The *Caenorhabditis elegans* GW182 protein AIN-1 interacts with PAB-1 and subunits of the PAN2-PAN3 and CCR4-NOT deadenylase complexes

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ABSTRACT

GW182 family proteins are essential for miRNA-mediated gene silencing in animal cells. They are recruited to miRNA targets via interactions with Argonaute proteins and then promote translational repression and degradation of the miRNA targets. The human and *Drosophila melanogaster* GW182 proteins share a similar domain organization and interact with PABPC1 as well as with subunits of the PAN2-PAN3 and CCR4-NOT deadenylase complexes. The homologous proteins in *Caenorhabditis elegans*, AIN-1 and AIN-2, lack most of the domains present in the vertebrate and insect proteins, raising the question as to how AIN-1 and AIN-2 contribute to silencing. Here, we show that both AIN-1 and AIN-2 interact with Argonaute proteins through GW repeats in the middle region of the AIN proteins. However, only AIN-1 interacts with *C. elegans* and *D. melanogaster* PABPC1, PAN3, NOT1 and NOT2, suggesting that AIN-1 and AIN-2 are functionally distinct. Our findings reveal a surprising evolutionary plasticity of the GW182 protein interaction network and demonstrate that binding to PABPC1 and deadenylase complexes has been maintained throughout evolution, highlighting the significance of these interactions for silencing.

INTRODUCTION

The proteins AIN-1 and AIN-2 have important and partially redundant functions in the miRNA pathway in *Caenorhabditis elegans* (*Ce*) (1–4). They are highly divergent members of the GW182 protein family, which plays an essential role in miRNA-mediated gene silencing in animals (5). In vertebrates and several invertebrate species, there are up to three GW182 paralogs with partially redundant functions, whereas there is only one orthologous protein in *Drosophila melanogaster* (*Dm* GW182).

GW182 family proteins are characterized by an N-terminal region (N-term) containing multiple glycine–tryptophan repeats (GW repeats), a central ubiquitin-associated (UBA) domain and a C-terminal RNA recognition motif (RRM) (Figure 1A) (2,6–9). Additional regions of the protein include a glutamine-rich (Q-rich) region, which is located between the UBA domain and the RRM, and a middle (Mid) and C-terminal (C-term) regions containing few or none GW repeats (Figure 1A) (6–9).

The N-term GW repeat region of the proteins mediates binding to the Argonaute (AGO) proteins and thus is essential for miRNA-mediated gene silencing (7,10–15). The Mid and C-term regions (collectively termed the silencing domain, SD) are also required for silencing (12,13,16,17). Indeed, GW182 protein mutants lacking the silencing domain fail to rescue silencing of several miRNA reporters in cells lacking endogenous GW182, even though these proteins interact with AGO proteins and are active in tethering assays (12,17–21).

The precise mechanism by which GW182 proteins mediate silencing is not completely understood. However, important insight was provided by the observation that these proteins interact with the cytoplasmic poly(A)-binding protein (PABPC1) and with subunits of the two major cytoplasmic deadenylase complexes (the PAN2-PAN3 and CCR4-NOT complexes) both in *D. melanogaster* and human cells (17,19–25).

Binding to PABPC1 is mediated by a conserved PAM2 motif (PABP-binding motif 2) located in the Mid region. This motif directly binds to the C-term MLLE domain of PABPC1 (17,22–24). Moreover, the protein sequences downstream of the PAM2 motif (termed M2) together with the C-term region mediate indirect binding to PABPC1 *in vivo* (17).

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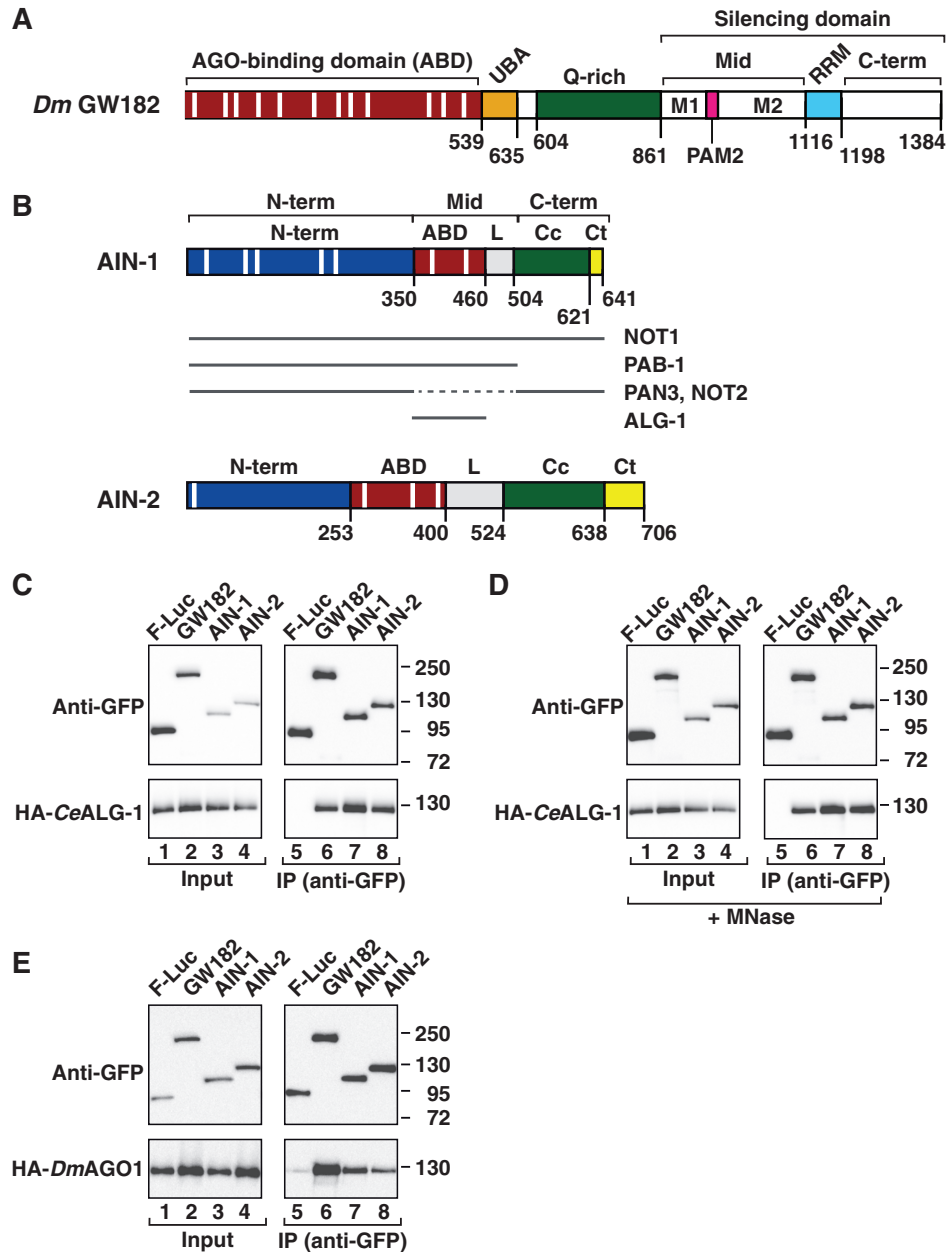
Figure 1. Interaction of AIN-1 and AIN-2 proteins with ALG-1. (**A**) Domain organization of *Dm* GW182. The N-term ABD is shown in red, with GW repeats shown as white vertical bars; Mid, Middle domain with PAM2 motif, M1 and M2 regions; The silencing domain includes the Mid and C-term regions but not the RRM, which is dispensable for silencing (38). (**B**) Domain organization of AIN-1 and AIN-2. Regions colored in red and green show limited similarity to the corresponding regions in *Dm* GW182. Cc, region with helical propensity. Gray lines underneath AIN-1 schematic represent protein fragments required for the interaction with the indicated partners. (**C–E**) S2 cells were cotransfected with plasmids expressing GFP-tagged *Dm* GW182, AIN-1 or AIN-2, and HA-tagged *Ce* ALG-1 or *Dm* AGO1. Cell lysates were immunoprecipitated using a polyclonal anti-GFP antibody. GFP-tagged firefly luciferase served as a negative control. For the HA-tagged proteins, inputs (1%) and immunoprecipitates (35%) were analyzed. For the GFP-tagged proteins, 3.5% of the inputs and 8% of the IPs were loaded. In panel D, cell lysates were treated with Micrococcal nuclease (MNase).

In addition to PABPC1, the silencing domains of human GW182 proteins (known as TNRC6A, B and C) confer direct binding to PAN3 and NOT1, which are subunits of the PAN2-PAN3 and CCR4-NOT deadenylase complexes, respectively (20,21,25). Binding to PAN3 is mediated by the M2- and C-term regions of the silencing domain (20), whereas NOT1 binding requires W-containing motifs in the M1, M2 and C-term regions (Figure 1A) (20,21,25). The interactions with the deadenylase complex subunits are conserved in *Dm* (20,21).

