

miRISC and the CCR4–NOT complex silence mRNA targets independently of 43S ribosomal scanning

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Abstract

miRNAs associate with Argonaute (AGO) proteins to silence the expression of mRNA targets by inhibiting translation and promoting deadenylation, decapping, and mRNA degradation. A current model for silencing suggests that AGOs mediate these effects through the sequential recruitment of GW182 proteins, the CCR4–NOT deadenylase complex and the translational repressor and decapping activator DDX6. An alternative model posits that AGOs repress translation by interfering with eIF4A function during 43S ribosomal scanning and that this mechanism is independent of GW182 and the CCR4–NOT complex in *Drosophila melanogaster*. Here, we show that miRNAs, AGOs, GW182, the CCR4–NOT complex, and DDX6/Me31B repress and degrade polyadenylated mRNA targets that are translated via scanning-independent mechanisms in both human and *Dm* cells. This and additional observations indicate a common mechanism used by these proteins and miRNAs to mediate silencing. This mechanism does not require eIF4A function during ribosomal scanning.

Keywords argonaute; DDX6; deadenylation; eIF4A; GW182

Subject Categories Protein Biosynthesis & Quality Control; RNA Biology

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Introduction

miRNAs are a class of small noncoding RNAs that assemble with Argonaute proteins into miRNA-induced silencing complexes (miRISCs) to mediate post-transcriptional repression of complementary mRNA targets (Ameres & Zamore, 2013). In animals, miRNA-mediated silencing is effected by a combination of translational repression and mRNA destabilization, with the latter accounting for most of the steady-state repression in mammalian cell cultures (Hendrickson *et al*, 2009; Guo *et al*, 2010; Eichhorn *et al*, 2014).

miRNA target degradation is catalyzed by the enzymes of the 5′-to-3′ mRNA decay pathway (Rehwinkel *et al*, 2005;

Behm-Ansmant *et al*, 2006; Eulalio *et al*, 2007, 2009; Chen *et al*, 2009; Piao *et al*, 2010). In this pathway, mRNAs are first deadenylated by the PAN2–PAN3 and the CCR4–NOT complexes, then decapped by the decapping enzyme DCP2, and finally degraded from the 5′-end by the XRN1 exonuclease (Jonas & Izaurralde, 2015). The GW182/TNRC6 proteins play a central role in this process by interacting with AGOs and recruiting the PAN2–PAN3 and the CCR4–NOT deadenylase complexes to miRNA targets, thereby accelerating their degradation (Fabian & Sonenberg, 2012; Jonas & Izaurralde, 2015).

In addition to accelerating mRNA degradation, miRNAs also trigger translational repression, but the precise repressive mechanism remains poorly understood, and several models have been proposed (Fabian & Sonenberg, 2012). One possible model is that the recruitment of the CCR4–NOT complex is sufficient to mediate silencing (Jonas & Izaurralde, 2015). This model is based on the following observations: First, the interaction of GW182 proteins with the CCR4–NOT complex is not only required for degradation but also required for translational repression of miRNA reporters (Braun *et al*, 2011; Chekulaeva *et al*, 2011; Fabian *et al*, 2011; Huntzinger *et al*, 2013; Zekri *et al*, 2013; Chen *et al*, 2014; Mathys *et al*, 2014). Second, like miRISC, the CCR4–NOT complex represses translation in the absence of deadenylation (Cooke *et al*, 2010; Braun *et al*, 2011; Chekulaeva *et al*, 2011; Bawankar *et al*, 2013; Zekri *et al*, 2013). Translational repression by the CCR4–NOT complex can, at least in part, be explained by a direct interaction between the NOT1 subunit and the DEAD-box ATPase DDX6 (also known as RCK). Human DDX6 and its *Drosophila melanogaster* (*Dm*) ortholog Me31B repress translation, activate decapping, and play a role in silencing (Chu & Rana, 2006; Eulalio *et al*, 2007; Nishihara *et al*, 2013; Chen *et al*, 2014; Mathys *et al*, 2014; Rouya *et al*, 2014).

An alternative model of silencing involves the translation initiation factor eIF4A. eIF4A proteins are DEAD-box RNA helicases that unwind secondary structures within mRNA 5′-UTRs, allowing the 43S preinitiation complex (PIC) to scan the 5′-UTR toward the start codon (Jackson *et al*, 2010). Several studies have indicated that miRISCs inhibit 43S scanning by interfering with eIF4A function (Meijer *et al*, 2013; Ricci *et al*, 2013; Fukao *et al*, 2014; Fukaya *et al*, 2014). How this interference is achieved remains unclear. One study reported that NOT1 interacts with eIF4A2 but not with its paralog eIF4A1 in human cells (Meijer *et al*, 2013).

It was suggested that this interaction locks eIF4A2 onto the mRNA 5'-UTR and represses translation by blocking 43S scanning (Meijer *et al*, 2013). However, the interaction between NOT1 and eIF4A2 was not confirmed in subsequent studies (Chen *et al*, 2014; Mathys *et al*, 2014; Rouya *et al*, 2014) and the knockout of eIF4A2 in human cells does not suppress silencing (Galicía-Vazquez *et al*, 2015).

Furthermore, in contrast to the model proposed by Meijer *et al*, two recent studies indicated that miRNAs repress translation by releasing rather than recruiting eIF4A2 and that both eIF4A1 and eIF4A2 are dislodged from silenced mRNAs (Fukao *et al*, 2014; Fukaya *et al*, 2014). Remarkably, *Dm* AGO1 promoted eIF4A displacement independently of GW182, and *Dm* GW182 caused eIF4A and eIF4E displacement independently of AGO1 (Fukaya *et al*, 2014). These and additional observations suggested that *Dm* AGO1 exhibits silencing activity independently of GW182 proteins in *D. melanogaster* (Fukaya & Tomari, 2012; Wu *et al*, 2013; Fukaya *et al*, 2014). Whether this is also the case for human AGOs has not been investigated.

In sum, the mechanism by which miRNAs repress translation is still not fully understood, and it is not known whether the recruitment of the CCR4–NOT complex fully explains silencing or whether parallel and potentially species-specific repressive mechanisms exist. To clarify these open questions, we adopted a comparative approach and investigated the silencing mechanisms in human and *Dm* cells. We found that miRNAs, AGO, GW182, and NOT1 do not require 43S ribosomal scanning to repress translation but require DDX6 in human cells. We further show that the repressive activity of AGOs depends on the integrity of the W-binding pockets, which in turn recruit GW182 proteins and the CCR4–NOT complex in both human and *D. melanogaster* cells. Collectively, our data indicate that CCR4–NOT is a major downstream effector complex in the miRNA pathway.

Results

The W-binding pockets of AGOs mediate binding to the GW182/TNRC6 proteins

Structural studies of human (*Hs*) AGO2 revealed the presence of tandem tryptophan (W)-binding pockets (pockets P1 and P2) on the surface of the AGO2 PIWI domain opposite to the miRNA-binding site (Appendix Fig S1A; Schirle & MacRae, 2012). These pockets represent potential binding sites for the GW182/TNRC6 proteins and are conserved in *Hs* AGO1, AGO3, and AGO4 as well as in *Dm* AGO1 (Appendix Fig S1B; Schirle & MacRae, 2012). To determine the contribution of the W-binding pockets to the interaction of AGOs with GW182 proteins, we designed mutations to disrupt the pockets in *Hs* AGO2 and *Dm* AGO1 (mutants P1 and P2, Appendix Fig S1A and B, Appendix Table S1).

Mutations in either of the two *Dm* AGO1 pockets abolished the interaction with endogenous *Dm* GW182 in coimmunoprecipitation assays in *Dm* Schneider cells (S2 cells; Fig 1A, lanes 8 and 9 versus 7). The mutant proteins were expressed at levels comparable to wild type and associated with endogenous miR-2a, indicating that the mutations do not disrupt the fold of the PIWI domain (Fig 1A). Similarly, combined mutations in the two pockets of *Hs* AGO2 abolished

its interaction with endogenous TNRC6A and GFP-tagged TNRC6B and TNRC6C in HEK293T cells (Fig 1B–D, lanes 10 versus 7). This AGO2 mutant no longer accumulated in P-bodies as expected because P-body localization requires the interaction with the TNRC6 proteins (Fig EV1A; Lazzaretti *et al*, 2009). Mutations in only one of the two pockets disrupted the interaction of *Hs* AGO2 with TNRC6C and strongly reduced TNRC6A binding (Fig 1B and D). The binding of TNRC6B was impaired by mutations in P2 but not in P1 (Fig 1C). These results reveal differences in the binding properties of the three TNRC6 proteins. Importantly, the mutations in the *Hs* AGO2 W-binding pockets did not interfere with miR-16 loading (Fig 1D). We concluded that the integrity of the W-binding pockets is required for *Hs* AGO2 and *Dm* AGO1 to interact with GW182/TNRC6 proteins.

The silencing activity of AGOs requires the integrity of the W-binding pockets

The availability of AGO mutants that do not interact with GW182 proteins enabled us to reinvestigate the question of whether AGOs exhibit silencing activity independently of GW182 using an approach orthogonal to knockdowns, which cannot be unambiguously interpreted (Fukaya & Tomari, 2012). We examined the silencing activity of the AGO mutants using previously described λ N- or MS2-based tethering assays (Pillai *et al*, 2004; Rehwinkel *et al*, 2005). Tethered *Dm* AGO1 and *Hs* AGO2 promoted degradation of polyadenylated mRNA reporters, as shown previously (Fig 2A–D; Pillai *et al*, 2004; Rehwinkel *et al*, 2005; Eulalio *et al*, 2008; Chen *et al*, 2009; Piao *et al*, 2010). Mutations in P2 were sufficient to abolish the activity of the *Dm* and *Hs* AGOs in tethering assays (Fig 2A–D). The activity of *Dm* AGO1 was also abolished by mutations in P1 (Fig 2A and B).

Because the reporters were degraded, it was possible that the recruitment of deadenylases by GW182/TNRC6 masked any additional repressive activity that AGOs might have independently of these proteins. Therefore, we next tested the activity of the AGO mutants using mRNA reporters that are refractory to deadenylation and subsequent decay. In *Dm* cells, we used an mRNA reporter with a 3'-end generated by a self-cleaving hammerhead ribozyme (HhR). Immediately upstream of the ribozyme cleavage site, the reporter contains an internal poly(A) stretch of 95 residues followed by a poly(C) stretch of 7 residues, which blocks deadenylation (F-Luc-5BoxB-A₉₅-C₇-HhR; Zekri *et al*, 2013). Tethered *Dm* AGO1 repressed this reporter predominantly at the translational level (Fig 2E and F). The mutations in either P1 or P2 abolished *Dm* AGO1 repressive activity (Fig 2E and F). The protein mutants were expressed at levels comparable to wild type (Fig 2G).

In human HEK293T cells, we used an R-Luc-5BoxB reporter containing an internal poly(A) stretch of 95 residues followed by the 3'-end of the MALAT1 noncoding RNA, which is processed by RNase P, and thus, it is not polyadenylated (Wilusz *et al*, 2012). *Hs* AGO2 repressed the expression of this reporter (Fig 2H–J). The repression was relieved by mutations in either of the two W-binding pockets (Fig 2H–J). The *Dm* or *Hs* AGO proteins did not repress the corresponding reporters that lacked the BoxB or MS2-binding sites (Fig EV1B–E). Taken together, our results indicate that the activity of tethered AGO proteins depends on the integrity of the W-binding pockets.

