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Two PABPC1-binding sites in GW182 proteins promote miRNA-mediated gene silencing

Eric Huntzinger, Joerg E Braun, Susanne Heimstädt, Latifa Zekri and Elisa Izaurralde*

Department of Biochemistry, Max Planck Institute for Developmental Biology, Tübingen, Germany

miRNA-mediated gene silencing requires the GW182 proteins, which are characterized by an N-terminal domain that interacts with Argonaute proteins (AGOs), and a C-terminal silencing domain (SD). In *Drosophila melanogaster* (Dm) GW182 and a human (Hs) orthologue, TNRC6C, the SD was previously shown to interact with the cytoplasmic poly(A)-binding protein (PABPC1). Here, we show that two regions of GW182 proteins interact with PABPC1: the first contains a PABP-interacting motif 2 (PAM2; as shown before for TNRC6C) and the second contains the M2 and C-terminal sequences in the SD. The latter mediates indirect binding to the PABPC1 N-terminal domain. In *D. melanogaster* cells, the second binding site dominates; however, in HsTNRC6A–C the PAM2 motif is essential for binding to both Hs and DmPABPC1. Accordingly, a single amino acid substitution in the TNRC6A–C PAM2 motif abolishes the interaction with PABPC1. This mutation also impairs TNRC6s silencing activity. Our findings reveal that despite species-specific differences in the relative strength of the PABPC1-binding sites, the interaction between GW182 proteins and PABPC1 is critical for miRNA-mediated silencing in animal cells.

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Introduction

GW182-family proteins are essential in animal cells for miRNA-mediated silencing (reviewed by Ding and Han, 2007; Eulalio *et al*, 2009a). Analysis of GW182 domains in *Drosophila melanogaster* (Dm) and human cells identified two domains that are required for silencing. The first is the N-terminal domain, which contains multiple glycine-tryptophan repeats (GW repeats) and confers binding to Argonaute proteins (AGOs; Behm-Ansmant *et al*, 2006; Till *et al*, 2007; Eulalio *et al*, 2008; Lazzaretti *et al*, 2009; Takimoto *et al*, 2009). The second is a bipartite silencing domain (SD), consisting of the Mid and C-terminal regions, which elicits translational repression and degradation of miRNA targets (Figure 1A; Eulalio *et al*, 2009b; Lazzaretti *et al*, 2009; Zipprich *et al*, 2009).

Exactly how the bipartite SD of GW182 proteins interferes with translation and accelerates mRNA degradation is not completely understood, but recent studies provide important insight by showing that these domains interact with the cytoplasmic poly(A)-binding protein PABPC1, both in *D. melanogaster* and human cells (Fabian *et al*, 2009; Zekri *et al*, 2009).

PABPC1 is a highly conserved eukaryotic protein that binds the poly(A) tail of mRNAs and stimulates translation through multiple interactions with translation factors (reviewed by Kahvejian *et al*, 2001; Derry *et al*, 2006). PABPC1 contains four N-terminal RNA recognition motifs (RRM1–4), a proline-rich unstructured linker and a C-terminal domain (termed PABC or MLLE, because of a conserved KITGMLLE signature motif in this domain; Figure 1A; Kozlov *et al*, 2010a). The MLLE domain recognizes a conserved motif termed PABP-interacting motif 2 (PAM2), which was first identified in the translational regulators Paip1 and Paip2 (PABP-interacting proteins 1 and 2) and is also present in multiple proteins involved in translation or mRNA decay (Khaleghpour *et al*, 2001; Roy *et al*, 2002; Albrecht and Lengauer, 2004; Kozlov *et al*, 2004, 2010a).

Interestingly, the SD of TNRC6C contains a PAM2 motif (previously termed conserved motif III or DUF; Figure 1A). This PAM2 motif in TNRC6C interacts directly with the PABPC1 MLLE domain in a way similar of those found in Paip1 and Paip2 (Fabian *et al*, 2009; Jínek *et al*, 2010; Kozlov *et al*, 2010b). In particular, when both TNRC6C and Paip2 bind to the MLLE domain, the invariant glutamate, phenylalanine and proline residues of the PAM2 motifs occupy structurally equivalent positions (Jínek *et al*, 2010; Kozlov *et al*, 2010b). Moreover, when the phenylalanine residue is substituted with alanine, the interaction of TNRC6C with the MLLE domain is abolished as shown before for Paip2 (Kozlov *et al*, 2004, 2010a, b).

The PAM2 motif is also conserved in DmGW182. Surprisingly, however, our previous studies showed that this motif is dispensable for PABPC1 binding in cell lysates (Zekri *et al*, 2009). DmGW182 instead binds PABPC1 via sequences downstream of the PAM2 motif (termed M2), plus sequences in the very C-terminal (C-term) region (Figure 1A). Nevertheless, the affinity of these regions for PABPC1 in-

*Corresponding author. Department of Biochemistry, Max Planck Institute for Developmental Biology, Spemannstrasse 35, 72076 Tübingen, Germany. Tel.: +49 7071 601 1350; Fax: +49 7071 601 1353; E-mail: elisa.izaurralde@tuebingen.mpg.de

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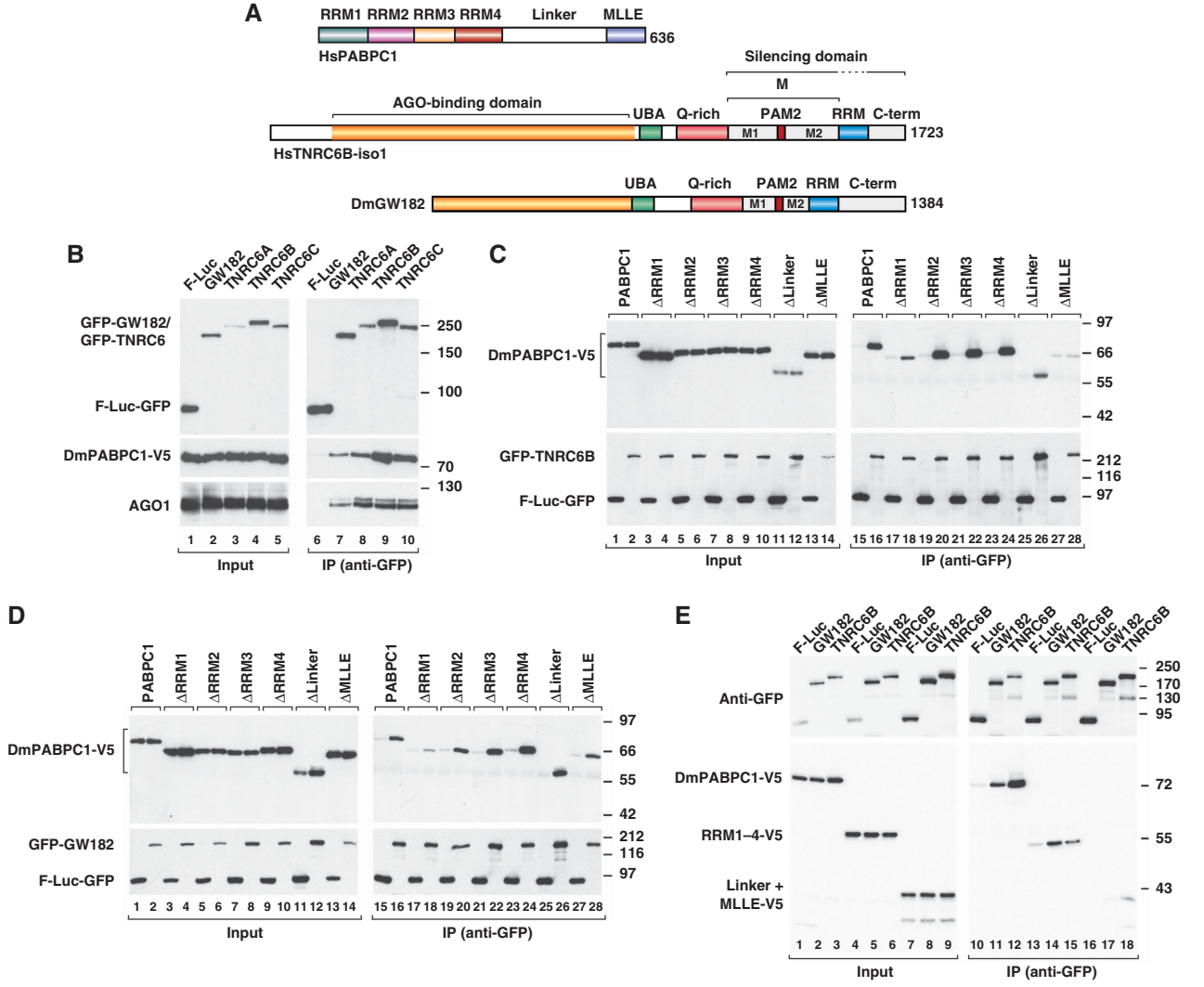