Remarkably, the *Ce* proteins AIN-1 and AIN-2 show <12% global sequence identity to *Dm* GW182 and human TNRC6s and do not contain sequences homologous to the

silencing domain (Figure 1B) (2,8,9). They also lack UBA and RRM domains, a PAM2 motif, as well as a defined Q-rich region. One common feature between AIN-1, AIN-2 and other GW182 proteins is the presence of GW repeats, which are dispersed within the N-term and Mid regions of AIN-1 and AIN-2. Accordingly, AIN-1 and AIN-2 coimmunoprecipitate *Ce* AGO-like proteins 1 and 2 (ALG-1 and ALG-2) (1,3). However, it is not known which GW repeats in AIN-1 and AIN-2 contribute to ALG-1 and ALG-2 binding.

The highly divergent sequences of AIN-1 and AIN-2 raise the question regarding how these proteins contribute to silencing and whether there are differences in the mechanisms of silencing between species. One possibility is that AIN-1 and AIN-2 perform similar functions as their vertebrate and insect homologs but have evolved different modes of interaction with PABPC1 and deadenylase complexes. Alternatively, AIN-1 and AIN-2 may serve as adaptor proteins between ALG-1,2 and other protein(s), which in turn mediate binding to PABPC1 and deadenylase complexes. Finally, miRNA-mediated gene silencing in *Ce* may occur by a different mechanism that does not require interactions with PABPC1 or deadenylase complexes, although miRNA targets have also been reported to undergo deadenylation in *Ce* (26).

In this study, we identified the GW repeats that are required for AIN-1 and AIN-2 to interact with ALG-1. Despite the low sequence conservation with *Drosophila* and human GW182 proteins, we found that AIN-1 interacts with *Ce* PABPC1 (PAB-1), PAN3, NOT1 and NOT2, whereas AIN-2 showed no significant interaction. Thus, despite strong sequence divergence, the interaction of AIN-1 with PABPC1 and deadenylase complexes has been conserved throughout evolution, underscoring their importance for miRNA-mediated gene silencing.

MATERIALS AND METHODS

Sequence analysis

Ce AIN-1 (WBGene00015547) and AIN-2 (WBGene000 15007) sequences were analyzed using sensitive methods such as PSI-BLAST and HHpred (27,28). The sequence identity of the paralogous sequences of AIN-1 and AIN-2 is ∼12%, but the Mid and C-term regions show some common features, as depicted in Figure 1A and B. Sequence homology to the AGO-binding region of *Dm* GW182 was detected using extensive PSI-BLAST searches (red region). Sequence homology for another region (green) to a region in *Dm* GW182 (amino acids 721–795) was also detected using HHpred. This region shows helical propensity and is annotated as Q-rich in *Dm* GW182.

Plasmids

Luciferase reporters and plasmids for expression of miRNAs, *Dm* AGO1, *Dm* GW182, *Dm* PABPC1 and subunits of the *Drosophila* deadenylase complexes were described before (7,19,20,29). The AGO1 F2V2 mutant carries valine substitutions of phenylalanines 594 (F594V) and 629 (F629V) (11). For expression of *Ce*

proteins, the corresponding cDNAs were cloned into the pAc5.1A–λN-HA or pAc5.1–EGFP vectors as described in Supplementary Table S1. Mutations in AIN-1 and AIN-2 were generated by site-directed mutagenesis using the QuickChange mutagenesis kit from Stratagene.

Coimmunoprecipitations and western blots

Coimmunoprecipitations were performed as previously described (17). Briefly, S2 cells were transfected in six-well plates using the Effectene Transfection Reagent (Qiagen). The transfection mixtures contained 1, 0.2 and 1 μg of plasmid expressing GFP-tagged *Dm* GW182, AIN-1 and AIN-2, respectively, or the corresponding mutants, and 0.5 μg of HA-tagged proteins (*Ce* PABP, *Ce* ALG-1, *Dm* AGO1 or deadenylation factors). *Dm* PABPC1 was expressed with a C-term V5 tag. Cells $(10–12 \times 10^6$ cells) were harvested 3 days after transfection, washed with PBS and lysed on ice in 0.5 ml of NET buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA and 0.1% Triton X-100] containing a protease inhibitor cocktail for 30 min. Cell lysates were supplemented with 2.5 mM $CaCl_2$ and treated with micrococcal nuclease for 30 min. After removal of insoluble proteins, cell lysates were incubated with homemade rabbit polyclonal anti-GFP antibodies (2 μl) for 1 h. Subsequently, Protein A-Sepharose beads (GE HealthCare, 50 μl) were added and samples were gently rotated for 1 h at 4°C. Beads were washed 3 times with NET buffer and once with NET buffer without Triton X-100. Proteins were eluted with SDS–PAGE sample buffer and analyzed by western blotting using conventional procedures. Small aliquots from cell lysates prior and after Micrococcal nuclease treatment were analyzed by native agarose gel electrophoresis to confirm that rRNA was efficiently degraded.

HA- and GFP-tagged proteins were detected using HRP-conjugated monoclonal anti-HA (Roche 3F10; 1:5000) and anti-GFP antibodies (Roche 11 814 460 001; 1:2000), respectively. V5-tagged proteins were detected with anti-V5 antibodies (Invitrogen, 1:5000). All western blots were developed with the ECL western blotting detection system (GE Healthcare) as recommended by the manufacturer.

Complementation and tethering assays in S2 cells

S2 cells were transfected in 24- or 6-well plates using Effectene transfection reagent (Qiagen). For the tethering assay, the following plasmids were cotransfected (amounts are given for six-well plates): 0.1 μg reporter plasmid (F-Luc-5BoxB or F-Luc without 5BoxB), 0.4 μg pAc5.1–R–Luc as transfection control and various amounts of plasmids expressing λN-HA-protein fusions (adjusted to obtain equal expression levels for all proteins tested). Firefly and *Renilla* luciferase activities were measured 3 days after transfection using the Dual-Luciferase Reporter Assay System (Promega).

AGO1 complementation assays were performed as described before (11). The following siRNAs were used: AGO1 5′-CGAAGGAGAUCAAGGGUUUUU-3′ and β-Gal 5′-CUACACAAAUCAGCGAUUUUU-3′; Dharmacon). GW182 complementation assays were performed

as previously described (12). For miRNA-mediated silencing assays, the transfection mixtures contained (amounts given for 24-well plates): 20 ng of firefly luciferase reporter plasmid, 80 ng of the *Renilla* transfection control and 40 ng of plasmids expressing miRNA primary transcripts or the corresponding vector without insert. When indicated, 10–100 ng of plasmid expressing recombinant protein was cotransfected.

Firefly and *Renilla* luciferase activities were measured 4 (AGO1 complementation) or 3 (GW182 complementation) days after transfection using the Dual-Luciferase Reporter Assay System (Promega). Total RNA was isolated using TriFast (Peqlab Biotechnologies) and analyzed as previously described (12).

RESULTS

AIN-1 and AIN-2 interact with ALG-1 through GW repeats in the Mid region

Sequence comparison and secondary structure predictions indicated that AIN-1 and AIN-2 consist of five different regions (Figure 1B): an N-term low-complexity region containing 5 and 1 GW repeats, respectively; a region of homology to the AGO-binding domain (ABD) of human TNRC6s and *Dm* GW182, containing additional 2 and 3 GW repeats, respectively; a linker region (L) rich in serine and glycine residues; a region with limited similarity to the Q-rich region of *Dm* GW182 (Cc), which exhibits helical propensity; and a C-terminal tail (Ct). Overall, the sequence identity between AIN-1 and AIN-2 and orthologous vertebrate and insect proteins is <12%, whereas AIN-1 and AIN-2 exhibit 22.4% identity (Supplementary Table S2).

To analyze the interaction of AIN-1 and AIN-2 with potential-binding partners, we used a heterologous system based on *Dm* S2 cells. We validated this approach by showing that AIN-1 and AIN-2 interacted with ALG-1 in these cells. Indeed, GFP-tagged versions of AIN-1 and AIN-2 coimmunoprecipitated HA-tagged ALG-1 in lysates from S2 cells coexpressing these proteins (Figure 1C and D, lanes 7 and 8). Interestingly, *Dm* GW182 also bound to ALG-1 (Figure 1C and D, lane 6), indicating that the mode of interaction is conserved. Conversely, both AIN-1 and AIN-2 coimmunoprecipitated *Dm* AGO1 (Figure 1E), consistent with the observation that ALG-1 and AGO1 exhibit 61% identity (Supplementary Table S3). The interactions with ALG-1 were observed in cell lysates treated with Micrococcal nuclease, suggesting that they are not mediated by RNA (Figure 1D, MNase).

To define the regions in AIN-1 required for ALG-1 binding, we generated mutants lacking either the N-term, Mid or C-term regions (Figure 1B) and tested them in coimmunoprecipitation assays. We observed that deletion of the AIN-1 Mid region abolished ALG-1 binding, whereas deletion of the N-term or C-term regions had no effect (Figure 2A). Furthermore, protein fragments containing only the AIN-1 Mid region or the Mid region without the linker (hereafter termed the AGO-binding domain, ABD) were sufficient for binding to ALG-1

(Figure 2A, lane 11; Figure 2B, lanes 7 and 8). These results indicate that the N-term GW repeats do not significantly contribute to the AIN-1/ALG-1 interaction.