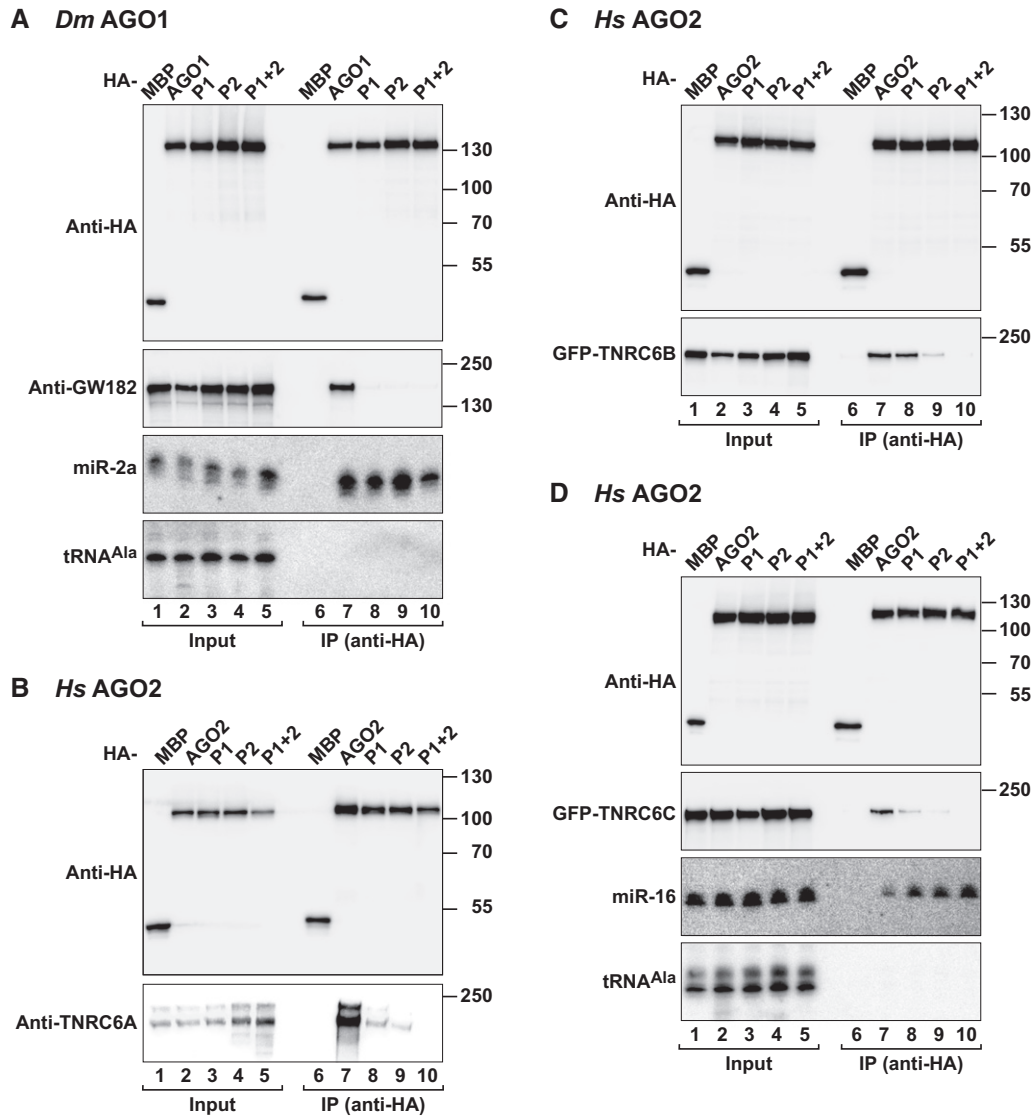


Figure 1. The W-binding pockets are required for AGOs to bind GW182/TNRC6 proteins.

- A** Lysates from S2 cells expressing HA-tagged versions of MBP or AGO1 (wild type or pocket mutants) were immunoprecipitated using an anti-HA antibody. Inputs (1% for the HA-tagged proteins and 2.5% for GW182) and immunoprecipitates (20 and 35%, respectively) were analyzed by Western blotting using an anti-HA antibody. Endogenous GW182 was detected using anti-GW182 antibodies. The association between HA-AGO1 and endogenous miR-2a was analyzed by Northern blotting. tRNA^{Ala} served as a loading control.
- B** Interaction of HA-tagged AGO2 (wild type or pocket mutants) with endogenous TNRC6A in HEK293T cells. HA-tagged MBP served as a negative control.
- C, D** Interaction of HA-tagged AGO2 (wild type or pocket mutants) with GFP-tagged TNRC6B and TNRC6C in HEK293T cells. HA-tagged MBP served as a negative control. Inputs (1.5% for the HA-tagged proteins and 2% for the GFP-tagged proteins) and immunoprecipitates (20 and 35%, respectively) were analyzed by Western blotting using the corresponding antibodies. The presence of endogenous miR-16 in the immunoprecipitates was determined by Northern blotting (D). tRNA^{Ala} served as a loading control. AGO mutants are described in Appendix Fig S1 and Appendix Table S1.

Source data are available online for this figure.

The W-binding pockets are required for *Dm* AGO1 to silence miRNA targets

Because tethering assays bypass some steps in silencing, we next tested the activity of the AGO mutants in complementation assays in *Dm* cells, wherein miRNAs predominantly associated with AGO1. For these assays, we used previously characterized firefly luciferase reporters containing 3'-UTRs of natural miRNA targets (e.g. *par-6* and *cg5281*, silenced by miR-1 and miR-12, respectively; and

nerfin-1 silenced by miR-9b and miR-279; Eulalio *et al*, 2007). The depletion of endogenous *Dm* AGO1 suppressed the silencing of all reporters, as expected, and both F-Luc expression and mRNA levels were restored (Figs 2K and L and EV1F and G). Western blot analysis indicated that AGO1 levels were reduced below 25% of their control levels in depleted cells (Fig EV1H and I).

In depleted cells, a siRNA-resistant version of *Dm* AGO1 fully restored silencing of all reporters (Figs 2K and L and EV1F and G). By contrast, the double P1+P2 mutant did not restore silencing. The

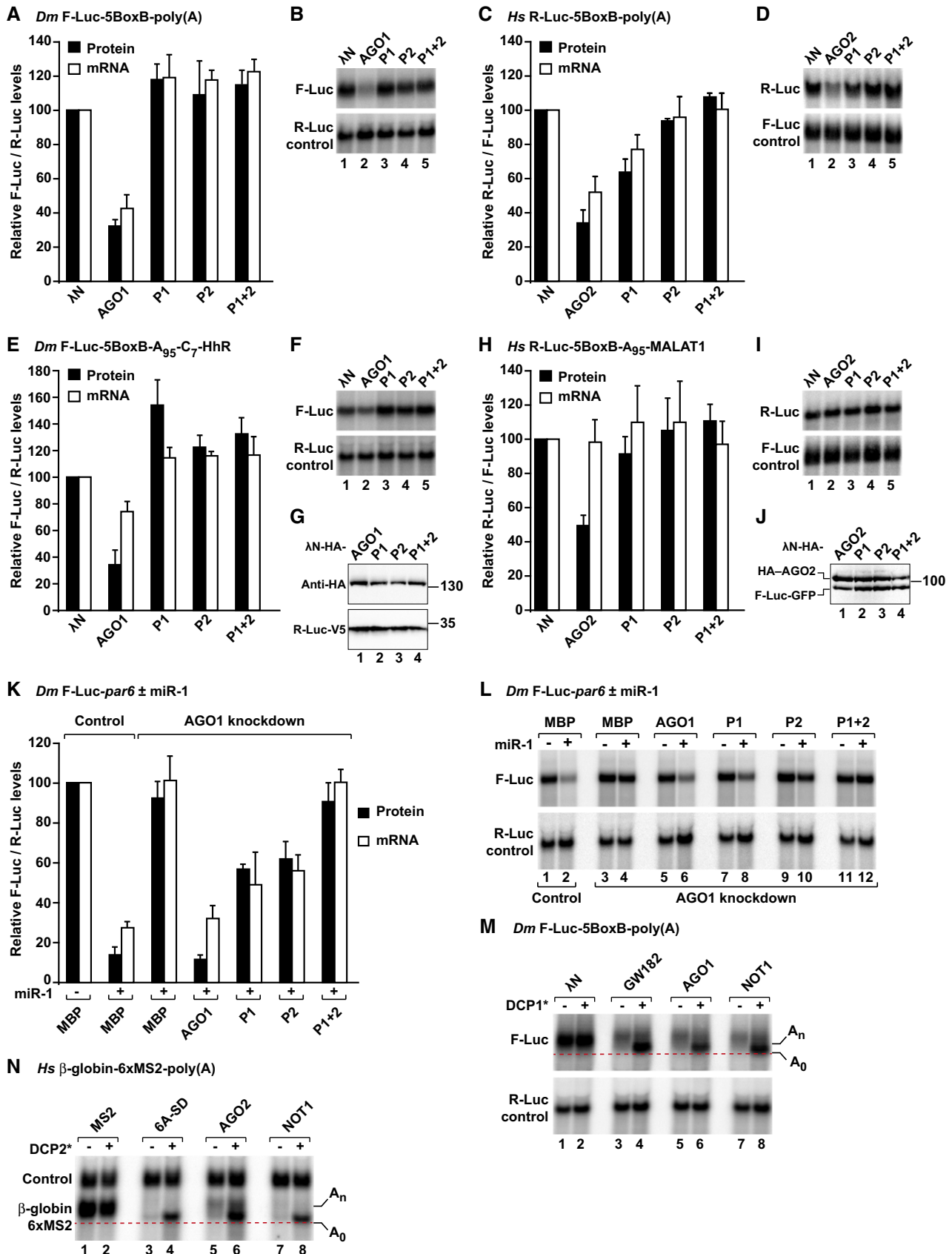


Figure 2.

Figure 2. The silencing activity of AGOs depends on the integrity of the W-binding pockets.