Figure 1 PABPC1 provides two binding sites for GW182 proteins. (**A**) Domain organization of HsPABPC1, HsTNRC6B isoform 1 and DmGW182. PABPC consists of four N-terminal RRM domains, a proline-rich unstructured linker, and a conserved C-terminal domain, termed MLLE. HsTNRC6B and DmGW182 consist of an N-terminal AGO-binding domain, which contains multiple GW-repeats (yellow); and a bipartite silencing domain (SD) which includes the Mid (M) and C-terminal regions but not the RRM. UBA, ubiquitin-associated domain; Q-rich, region rich in glutamine; PAM2, PABP-interacting motif 2; RRM, RNA recognition motif; M1 and M2, regions within the Mid (M) domain; C-term, C-terminal region. (**B**) S2 cells were transfected with a plasmid expressing V5-tagged DmPABPC1 together with plasmids for expression of GFP-tagged proteins (DmGW182, human TNRC6A–C, or firefly luciferase (F-Luc, which served as a negative control)). Three days after transfection, cells were lysed and proteins were immunoprecipitated using a polyclonal anti-GFP antibody. Inputs and immunoprecipitates were analysed by western blotting using anti-GFP and anti-V5 antibodies. The presence of endogenous AGO1 in the immunoprecipitates was analysed using a specific anti-AGO1 antibody. (**C**–**E**) The interaction of GFP-TNRC6B or GFP-GW182 with full-length DmPABPC1-V5 or the indicated PABPC1 deletion mutants (V5 tagged) was analysed as described in panel (**B**). Note that in panel (**E**), cell lysates were treated with micrococcal nuclease prior to immunoprecipitation.

creases when the PAM2 motif is included (Zekri *et al*, 2009); however, deleting the PAM2 motif does not affect the DmGW182 silencing activity *in vivo* (Eulalio *et al*, 2009b). The M2 and C-term regions of DmGW182 do not interact with the MLLE domain of DmPABPC1 but rather interact with the N-terminal PABPC1 RRM domains (Zekri *et al*, 2009). These observations raise a key question: Do the differences in human TNRC6C and DmGW182 reflect differences in the mechanisms of silencing between these distant species?

Another important question that remains open is to what extent the interaction between GW182 proteins and PABPC1 contributes to silencing *in vivo*. Currently, two lines of evidence support a role for PABPC1 in silencing. First, overexpressing PABPC1 in both *D. melanogaster* and human cells suppresses silencing (Zekri *et al*, 2009; Walters *et al*, 2010). Second, depleting PABPC1 from cell-free extracts abolishes miRNA-mediated deadenylation (Fabian *et al*, 2009). It has been difficult to obtain more direct evidence of a role for PABPC1 in the miRNA pathway (i.e. using RNAi knockdowns) because depleting PABPC1 causes rapid cell death and general mRNA destabilization (Behm-Ansmant *et al*, 2007).

In this study, we investigate further the interaction of DmGW182 and human TNRC6A–C proteins with PABPC1. We show that PABPC1 provides two binding sites for GW182 proteins: one on the MLLE domain and another on the RRM domains. Conversely, GW182 proteins contain two PABPC1-binding sites: the PAM2 motif, which confers direct binding to the MLLE domain (as shown before for TNRC6C; Fabian *et al*,

2009; Jínek *et al*, 2010; Kozlov *et al*, 2010b), and a less-defined sequence comprising the M2 and C-term regions, which interacts indirectly with the PABPC1 RRMs (as shown before for DmGW182; Zekri *et al*, 2009). Both sites contribute to PABPC1-binding *in vivo*, but for the human proteins, the dominant interaction is between PABPC1 MLLE and PAM2, whereas for DmGW182, the critical interaction is with PABPC1 RRMs. These results reconcile the apparent discrepancy between earlier studies in human and *D. melanogaster* cells (Fabian *et al*, 2009; Zekri *et al*, 2009).

We also show that in *D. melanogaster* cells depleted of endogenous GW182, human TNRC6B can rescue silencing. Remarkably, this ability to restore silencing is abrogated by a single amino acid substitution in the PAM2 motif of TNRC6B. This mutation also abolishes TNRC6s binding to both Dm and HsPABPC1. Moreover, a chimeric DmGW182 construct containing the PAM2 motif, plus the M2 and C-term regions of human TNRC6B, requires the PAM2 motif to interact with PABPC1. Importantly, a phenylalanine to alanine substitution within the PAM2 motif abrogated both PABPC1 binding and the silencing activity of the chimeric protein. Finally, we show that a TNRC6A protein lacking the PAM2 motif or carrying a single amino acid substitution in this motif does not interact with HsPABPC1 and is strongly impaired in restoring silencing in human cells depleted of endogenous TNRC6A and TNRC6B. Together, our results definitively establish a crucial role for GW182–PABPC1 interaction in the miRNA pathway.

Results

PABPC1 provides two binding sites for GW182 proteins

Previous studies reported that DmGW182 and TNRC6C interact with different PABPC1 domains (see Introduction). Therefore, we wished to determine whether, the species-specific binding differences reside in the GW182 proteins, in PABPC1 or both. To do this, we examined the interaction of human TNRC6s with DmPABPC1 in *D. melanogaster* Schneider-2 cells (S2 cells). In S2 cells, the expression level of TNRC6B was comparable to that of DmGW182, whereas human TNRC6A and TNRC6C were expressed at lower levels (Figure 1B, lanes 2–5). Nevertheless, considering the amount of proteins detected in the immunoprecipitates, the three human proteins coimmunoprecipitated DmPABPC1 and endogenous DmAGO1 more efficiently than DmGW182 (Figure 1B, lanes 7–10).

To define the domains in DmPABPC1 that are important for the interaction with either DmGW182 or human TNRC6B, we tested each with a series of DmPABPC1 deletion mutants. PABPC1 contains four RRMs connected to the C-terminal MLLE domain by a flexible linker (Figure 1A; Derry *et al*, 2006). Deleting RRM1 reduced the PABPC1 interaction with both DmGW182 and TNRC6B (Figure 1C and D, lane 18 versus lane 16). In contrast, deleting the MLLE domain inhibited PABPC1 from binding to TNRC6B but not to DmGW182 (Figure 1C and D, lane 28). Deleting RRM2, RRM3, RRM4 or the linker region had no effect in any of these interactions (Figure 1C and D, lanes 19–26). Together, these results indicate that PABPC1 has two binding sites for GW182 proteins: one that is contributed by the RRM1 domain and another by the MLLE domain.

We next asked whether PABPC1 RRMs or the MLLE domain were sufficient for binding to DmGW182 and TNRC6B, respectively. We observed that TNRC6B interacted with PABPC1 fragments comprising either the RRMs or the MLLE domain (Figure 1E, lanes 15 and 18). However, these interactions were less efficient than those observed with full-length PABPC1 (Figure 1E, lane 12), suggesting that the RRM and the MLLE domains contribute (additively or synergistically) to the interaction with TNRC6B. Furthermore, we confirmed that, in cell lysates, DmGW182 interacts with the PABPC1 RRMs but not the MLLE domain as shown before (Figure 1E, lanes 14 and 17; Zekri *et al*, 2009). Importantly, the interactions shown in Figure 1E were observed in cell lysates treated with micrococcal nuclease, suggesting that they are not mediated by RNA.

GW182 proteins interact with PABPC1 through two distinct binding sites

We next tested how the various sequences within the SDs of DmGW182 and TNRC6B contribute to PABPC1 binding. Both SDs consist of four segments: M1, PAM2 motif, M2 and C-term (Figures 1A and 2A). Deleting the TNRC6B PAM2 motif abolished the interaction with PABPC1, whereas no effect was observed when the M2 and C-term regions were deleted individually (Figure 2B, lanes 10–12). When, however, the M2 and C-term regions were both deleted, then PABPC1 binding was reduced, suggesting that these regions work in concert to bind PABPC1 (Figure 2B, lane 13). Thus, for TNRC6B, although the PAM2 motif is the high-affinity PABPC1-binding site, the M2 and C-term regions also contribute.

In the case of DmGW182, we previously reported that the same three regions (PAM2 motif and the M2 and C-term regions) contribute to PABPC1 binding (Zekri *et al*, 2009). However, the contribution of the PAM2 motif and the M2 region was apparent only when binding to PABPC1 was impaired, for example by deleting the C-term region (Zekri *et al*, 2009). The results shown in Figure 2C confirm and extend these previous observations. Indeed, we confirmed that the interaction of GW182 with PABPC1 is not affected when the M2 region or the PAM2 motif are deleted individually (Figure 2C, lanes 13 and 14; Zekri *et al*, 2009). In contrast, deleting the C-term region reduced PABPC1 binding (Figure 2C, lane 15). PABPC1 binding was further decreased when the C-term region was deleted in combination with the M2 region or the PAM2 motif (Figure 2C, lanes 16 and 17; Zekri *et al*, 2009). PABPC1 binding was abolished when all three regions were deleted (i.e. PAM2, M2 and C-term; Figure 2C, lane 19; Zekri *et al*, 2009). Similar results were obtained when cell lysates were treated with micrococcal nuclease (Supplementary Figure S1).

