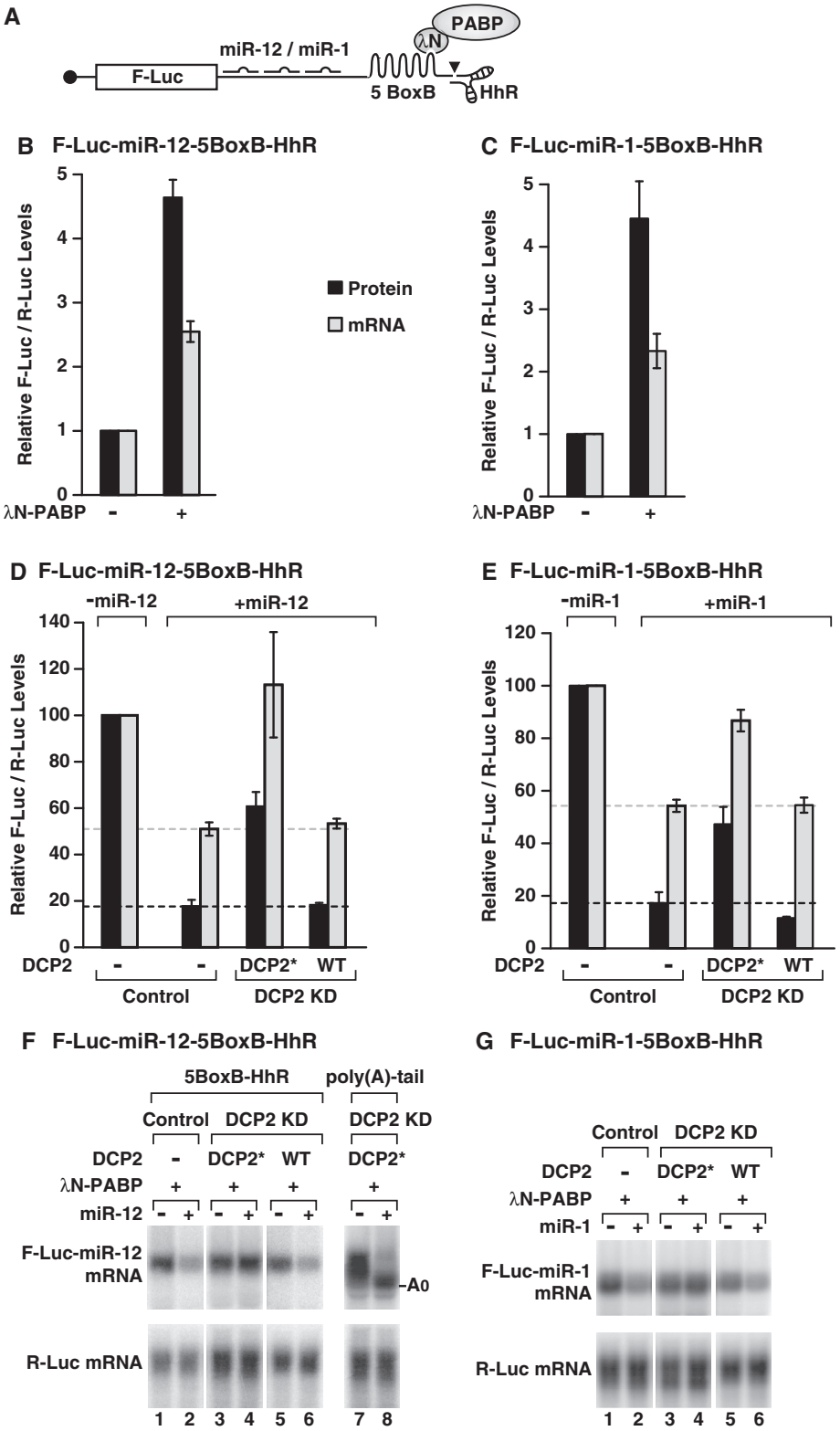


**Figure 5.** miRISC induces deadenylation-independent decapping. (**A**) Schematic representation of the F-Luc-miRNA-5BoxB-HhR reporter containing miR-12 or miR-1-binding sites, five BoxB hairpins and a self-cleavable HhR. (**B** and **C**) The graphs represent F-Luc activities (black bars) and mRNA levels (gray bars) normalized to those of *Renilla* luciferase in the absence or presence of λN-PABPC1 in control cells in the absence of miRNAs. The values were set to 1 in the absence of λN-PABPC1. (**D** and **E**) The graphs represent F-Luc activities (black bars) and mRNA levels (gray bars) normalized to those of *Renilla* luciferase in the absence or presence of miRNAs in control cells or in cells depleted of DCP2 expressing a dsRNA-resistant version of DCP2 (wild-type, WT or the catalytic DCP2* mutant). For each condition, the normalized values of F-Luc activity and mRNA levels were set at 100 in the absence of the miRNA. The mean values ± standard deviations from three independent experiments are shown in all panels. Dashed lines indicate F-Luc activity (black) and mRNA levels (gray) in control cells expressing the miRNA. (**F** and **G**) Northern blots of representative RNA samples corresponding to the graphs shown in panels (C) and (F), respectively.