- A Tethering assay using the F-Luc-5BoxB-poly(A) reporter and λ N-HA-AGO1 (wild type or pocket mutants) in S2 cells. A plasmid expressing R-Luc served as a transfection control. (A) F-Luc activity and mRNA levels were normalized to those of the R-Luc transfection control and set to 100% in cells expressing the λ N-HA peptide.
- B Northern blot of representative RNA samples corresponding to the experiment shown in (A).
- C, D Tethering assay using the R-Luc-5BoxB-poly(A) reporter and λ N-HA-AGO2 (wild type or mutants) in human HEK293T cells. A plasmid expressing F-Luc served as a transfection control. R-Luc activity and mRNA levels were normalized to those of the F-Luc transfection control and analyzed as described in panels (A, B).
- E, F Tethering assay using the F-Luc-5BoxB-A₉₅-C₇-HhR reporter and λ N-HA-AGO1 (wild type or mutants) in S2 cells. Samples were analyzed as described in panels (A, B).
- G Western blot analysis showing the equivalent expression of the λ N-HA-AGO1 proteins used in the tethering assays shown in panels (A, B, E, F).
- H, I Tethering assay using the R-Luc-5BoxB-A₉₅-MALAT1 reporter and λ N-HA-AGO2 (wild type or mutants) in HEK293T cells.
- J Western blot analysis showing the equivalent expression of the λ N-HA-AGO2 proteins used in the tethering assays shown in (C, D, H, I).
- K, L Complementation assays using F-Luc-*par6* reporter in S2 cells depleted of endogenous AGO1. Plasmids encoding HA-AGO1 (wild type or mutants) or HA-MBP (as negative control) and miR-1 primary transcript (or empty vector) were included in the transfection mixtures as indicated. For each condition, firefly luciferase activities and mRNA levels were normalized to those of the *Renilla* luciferase transfection control and set to 100% in the absence of miR-1 (100% values are only shown for control cells). (K) Normalized firefly luciferase activities and mRNA levels. (L) Northern blot of representative RNA samples.
- M A tethering assay using the F-Luc-5BoxB-poly(A) reporter was performed in S2 cells. The transfection mixtures included plasmids expressing GFP or GFP-DCP1* as indicated. The panel shows a Northern blot of representative RNA samples. The positions of the polyadenylated (A_n) and deadenylated (A₀, dashed red line) mRNA reporter are indicated on the right.
- N A tethering assay using the β -globin-6xMS2 reporter and MS2-tagged proteins was performed in HEK293T cells. The transfection mixtures contained plasmids expressing wild-type DCP2 (–) or the catalytic DCP2* mutant (+). The panel shows a Northern blot of representative RNA samples analyzed as described in (M).
- Data information: In all panels, bars represent mean values and error bars represent standard deviations from at least three independent experiments. Source data are available online for this figure.

single-pocket mutants partially rescued silencing to different extents depending on the reporters (Figs 2K and L and EV1F and G), suggesting that although these mutants do not bind to GW182 in coimmunoprecipitation assays, they may still interact transiently *in vivo*. The AGO1 proteins were expressed at similar levels (Fig EV1I). These levels were comparable to the levels of endogenous AGO1 in control cells (Fig EV1I, lane 3 versus 1). We concluded that the integrity of the two W-binding pockets is also required for *Dm* AGO1 to silence miRNA targets in complementation assays.

AGOs, GW182/TNRC6s, and CCR4–NOT share a common mechanism to degrade mRNAs

It is well established that GW182/TNRC6 proteins and AGOs induce mRNA degradation through the 5'-to-3' decay pathway (Rehwinkel *et al*, 2005; Behm-Ansmant *et al*, 2006; Eulalio *et al*, 2007; Chen *et al*, 2009; Piao *et al*, 2010). In this pathway, deadenylation precedes decapping. Consequently, mRNAs degraded through this pathway accumulate in a deadenylated form in cells in which decapping is inhibited (Eulalio *et al*, 2007).

To investigate whether GW182/TNRC6, AGOs, and NOT1 all elicit first deadenylation and then decapping, we sought to inhibit decapping. To this end, we overexpressed a DCP1 mutant that inhibits decapping in a dominant negative manner (DCP1* mutant; Chang *et al*, 2014; Appendix Table S1). Tethered *Dm* GW182, AGO1, and NOT1 degraded the F-Luc-5BoxB-poly(A) reporter (Fig 2M, lanes 3, 5, and 7 versus 1). Overexpression of the DCP1 mutant prevented this degradation in S2 cells (Fig 2M, lanes 4, 6, and 8 versus 2), without affecting the expression of the tethered proteins (Fig EV1J). The reporter accumulated as a fast migrating form, which corresponds to the deadenylated decay intermediate (Eulalio *et al*, 2007). Thus, as observed previously for GW182 and AGOs, tethered NOT1 caused deadenylation-dependent decapping.

Likewise, in HEK293T cells, MS2-tagged TNRC6A silencing domain (SD), AGO2, and NOT1 degraded a β -globin reporter containing 6 binding sites for the MS2 protein in the 3'-UTR (Fig 2N, lanes 3, 5, and 7 versus 1). This degradation was prevented in cells

expressing a DCP2 catalytically inactive mutant (DCP2*), and the reporter accumulated in the deadenylated form (Fig 2N, lanes 4, 6, and 8). The expression of the tethered proteins was not affected (Fig EV1K). Our results indicate that miRNAs, AGOs, GW182/TNRC6 proteins, and the CCR4–NOT complex share a common mechanism to degrade target mRNAs.

AGOs, GW182/TNRC6s, and CCR4–NOT silence mRNAs translated via a scanning-independent mechanism

Several studies reported that miRNAs silence gene expression by targeting eIF4A and interfering with ribosome scanning (Meijer *et al*, 2013; Ricci *et al*, 2013; Fukao *et al*, 2014; Fukaya *et al*, 2014). However, there were considerable disagreements between these studies. Ricci *et al* found that translation driven by the EMCV IRES is relatively refractory to miRNA silencing, whereas Meijer *et al* found that it is efficiently silenced by miRNAs. Furthermore, the results of experiments in which miRNA-mediated translational repression is measured in the presence of drugs that target eIF4A and inhibit scanning (e.g. silvestrol or hippuristanol) are difficult to interpret because the transfection control is also inhibited. Therefore, to test whether AGO, GW182/TNRC6, and CCR4–NOT all require ribosome scanning to repress translation, we opted for an orthogonal approach and generated reporters translated via a scanning-independent mechanism.

The reporter for expression in *Dm* cells was derived from the F-Luc-5BoxB-A₉₅-C₇-HhR reporter but contained a 5'-UTR of only eight nucleotides (8nt-F-Luc-5BoxB-A₉₅-C₇-HhR (Fig 3A, Appendix Tables S2 and S3)). The transcription start site used in S2 cells was confirmed by 5' RACE (Fig EV2A). Notably, the translation efficiency of this reporter (F-Luc activity normalized to mRNA levels) was reduced only 1.4-fold relative to the parental reporter containing a 109-nt 5'-UTR (Fig EV2B–E). Given that F-Luc proteins lacking the N-terminal 3–10 amino acids are inactive (i.e. when translation starts at the next in-frame methionine, Met31; Fig EV2F–H; Sung & Kang, 1998), and considering that the translation efficiencies of the reporters with a short and a long

5'-UTR are comparable (Fig EV2B–E), it is reasonable to assume that a significant fraction of ribosomes initiate translation at the first AUG (or minimally at codon 3) on the mRNA with a 8-nt 5'-UTR. Consequently, translation of the 8nt-F-Luc-5BoxB-A₉₅C₇-HhR occurs without scanning. Accordingly, when the F-Luc ORF was replaced by HA–GST, the HA-tagged protein could be detected by Western blotting using anti-HA antibodies further validating the conclusion that a substantial fraction of ribosomes start translation at the first AUG (Fig EV2J).

Remarkably, the reporter containing a 8-nt 5'-UTR was repressed by tethered AGO1, GW182, and NOT1 (Figs 3B and C and EV2J). The repression was comparable to that observed for the parental reporter containing a 109-nt long 5'-UTR as well as for the reporter also containing a poly(A) tail, which was consequently degraded (Figs 3B and C and EV2J).

To further confirm that the reporter was translated via a scanning-independent mechanism and to prevent leaky initiation, we replaced all in-frame methionines (except the initiating Met) in the ORF of the F-Luc-5BoxB-A₉₅C₇-HhR reporter with leucine and inserted a V5 tag C-terminally to detect the protein product (F-Luc(Leu)-V5) because the substitutions abrogated F-Luc activity. *Dm* AGO1, GW182, and NOT1 (as well as Me31B, see below) repressed the expression of the F-Luc(Leu)-V5 protein independently of whether it was translated from a mRNA reporter containing a 109-nt or 8-nt 5'-UTR. mRNA levels were not affected (Figs 3D and E and EV2K–N).

To analyze the requirement for 43S scanning in human cells, we replaced the 5'-UTR of the R-Luc-6xMS2-A₉₅-MALAT1 reporter with a TISU motif (translation initiator of short 5'-UTR, Fig 3F, Appendix Tables S2 and S3), which directs efficient cap-dependent translation initiation of very short 5'-UTRs (9-nt) via a scanning- and eIF4A-independent mechanism (Elfakess *et al*, 2011; Sinvani *et al*, 2015). The 5'-UTR of the TISU reporter was confirmed by 5' RACE (Fig EV3A and Appendix Fig S2).

The translation efficiency of the TISU reporter was 16-fold lower relative to the parental reporter containing a 216-nt long 5'-UTR and the MALAT1 3'-end (Fig EV3B–E, compare reporters c and g). Nevertheless, TNRC6A-SD, AGO2, and NOT1 repressed the expression of these two reporters and that of a corresponding polyadenylated reporter to comparable extents. The polyadenylated reporter was degraded, whereas the reporters containing the MALAT1 3'-end were resistant to degradation (Figs 3G and H and EV3F). Notably, insertion of an HA-tag immediately downstream of the first AUG in the TISU reporter enabled detection of R-Luc

by Western blotting using anti-HA antibodies (Fig EV3G), confirming that TISU directs translation initiation at the first AUG and therefore in the absence of scanning.

We further confirmed that translation of the TISU reporter was independent of scanning, and thus of eIF4A activity, using silvestrol, a compound that stimulates eIF4A1/2 RNA-binding activity, reducing the pool of the protein available for translation (Bordeleau *et al*, 2008; Cencic *et al*, 2009). Silvestrol inhibited cap-dependent translation of *Renilla* luciferase, but the translation driven by the TISU sequence was only marginally inhibited (Fig 4A) as expected (Elfakess *et al*, 2011). In contrast, silvestrol stimulated HCV IRES-driven translation of an F-Luc reporter, which was included as a transfection control (Fig EV3H). Notably, the inhibitory effect of silvestrol in translation was observed despite a two- to fivefold increase of reporter mRNA levels in HEK293 cells (Fig EV3I and J).

Silencing does not correlate with 5'-UTR secondary structure

To further investigate the dependence on eIF4A for silencing, we generated reporters containing 5'-UTRs with different degrees of secondary structure, which were expected to exhibit different eIF4A requirements (Appendix Tables S2 and S3). In particular, we generated a reporter containing a 5'-UTR consisting of 18 CAA repeats, which is unlikely to adopt secondary structures, and its translation is thought to be independent of eIF4A (Meijer *et al*, 2013). This 5'-UTR was also shown to confer resistance to silencing (Meijer *et al*, 2013). Unexpectedly, translation of this reporter was partially inhibited by silvestrol (Figs 4A and EV3H). TNRC6A-SD, AGO2, and NOT1 repressed the CAA reporter without causing mRNA degradation due to the presence of the MALAT1 3'-end (Fig 4B and C).

We also tested a 5'-UTR that contains four 12-nt guanine quartet (CGG)₄ motifs in tandem and is predicted to form G-quadruplex structures and thus requires eIF4A for translation (Cencic *et al*, 2009; Wolfe *et al*, 2014; Appendix Tables S2 and S3). Accordingly, the translation of this reporter was sensitive to silvestrol treatment (Figs 4A and EV3H). TNRC6A-SD, AGO2, and NOT1 repressed the CGG reporter to an extent comparable to that of the CAA reporter (Fig 4D and E versus Fig 4B and C), despite a eightfold difference in translation efficiency (Fig EV3B–E).

In sum, AGO, GW182/TNRC6s, and NOT1 silence mRNA targets independently of whether their translation requires ribosome scanning and independently of the 5'-UTR secondary structure.