An important implication of the results shown in Figure 2C is that the GW182 C-term region provides a major PABPC1-binding sites in cell lysates, however, the M2 region and the PAM2 motif also contribute, although on their own they are not sufficient. This conclusion is further supported by experiments aimed at defining the minimal PABPC1-binding domain in DmGW182. We observed that a protein fragment containing the PAM2 motif and the M2 and C-ter regions was sufficient for PABPC1 binding (Supplementary Figure S2A, lane 17 versus lane 13; Zekri *et al*, 2009), whereas fragments containing one or two of these sequences exhibited reduced

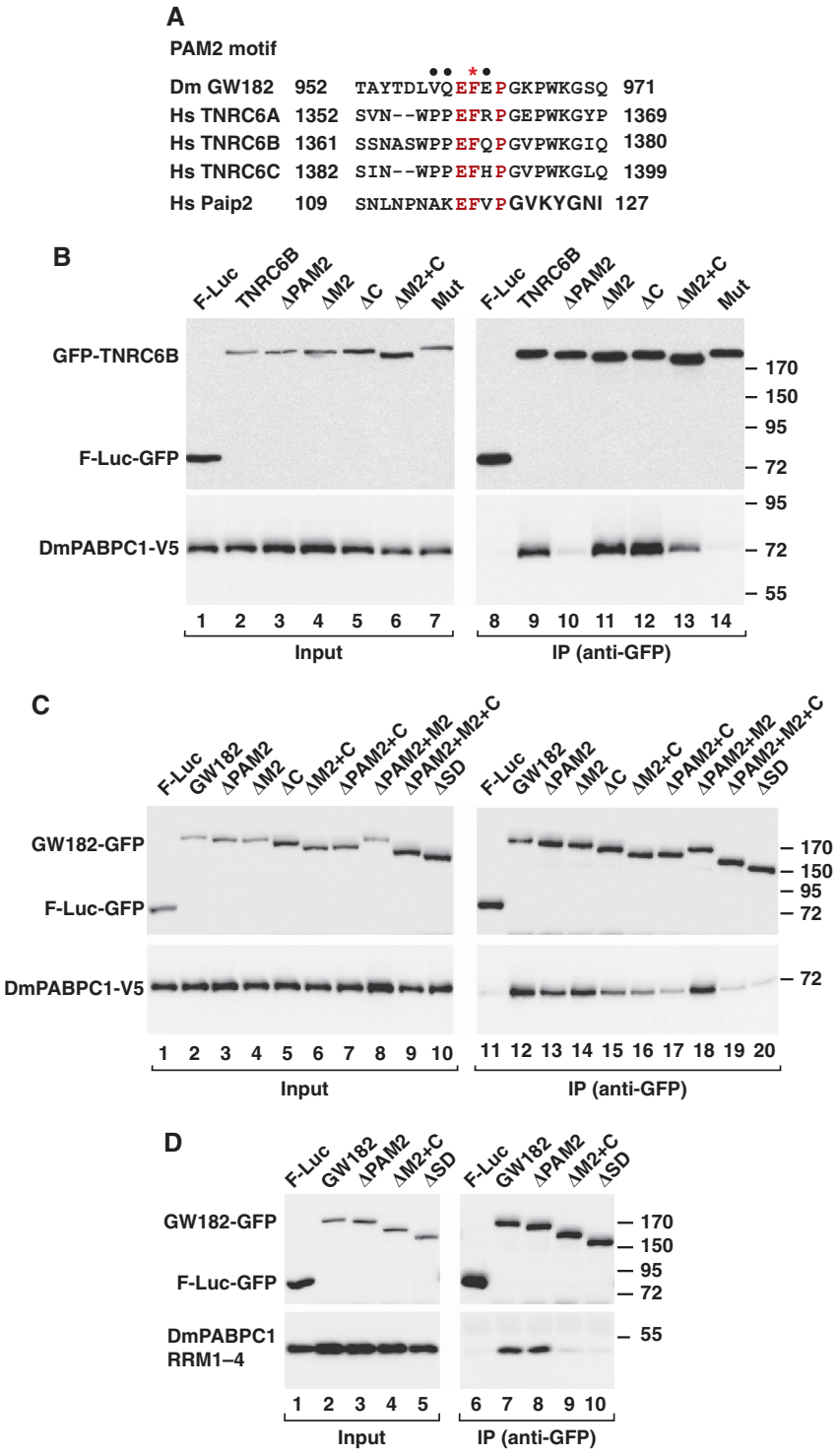

Figure 2 GW182 proteins contain two PABPC1-binding sites. (**A**) Sequence alignment of PAM2 motifs of human TNRC6A–C, Paip2 and DmGW182. Invariant residues are shown in red. The asterisk indicates the phenylalanine residue that is substituted with alanine in our mutants (Mut). Dots indicate the residues in the PAM2 motif of GW182 that were substituted in the experiment shown in Figure 3B. (**B**, **C**) S2 cells expressing GFP-TNRC6B, GFP-DmGW182 or the corresponding protein mutants together with DmPABPC1-V5 were lysed 3 days after transfection. Proteins were immunoprecipitated from cell lysates using a polyclonal anti-GFP antibody and analysed by western blotting as described in Figure 1. (**D**) Interaction of GFP-DmGW182 or the indicated mutants with a V5-tagged DmPABPC1 fragment containing RRM1–4.

or no affinity for PABPC1 in cell lysates (Supplementary Figure S2A, lanes 15, 16 and 18–22). These results remained unchanged in the presence of micrococcal nuclease (Supplementary Figure S2B).

The results described above, together with the observation that DmGW182 interacts with the RRM domains as efficiently with full-length PABPC1, suggest that the role of the M2 and C-term regions is to confer binding to PABPC1 RRMs.

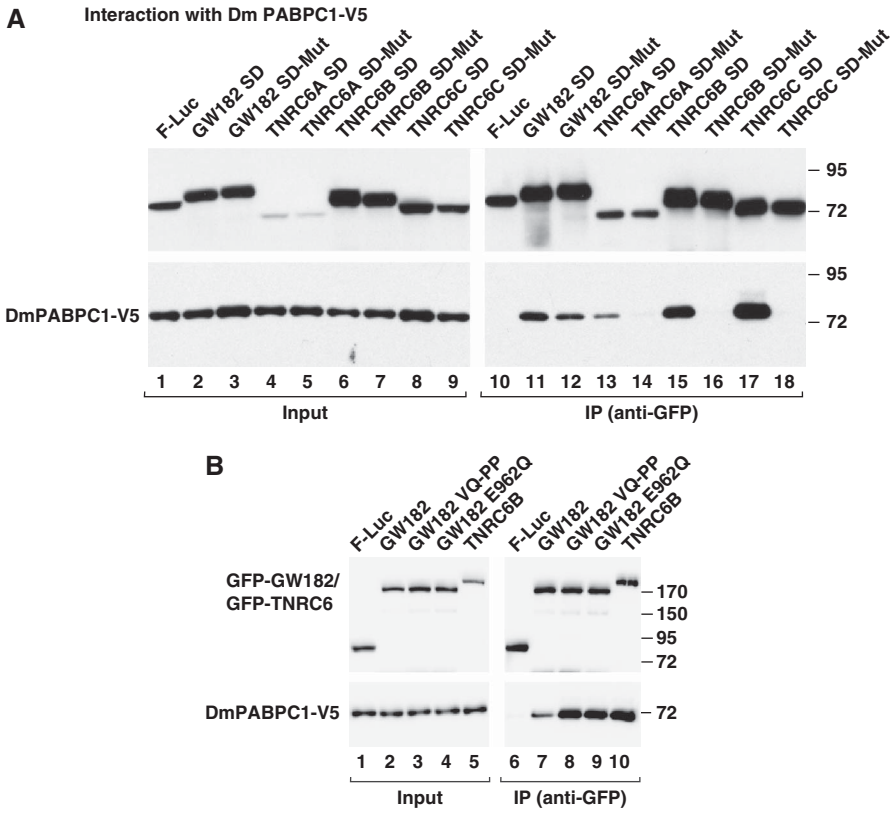

Figure 3 The invariant phenylalanine residue in human PAM2 motifs is essential for binding to DmPABPC1. (**A**) The interaction of DmPABPC1-V5 with GFP-tagged DmGW182 or human TNRC6A–C silencing domains was analysed by coimmunoprecipitation assays as described in Figure 1B. Protein mutants (Mut) contain an alanine to phenylalanine substitution in the PAM2 motif (see Figure 2A, asterisk). (**B**) S2 cells transiently expressing DmPABPC1-V5 together with GFP-GW182 wild type or the indicated GW182 mutants were lysed 3 days after transfection. Proteins were immunoprecipitated from cell lysates using anti-GFP antibodies and analysed as described in Figure 1B. GW182 mutants carry substitutions of the amino acids that are indicated with dots in Figure 2A.

Therefore, we constructed a PABPC1 mutant containing only the RRM1–4 domains and tested how well it interacts with various GW182 deletion mutants. We observed that deleting the M2 and C-term regions inhibited DmGW182 from interacting with the PABPC1 RRMs as efficiently as deleting the entire SD (which includes the PAM2 motif); in contrast, deleting the PAM2 motif alone had no effect (Figure 2D, lanes 8–10). Collectively, these results clearly demonstrate that the M2 and C-term regions interact with DmPABPC1 RRM domains in cell lysates.