The ABD of AIN-1 contains two GW repeats; to examine their contribution to ALG-1 binding, we substituted the tryptophan residues in each repeat with alanines. These substitutions slightly reduced AIN-1 binding to ALG-1 (Figure 2C, lanes 10 and 11). A stronger reduction was observed when the substitutions were combined (Figure 2C, lane 12). However, the AIN-1 double mutant retained some residual ALG-1 binding relative to the AIN-1 mutant lacking the entire Mid region (Figure 2C, lane 12 versus 9), indicating that additional residues in the Mid region contribute to the interaction.

A similar analysis of the interaction of AIN-2 with ALG-1 revealed that the corresponding ABD is essential for this interaction (Figure 2D, lane 13). The AIN-2 ABD contains 3 GW repeats. We observed that alanine substitution of the tryptophan residue in the first repeat (GW1-A) reduced binding to ALG-1, whereas substitutions in repeats 2 and 3 had no effect (Figure 2D, lanes 14–16). ALG1 binding was abolished when repeats 1 and 2 were substituted simultaneously (Figure 2D, lane 17), whereas substitutions in repeats 1 and 3 or 2 and 3 reduced but did not abolish binding (Figure 2D, lanes 18 and 19). We conclude that the GW repeats in the ABD are essential for AIN-2 to interact with ALG-1. These repeats contribute to ALG-1 binding in an additive manner as previously observed for the human and *Dm* GW182 proteins (10,12,13,15,30). These results also indicate that a GW repeat present in the AIN-2 N-term region does not detectably contribute to the interaction with ALG-1.

AIN-1 interacts with the RRM domains of PAB-1

We next tested whether AIN-1 and AIN-2 interact with *Ce* PAB-1. Remarkably, only AIN-1 coimmunoprecipitated HA-tagged *Ce* PAB-1 (Figure 3A and B, lane 7). In contrast, AIN-2 did not detectably interact with PAB-1, although it was expressed at similar levels (Figure 3A and B, lane 8). Interestingly, although *Ce* and *Dm* PABPC1 exhibit 51.5% identity (Supplementary Table S3), *Dm* GW182 did not interact with *Ce* PAB-1 (Figure 3A and B, lane 6). In contrast, AIN-1 interacted with *Dm* PABPC1 (Figure 3C, lanes 11 and 15). The interaction of AIN-1 with *Ce* or *Dm* PABPC1 was observed in extracts treated with Micrococcal nuclease, suggesting that this association is not mediated by RNA (Figure 3B and C).

PABPC1 contains four N-term RRM1–4, a proline-rich unstructured linker, and a C-term domain [termed PABC or MLLE because of a conserved KITGMLLE signature motif in this domain; Figure 3D; (24)]. Human TNRC6 and *Dm* GW182 interact directly with the C-term MLLE domain of PABPC1 through a conserved PAM2 motif located in the Mid region of the silencing domain [see Figure 1A; (17,22–24)]. However, we could not identify a PAM2 motif in AIN-1. To map the sequences in AIN-1 required for PAB-1 binding, we performed coimmunoprecipitations using the deletion mutants
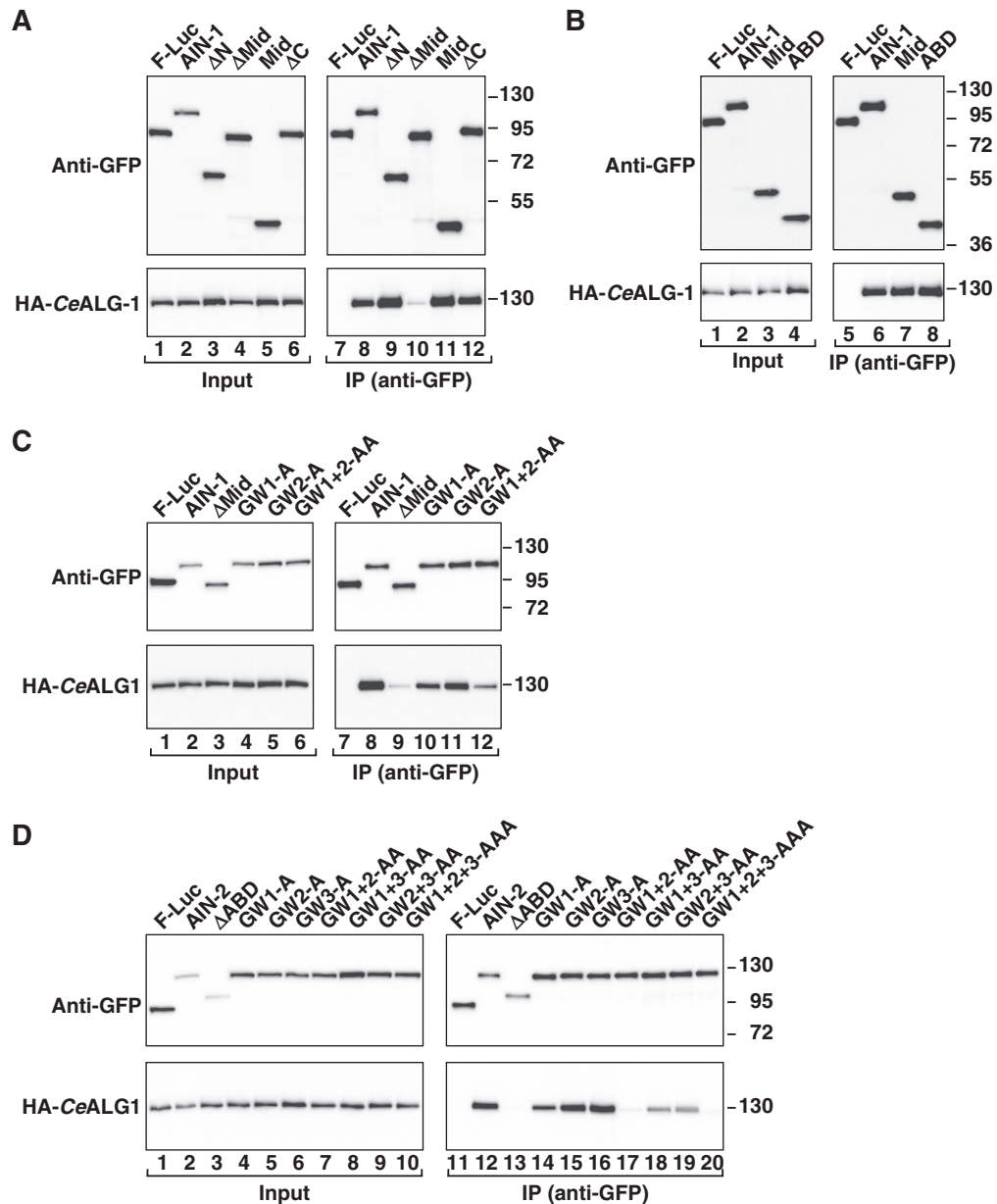
Figure 2. GW repeats in the ABD domains of AIN-1 and AIN-2 mediate binding to ALG-1. (**A–D**) S2 cells coexpressing HA-ALG-1 together with GFP-tagged AIN-1 or AIN-2 (wild-type or mutants) were lysed 3 days after transfection. Cell lysates were immunoprecipitated with a polyclonal anti-GFP antibody and analyzed as described in Figure 1. In panel (C), AIN-1 mutants GW1-A, GW2-A and GW1+2-AA carry alanine substitutions of tryptophan residues in the first, second or both GW repeats, respectively, of the ABD. The same nomenclature was used to describe AIN-2 mutants in panel (D).

described above. We observed that deletion of the N-term or Mid regions of AIN-1 reduced the interaction with PAB-1, whereas deletion of the C-term region was inconsequential (Figure 3E, lanes 9, 10 and 12). However, neither the N-term nor the Mid regions in isolation were sufficient for binding (Figure 3E and F). Thus, both the N-term and Mid regions contribute to PAB-1 binding (Figure 1B).

We next investigated whether AIN-1 interacts with the MLLE domain of PAB-1 despite the lack of a PAM2 motif. We observed that AIN-1 interacted preferentially with the RRM domains of PAB-1 but not with the MLLE

domain (Figure 3G). We conclude that the interaction of GW182 proteins with PABP is conserved, although the interaction mode may differ between species.