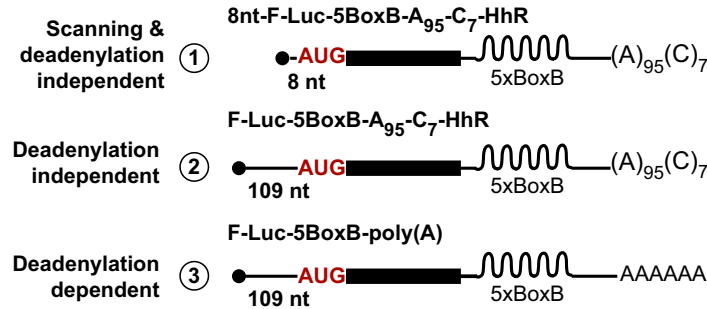
Figure 3. AGO, GW182/TNRC6, and NOT1 silence mRNA reporters translated via a scanning-independent mechanism.

- A Schematic representation of the *Dm* reporters used in panels (B, C).
 B, C Tethering assay using the F-Luc-5BoxB reporters shown in panel (A) and λN-HA-tagged GW182, AGO1, and NOT1 in S2 cells. F-Luc activity and mRNA levels were normalized to those of the R-Luc transfection control and analyzed as described in Fig 2A and B. Quantification of the corresponding Northern blots is shown in Fig EV2J.
 D, E Tethering assay with the indicated reporters containing a F-Luc(Leu)-V5 fusion in which all in-frame methionines were replaced with leucine. The corresponding Western and Northern blots are shown in Fig EV2K–N.
 F Schematic representation of the reporters used in panels (G, H).
 G, H Tethering assay using the R-Luc-6xMS2 reporters shown in panel (F) and HA-MS2-tagged TNRC6A-SD, AGO2, and NOT1 in HEK293T cells. R-Luc activity and mRNA levels were normalized to those of the F-Luc transfection control and analyzed as described in Fig 2C and D. The corresponding quantification of mRNA levels is shown in Fig EV3F.

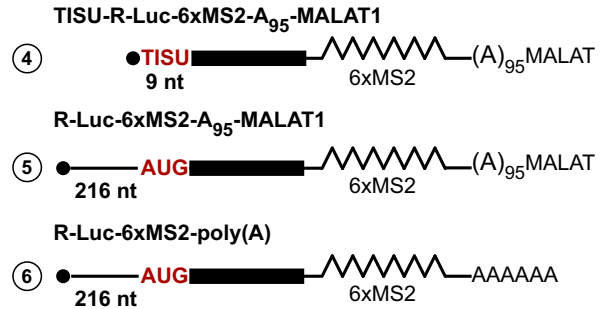
Data information: In all panels, bars represent mean values and error bars represent standard deviations from at least three independent experiments.

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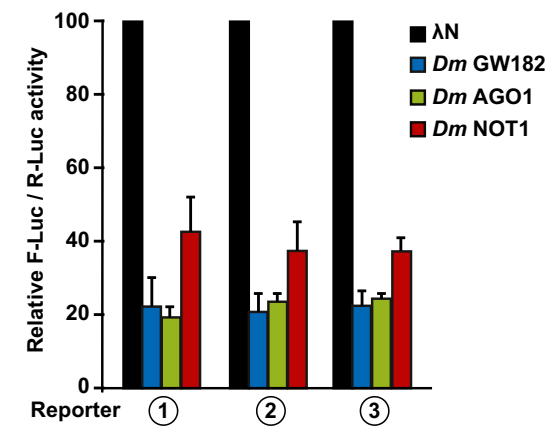
A Dm reporters



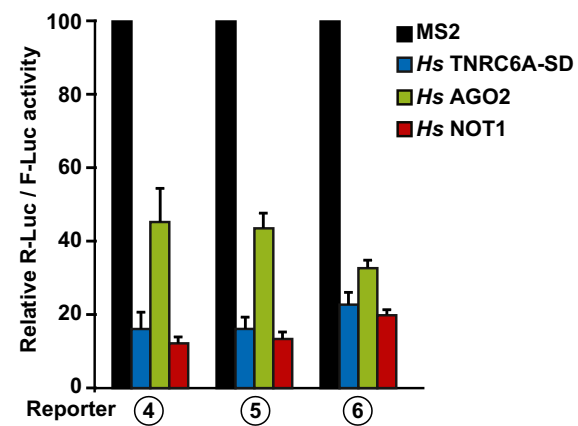
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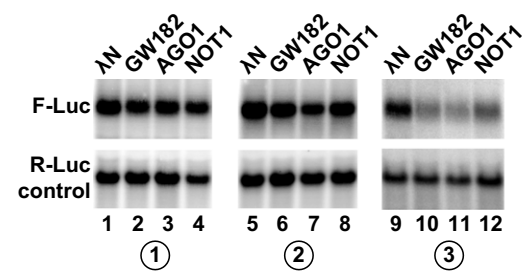
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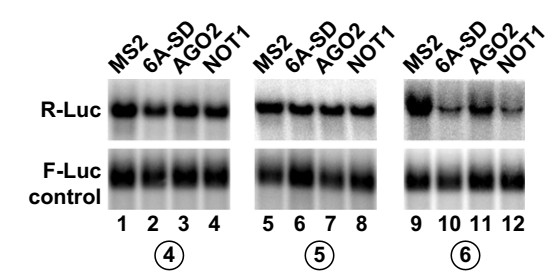
G Hs cells



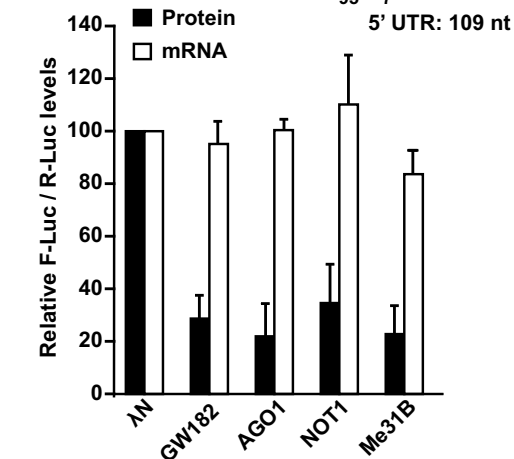
C Dm cells



H Hs cells



D Dm F-Luc(Leu)-V5-5BoxB-A95-C7-HhR



E Dm 8nt-F-Luc(Leu)-V5-5BoxB-A95-C7-HhR

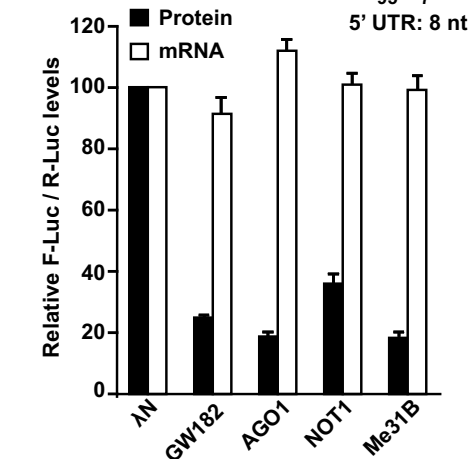


Figure 3.

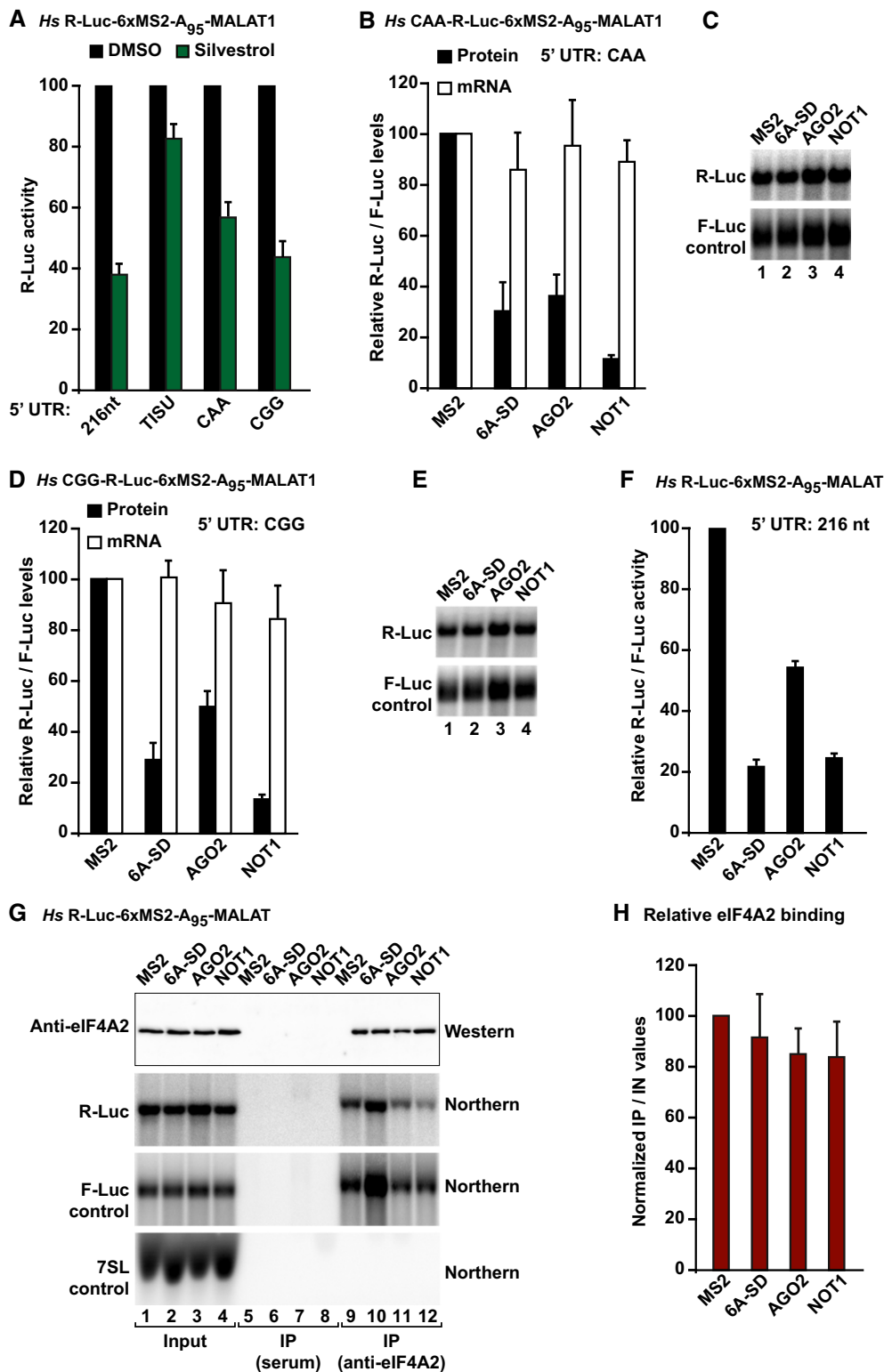


Figure 4.

eIF4A2 binding to mRNAs does not correlate with silencing efficiency

eIF4A2 was reported to be either recruited to or dislodged from silenced miRNA targets (Meijer *et al*, 2013; Fukao *et al*, 2014; Fukaya

et al, 2014). To resolve this discrepancy, we investigated eIF4A2 association with the R-Luc-6xMS2-A₉₅-MALAT1 reporter containing a 5'-UTR of 216 nt. The translation of this reporter is sensitive to silvestrol treatment (Fig 4A). The reporter was silenced to different extents by tethered AGO2, TNRC6A-SD, or NOT1 (Fig 4F). Nevertheless, eIF4A2

Figure 4. eIF4A2 binding to mRNAs is not influenced by silencing.