A single amino acid substitution in the PAM2 motif of human TNRC6s abolishes binding to DmPABPC1

The PAM2 motifs from diverse proteins contain three invariant residues EF(X)P that occupy equivalent structural positions when bound to an MLLE domain (Figure 2A; Jínek *et al*, 2010; Kozlov *et al*, 2010a, b). In particular, the invariant phenylalanine residue is critical for this interaction: if this phenylalanine is substituted with alanine then the Paip2 and TNRC6C PAM2 motifs cannot bind PABPC1 (Kozlov *et al*, 2004, 2010a, b). Similarly, this substitution abolishes the TNRC6B interaction with DmPABPC1 as efficiently as deleting the entire PAM2 motif (Figure 2B, lane 14; Figure 3A, lane 16). The critical role of the invariant phenylalanine residue in the interaction with DmPABPC1 was confirmed for TNRC6A and TNRC6C SDs (Figure 3A, lanes 14 and 18). The equivalent substitution in the PAM2 motif of DmGW182 had only a minor effect on DmPABPC1 binding, as expected (Figure 3A, lane 12).

The coimmunoprecipitation assays suggest that the *D. melanogaster* PAM2 motif has a relatively lower affinity for the DmPABPC1 MLLE domain. This might be because the motif lies in a suboptimal sequence context. However, we consider this possibility unlikely because the human PAM2 motif is functional in the context of DmGW182 (i.e. it enhances DmGW182 binding to PABPC1; see below Figure 8A). An alternative explanation is that the affinity may be lower because a negatively charged residue sits at the core of the PAM2 motif (between the invariant phenylalanine and proline residues, Figure 2A). Indeed, negatively charged residues are extremely rare at the equivalent position in PAM2 motifs from diverse proteins and are absent in all PAM2 motifs validated experimentally (Albrecht and Lengauer, 2004; Kozlov *et al*, 2010a). Additionally, in the human TNRC6A–C, the invariant residues of the PAM2 motifs are preceded by proline residues that establish hydrophobic interactions with the MLLE domain (Kozlov *et al*, 2010b), these residues are substituted with valine and glutamine in DmGW182 (Figure 2A).

We therefore tested whether substituting residues in the PAM2 motif of GW182 with the residues present in the human proteins could enhance PABPC1 binding. Substituting the GW182 PAM2-motif amino acids V958 and Q959 with prolines enhanced GW182 binding to DmPABPC1

(Figure 3B, lane 8 versus lane 7). Similar results were obtained when the negatively charged residue E962 was substituted with glutamine as in TNRC6B (Figure 3B, lane 9). The two GW182 mutants interacted with DmPABPC1 as efficiently as TNRC6B (Figure 3B, lane 10). Thus, the differences in the amino acid sequences of these PAM2 motifs can account for the different affinities for the DmPABPC1 MLLE domain.

The PAM2 motifs of human TNRC6s are essential for binding to HsPABPC1

We next analysed whether the human proteins interact in a similar way with HsPABPC1. We transfected human HEK-293 cells with plasmids expressing either wild-type TNRC6A–C SDs or the corresponding mutants carrying the phenylalanine to alanine substitution in the PAM2 motifs. We observed that the single amino acid substitution in the PAM2 motifs was sufficient to abolish the interaction with endogenous PABPC1 (Figure 4A–C). Thus, the PAM2 motifs of TNRC6A–C are essential in mediating binding to both Hs and DmPABPC1.

We also tested whether the M2 and C-term regions of human TNRC6s contribute to PABPC1 binding in human cell lysates. Remarkably, deleting either the M2 or C-terminal regions in the TNRC6C SD reduced the interaction with PABPC1, whereas deleting the RRM from TNRC6C had no effect (Figure 4D, lanes 12–14). As a control, we confirmed that deleting the PAM2 motif (or the entire Mid domain) abrogated PABPC1 binding (Figure 4D, lanes 10 and 11). Similar results were obtained when cell lysates were treated with micrococcal nuclease (data not shown). These findings demonstrate that TNRC6s contain two PABPC1-binding sites: the PAM2 motif and the M2 plus C-term regions.

To investigate whether the interaction mediated by the M2 and C-term regions of GW182 proteins is direct, we performed glutathione S-transferase (GST) pull-down assays with recombinant proteins expressed in *Escherichia coli*. These experiments revealed the following observations:

First, we observed that a GST-tagged C-terminal fragment of TNRC6B containing the PAM2 motif and the downstream protein sequences (i.e. M2, RRM and C-term) interacted with both human and DmPABPC1, but did not interact with the corresponding PABPC1 mutants lacking the MLLE domain (Figure 4E, lanes 11 versus 14, and 27 versus 30, respectively). Second, deleting the PAM2 motif abolished the interaction of TNRC6B SD with both Hs and DmPABPC1 (Figure 4E, lanes 12 and 28, respectively; Figure 5A, lane 10), in agreement with the coimmunoprecipitation assays. In contrast, deleting the M2 and C-terminal regions did not affect PABPC1-binding *in vitro* (Figure 5A, lane 13). Surprisingly, *in vitro*, the interaction of DmGW182 SD with DmPABPC1 was mediated by the PAM2 motif (Figure 5B, lane 10 versus lane 9). Accordingly, a GW182 SD mutant lacking the M2 and C-terminal regions pulled down DmPABPC1 (Figure 5B, lane 13). Together, these results indicate that the PAM2 motifs of GW182 proteins mediate direct binding to PABPC1, whereas the M2 and C-term regions interact with PABPC1 indirectly, most likely through additional proteins present in cell lysates. Alternatively, the interaction of the M2 and C-term regions with PABPC1 may require post-translational modifications that do not occur in bacteria. Importantly, the observation that the DmGW182 PAM2 motif directly interacts with DmPABPC1 *in vitro*, but is neither sufficient nor necessary for binding to DmPABPC1 in cell lysates, suggest that the DmPAM2 motif might not be able to efficiently compete with additional PAM2-containing proteins for binding to PABPC1 in *D. melanogaster* cells.

GW182 silencing activity correlates with PABPC1 binding

To evaluate how the interaction between GW182 and PABPC1 contributes to silencing, we tested whether DmGW182 mutants that are impaired in PABPC1 binding in cell lysates could complement silencing in cells lacking endogenous GW182. To this end, we used a complementation assay described before (Eulalio *et al*, 2009b). In this assay, endogenous GW182 is depleted using a dsRNA that targets the coding sequence of the *GW182* mRNA. This depletion inhibits miRNA-mediated silencing (Figure 6A–G). GW182 mutants were then tested for the ability to restore silencing in GW182-depleted cells. Transcripts encoding the recombinant proteins were made resistant to the dsRNA by introducing mutations that disrupt basepair interactions with the dsRNA without altering the protein sequence.

We monitored miRNA activity using two different reporters: the F-Luc-Par-6 reporter which is degraded in the presence of miR-1 and the F-Luc-Nerfin reporter which is silenced by miR-9b or miR-279 mainly at the translational level (Behm-Ansmant *et al*, 2006; Eulalio *et al*, 2007). We observed that, independently of the reporter, a DmGW182 mutant lacking the PAM2 motif fully rescued silencing (Figure 6A–F). Deleting the M2 region had a slight inhibitory effect (particularly for the F-Luc-Par-6 reporter), whereas deleting the C-term region impaired silencing for all reporters as shown before (Figure 6A–F; Eulalio *et al*, 2009b). When in addition to the C-term region, the PAM2 motif and the M2 region were deleted, the silencing activity of the protein decreased further and was comparable to that of the protein lacking the entire SD (Figure 6A–F). Note that these deletion mutants did not rescue silencing even though they were expressed at higher levels than the wild type (Figure 6H). Moreover, the activity of wild-type GW182 and mutants remained unchanged when the amounts of transfect plasmid were increased up to 10-fold (Supplementary Figure S3). We conclude that the silencing activity of GW182 mutants strongly correlates with the ability to bind to PABPC1. Nevertheless, because several regions of DmGW182 must be deleted to abolish PABPC1-binding *in vivo*, we cannot rule out that these regions are also required for additional functions.

Human TNRC6s complement silencing in D. melanogaster cells

Next, we investigated whether the human proteins could restore silencing in S2 cells depleted of endogenous DmGW182. As shown above, depleting endogenous GW182 suppresses silencing of the F-Luc-Par-6 reporter, leading to a five- to nine-fold increase in firefly luciferase expression (Figure 6A and B; Supplementary Figure S4). Expressing a dsRNA-resistant form of GW182 fully restored silencing, over a broad range of transfected expression plasmid (from 10 to 200 ng; Supplementary Figures S3 and S4). Similarly, expressing TNRC6B restored silencing almost as efficiently as GW182 (Supplementary Figure S4). In contrast, TNRC6A and TNRC6C rescued silencing significantly only when the