AIN-1 interacts with the PAN3 C-term kinase-like domain

We next studied whether AIN-1 and AIN-2 interact with *Ce* PAN3. As observed for PAB-1, only AIN-1 interacted with *Ce* PAN3 in an RNA-independent manner (Figure 4A and B, lane 7), whereas AIN-2 or GW182 did not bind (Figure 4A and B, lanes 6 and 8). In
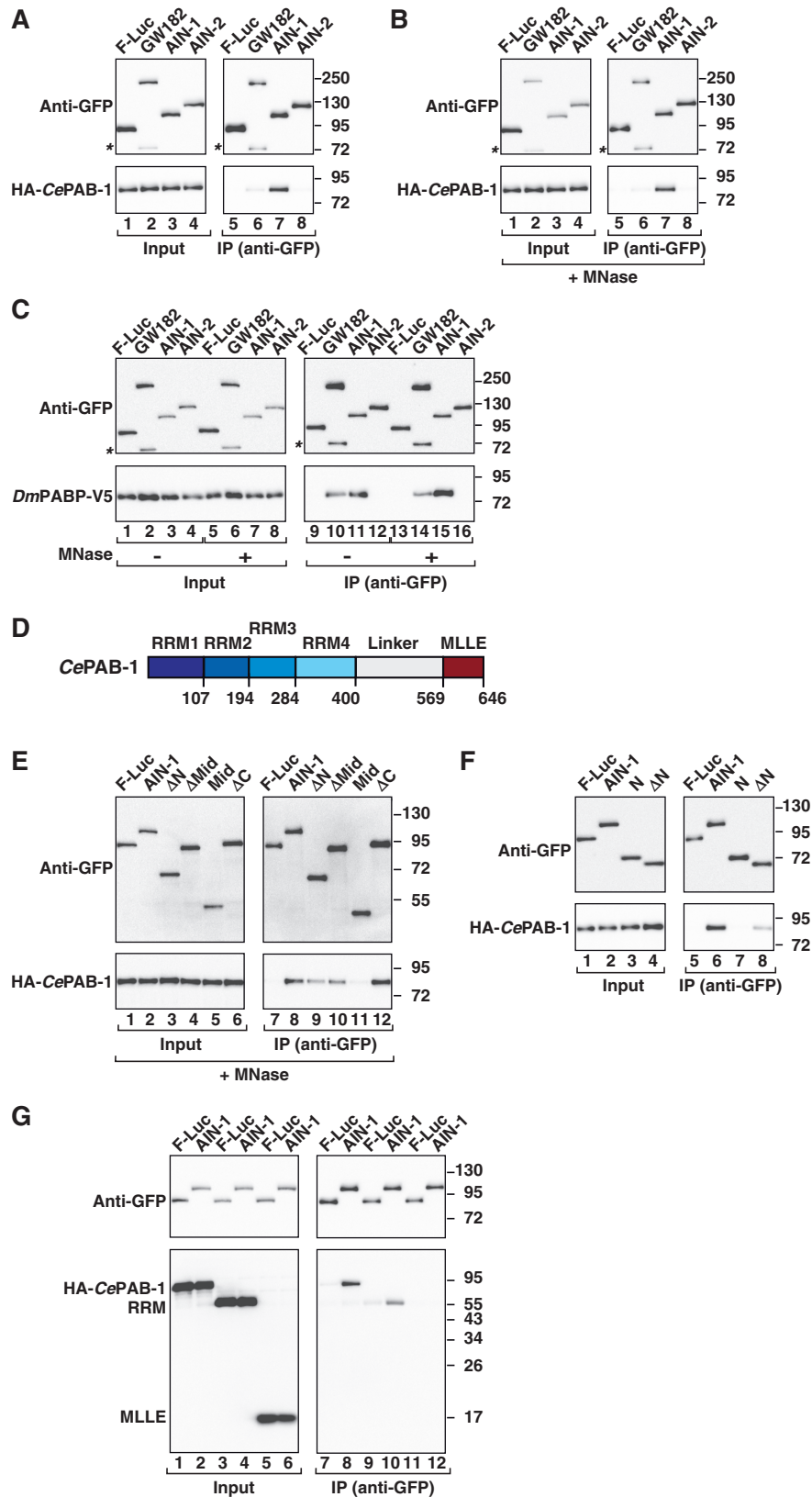
Figure 3. AIN-1 interacts with the RRM domains of PAB-1. (**A–C**) S2 cells were cotransfected with plasmids expressing GFP-tagged GW182, AIN-1 or AIN-2, and HA-tagged *Ce* PAB-1 or V5-tagged *Dm* PABP as indicated. Cell lysates were immunoprecipitated and analyzed as described in Figure 1. Asterisks indicate a *Dm* GW182 degradation product. (**D**) Domain organization of *Ce* PAB-1. PAB-1 consists of four N-term RRM1–4, a proline-rich unstructured linker, and a conserved C-term domain, termed MLLE. (**E–G**) The interaction of GFP-tagged AIN-1 (wild type or mutants) with full-length HA-tagged *Ce* PAB-1 or fragments was analyzed as described in Figure 1. In panels B and E, cell lysates were treated with Micrococcal nuclease.
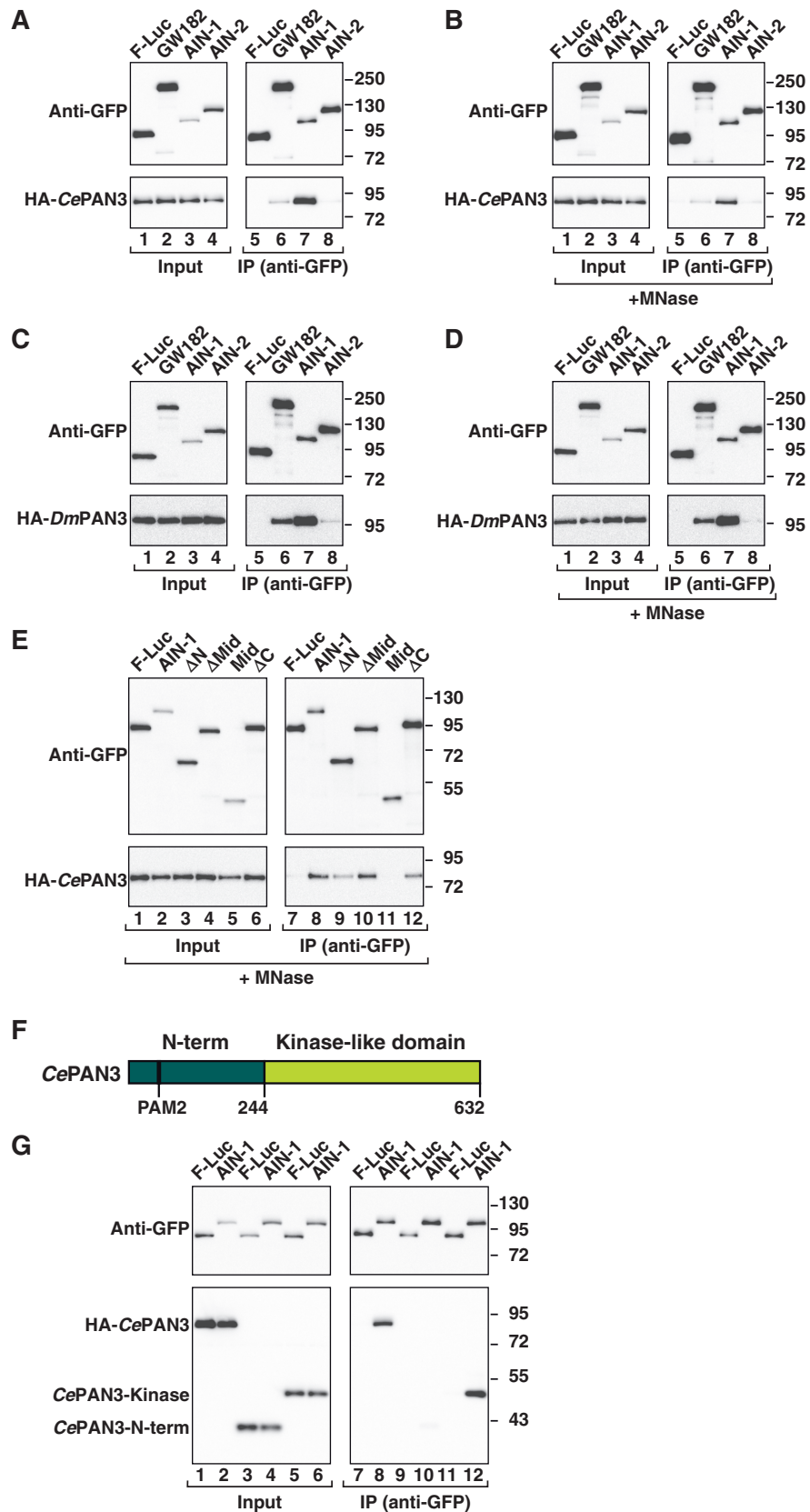
Figure 4. AIN-1 interacts with the PAN3 kinase-like domain. (**A–D**) The interaction of GFP-tagged GW182, AIN-1 or AIN-2 with HA-tagged *Ce* or *Dm* PAN3 was analyzed as described in Figure 1. (**E**) Interaction of GFP-tagged AIN-1 wild type or mutants, and HA-tagged *Ce* PAN3. (**F**) Domain organization of *Ce* PAN3. PAN3 consists of an N-term region containing a PAM2 motif, and a conserved C-term domain related to protein kinases. (**G**) Interaction of GFP-tagged AIN-1 with HA-tagged wild-type *Ce* PAN3 or deletion mutants.

contrast, AIN-1 coimmunoprecipitated *Dm* PAN3 more efficiently than *Dm* GW182 (Figure 4C and D).