- A HEK293T cells were transfected with the indicated R-Luc-6xMS2-A₉₅-MALAT1 reporters containing the indicated 5'-UTRs. An F-Luc reporter whose translation was dependent on the hepatitis C virus (HCV) IRES was included as a control. One day after transfection, cells were treated with DMSO or silvestrol. R-Luc activity in silvestrol-treated cells was normalized to that measured in control cells. The corresponding values for the HCV IRES-F-Luc reporter are shown in Fig EV3H. The panel shows mean values ± standard deviations from three independent experiments.
- B–E Tethering assays using the indicated R-Luc-MS2-A₉₅-MALAT1 reporters and MS2-HA-tagged TNRC6A-SD, AGO2, and NOT1 in HEK293T cells. R-Luc activity and mRNA levels were normalized to those of the F-Luc transfection control and set to 100% in cells expressing MS2-HA. Panels (B, D) show mean values ± standard deviations from three independent experiments. Panels (C, E) show Northern blots of representative RNA samples.
- F–H Lysates from HEK293T cells expressing R-Luc-MS2-A₉₅-MALAT1 reporter and MS2-HA-tagged proteins were immunoprecipitated using anti-eIF4A2 antibody. The RNAs coimmunoprecipitating with eIF4A2 were analyzed by Northern blot. 7SL RNA served as a loading control. R-Luc mRNA levels were normalized to those of the F-Luc control. The normalized values in the IP were divided by those in the input and set to 100 for cells expressing MS2 peptide. (F) Normalized R-Luc activities. The panel shows mean values ± standard deviations from five independent experiments. (G) Representative Western and Northern blots of input and IP fractions. For the Western blot, 2% of the input and 6% of the IP fraction were analyzed. For the Northern blots, 2% of the input and 40% of the IP were analyzed. (H) Efficacy of the immunoprecipitation. Error bars represent standard errors from five independent experiments.

Source data are available online for this figure.

associated with the reporter to comparable levels (Fig 4G and H). Moreover, the association was comparable to that observed for the unrepressed reporter in cells expressing the MS2 protein alone (Fig 4G and H and Appendix Fig S3). The possibility that the observed eIF4A2 binding is unspecific is unlikely, as eIF4A2 did not associate with 7SL RNA, and the reporters were not precipitated by an unrelated rabbit serum (Fig 4G). Thus, eIF4A2 in association with mRNAs is neither enhanced nor reduced by silencing.

Ribosome scanning is not required for mRNA degradation caused by tethered AGOs, GW182/TNRC6s, and NOT1

Because it has been reported that ribosome scanning is a prerequisite for miRNAs to degrade their targets (Meijer *et al*, 2013), we next analyzed whether GW182/TNRC6, AGO, and NOT1 degraded polyadenylated mRNA reporters that are translated via a scanning-independent mechanism. In *Dm* cells, a polyadenylated reporter containing an 8-nt 5'-UTR was degraded by tethered GW182, AGO1, and NOT1 (Fig 5A and B). Similarly, the TISU-R-Luc-6xMS2-poly(A) reporter was also degraded by AGO2, TNRC6A-SD, and NOT1 in HEK293T cells (Fig 5C and D). Thus, in the absence of scanning, deadenylation and subsequent mRNA decay still occur.

We also investigated whether a β-globin reporter containing a 114-nt 5'-UTR could be degraded in the presence of silvestrol. This reporter was selected because it is very efficiently degraded by tethered TNRC6A-SD, AGO2, and NOT1 in human cells. This degradation was not prevented by silvestrol (Fig 5E–G), although translation of a cotransfected R-Luc reporter was inhibited 2.5-fold (Fig 5E) and the mRNA reporters were stabilized by the silvestrol treatment in the absence of the tethered proteins (Fig 5F, lane 5 versus 1). Taken together, our results indicate that mRNA degradation induced by tethered AGO, GW182, and NOT1 does not require prior ribosomal scanning.

miRNAs silence reporters translated via a scanning-independent mechanism

Next, we investigated whether miRNAs (as opposed to tethered silencing factors) also repress reporters translated via a scanning-independent mechanism. To this end, we generated F-Luc-*nerfin-1* and F-Luc-*par6* reporters containing an 8-nt 5'-UTR. These reporters were efficiently silenced by the corresponding miRNAs as the parental reporters, which contained 109-nt long

5'-UTRs (Fig 6A–D). Furthermore, the *nerfin-1* reporter was repressed predominantly at the translational level whereas the *par-6* reporter was predominantly degraded independently of the length of the 5'-UTR (Fig 6A–D).

For expression in human cells, we generated a polyadenylated R-Luc reporter containing the TISU motif or a 216-nt 5'-UTR and eight *let-7* miRNA-binding sites in the 3'-UTR. Both of these reporters were efficiently silenced and partially degraded by endogenous *let-7* independently of the length of the 5'-UTR in HeLa cells (Fig 6E and F). We conclude that miRNAs also silence mRNA reporters translated via scanning-independent mechanisms in both human and *Dm* cells.

Finally, we also tested the silencing of a *let-7* reporter containing the MALAT1 3' end (R-Luc-8x*let-7*-A₉₅-MALAT reporter) in the presence of silvestrol in HeLa cells. The silvestrol treatment partially suppressed silencing, as observed for hippuristanol treatment in previous studies. However, we observed a more than 10-fold increase in mRNA levels both for the R-Luc reporter and the F-Luc control in HeLa cells treated with silvestrol (Fig EV3K–M). These results indicate that experiments in which miRNA-mediated translational repression is measured in the presence of drugs that inhibit general translation should be interpreted with caution due to indirect effects on mRNA stability and the lack of appropriate transfection controls that are not affected by the treatment.

The CCR4–NOT complex requires DDX6 to repress translation

We and others have shown that DDX6 acts downstream of the CCR4–NOT complex to mediate translational repression and stimulate decapping in human cells (Chen *et al*, 2014; Mathys *et al*, 2014; Rouya *et al*, 2014). To further confirm that DDX6 function lies downstream of CCR4–NOT, we used tethering assays to analyze the repressive activity of a NOT1 mutant that it is impaired in binding to DDX6 but binds to CNOT2 and CNOT3 (Fig 6G, NOT1 Mut; Appendix Table S1). The NOT1 mutant was impaired in repressing the translation of the R-Luc-5BoxB-MALAT1 mRNA, which is not degraded (Fig 6H and I). These results indicate that full-length NOT1 requires interaction with DDX6 to effectively repress translation of a reporter that is resistant to deadenylation. However, the NOT1 mutant was active when tethered to the corresponding polyadenylated reporter, and the reporter was degraded (Fig 6J and K). The NOT1 proteins were expressed at comparable levels (Fig 6L). Thus,

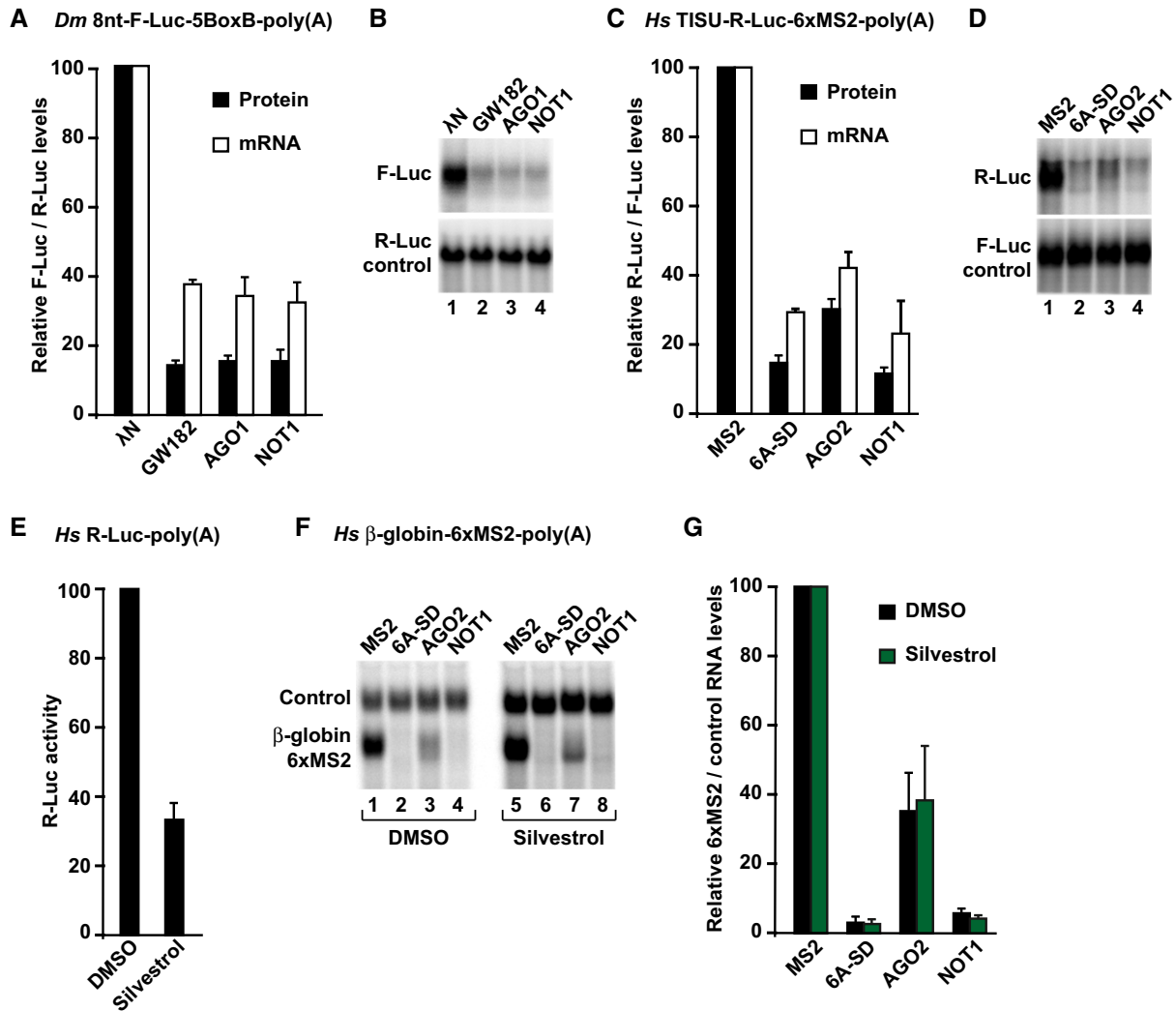


Figure 5. Ribosome scanning is not required for degradation of silenced mRNAs.

A, B Tethering assay using the 8nt-F-Luc-5BoxB-poly(A) reporter in S2 cells. Firefly luciferase activities and mRNA levels were normalized to those of the *Renilla* luciferase and analyzed as described in Fig 2A and B.