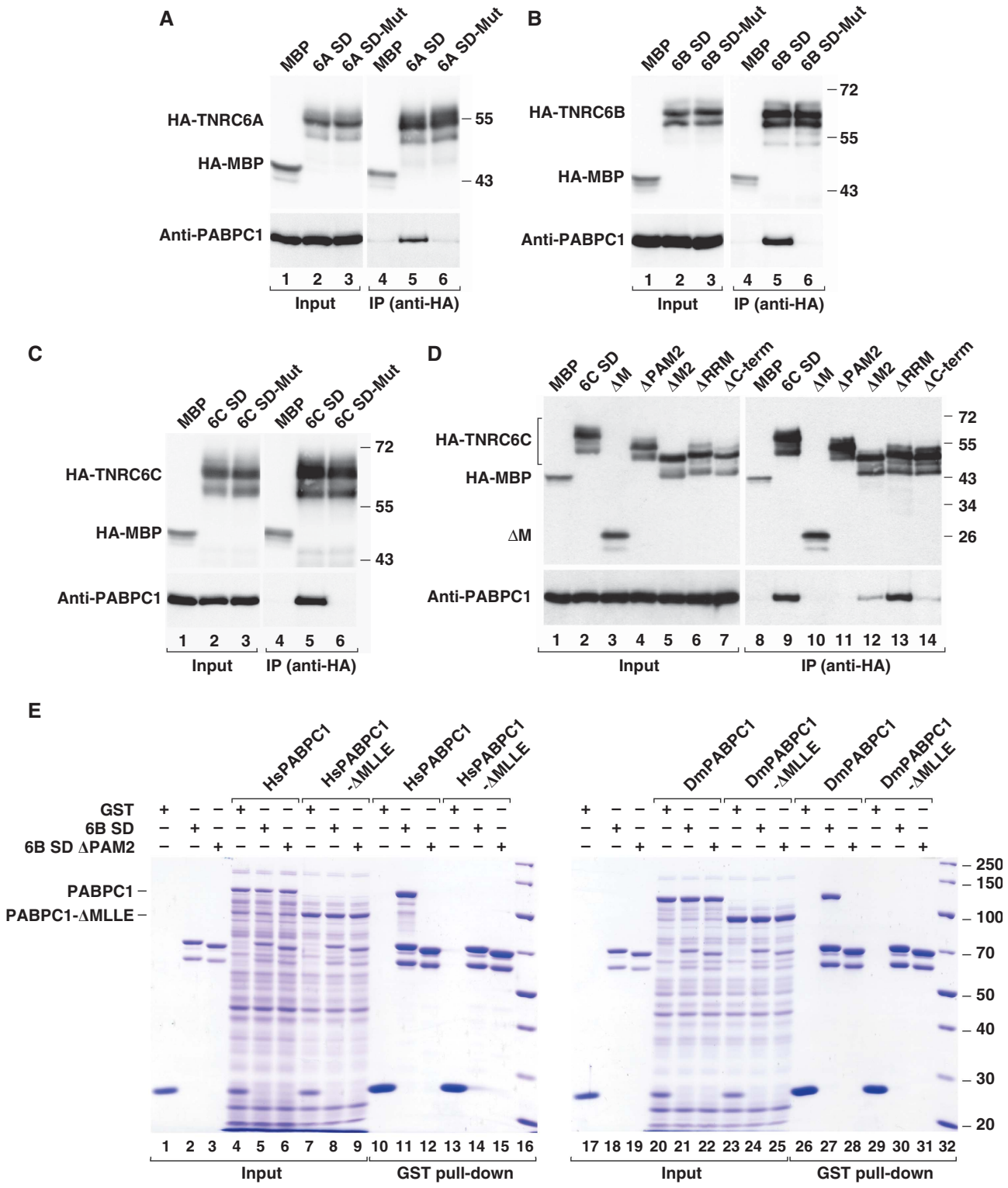

Figure 4 The PAM2 motifs of TNRC6A–C are essential for binding to HsPABPC1. (**A**–**C**) Human HEK-293 cells transiently expressing HA-tagged silencing domains of TNRC6A–C or the indicated mutants were lysed 2 days after transfection. Proteins were immunoprecipitated from RNaseA-treated cell lysates using anti-HA antibodies. HA-tagged maltose-binding protein (MBP) served as a negative control. Inputs and immunoprecipitates were analysed by western blotting using anti-HA antibodies. The presence of endogenous PABPC1 in the immunoprecipitates was analysed using an anti-PABPC1 antibody. (**D**) Interaction of HA-TNRC6C silencing domain or the indicated deletion mutants with endogenous PABPC1, in human cells. Immunoprecipitations were performed as described in panels (**A**–**C**). Note that TNRC6A–C silencing domains are unstable in human cells and show several degradation products (panels **A**–**D**). (**E**) Interaction of recombinant MBP-tagged HsPABPC1 (lanes 1–16), DmPABPC1 (lanes 17–32) or the corresponding mutants lacking the MLLE domain with GST-TNRC6B-SD (6B-SD) or a mutant lacking the PAM2 motif. Inputs (5%) and bound fractions (50%) were analysed on a 10% SDS–PAGE. GST served as a negative control.

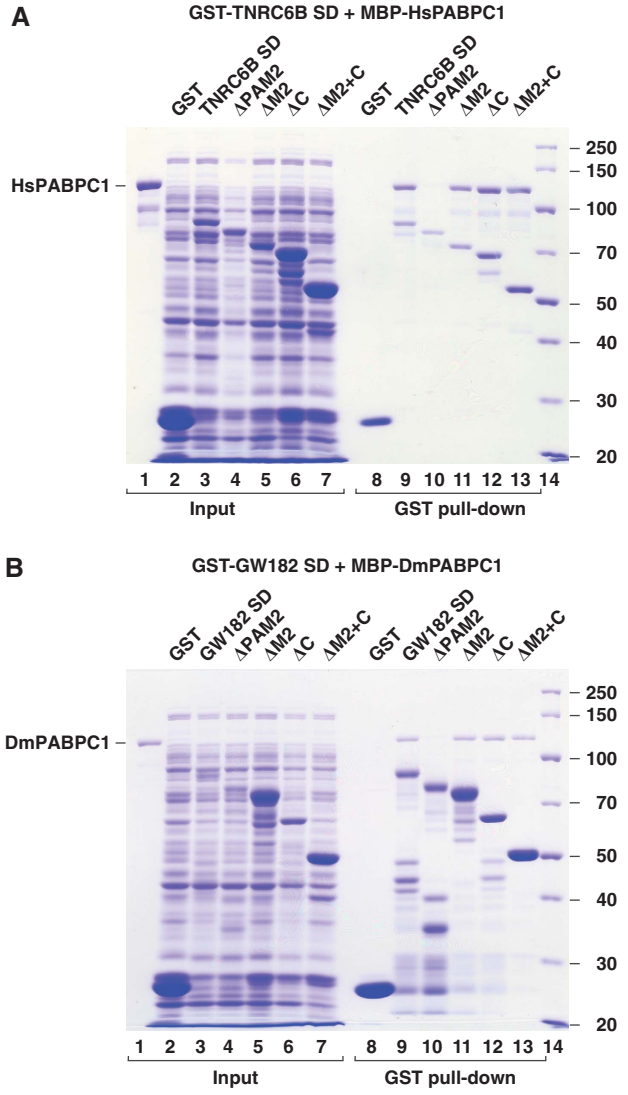

Figure 5 The PAM2 motifs of GW182 proteins confer direct binding to PABPC1 *in vitro*. (**A**) Interaction of recombinant MBP-HsPABPC1 with GST-TNRC6B-SD or the indicated deletion mutants. (**B**) Interaction of recombinant MBP-DmPABPC1 with GST-GW182-SD or the indicated deletion mutants. Inputs (20%) and bound fractions (50%) were analysed on a 10% SDS–PAGE. GST served as a negative control.

highest amount of expression plasmid was transfected (Supplementary Figure S4). The differences in silencing activity between TNRC6s could be due to differences in protein expression levels (see Figure 1B) and not to incompatibility with the *D. melanogaster* silencing machinery, as all three human proteins interact with DmAGO1 and PABPC1 (Figure 1B). However, it is possible that TNRC6A and