Our previous studies showed that human TNRC6 proteins interact with PAN3 through the M2 and C-term regions of the silencing domains (20). We observed that deletion of either the N-term or C-term regions of AIN-1 reduced binding to PAN3 (Figure 4E and Supplementary Figure S1A). In contrast, a protein fragment containing both the N-term and C-term regions but lacking the Mid region (ΔMid) interacted with PAN3 to a similar extent as full-length AIN-1 (Figure 4E, lane 10 and Supplementary Figure S1A), indicating that the N-term and C-term regions both contribute to the interaction. Accordingly, the isolated N-term or C-term regions did not interact with PAN3 (Supplementary Figure S1A).

PAN3 proteins contain an N-term region and a C-term domain with homology to protein kinases (Figure 4F). The PAN3 N-term region contains a canonical PAM2 motif and interacts with PABPC1, whereas the C-term kinase-like domain is required for PAN3 binding to PAN2, the catalytic subunit of the PAN2–PAN3 deadenylase complex (31–33). Our previous studies showed a direct interaction between human TNRC6 proteins and the kinase-like domain of PAN3 (20). Likewise, AIN-1 interacted with the PAN3 kinase-like domain but not with the N-term region (Figure 4G). Thus, the interaction of GW182 proteins with the kinase-like domain of PAN3 is conserved. Since the PAN3 kinase-like domain does not interact with PABPC1, we conclude that AIN-1 interacts with PAN3 independently of PABPC1, as previously reported for human TNRC6s (20).

AIN-1 interacts with NOT1 and NOT2

We also examined the interaction of AIN-1 and AIN-2 with NOT1 and NOT2, which are the subunits of the CCR4-NOT complex that exhibit the strongest interaction with *Dm* GW182 (20). We observed that AIN-1 interacted with *Ce* NOT1 and *Ce* NOT2 (a.k.a. NTL-2) (Figure 5A–C, lane 7), as well as with *Dm* NOT1 and *Dm* NOT2 (Figure 5D and E), whereas AIN-2 showed very weak binding to *Ce* NOT1 (Figure 5B, lane 8). Immunoprecipitations with AIN-1 deletion mutants, revealed that full-length AIN-1 is required for binding to NOT1, as deletion of either the N-term, Mid or C-term regions reduced binding and none of these regions in isolation was sufficient for binding (Figure 5F). In contrast, the AIN-1 interaction with NOT2 is mediated by the N-term and C-term regions, as an AIN-1 mutant lacking the Mid domain coimmunoprecipitated NOT2 to a similar extent as the full-length protein (Figure 5G, lane 14 versus 10; see Figure 1B).

Recent work showed that the interaction of human TNRC6s with NOT1 is mediated by W-containing motifs in the M1, M2 and C-term regions of the silencing domains (21,25). These motifs contribute to the affinity of the interaction in an additive manner. AIN-1 contains a total of 22 tryptophan residues (of which 7 are in GW repeats) in the N-term and Mid regions, whereas the C-term region lacks tryptophan residues. To investigate the potential contribution of W-containing motifs to

deadenylase binding, we generated an AIN-1 mutant in which all tryptophan residues were substituted with alanines (AIN-1-22×W-A). This mutant displayed lower mobility in SDS–PAGE and no longer interacted with NOT1 and NOT2 (Supplementary Figure S1B and S1C). As expected, the substitutions abrogated AGO1 and ALG-1 binding (Supplementary Figure S1D and S1E). Unexpectedly, interaction with PAB-1 and PAN3 was also abolished (Supplementary Figure S1F and S1G), suggesting that simultaneous substitution of 22 tryptophan residues by alanines non-specifically disrupted protein–protein interactions. Nevertheless, our data do not rule out that a subset of tryptophan residues is involved in mediating NOT1 binding, as shown recently (21, 25).

## ALG-1 rescues silencing in cells depleted of AGO1

Given that the interactions of GW182 proteins with AGOs are conserved and that *Dm* GW182 interacts with *Ce* ALG-1, we tested if ALG-1 could rescue silencing in S2 cells depleted of AGO1, which is the AGO protein dedicated to the miRNA pathway in *D. melanogaster* (34). To this end, we made use of a complementation assay as previously described (11). In this assay, S2 cells were transfected with either siRNA specific to AGO1 or a control siRNA against β-Gal. We then tested ALG-1 for its ability to restore silencing in AGO1-depleted cells.

We monitored miRNA activity using previously characterized firefly luciferase reporters, including the F-Luc-Par-6 reporter (silenced by miR-1) and the F-Luc-Nerfin-1 reporter [silenced by miR-9b; (7,29)]. Depletion of endogenous AGO1 suppressed silencing of the reporters, leading to a 6- to 7-fold increase in firefly luciferase expression (Figure 6A–D). Transfecting AGO1-depleted cells with a plasmid expressing a siRNA-resistant form of AGO1 fully restored silencing (Figure 6A–D) as reported previously (11). Interestingly, ALG-1 also restored silencing in AGO1-depleted cells (Figure 6A–D) when expressed at similar levels (Figure 6E). In contrast, neither ALG-1 nor AGO1 rescued silencing in cells in which AGO1 and *Dm* GW182 were codepleted (Figure 6F and G), indicating that both AGO1 and ALG-1 interact with *Dm* GW182 to silence miRNA targets. Accordingly, coexpression of AGO1 or ALG-1 with a dsRNA-resistant form of *Dm* GW182 rescued silencing in cells codepleted of AGO1 and GW182 (Figure 6F and G). These results indicate conservation in the mechanisms of miRNA loading and target silencing.