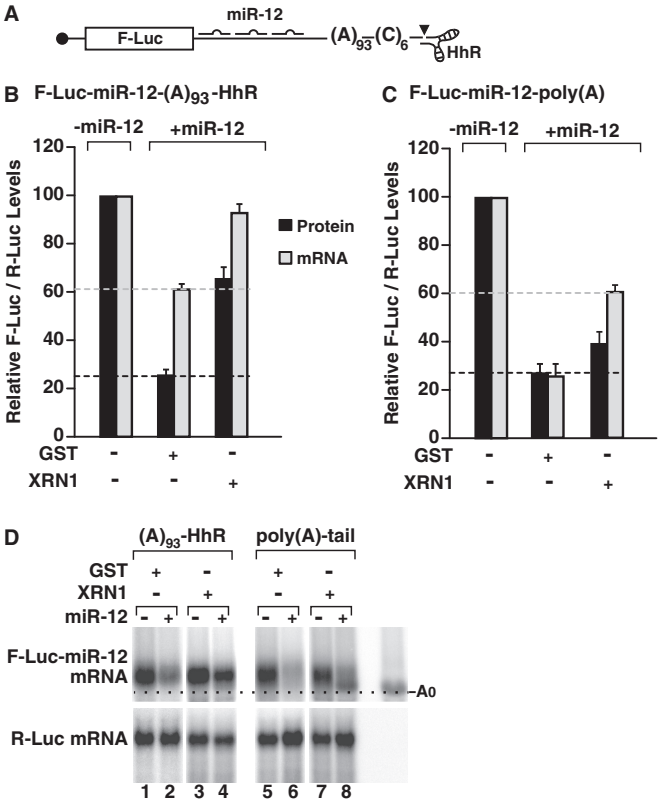


**Figure 6.** Schematic representation of the F-Luc-miR-12-$(A)_{93}$-HhR reporter. The F-Luc-miR-12-$(A)_{93}$-HhR reporter contains three miR-12-binding sites, consecutive poly(A) and poly(C) stretches of 93 and 6 residues, respectively, and a self-cleavable HhR. (**B–D**) S2 cells were transfected with a mixture of three plasmids: one expressing the indicated F-Luc reporters, another expressing the miR-12 primary transcript or the corresponding empty vector (-miR-12) and a third expressing *Renilla* luciferase (R-Luc). The transfection mixtures contained plasmids expressing GST-XRN1 or GST as a negative control. F-Luc activity (black bars) and mRNA levels (gray bars) were normalized to those of the *Renilla* luciferase and set at 100 in the absence of miR-12. The mean values ± standard deviations from three independent experiments are shown in panels (B and C). (D) Northern blot of representative RNA samples.

reporters was strictly miRNA dependent and stimulated by GW182. miRNAs and GW182 also stimulated the association of HPat and Me13B with the Ev*Alu*ator reporters, indicating that these decapping factors interact with miRISC components that are bound to Ev*Alu*ator RNA. However, DCP1 and Me31B did not interact with isolated AGO1 or GW182 in co-immunoprecipitation assays (Supplementary Figure S4), suggesting that the interaction of decapping factors with miRISC is indirect or that DCP1 and Me31B recognize AGO1 and GW182 as a complex. Indeed, it is possible that the decapping factors are recruited by the PAN2-PAN3 or CCR4-NOT deadenylase complexes, which interact with GW182 proteins directly (7–9). Alternatively, DCP1 and Me31B might recognize AGO1 or GW182 only in a certain conformation that is adopted on target binding. Although HPat did interact with AGO1 and GW182 in co-immunoprecipitation assays, these interactions were apparently not sufficient to enhance the association of HPat and a polyadenylated miRNA target (Figure 4A). Nevertheless, it is possible that these interactions contribute to the recruitment of HPat to deadenylated or oligoadenylated targets.

A previous study in human cells reported that EDC4 co-localized with a specific miRNA target in a miRNA-dependent manner, whereas DCP1 and RCK (the human ortholog of *Dm* Me31B) associated with the target, regardless of the presence of the miRNA (23). In agreement with this study, we observed that decapping factors associate with miRNA targets in the absence of the miRNA; however, we found that their binding is enhanced by the cognate miRNA. This enhancement was observed for targets that are not degraded or when degradation of the target was partially inhibited and may have escaped detection in co-localization studies.