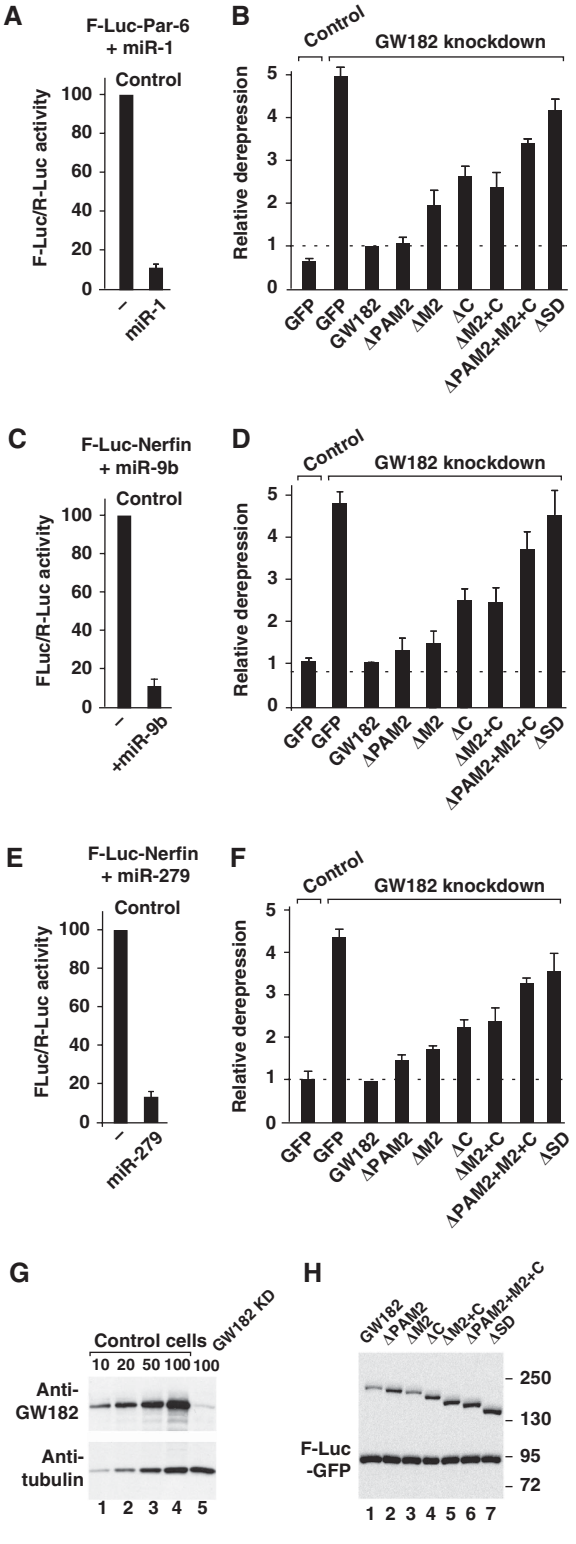

Figure 6 DmGW182 silencing activity correlates with the ability to bind PABPC1 in cell lysates. (**A**–**F**) S2 cells were treated with dsRNA targeting the coding sequence of *GW182* mRNA. Control cells were treated with GST dsRNA. These cells were subsequently transfected with a mixture of three plasmids: one expressing the indicated F–Luc miRNA reporters; another expressing miRNA primary transcripts or the corresponding empty vector (–); and a third expressing *Renilla* luciferase (R–Luc). Plasmids (10 ng) encoding wild-type GFP-GW182 or various deletion mutants 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). (**A**, **C**, **E**) Normalized firefly luciferase activities in the absence or presence of miRNAs in control cells (i.e. cells treated with GST dsRNA and expressing GFP). (**B**, **D**, **F**) The relative fold derepression for each condition. Mean values ± s.d. from three independent experiments are shown. (**G**) The effectiveness of the GW182 depletion was analysed by western blotting using anti-GW182 antibodies. In lanes 1–4, dilutions of untreated cells (control) are loaded. Blots were probed with anti-tubulin antibodies to test for equal loading. (**H**) Expression levels of full-length GW182 and mutants. F-Luc-GFP served as a transfection control.

TNRC6C are impaired for interaction with other factors required for silencing.

The TNRC6s–PABPC1 interaction is required for silencing

As TNRC6B can complement silencing in S2 cells and a single point mutation in TNRC6B is sufficient to prevent binding to PABPC1 both *in vivo* and *in vitro*, we had the opportunity to test whether the TNRC6B–PABPC1 interaction is relevant for silencing in a cellular context. In complementation assays in S2 cells, we observed that a TNRC6B mutant lacking the PAM2 motif was strongly impaired (Figure 7A–F). More importantly, a protein carrying the phenylalanine to alanine substitution in the PAM2 motif (Mut, F1370A) also failed to rescue silencing (Figure 7A–F). The equivalent mutation in GW182 (Mut, F961A) had no effect (Figure 7A–F). Deleting the TNRC6B M2 and C-term regions also affected silencing (Figure 7A–F); however, whether this effect reflects that these regions indirectly contribute to PABPC1 binding or that they have additional functions in silencing is unknown.

For the F-Luc-Par-6 reporter that is directed to degradation by miR-1, we analysed transcript levels by Northern blotting. In control cells expressing miR-1, F-Luc-Par-6 mRNA levels were strongly reduced (Figure 7G, lane 2 versus lane 1). Depleting GW182 inhibited miR-1-mediated mRNA degradation as reported before (Figure 7G, lane 4; Eulalio *et al*, 2008). Transfecting GW182-depleted cells with a plasmid expressing either wild-type GW182 or TNRC6B, restored mRNA degradation (Figure 7G, lanes 6 and 12). The GW182 mutant lacking the PAM2 motif also mediated mRNA degradation (Figure 7G, lane 8). In contrast, the TNRC6B mutant lacking the PAM2 motif could not trigger reporter degradation (Figure 7G, lane 14). The control, a GW182 lacking the M2 and C-term regions, failed to restore mRNA degradation (Figure 7G, lane 10). The mutant proteins were expressed at comparable or slightly higher levels than wild type (Figure 7H). We conclude that the TNRC6B–PABPC1 interaction is required for silencing of miRNA targets, regardless of whether the target is degraded (F-Luc-Par-6) or translationally repressed (F-Luc-Nerfin).

A chimeric GW182 protein requires the interaction with PABPC1 to elicit silencing

To further investigate how the GW182–PABPC1 interaction is relevant in silencing, we sought to engineer a DmGW182 protein that requires the PAM2 motif to bind to PABPC1. Initially, we generated a DmGW182 variant in which the PAM2 motif was substituted with the TNRC6B PAM2 motif (GW182-PAM2^{6B}). This chimeric protein interacted with Dm PABPC1 more efficiently than wild-type GW182 (Figure 8A, lane 13 versus lane 11); however, if the F1370A substitution in the PAM2 motif was introduced, binding to PABPC1 was comparable to that of wild-type GW182 (Figure 8A, lane 14 versus lane 11). Accordingly, in cells depleted of endogenous GW182, the chimeric protein complemented silencing regardless of the F1370A substitution (Figures 8B–G). These results further support the conclusion that in the context of DmGW182, the PAM2 motif is dispensable for both PABPC1 binding and silencing.

Next, in addition to the PAM2 motif, we substituted the M2, RRM and C-term sequences of DmGW182 with those from TNRC6B (GW182-SD^{6B}). This chimeric protein behaved like TNRC6B with respect to PABPC1 binding (Figure 8A). Indeed, relative to wild-type GW182, the PABPC1-binding efficiency increased (Figure 8A, lane 15 versus lane 11). More importantly, the F1370A substitution strongly reduced the interaction with PABPC1 (Figure 8A, lane 16 versus lane 15). Thus, as for TNRC6B, the chimeric protein relies mainly on the PAM2 motif to interact with PABPC1. In complementation assays, the chimeric protein carrying the F1370A substitution was impaired (Figure 8B–G), indicating that the interaction of GW182 proteins with PABPC1 is critical for silencing of miRNA targets.

The interaction of TNRC6A with PABPC1 is critical for silencing in human cells

So far, we demonstrated that the interaction between TNRC6s and PABPC1 has a critical function in silencing in *D. melanogaster* S2 cells. To determine whether this is also true in human cells, we examined whether overexpressing TNRC6C in HeLa cells stimulated silencing of an R-Luc reporter containing three let-7-binding sites in the 3′ UTR (Pillai *et al*, 2005). We found that TNRC6C enhanced silencing, decreasing luciferase activity an additional two-fold relative to control cells (Figure 9A and B). In contrast, the TNRC6C mutant carrying the F1389A substitution failed to stimulate silencing (Figure 9A and B).

Next, we depleted TNRC6 proteins using specific siRNAs and tested whether siRNA-resistant forms of the TNRC6s could complement silencing in depleted cells. We observed that siRNAs targeting TNRC6A and TNRC6B efficiently suppressed silencing of the let-7 reporter in HeLa cells (Figure 9C and D; other combinations of siRNAs and protein expression were less effective, data not shown). In cells codepleted of TNRC6A and TNRC6B, the expression of wild-type TNRC6A partially restored silencing, while the TNRC6A mutant either lacking the PAM2 motif or carrying the F1359A substitution were impaired in restoring silencing, although they were expressed at comparable levels as the wild type (Figure 9C–E). A TNRC6A mutant lacking the entire SD was inactive in the complementation assay, although this mutant was expressed at higher levels (Figure 9C–E). Altogether, these data support the idea that the interaction of TNRC6s with PABPC1 is also critical for silencing miRNA targets in human cells.

Discussion

Proteins of the GW182 family have an essential function in the miRNA pathway in diverse organisms (reviewed by Ding and Han, 2007; Eulalio *et al*, 2009a). The GW182 N-terminal and C-terminal domains interact with AGOs and PABPC1, respectively (reviewed by Tritschler *et al*, 2010). Here, we show that the GW182–PABPC1 interaction plays a crucial role in miRNA-mediated gene silencing.