C, D Tethering assay using the TISU-R-Luc-6xMS2-poly(A) reporter in human HEK293T cells. Samples were analyzed as described in Fig 2C and D.

E–G Tethering assay using the β -globin-6xMS2-poly(A) reporter in human HEK293T cells treated with silvestrol or DMSO. Panel (E) shows the inhibitory effect of silvestrol on the translation of a cotransfected R-Luc reporter. In panels (F, G), samples were analyzed as described in Fig 2C and D.

Data information: Bars represent mean values \pm standard deviations from three independent experiments.

Source data are available online for this figure.

the deadenylase subunits degrade mRNA poly(A) tails even when the interaction with DDX6 is impaired.

Additionally, DDX6 depletion suppressed NOT1-mediated silencing of the R-Luc-5BoxB-MALAT1 reporter in HeLa cells (Fig EV4A and B), supporting the conclusion that NOT1 requires DDX6 to repress translation. In contrast to the results obtained in HeLa cells, depletion of Me31B in S2 cells did not completely suppress the silencing activity of AGO1, GW182, and NOT1 tethered to a reporter that is not degraded (Fig EV4C and D). These results suggest the existence of alternative silencing mechanisms in these cells. Alternatively, the residual amounts of Me31B in depleted cells (< 10% of control) might still be sufficient for silencing (Fig EV4D).

DDX6 represses translation initiation independently of 43S scanning

If DDX6/Me31B acts downstream of AGOs, GW182/TNRC6, and CCR4–NOT, it is expected that they also repress reporters translated via a scanning-independent mechanism. Consistent with this expectation, tethered DDX6 repressed the expression of all reporters containing the MALAT1 3'-end, including the TISU reporter, in HEK293T cells (Fig 7A–C). Thus, DDX6 represses translation in the absence of 43S scanning and deadenylation. DDX6 activity was only slightly impaired by the mutations that prevent binding to NOT1 (DDX6 Mut1), consistent with the notion that DDX6 acts downstream of the CCR4–NOT complex (Fig 7A–D). Similarly, *Dm*

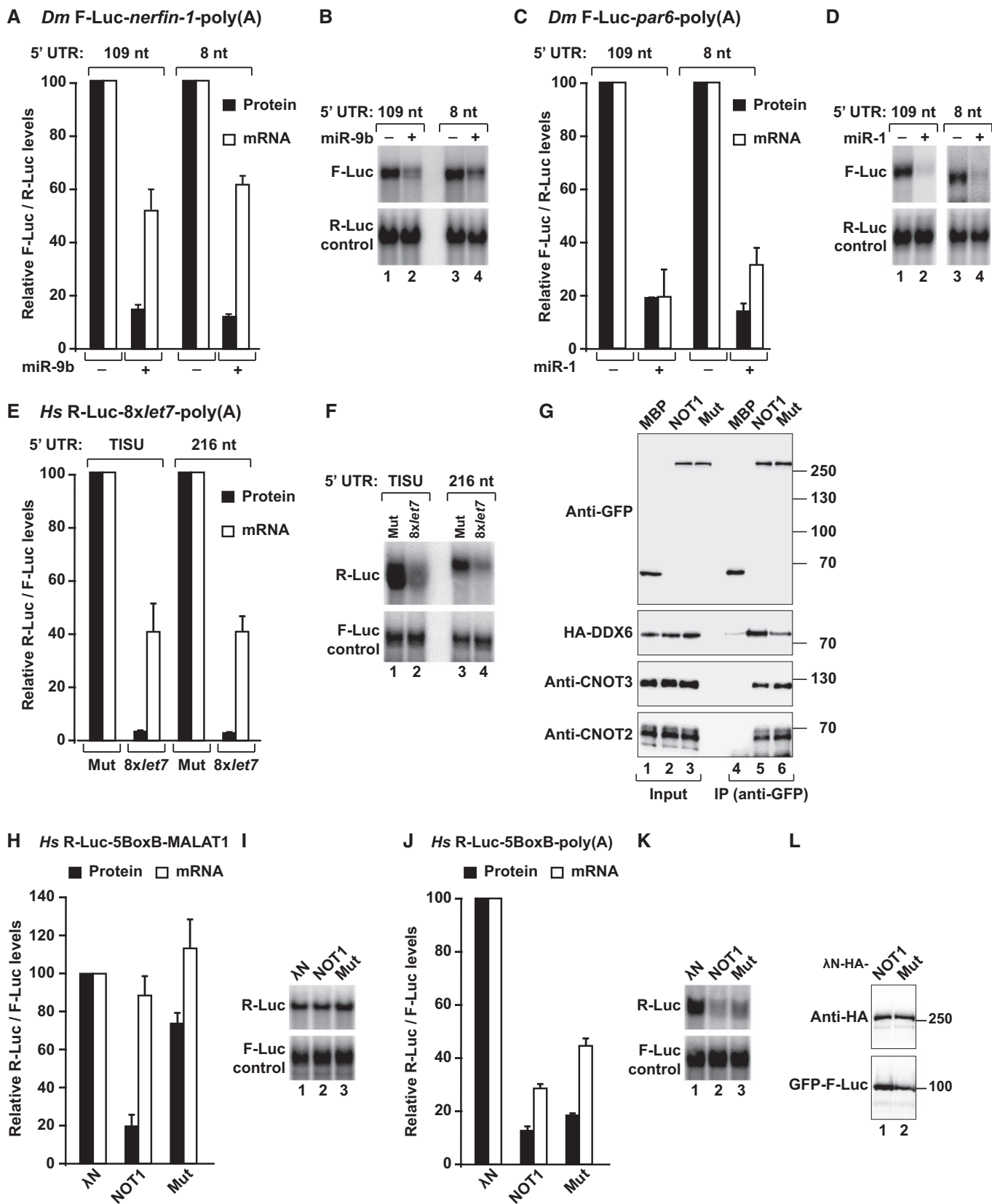


Figure 6.

Figure 6. Ribosome scanning is not required for miRNA target degradation.

A–D S2 cells were transfected with polyadenylated F-Luc-*nerfin-1* or F-Luc-*par6* reporters containing an 8-nt or 109-nt 5'-UTR and the corresponding miRNAs. A plasmid expressing *Renilla* luciferase (R-Luc) served as transfection control. Panels (A, C) show normalized firefly luciferase activities and mRNA levels. Northern blot analyses of representative RNA samples are shown in (B, D).

E, F HeLa cells were transfected with plasmids expressing the indicated R-Luc-*let-7* reporters or the corresponding reporters carrying mutations in the *let-7*-binding sites and a plasmid expressing F-Luc as a transfection control. *Renilla* luciferase activities were normalized to those of the F-Luc and set to 100% in cells transfected with the reporter lacking the *let-7*-binding sites and analyzed as described in (A–D).

G Interaction of GFP-NOT1 (wild type or a mutant that does not bind DDX6, Mut) with HA-DDX6 and endogenous CNOT2 and CNOT3 in HEK293T cells.

H–K Tethering assay using the R-Luc-5BoxB reporters containing the MALAT1 3'-UTR (H, I) or a poly(A) tail (J, K) and λ N-HA-NOT1 (wild type or Mut). R-Luc activity and mRNA levels were normalized to those of the F-Luc transfection control and analyzed as described in Fig 2C and D.

L Expression levels of wild type and mutant NOT1.

Data information: Bars represent mean values \pm standard deviations from three independent experiments.
Source data are available online for this figure.

Me31B repressed the F-Luc-5BoxB-A₉₅-C₇-HhR reporter independently of the length of the 5'-UTR (Fig 7E), indicating that the ability to repress translation in the absence of scanning is conserved among DDX6 orthologs.

In yeast, Dhh1 (the DDX6 ortholog) was shown to repress translation at initiation (Coller & Parker, 2005) or at elongation (Sweet *et al*, 2012). To help to resolve this discrepancy, we analyzed the association of the R-Luc-6xMS2-A₉₅-MALAT1 reporter with polysomes upon repression by DDX6. Sucrose gradient analyses indicated that tethered DDX6 changed the distribution of the reporter in the polysome profile toward lighter polysomes/nonpolysomal fractions without causing mRNA degradation, indicating that DDX6 represses translation initiation in HEK293T cells (Figs 7G and EV4E and F).

DDX6 requires interaction with NOT1 and the integrity of the FDF-binding surface to silence miRNA reporters

To obtain further insight on how DDX6 represses translation, we compared the ability of DDX6 mutants to rescue silencing in HeLa cells depleted of DDX6. In particular, we used a DDX6 mutant (Mut1, Appendix Table S1) that does not interact with NOT1 but interacts with 4E-T and decapping factors (namely PatL1, EDC3, and LSM14; Fig 8A–E). We also tested a mutant with substitutions on the FDF-binding surface of DDX6 (Mut2, Appendix Table S1). These mutations prevent the interaction with 4E-T and decapping factors, which all bind to the FDF-binding surface of DDX6 in a mutually exclusive manner (Fig 8A–D; Tritschler *et al*, 2009; Ozgur *et al*, 2015). However, DDX6 Mut2 still binds to NOT1 (Fig 8E), indicating that the mutations do not alter the protein fold. We observed that only wild-type DDX6 rescued silencing of the

R-Luc-8xlet7-poly(A), whereas DDX6 Mut1 or Mut2 were inactive (Fig 8F). Similarly, a DDX6 DEAD-box mutant (E236Q) did not rescue silencing, as reported before (Mathys *et al*, 2014). DDX6 was depleted to < 25% of wild type (Fig 8G). Together, these results indicate that DDX6 requires interaction with NOT1 and additional factors that bind to the FDF-binding surface to mediate silencing of miRNA targets.

Previous studies indicated that depletion of decapping factors prevents miRNA target degradation but does not suppress miRNA-mediated translational repression because the reporters accumulated in a deadenylated form, which is not translated efficiently (Eulalio *et al*, 2007). Therefore, we tested whether 4E-T could mediate the repression. However, depletion of 4E-T did not suppress silencing of this reporter (Fig 8H). 4E-T was depleted to < 25% of wild type (Fig 8I). Thus, it is possible that other, not-yet-identified, factors interact with the FDF-binding surface of DDX6 to mediate silencing or that the known binding partners act redundantly to promote silencing.

Discussion**AGOs require the W-binding pockets to mediate silencing**

Previous studies reported that *Dm* AGO1 can repress miRNA targets independently of GW182/TNRC6 proteins and of the CCR4–NOT complex (Fukaya & Tomari, 2012; Wu *et al*, 2013; Fukaya *et al*, 2014). Here, we show that amino acid substitutions in the two W-binding pockets present on the surface of the PIWI domain of *Dm* AGO1 and *Hs* AGO2 (Schirle & MacRae, 2012) abolish the binding of AGOs to GW182/TNRC6 proteins without disrupting

Figure 7. DDX6 represses translation independently of ribosome scanning.

A–C Tethering assay using the R-Luc-6xMS2-A₉₅-MALAT1 reporters containing the indicated 5'-UTRs and MS2-HA-tagged DDX6 or a DDX6 mutant (Mut1) that does not bind to NOT1 in HEK293T cells. R-Luc activity and mRNA levels were normalized to those of the F-Luc control and analyzed as described in Fig 2C and D.