## miRISC enhances deadenylation-independent decapping

A functional implication for the association of decapping factors with miRNA-targets is that miRNA targets can be decapped and degraded even in the absence of a poly(A) tail or ongoing deadenylation. In combination with previously published data (1–4), our results suggest that miRISC has multiple and redundant activities to ensure robust gene regulation: it induces translational repression, deadenylation and decapping, the latter in both a deadenylation-dependent and -independent manner.

Under which circumstances can deadenylation-independent decapping contribute to silencing? Decapping might play a role in silencing specific miRNA targets when deadenylation is blocked or when decapping is blocked and targets that have undergone deadenylation accumulate. Indeed, deadenylation and decapping can be uncoupled on specific mRNAs, in different cell types and under various cellular conditions, leading to the accumulation of deadenylated repressed mRNAs (45). These mRNAs can re-enter the translational pool on polyadenylation or might be degraded in a deadenylation-independent manner once decapping resumes. For example, in immature mouse oocytes, DCP2 and DCP1 are not detectable, but their expression increases during oocyte maturation (46). Consequently, in immature oocytes, many maternal mRNAs (most likely including miRNA targets) accumulate in a deadenylated silenced form. These mRNAs may be polyadenylated and translated at later stages of oogenesis or embryogenesis. However, a fraction of these deadenylated targets may be degraded through decapping when DCP2 and DCP1 are expressed. Additionally, DCP1 and DCP2 are phosphorylated under cellular stress conditions (47,48), and DCP1 is hyperphosphorylated during mitosis (49). Under these conditions, a subset of mRNAs is stabilized, suggesting that DCP1 and DCP2 phosphorylation inhibits decapping. Thus, it is possible that under various stress conditions, miRNA targets accumulate in a deadenylated form because decapping is inhibited and that deadenylation-independent decapping is required for the clearance of these targets on return to normal cellular conditions.

Notably, in addition to their role in target degradation, decapping activators act as general repressors of translation even in the absence of decapping (50,51). Therefore, these factors could play a more direct role in the translational repression of miRNA targets in the absence of mRNA degradation.

### Reversibility of silencing

In contrast to translational repression and deadenylation, decapping irreversibly shuts down translation initiation and commits mRNA to full degradation. Thus, decapping prevents the reversal of miRNA-mediated silencing. However, some miRNA targets have been shown to be released from miRNA-mediated repression in response to extracellular signals (28,52,53), suggesting that decapping is somehow blocked for these targets to allow for a fast reversal of their repression. How decapping is prevented in a target-specific manner remains unclear, but it can reasonable be expected that proteins associated with these targets block decapping *in cis* by preventing DCP2 access to the cap structure. These proteins may bind the cap structure directly or may act indirectly, for example, by stabilizing binding of the cap-binding protein eIF4E to the mRNA. Proteins that act as inhibitors of DCP2-mediated decapping have been described and include Variable Charged X chromosome VCX-A protein, YB-1, Y14 and *Dm* CUP (33,54–56). Thus, it is possible that additional proteins that prevent the decapping of specific mRNAs are present in eukaryotic cells. Such mRNA-specific decapping regulators would be likely to play an important role in controlling the reversibility of silencing. Alternatively, mRNAs can be recapped in the cytoplasm (57); however, how recapping is regulated remains unknown.

In addition to the aforementioned sequence-specific decapping regulators, the cap-binding protein eIF4E acts as a general inhibitor of decapping by limiting DCP2 access to the cap structure (58,59). Therefore, for decapping to occur, eIF4E needs to dissociate from the 5′ end of the mRNA. We show that eIF4E remains bound to at least a fraction of silenced miRNA targets in cells in which decapping is blocked. Furthermore, the DCP2 catalytic mutant did not detectably associate with the mRNA target, even though its overexpression inhibited decapping. These observations suggest that DCP2 does not stably associate with miRNA targets. Similarly, DCP2 did not co-localize with miRNA targets in human cells, although in these cells, EDC4 co-localized with the target in a miRNA-dependent manner (23). Thus, the process of decapping may involve multiple consecutive steps, including the association of decapping activators with the target mRNA in the absence of DCP2, eIF4E dissociation, DCP2 recruitment and cap hydrolysis. Our results suggest that miRISC facilitates an early decapping step by increasing the local concentration of decapping factors on mRNA targets, promoting decapping independently of deadenylation. Further studies are necessary to determine whether, in addition to recruiting decapping factors, miRISC plays a more direct role in accelerating the chemical catalysis step of decapping.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

## ACKNOWLEDGEMENTS

The authors are grateful to Oliver Weichenrieder for helpful suggestions and comments on the manuscript.

## FUNDING

Max Planck Society, Deutsche Forschungsgemeinschaft [DFG, FOR855 and the Gottfried Wilhelm Leibniz Program awarded to E.I.]; Toyobo Biotechnology Foundation (to T.N.). Funding for open access charge: Max Planck Society.

*Conflict of interest statement*. None declared.

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