GW182 proteins are PABP-interacting proteins (Paips)

We found GW182 proteins are similar to Paip1 and Paip2 in that they contain two binding sites for PABPC1: one in the PAM2 motif and another in the M2 and C-terminal regions (Figure 9F). In human TNRC6A–C and DmGW182, the PAM2 motif interacts directly with the C-terminal MLLE domain of PABPC1. Previous structural and functional studies indicated that the PAM2 motifs in TNRC6C, Paip1 and Paip2 are

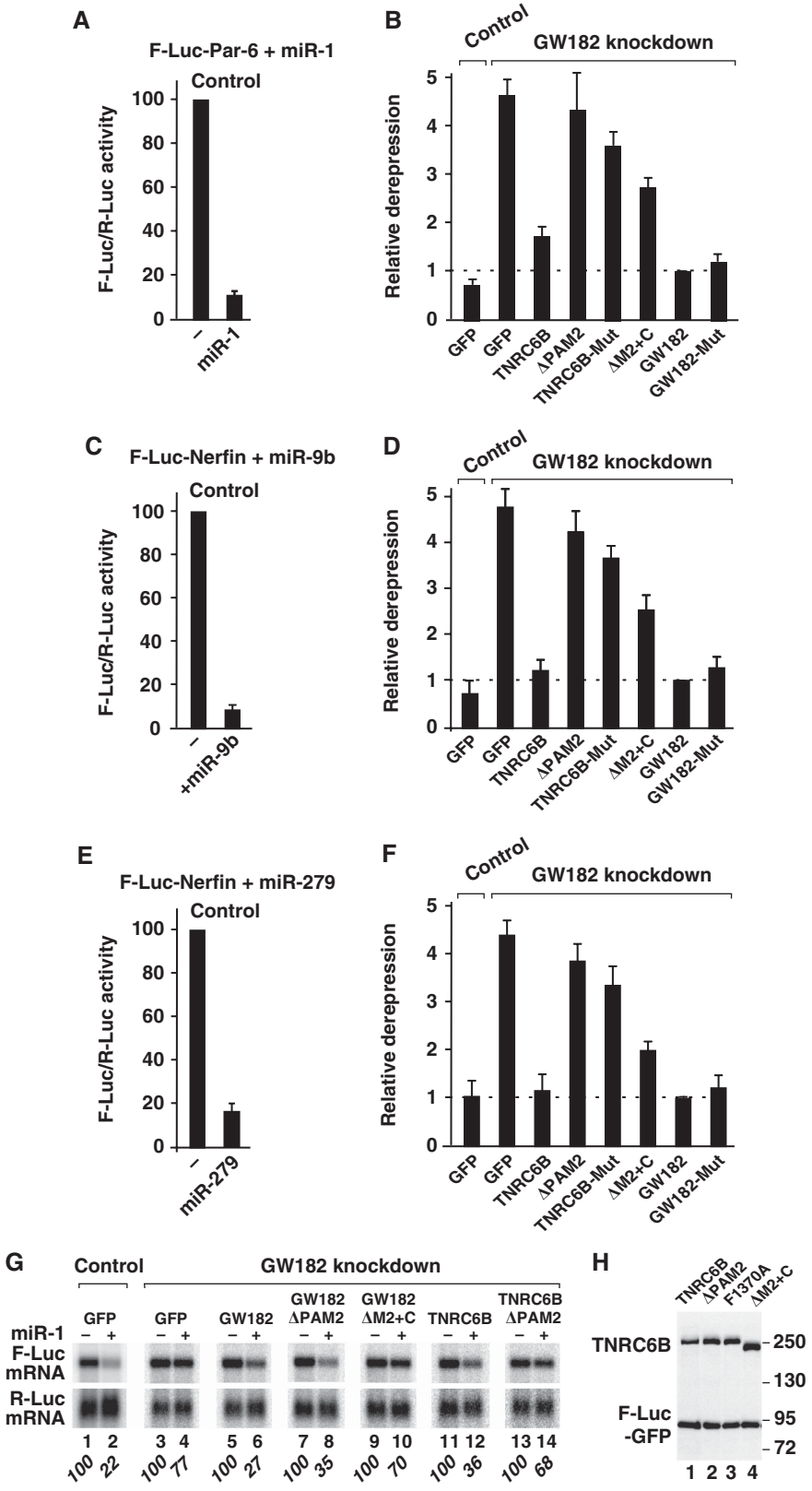

Figure 7 miRNA-mediated gene silencing requires TNRC6B interaction with PABPC1. (**A**–**F**) Complementation assays were performed with wild-type TNRC6B and various TNRC6B mutants (10 ng) and the indicated miRNA reporters, as described in Figure 6. Wild-type GW182 and the corresponding F961A mutant served as controls. (**G**) Northern blot analysis of representative RNA samples are shown in panels (**A**, **B**). Numbers in italics below the panels represent firefly luciferase mRNA levels normalized to those of the *Renilla* and set to 100 in cells transfected with the empty vector (i.e. in the absence of miR-1, (−)) for each condition. (**H**) Western blotting showing the expression levels of recombinant proteins.

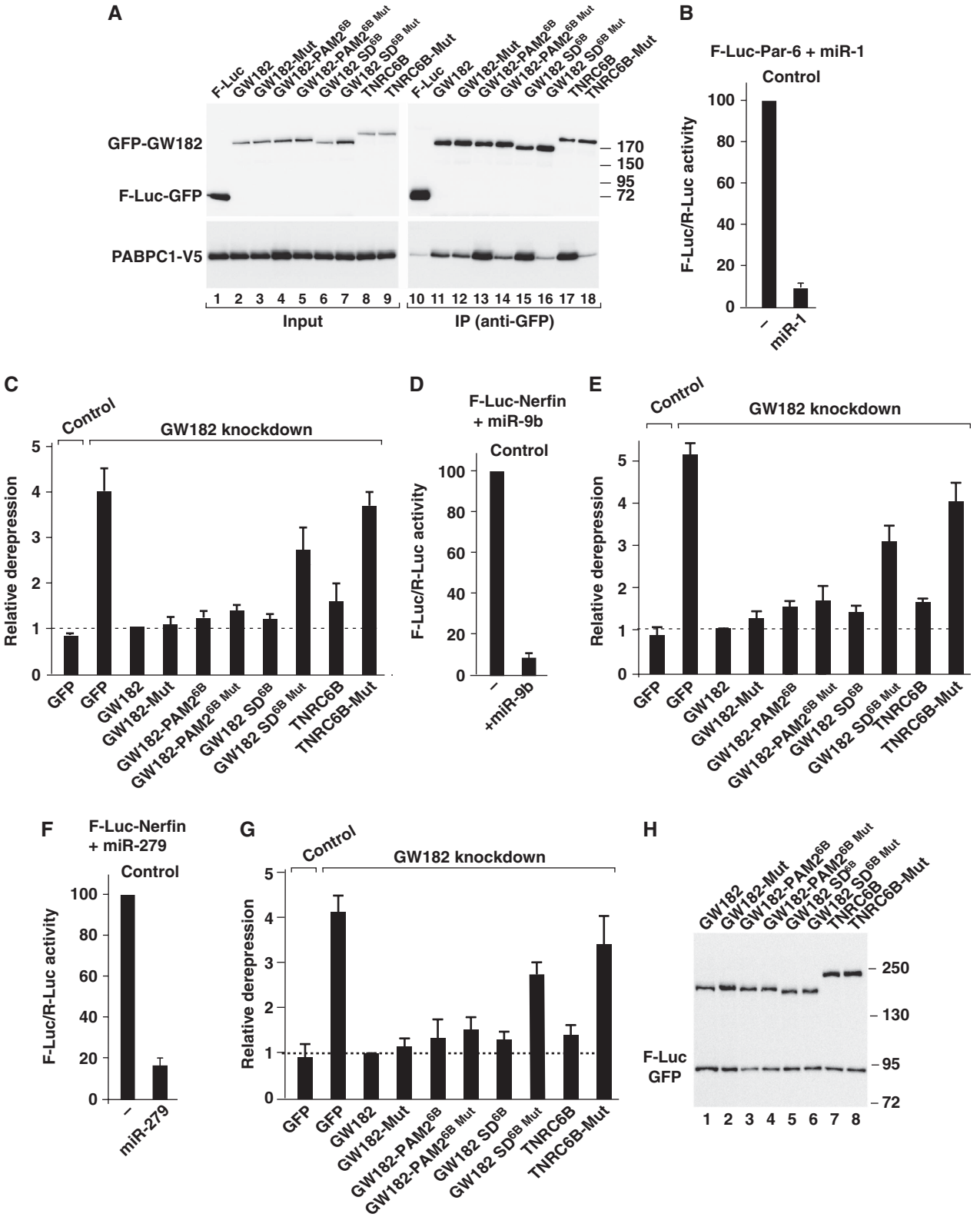

Figure 8 Generation of a chimeric GW182 protein that depends on the PAM2 motif for PABPC1 binding. (**A**) Two DmGW182-TNRC6B chimeric proteins were generated. The protein GW182-PAM2^{6B} consist of DmGW182 protein sequences in which the PAM2 motif was replaced with the PAM2 motif of human TNRC6B. The GW182-SD^{6B} protein contains (in addition to the PAM2 motif), the M2, RRM and C-term regions of TNRC6B. The F1370A substitution was introduced in the two chimeric proteins as indicated (Mut). The ability of the proteins to interact with DmPABPC1 was tested as described in Figure 1B. Wild-type GW182, TNRC6B and the corresponding PAM2 mutants (F961A and F1370A, respectively) were included for comparison. (**B**–**G**) The activity of the chimeric proteins in complementation assays was tested as described in Figure 6. (**H**) Western blotting showing the expression levels of recombinant proteins.

functionally equivalent (Fabian *et al*, 2009; Jínek *et al*, 2010; Kozlov *et al*, 2010b). Our findings extend this conclusion to the PAM2 motifs of TNRC6A and TNRC6B. Indeed, substituting alanine for the invariant phenyalanine in the PAM2 motif abolished binding to Hs or DmPABPC1 for all three human TNRC6 proteins (Figures 2B, 3A and 4). Nevertheless, the M2 and C-term regions also contribute to PABPC1 binding in cell lysates (Figures 2B and 4D). In DmGW182, the M2 and C-terminal site mediates binding to the RRM domains at the PABPC1 N-terminus (Figure 2D); this binding is most likely mediated by additional proteins and not by RNA because it is also observed in cell lysates treated with micrococcal nuclease.