AIN-1 and AIN-2 repress expression of bound mRNAs

To investigate the conservation of the pathway downstream of AGOs, we tested whether AIN-1 and AIN-2 could repress expression of target mRNAs using a tethering assay as previously described (35). AIN-1 and AIN-2 were expressed with two tags: a peptide derived from the bacteriophage λ N protein (λN tag) to enable tethering to a firefly luciferase (F-Luc) reporter and an HA tag (hemagglutinin) to allow detection of the expressed protein by western blot. The F-Luc reporter contains five Box B hairpins (5BoxB) inserted in the 3'-UTR; these bind the λN tag with high affinity and
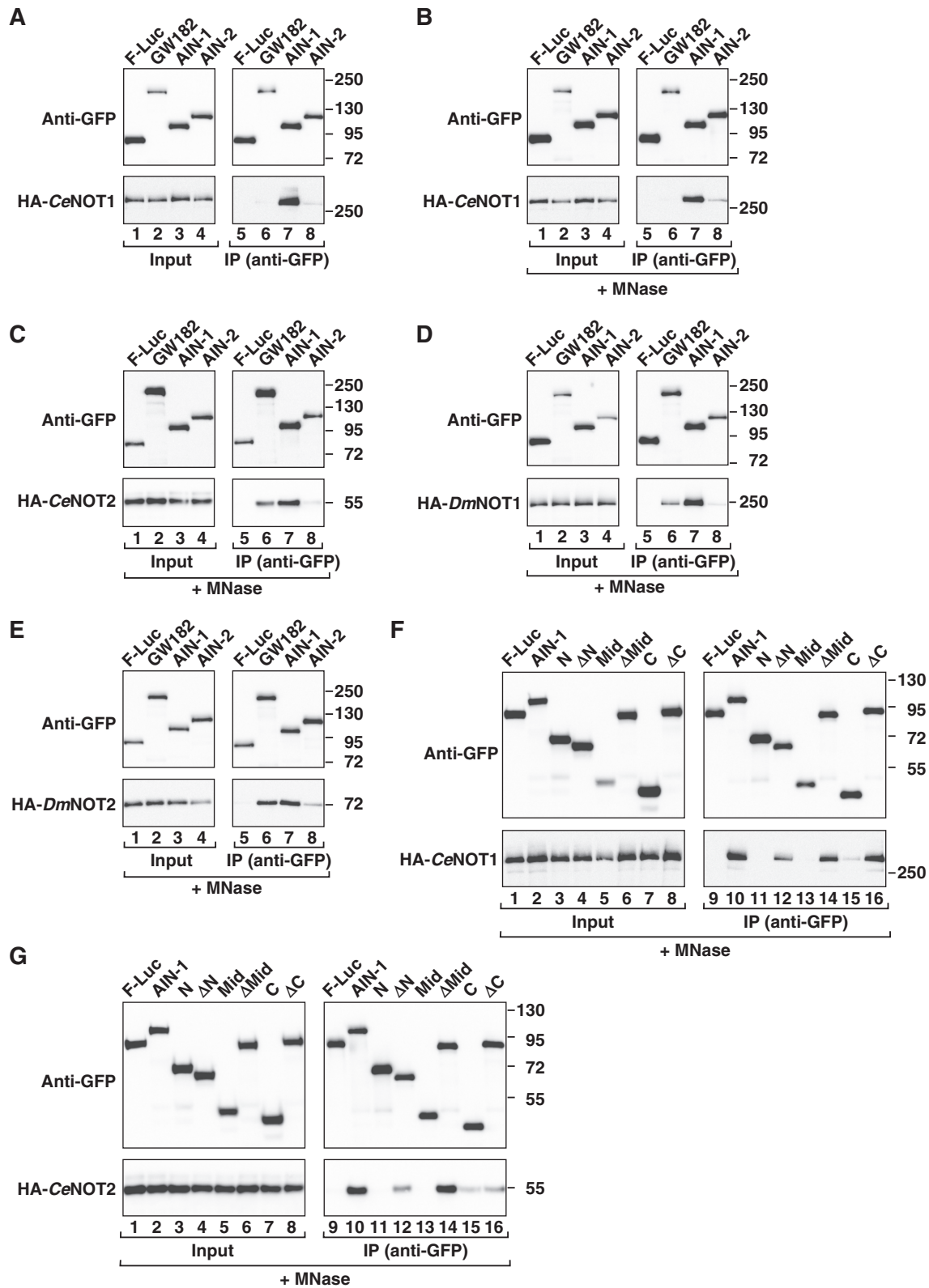
Figure 5. AIN-1 interacts with NOT1 and NOT2. (**A–E**) Interaction of GFP-tagged GW182, AIN-1 or AIN-2 with HA-tagged NOT1 or NOT2. For *Ce* NOT1 and *Ce* NOT2, 1% of the input and 30% of the IPs were loaded. (**F** and **G**) Interaction of GFP-tagged AIN-1 (wild-type or mutants) with HA-tagged *Ce* NOT1 or *Ce* NOT2. Cell lysates were treated with Micrococcal nuclease as indicated.
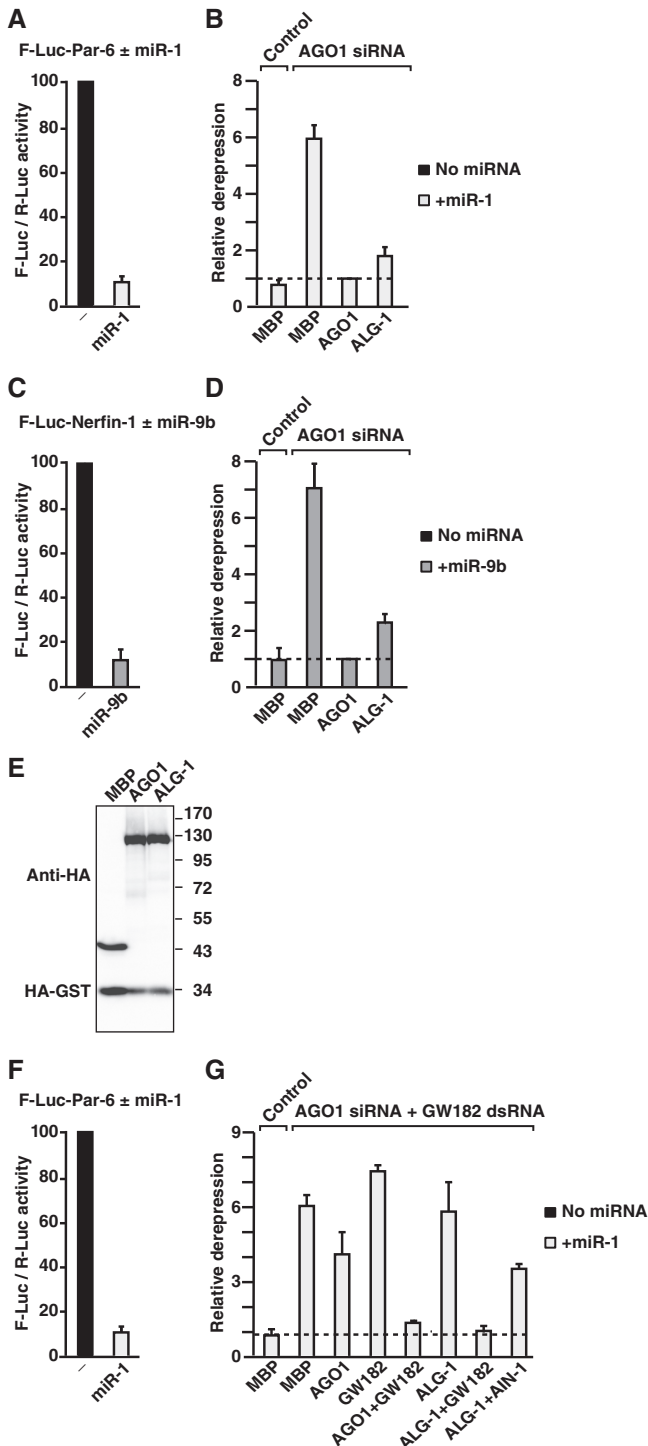
Figure 6. ALG-1 rescues silencing in S2 cells depleted of AGO1. (**A–D**) S2 cells were transfected with a siRNA targeting *AGO1* mRNA. Control cells were treated with an siRNA targeting *β-Gal*. The siRNAs were cotransfected with a mixture of three plasmids: one expressing the indicated F-Luc reporters; another expressing miRNA primary transcripts or the corresponding empty vector (−); and a third expressing *Renilla* luciferase (R-Luc). Plasmids expressing HA-AGO1 (siRNA resistant), HA-ALG-1 or HA-MBP (Maltose-binding protein) were included in the transfection mixtures as indicated. Firefly luciferase activities were normalized to those of the *Renilla* luciferase transfection control and set to 100 in cells transfected with the empty vector (i.e. in the absence of miRNA) for each condition. Panels (A and C) show normalized firefly luciferase activities in the absence or

thus recruit AIN-1 or AIN-2 to the F-Luc-5BoxB mRNA. A plasmid encoding *Renilla* luciferase served as a transfection control (R-Luc).

We observed that both AIN-1 and AIN-2 repressed expression of the F-Luc-5BoxB reporter relative to the λN-HA tag or a negative mutant of *Dm* AGO1 termed F2V2 [Figure 7A–C; (11)]. The repression was similar to that observed with *Dm* GW182, which served as the positive control (Figure 7A–C). Furthermore, the repression was specific; the expression of an F-Luc reporter lacking the BoxB hairpins was unaffected by *Dm* GW182, AIN-1 or AIN-2 expression (Supplementary Figure S2A–C). Importantly, AIN-1 and AIN-2 silenced the F-Luc-5BoxB reporter in AGO1-depleted cells, indicating that their activity in tethering assays is not mediated by AGO1 (Supplementary Figure S2D and E). These results are not due to inefficient depletion because silencing of the F-Luc-Par-6 reporter by miR-1 was completely suppressed in these cells (Supplementary Figure S2E). To confirm that AIN-1 and AIN-2 activities are independent of AGO proteins, we tested protein mutants that do not interact with ALG1 (i.e. carrying deletions of the Mid domain or mutations in the GW repeats of the ABD). All mutants repressed the F-Luc-5BoxB reporter similar to the wild-type protein (Figure 7D and Supplementary Figure S2F), but did not affect the expression of a reporter lacking the BoxB hairpins (Supplementary Figure S2G–S2I). In contrast, the AIN-1 mutant with 22 tryptophan-to-alanine substitutions was inactive in this assay (Figure 7D, 22 × W-A).

Northern blot analysis of the F-Luc-5BoxB mRNA revealed that both AIN-1 and AIN-2 reduced the abundance of the F-Luc-5BoxB mRNA by ∼2.5-fold relative to the R-Luc mRNA transfection control (Figure 7A and B). Remarkably, all of the tested AIN-1 or AIN-2 protein fragments that were active in the tethering assays promoted mRNA degradation (Figure 7D and E). However, the decrease in mRNA levels was smaller than the reduction observed in firefly luciferase activity, suggesting a net contribution of translational repression. Similarly, previous studies showed that almost all fragments of *Dm* GW182 promoted translational repression and mRNA degradation in tethering assays, including N-term fragments that do not interact with PABPC1 (18,21,36). Therefore, we conclude that the activity of the GW182 protein fragments in tethering assays is not correlated with AGO, deadenylase or PABPC1 binding in coimmunoprecipitation assays. However, we cannot rule out that transient and weak interactions occur *in vivo* that are not detectable under the conditions used for coimmunoprecipitations.