D Immunoprecipitation assay showing the interaction of DDX6 or DDX6 Mut1 with GFP-NOT1 in HEK293T cells. Inputs (1% for the HA-tagged proteins and 3% for the GFP-tagged proteins) and immunoprecipitates (20 and 15%, respectively) were analyzed by Western blotting using anti-HA and anti-GFP antibodies. Endogenous CNOT2 and CNOT3 (input 0.5% and IP 10%) were detected using specific antibodies (Appendix Table S4).

E Tethering assay using the F-Luc-5BoxB-A₉₅-C₇-HhR reporters containing an 8-nt or a 109-nt 5'-UTR and λ N-HA-Me31B in S2 cells. F-Luc activity and mRNA levels were analyzed as described in Fig 2A and B.

F, G Tethering assay using the R-Luc-6xMS2-A₉₅-MALAT1 reporter and MS2-DDX6 in HEK293T. In panel (F), luciferase activity was analyzed as described in Fig 2C. The association of the R-Luc reporter and the F-Luc control with polysomes was analyzed by sedimentation through a sucrose gradient. Panel (G) shows the amount of the R-Luc mRNA in each fraction normalized to the total amount across all fractions in cells expressing MS2 (black curve) or MS2-DDX6 (red curve). Lower panels show representative Northern blots. The data corresponding to the F-Luc control and the corresponding ribosomal RNA profiles are shown in Fig EV4E and F.

Data information: Bars represent mean values \pm standard deviations from three independent experiments.
Source data are available online for this figure.

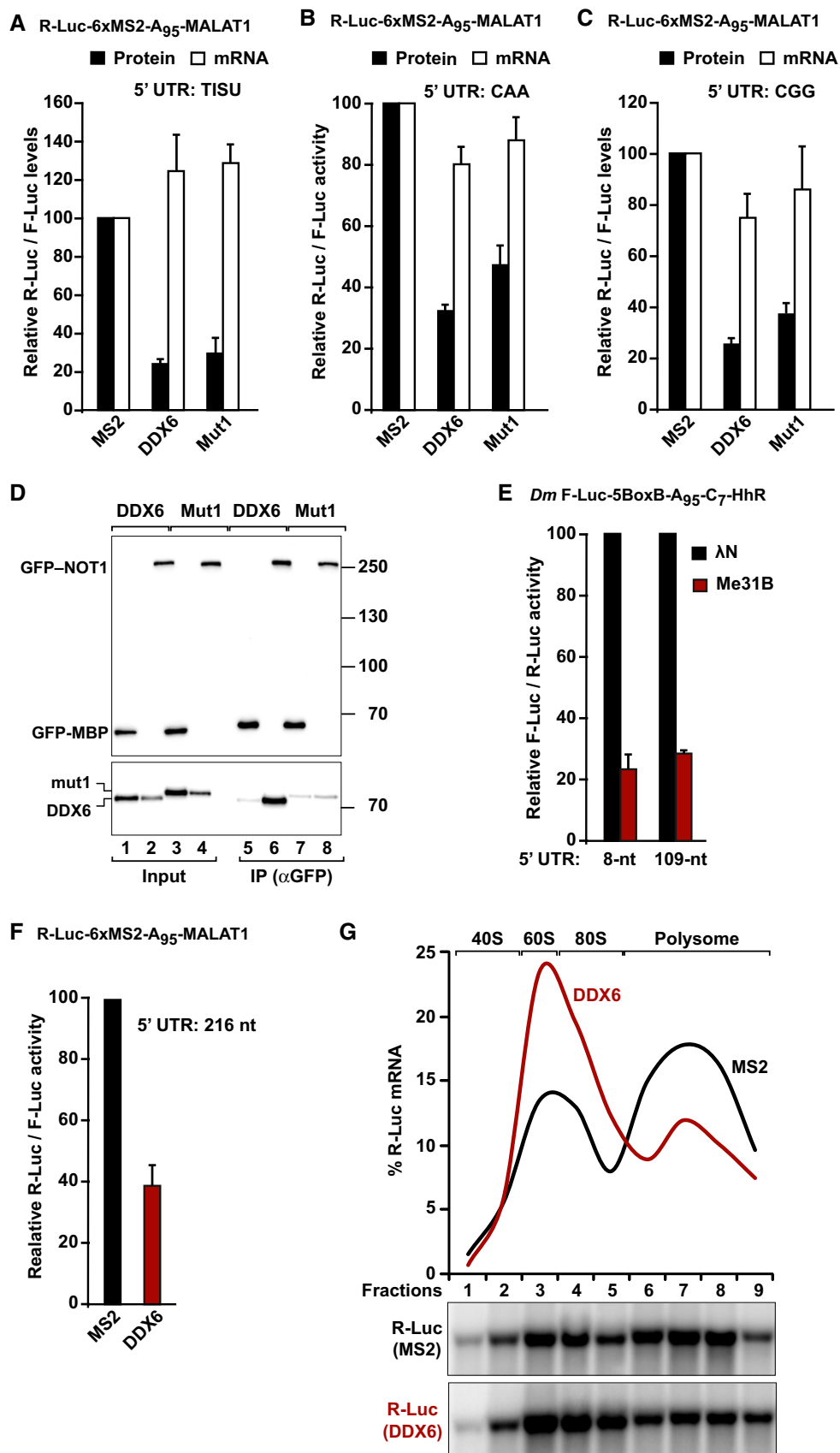


Figure 7.

miRNA loading. These mutations also abolish the silencing activity of AGOs in both tethering and complementation assays. The most parsimonious explanation for our results is that AGOs require interaction with GW182/TNRC6 proteins to mediate silencing. Although we cannot rule out the possibility that AGOs interact with another, yet-unidentified, protein partner using a mode of interaction similar to that used with GW182/TNRC6, we consider this possibility unlikely because AGOs, GW182/TNRC6, NOT1, and miRNAs degrade polyadenylated mRNA targets via the 5'-to-3' decay pathway. Furthermore, all three proteins as well as miRNAs repress the translation of mRNA targets that are refractory to deadenylation. Finally, we show that all three proteins and miRNAs repress and/or degrade mRNA targets that do not require 43S ribosomal scanning for translation. These results point to a common mechanism used by these proteins and miRNAs to mediate silencing.

Ribosomal scanning is not a prerequisite for silencing

A previous study reported that mRNAs with highly structured 5'-UTRs, which depend on eIF4A for translation, are susceptible to silencing whereas mRNAs with unstructured 5'-UTRs are refractory to silencing in human cells (Meijer *et al*, 2013). By contrast, Ricci *et al* (2013) failed to detect a direct correlation between silencing and 5'-UTR secondary structure in rabbit reticulocyte lysates. Furthermore, it has been reported that 5'-UTR features such as length and secondary structure do not reliably correlate with the extent of miRNA-mediated repression (Agarwal *et al*, 2015). We found no evidence for the requirement of structured 5'-UTRs for silencing in either human or *Dm* cells. Our conclusions are based on the use of reporters that have very short 5'-UTRs (in *Dm* cells) or contain the TISU motif (human cells). These reporters direct translation independently of scanning. We could also not confirm that a reporter containing 18 CAA repeats in the 5'-UTR is resistant to silencing, as reported by Meijer *et al* (2013). Furthermore, the CAA reporter was sensitive to silvestrol treatment, indicating that it partially depends on eIF4A activity for translation as reported by Pestova and Kolupaeva (2002).

In summary, all reporters tested in our study were partially degraded when they contained a poly(A) tail or were repressed predominantly at the translational level when they contained 3' termini that conferred resistance to deadenylation. Similar results were obtained for tethered human AGO2, TNRC6, NOT1, DDX6 and their *Dm* orthologs as well as for miRNAs in the absence of tethering, indicating that 43S scanning is not a prerequisite for translational repression, deadenylation, and decay of miRNA targets.

Silencing does not require eIF4A function during scanning

Previous studies made use of eIF4A inhibitors (hippuristanol, silvestrol, or pateamine A) to demonstrate a role for eIF4A in silencing. However, this approach has yielded conflicting results, as these drugs were reported either to have no effect (Petersen *et al*, 2006) or to partially inhibit miRNA-mediated silencing (Meijer *et al*, 2013; Fukao *et al*, 2014). These discrepancies most likely result from the difficulty of finding appropriate normalization controls that are not affected by the treatment or by indirect effects on mRNA stability that have been overlooked. By contrast, our conclusion that the scanning function of eIF4A is not required for silencing is based on the results obtained with the reporters containing a very short 5'-UTR (in *Dm* cells) or the TISU motif (human cells), which are not subjected to the normalization problem linked to the use of translational inhibitors. Using these reporters, we demonstrated that 43S scanning and thus eIF4A activity in this process are not required for silencing.

We further show that AGO2, GW182/TNRC6, and NOT1 degrade polyadenylated reporters in the presence of silvestrol, although the treatment causes an increase in reporter mRNA levels in the absence of the tethered proteins. These results indicate that silencing complexes assemble and degrade the mRNA target when translation is inhibited by silvestrol. In accordance with these results, miRNAs, AGO, GW182/TNRC6, and NOT1 degrade polyadenylated reporters that do not require scanning for translation.

Finally, we show that miRNAs can repress translation by a mechanism that does not involve either eIF4A2 dissociation or recruitment.

Figure 8. DDX6 requires interaction with NOT1 and the integrity of the FDF-binding surface to mediate silencing.

- A–D Immunoprecipitation assay showing the interaction of GFP-tagged DDX6 (wild type or the indicated mutants) with HA-tagged decapping factors and 4E-T in HEK293T cells. Inputs (1% for the HA-tagged proteins and 3.5% for the GFP-tagged proteins) and immunoprecipitates (20 and 7%, respectively) were analyzed by Western blotting using anti-HA and anti-GFP antibodies. GFP-MBP served as a negative control.
- E Immunoprecipitation assay showing the interaction of GFP-tagged NOT1 with HA-tagged DDX6 (wild type or the indicated mutants) in HEK293T cells. Samples were analyzed as described in (A–D).
- F Control HeLa cells (transfected with a scrambled shRNA) or cells depleted of DDX6 were transfected with a mixture of three plasmids: the R-Luc-8xlet-7-poly(A) or the corresponding reporter carrying mutations in let-7-binding sites (R-Luc-Mut, Mut), a plasmid expressing F-Luc as a transfection control, and a plasmid expressing shRNA-resistant versions of GFP-DDX6 (wild type or the indicated mutants) or MBP. For each condition, *Renilla* luciferase activity was measured, normalized to that of the F-Luc transfection control and set at 100% in cells expressing R-Luc-Mut. The left panel shows the normalized *Renilla* luciferase activities in control cells (i.e. cells treated with scrambled shRNA and expressing MBP). The right panel shows relative fold derepression for each condition for the R-Luc-8xlet-7-poly(A) after normalization. Mean values \pm standard deviations from 4 independent experiments are shown.
- G Western blot showing the efficiency of the DDX6 knockdown. Dilutions of control cell lysates were loaded in lanes 1–4 to estimate the efficacy of the depletion. PABP served as a loading control.
- H Control HeLa cells (transfected with a scrambled shRNA) or cells depleted of 4E-T were transfected with a mixture of three plasmids as described in panel (F). The left panel shows normalized *Renilla* luciferase activities in control cells (i.e. cells treated with scrambled shRNA). The right panel shows the relative fold derepression for the R-Luc-8xlet-7-poly(A) (after normalization) in 4E-T-depleted cells relative to control cells. Mean values \pm standard deviations from three independent experiments are shown.
- I Western blot showing the efficiency of the 4E-T knockdown. Dilutions of control cell lysates were loaded in lanes 1–4 to estimate the efficacy of the depletion. Tubulin served as a loading control.