presence of miRNAs in control cells (i.e. cells treated with β-Gal siRNA and expressing MBP). (B and D) show the relative fold derepression for each condition. Mean values ± standard deviations from three independent experiments are shown. (**E**) Western blot analysis showing equivalent expression of the HA-tagged proteins. (**F** and **G**) S2 cells were codepleted of AGO1 and GW182, transfected with the indicated plasmids and analyzed as described in panels (A–D).
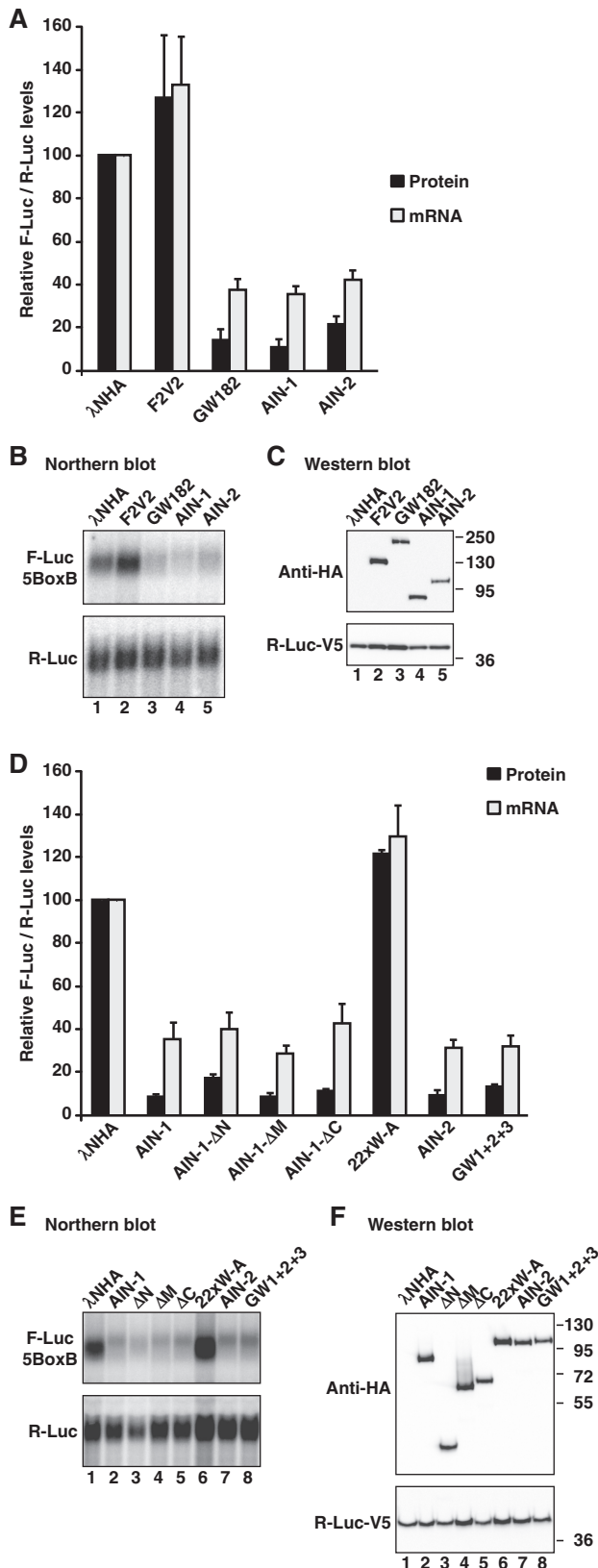
Figure 7. AIN-1 and AIN-2 promote degradation of bound mRNAs. (**A–F**) S2 cells were transfected with a mixture of three plasmids: one expressing the F–Luc-5BoxB reporter; another expressing the λN-HA peptide or the indicated λN-HA-tagged proteins; and a third expressing *Renilla* luciferase (R–Luc). Firefly luciferase activity and mRNA levels

AIN-1 and AIN-2 do not rescue silencing in cells depleted of GW182

As mentioned above, previous reports documented that multiple and non-overlapping fragments of *Dm* GW182 are active in tethering assays including N-term fragments (18,21,36). However, despite their activity in tethering assays, GW182 N-term protein fragments did not rescue silencing in cells lacking endogenous GW182 in complementation assays (except for the Nerfin reporter silenced by miR-9b), but rather inhibited silencing in a dominant-negative manner (12,18). Therefore, we next tested the silencing activity of AIN-1 and AIN-2 in complementation assays.

Knockdown of endogenous *Dm* GW182 in S2 cells was achieved using dsRNA targeting *GW182* mRNA. This depletion inhibited silencing of the reporters tested, leading to a 2.5- to 3-fold increase in firefly luciferase expression (Figure 8A–D). GW182-depleted cells were then transfected with a plasmid expressing a dsRNA-resistant version of GW182, which restored silencing (Figure 8A–D) as previously reported (12). Despite similar expression levels, neither AIN-1 nor AIN-2 restored silencing in GW182-depleted cells (Figure 8A–E and Supplementary Figure S3A–S3D). Moreover, coexpression of AIN-1 and AIN-2 also failed to rescue silencing (Figure 8A–D). Likewise, coexpression of AIN-1 with *Ce* ALG-1, PAN3, NOT1 or PAB-1 did not rescue silencing (Supplementary Figure S3A–S3D). Additionally, both AIN-1 and AIN-2 inhibited silencing in a dominant-negative manner in control cells (Supplementary Figure S4A and S4B). This dominant-negative effect was reduced when AIN-1 and AIN-2 mutants that do not interact with ALG-1 were tested (Supplementary Figure S4A), suggesting that AIN-1 and AIN-2 inhibit silencing at least in part by competing with GW182 for AGO1 binding.

Finally, coexpression of AIN-1 and ALG-1 did not rescue silencing in S2 cells codepleted of *Dm* GW182 and AGO1, whereas coexpression of *Dm* GW182 with ALG-1 did (Figure 6F and G). Thus, although AIN-1 and AIN-2 were active in tethering assays (Figure 7), both proteins were inactive in complementation assays (Figure 8).

Together, our results indicate that AIN-1 and AIN-2 are probably too divergent to rescue silencing in GW182-depleted cells despite that AIN-1 interacts with *Dm* AGO1, PABPC1, NOT1, NOT2 and PAN3.

DISCUSSION

GW182 proteins play a central role in the miRNA pathway in animal cells. Vertebrate and insect members

were normalized to those of *Renilla* luciferase. The normalized values of F–Luc activity and mRNA levels were set to 100 in the presence of the λN-HA peptide. Mean values ± standard deviations from three independent experiments are shown. Panels (B and E) show a northern blot of representative RNA samples quantified in panels (A) and (D). Panels (C and F) show equivalent expression of the λN-HA-tagged proteins. 22×W-A: AIN-1 mutant in which all tryptophan residues were substituted with alanines. GW1+2+3: AIN-2 mutant in which tryptophan residues in the three GW repeats of the ABD were substituted with alanine.
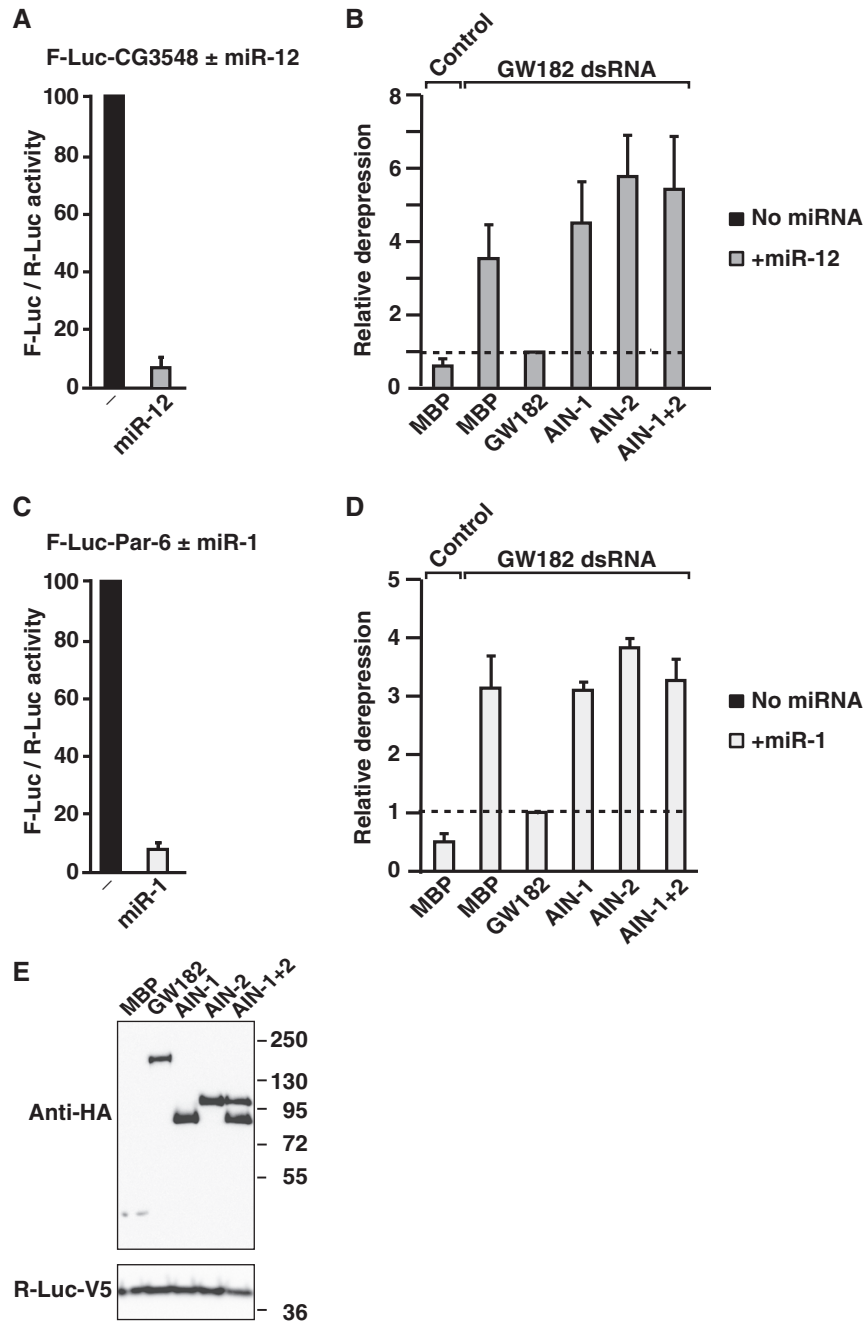
Figure 8. AIN-1 does not rescue silencing in S2 cells depleted of GW182. (**A–D**) S2 cells were treated with a dsRNA targeting *GW182* mRNA. Control cells were treated with *GFP* dsRNA. These cells were subsequently transfected with a mixture of three plasmids: one expressing the indicated F-Luc reporters; another expressing miRNA primary transcripts or the corresponding empty vector (−); and a third expressing *Renilla* luciferase (R-Luc). Plasmids expressing wild-type HA-GW182 (dsRNA resistant), HA-AIN-1, HA-AIN-2 or HA-MBP were included in the transfection mixtures, as indicated. Firefly luciferase activities were normalized to those of the *Renilla* luciferase transfection control and set to 100 in cells transfected with the empty vector (i.e. in the absence of miRNAs) for each condition. Panels (A and C) show normalized firefly luciferase activities in the absence or presence of miRNAs in control cells (i.e. cells treated with *GFP* dsRNA and expressing MBP). Panels (B and D) show the relative fold derepression for each condition. Mean values ± standard deviations from three independent experiments are shown. (**E**) Western blot showing that the HA-tagged proteins were expressed to equivalent levels.

of the family interact with the AGO proteins, PABPC1 and subunits of the two cytoplasmic deadenylase complexes (the PAN2-PAN3 and the CCR4-NOT complexes) (5,20,21,25). Despite strong sequence divergence, we demonstrated that *Ce* AIN-1 interacts with *Ce* or *Dm* PABPC1, PAN3, NOT1 and NOT2, whereas AIN-2 shows no significant interactions. In contrast, both AIN-1 and AIN-2 interact with ALG-1, as previously reported (1,3). Our findings suggest that AIN-1 and AIN-2 are functionally distinct and may silence

miRNA targets using different mechanisms. Indeed, AIN-2 may not promote miRNA target deadenylation and subsequent degradation, potentially explaining why AIN-2 depletion has no overt phenotype but enhances the delayed heterochronic phenotype observed in worms lacking AIN-1 (2,3). This phenotype includes defects in alae formation, drastic increase in seam-cell numbers and protruding vulva (2,3). Alternatively, AIN-2 may be expressed at lower levels and/or regulate fewer targets. Finally, AIN-2 may use a similar mechanism but may recruit PABPC1 and deadenylases through transient low-affinity interactions or through an adaptor protein(s).

Evolutionary plasticity of the GW182 protein interaction network

The finding that AIN-1 interacts with PABPC1, PAN3, NOT1 and NOT2 is surprising given that AIN-1 shows <12% sequence identity with *Dm* GW182 or human TNRC6s, whereas the binding partners (e.g. PABPC1, PAN3, NOT1 and NOT2) are conserved (Supplementary Tables S2 and S3). However, although the interactions of GW182 proteins with PABPC1, NOT1 and PAN3 are conserved in humans and *D. melanogaster*, the mode of interaction is not. For example, human TNRC6s interact with PABPC1 through a PAM2 motif in the silencing domain (17,22–24). This motif is also present in *Dm* GW182 and interacts with PABPC1 *in vitro*; nevertheless, deletion of the *Dm* GW182 PAM2 motif does not abrogate PABPC1 binding *in vivo* (17). In our previous studies, we showed that *Dm* GW182 interacts with PABPC1 through additional regions in the silencing domain. Although indirect, this interaction is dominant over that mediated by the PAM2 motif *in vivo* (17,19).

Another example of change in the binding mode is the interaction of GW182 proteins with NOT1. Indeed, recent work showed that binding of human TNRC6s to NOT1 is mediated by short motifs in the M1, M2 and C-term regions of the silencing domains, which contribute to the interaction in an additive manner (20,21,25). The motif in the M1 region (termed CCR4-interacting motif 1, CIM-1) is conserved in *Dm* GW182 (25). However, in contrast to human TNRC6s, the silencing domain of *Dm* GW182 is not sufficient for binding to NOT1 (our unpublished results), suggesting that additional motifs upstream of the silencing domain are present in *Dm* GW182.

Finally, although the interaction of GW182 proteins with AGOs is mediated by GW repeats (i.e. GW, WG or GWG), the exact location and number of repeats is not conserved (10,12,13,15,30). Here, we show that only a few repeats in the Mid region of AIN-1 and AIN-2 contribute to their interaction with ALG-1; in contrast, 12 and >20 GW repeats in the N-term regions of *Dm* GW182 and human TNRC6s, respectively, contribute to AGO protein binding (12,13).

How can the GW182 protein interaction network be conserved while the location of the binding sites appears to be rapidly evolving? The regions of GW182 proteins mediating the interactions with AGO1, PABPC1 and deadenylase subunits are predicted to be unstructured. These unstructured regions interact with (predicted or known) globular domains in AGO1, PABPC1, PAN3, NOT1 and NOT2, suggesting that the interactions might be mediated by short linear motifs [SLiMs; (37)] in the GW182 unstructured regions. This provides one explanation for why the location of the binding sites is rapidly evolving. Linear motifs are evolutionarily plastic, as only a small number of point mutations in a disordered region of a protein sequence are required to relocate these motifs. In doing so, the interactions between GW182 and binding partners can be maintained, but the details can change during evolution. Additionally, the possibility that GW182 proteins interact with their partners via SLiMs, potentially explains why the binding of AIN-1 to PABPC1, PAN3, NOT1 and NOT2 requires multiple protein regions (Figure 1B), as SLiMs may be dispersed along the protein sequence and collectively contribute to high-affinity interactions. In this context, we can envision a scenario in which nematode AIN-1 proteins gained additional motifs to interact with PABP and deadenylases, so that the silencing domain became progressively redundant and was eventually lost.

Conservation of silencing mechanisms

The present work shows that ALG-1 can rescue silencing in *Drosophila* cells depleted of AGO1, demonstrating conservation in the miRNA pathway including the mechanism of AGO loading, target recognition and target silencing. However, despite conservation of the basic interactions, AIN-1 could not rescue silencing in cells depleted of *Dm* GW182. Furthermore, both AIN-1 and AIN-2 inhibited silencing in a dominant-negative manner in wild-type cells. This effect was at least in part mediated by the ABD, suggesting that these proteins compete with GW182 for binding to AGO1 but assemble non-functional miRISC complexes. Why are these complexes non-functional? One possibility is that the assembly of functional silencing complexes requires GW182 proteins to interact with unknown partners (other than PABPC1 and deadenylase complexes), and that these interactions are not conserved. Alternatively, changes in protein conformation may be required to activate silencing complexes but may not occur for complexes assembled with AIN-1 or AIN-2 proteins in S2 cells.

An important message from our study is that AIN-1 and AIN-2 do not complement the *Dm* GW182 depletion, although both proteins silence an mRNA reporter to which they are artificially tethered. Furthermore, there is no correlation between activity in tethering assays and binding to AGO, deadenylases or PABPC1. Similarly, non-overlapping fragments of *Dm* GW182, including N-term fragments that do not interact with PABPC1, have been shown to be active in tethering assays (18,21,36). These results were interpreted as evidence that interactions of GW182 proteins with PABPC1 are not required for silencing (36). However, in complementation assays, TNRC6 mutants that do not interact with PABPC1 (i.e. PAM2 mutants) are impaired in silencing both in human and S2 cells, indicating that the PABPC1 interaction is critical for silencing *in vivo* (17,20). One possible explanation for the discrepancies between

tethering assays and complementation assays is that tethering assays involve direct high-affinity binding of multiple GW182 proteins to the mRNA target and thus bypass upstream steps in the silencing pathway. Therefore, although tethering assays are an invaluable tool in the dissection of the role of GW182 protein domains in silencing, conclusions from these assays should be validated in complementation assays.

In summary, our work extends and confirms the evolutionary conservation of GW182 interactions with PABPC1 and deadenylase complexes, unveiling their importance for silencing. Further elucidating the molecular basis for these interactions will be key to understanding their roles in both miRNA-mediated translational repression and mRNA target degradation.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables S1–S3 and Supplementary Figures S1–S4.

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