Source data are available online for this figure.

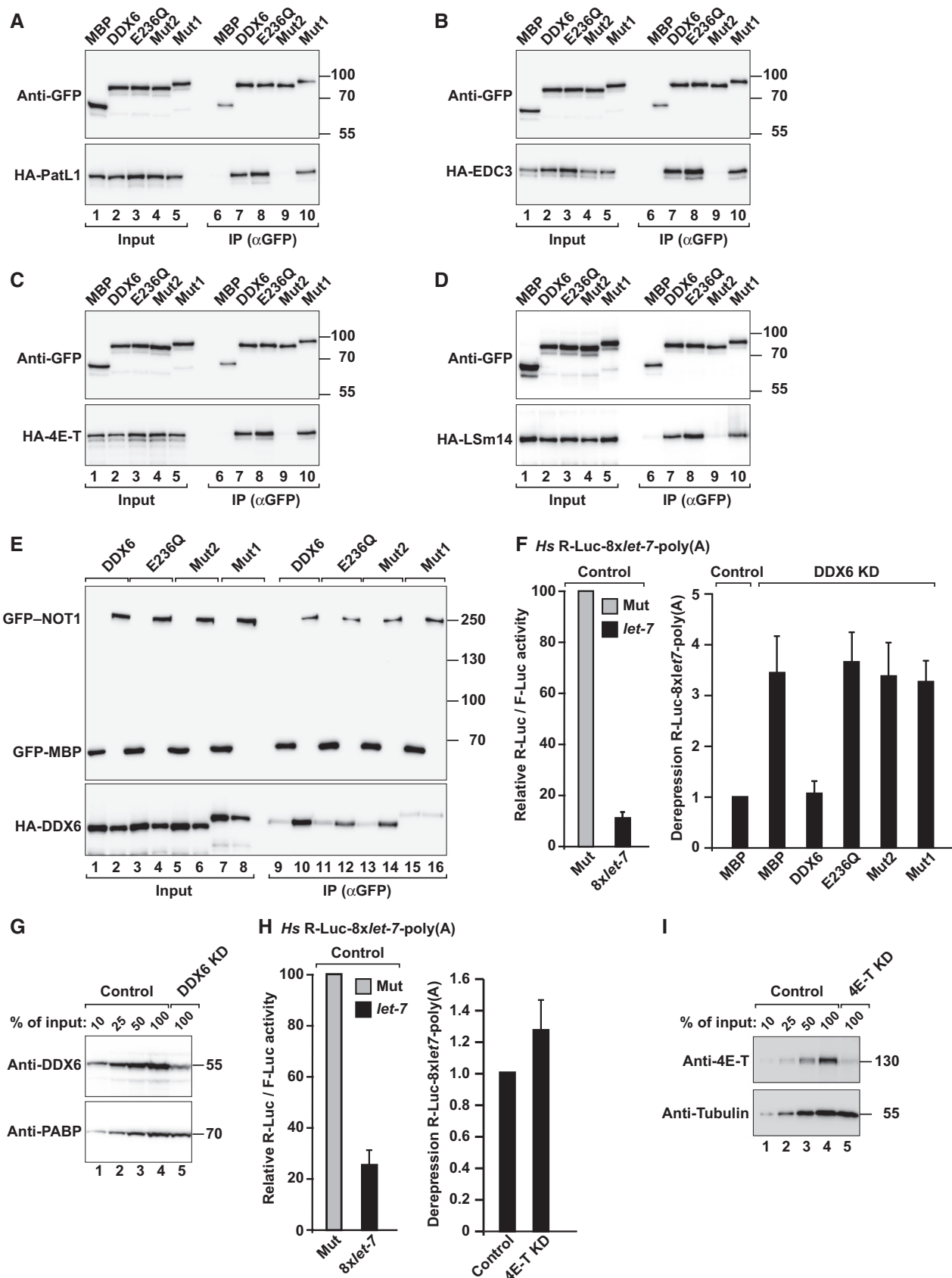


Figure 8.

Together with the observation that the knockout of eIF4A2 in human cells does not suppress silencing (Galicía-Vazquez *et al*, 2015), our results indicate that eIF4A2 is unlikely to act as a repressor in the miRNA pathway.

Our results do not rule out an involvement of eIF4A1 in silencing (Fukao *et al*, 2014; Fukaya *et al*, 2014) but reveal the existence of a mechanism that acts independently of eIF4A1 (i.e. when eIF4A1 is inhibited) or downstream of eIF4A1 dissociation.

The role of DDX6 in silencing

Our data together with previous studies indicate that human DDX6 is required for translational repression mediated by the CCR4–NOT complex in human cells (Chen *et al*, 2014; Mathys *et al*, 2014; Rouya *et al*, 2014). The contribution of DDX6 to this repression becomes apparent in particular for reporters that lack a poly(A) tail and hence are not degraded. In the presence of a poly(A) tail, the dominant effect of the CCR4–NOT complex is deadenylation and decay in human and *Dm* cells. mRNA destabilization is observed even when the NOT1–DDX6 interaction is impaired, suggesting that the recruitment of decapping factors and XRN1 still occurs. Therefore, it is possible that in addition to DDX6, other factors are involved in coupling deadenylation with decapping. For example, the decapping activator PatL1 interacts with the CCR4–NOT complex (Jonas & Izaurralde, 2013) and can also facilitate the recruitment of decapping factors to mRNAs undergoing deadenylation. Alternatively, the residual amount of DDX6 in depleted cells may be sufficient for deadenylation by the CCR4–NOT complex but not for translational repression.

A question that remains unanswered is how DDX6 represses translation. Our polysome profiles together with previous studies indicate that DDX6 orthologs inhibit translation predominantly at initiation (Coller & Parker, 2005; Minshall *et al*, 2009). Furthermore, we found that DDX6-mediated repression does not require ribosomal scanning, suggesting that DDX6 may interfere with eIF4E or eIF4G function. In this context, it has been shown that DDX6 interacts with the eIF4E-transporter protein (4E-T; Nishimura *et al*, 2015; Ozgur *et al*, 2015), an eIF4E-binding protein that competes with eIF4G for binding to eIF4E and represses translation initiation. However, depletion of 4E-T only partially alleviates silencing (this study; Kamenska *et al*, 2014; Nishimura *et al*, 2015), which implies that DDX6 may employ additional mechanisms to repress translation that are thus far unknown. Accordingly, we observed that the integrity of the FDF-binding surface of DDX6 is required for silencing. Because this surface interacts with decapping factors in addition to 4E-T, it is possible that these factors play a role in translational repression in addition to mRNA degradation. It is also possible that other, not-yet-identified, DDX6 partners also use the FDF-binding surface to interact with DDX6.

Through its interaction with the CCR4–NOT complex, DDX6 is likely to be involved in the repression of many mRNAs in addition to miRNA targets. Indeed, the CCR4–NOT complex is recruited to specific mRNAs by numerous RNA-associated proteins, including Bicardal-C, Smaug, CUP, Nanos, Pumilio, and tristetraprolin (TTP; Jonas & Izaurralde, 2015). Thus, elucidating the precise mechanism by which DDX6 represses translation is expected to provide valuable

insight into a widespread post-transcriptional repressive mechanism operating in eukaryotic cells.

Materials and Methods

DNA constructs

Plasmids for the expression of λ N-HA-, GFP-, and MS2-tagged proteins in *Dm* S2 and human cells, as well as miRNA reporters and luciferase reporters for tethering assays, have been previously described (Rehwinkel *et al*, 2005; Eulalio *et al*, 2007, 2008; Braun *et al*, 2011; Zekri *et al*, 2013; Chen *et al*, 2014). We generated *Hs* AGO2 and *Dm* AGO1 mutants via site-directed mutagenesis. Protein mutants used in this study are listed in Appendix Table S1. The reporters lacking the 5'-UTR or containing the TISU motif, the (CAA)₁₈ repeats, and the 12-nucleotide guanine quartet (CGG)₄ were generated by site-directed mutagenesis using the corresponding parental plasmids as template. The sequences of the corresponding 5'-UTRs are listed in Appendix Table S3. Additional constructs used in this study are described in the Appendix Table S2.

Cell culture and transfections

S2 cells were transfected in 6-well plates using Effectene transfection reagent (Qiagen). Human cells were transfected using Lipofectamine 2000 or TurboFect reagents (Life Technologies). In all experiments, firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega), 3 days after transfection of the S2 cells and 48 h after transfection of the human cells. Total RNA was isolated from the S2 and human cells using TriFast (Peqlab Biotechnologies) and analyzed as described previously (Behm-Ansmant *et al*, 2006). All Western blots were developed using the ECL Western blotting detection system (GE Healthcare). Antibodies used in this study are listed in Appendix Table S4. Silvestrol (MedChem Express, HY-13251) was resuspended in DMSO at 1 mg/ml concentration and added to the cells at a final concentration of 0.25 μ M. The treatment was for 16 h.

Tethering, complementation, and coimmunoprecipitation assays

The interaction of AGO (wild type or mutants) with endogenous miRNAs and GW182/TNRC6 was tested as described previously (Rehwinkel *et al*, 2005; Eulalio *et al*, 2008). Tethering assays in S2 and HEK293T cells were performed as described before (Braun *et al*, 2011). Detailed protocols can be found in the Appendix Supplementary Methods. AGO1 complementation assays in S2 cells were performed as described previously (Huntzinger *et al*, 2013).

Polysome profiling in HEK293T cells

HEK293T cells (9×10^6 /15-cm dish) were transfected with Lipofectamine 2000. The transfection mixtures contained 20 μ g of the R-Luc-6xMS2-(A)₉₅-MALAT1 reporter, 4 μ g of the pEGFP-N3-F-Luc transfection control, and 6 μ g of the plasmids expressing MS2-HA

or MS2-HA-DDX6. Cells were treated with cycloheximide 48 h after transfection at a final concentration of 50 µg/ml for 30 min. Cell lysis and sucrose gradients were performed as described in the Appendix Supplementary Methods.

RNA immunoprecipitation in HEK293T cells

To study the association of eIF4A2 with mRNA reporters in HEK293T cells, cells (4×10^6 /10-cm dish) were transfected with Lipofectamine 2000. The transfection mixtures contained 5 µg of the pEGFP-N3-F-Luc transfection control reporter and any one of the following reporters: pCIneo-R-Luc-6xMS2-A₉₅-MALAT1 (10 µg), pCIneo-TISU-R-Luc-6xMS2-MALAT1 (8 µg), pCIneo-CAA-R-Luc-6xMS2-A₉₅-MALAT1 (15 µg), or pCIneo-CGG-R-Luc-6xMS2-A₉₅-MALAT1 (8 µg). A detailed description of the precipitation procedure is found in the Appendix Supplementary Methods.

Expanded View for this article is available online.

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Author contributions

DKO developed the reporters used in this study and coordinated the experimental work. DKO, DB, EH, MF, and SH performed the experiments. DKO and DB analyzed and interpreted the data. EI designed and supervised the project. EI wrote the manuscript with the contribution from DKO and DB.

Conflict of interest

The authors declare that they have no conflict of interest.

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