For human TNRC6A–C, PABPC1 binding is mediated predominantly by the PAM2 motifs (Figure 9F). In *D. melanogaster* cell lysates, in contrast, the GW182 PAM2 motif is dispensable for PABPC1 binding. Nevertheless, the *D. melanogaster* PAM2 motif contributes to PABPC1 binding, because when it is deleted from a protein lacking the C-term region, PABPC1-binding efficiency decreases further (Figure 2C). Thus, although the human and *D. melanogaster* PAM2 motifs and M2 and C-term regions differ in their contribution to PABPC1-binding *in vivo* (Figure 9F), it is likely that TNRC6s and GW182 form complexes with PABPC1 that are functionally equivalent. Accordingly, human TNRC6CA–C can complement silencing in S2 cells depleted of endogenous DmGW182 (Figure 7; Supplementary Figure S4), indicating that silencing mechanisms are conserved between these organisms.

How does the GW182–PABPC1 interaction contribute to silencing?

Although both Paip1 and Paip2 contain PAM2 motifs, and interact with PABPC1 in a similar manner, they affect translation in opposite ways: Paip1 stimulates translation whereas Paip2 inhibits translation (Kahvejian *et al*, 2001; Khaleghpour *et al*, 2001; Derry *et al*, 2006). Clearly, GW182 proteins are likely to act like Paip2 and interfere with PABPC1 function in translation and/or mRNA stabilization. So, how do GW182

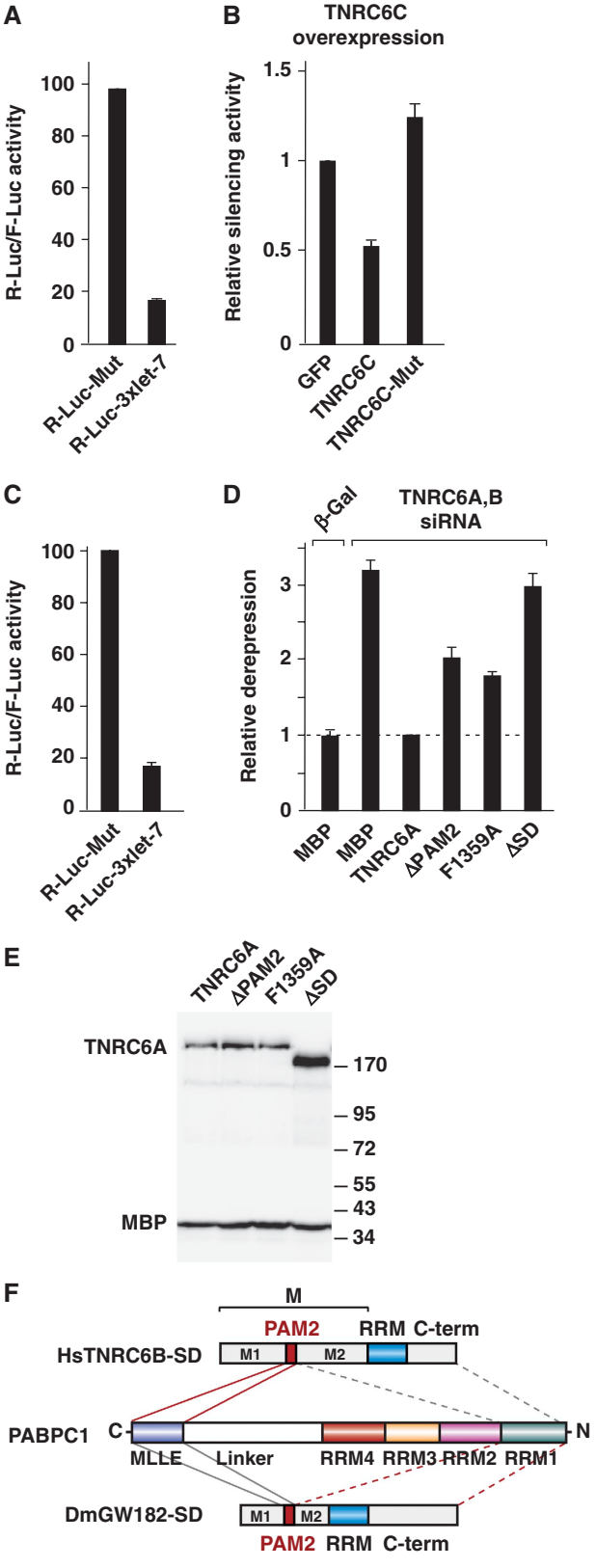

Figure 9 The interaction of TNRC6s with PABPC1 contributes to silencing in human cells. (**A**, **B**) Human HeLa cells were transfected with a mixture of three plasmids: the R-Luc-3xlet-7 or the corresponding reporter lacking let-7-binding sites (R-Luc-Mut), a plasmid expressing firefly luciferase as a transfection control, and plasmids expressing GFP or the indicated GFP-tagged proteins. *Renilla* luciferase activity was normalized to that of the firefly luciferase and set to 100 in cells expressing the reporter lacking the let-7-binding sites for each condition. (**A**) Normalized *Renilla* luciferase activities in control cells (i.e. cells expressing GFP). (**B**) Relative silencing activity for each condition. Mean values ± s.d. are shown. (**C**–**E**) HeLa cells were transfected with a control β-Gal siRNA or a mixture of siRNAs targeting TNRC6A and TNRC6B. Two days later, cells were re-transfected with the same siRNAs and a mixture of three plasmids: the R-Luc-3xlet-7 or the corresponding reporter lacking let-7-binding sites (R-Luc-Mut), a plasmid expressing F-Luc as a transfection control, and plasmids expressing MBP or siRNA-resistant versions of HA-TNRC6A wild type or mutants. Cells were harvested 48 h after the second transfection. *Renilla* luciferase activity was measured and normalized to that of the F-Luc and set to 100 in cells expressing the reporter lacking the let-7-binding sites for each condition. (**C**) Normalized *Renilla* luciferase activities in control cells (i.e. cells treated with β-Gal siRNA and expressing MBP). (**D**) The relative fold derepression for each condition. Mean values ± s.d. are shown. (**E**) Protein expression levels. (**F**) Schematic diagram showing the interaction of HsTNRC6B and DmGW182 silencing domains with the N- and C-terminal domains of PABPC1. Protein domains are as described in Figure 1A. Red lines indicate the dominant interactions observed in cell lysates. Dashed lines indicate interactions observed only in cell lysates but not with recombinant proteins expressed in *E. coli*.

proteins affect PABPC1 function? One possible mechanism is that GW182 proteins prevent mRNA circularization as described previously with Paip2 (Karim *et al*, 2006). This idea is based on the observation that the SD of DmGW182 competes with eukaryotic initiation factor 4G (eIF4G) for binding to PABPC1 in cell lysates (Zekri *et al*, 2009). eIF4G interacts with the N-terminal RRMs of PABPC1; this interaction stimulates translation by enabling the mRNA to adopt a closed-loop conformation (reviewed by Kahvejian *et al*, 2001). Consequently, by interfering with PABPC1–eIF4G interaction, GW182 proteins could inhibit translation. Moreover, when an mRNA is in the open conformation, the 5′ cap and poly(A) tail could be more accessible to the mRNA decay enzymes leading to mRNA degradation.

Another mechanism by which the PABPC1–GW182 interaction could contribute to silencing is by reducing PABPC1 affinity for the poly(A) tail as described for Paip2 (Khaleghpour *et al*, 2001). This could render the poly(A) tail more accessible to deadenylases and thus indirectly interfere with mRNA circularization.

Finally, it is notable that not all proteins containing a PAM2 motif act directly on PABPC1, as shown for Paip1 and Paip2. Other proteins such as human TOB for example, just use PABPC1 as a binding platform that allows them to hook onto mRNAs using a PAM2 motif. TOB also interacts with the CAF1–CCR4–NOT mRNA deadenylase complex, and thus through its interaction with PABPC1, it can promote deadenylation of mRNAs (Ezzeddine *et al*, 2007). Analogously, it is possible that a GW182–PABPC1 complex might provide a binding platform for additional interactions required in silencing; these could include interactions with the CAF1–CCR4–NOT1 deadenylase complex. Indeed, Fabian *et al* (2009) showed that PABPC1 is required for the accelerated deadenylation of miRNA targets observed *in vitro*. Here we show that the role of GW182–PABPC1 interaction is not restricted to promoting deadenylation but rather this interaction is required for silencing independently of whether or not the target is degraded. Thus GW182–PABPC1 interaction may contribute to silencing through multiple mechanisms. Independently of the precise molecular mechanism, our findings indicate that PABPC1 has a crucial function in miRNA-mediated gene silencing in animal cells.

Materials and methods

DNA constructs

Luciferase reporters and plasmids for expression of miRNAs and epitope-tagged proteins were described before (Zekri *et al*, 2009; Eulalio *et al*, 2007, 2008, 2009b). cDNAs encoding human TNRC6A and C were cloned into the *Hin*dIII and *Xho*I site of plasmid pAc5.1-EGFP. Human TNRC6B cDNA was cloned into the *Hin*dIII and *Xba*I sites of plasmid pAc5.1-EGFP. Mutations in DmGW182, DmPABPC1 and human TNRC6A–C were generated by site-directed mutagenesis using the Quick Change mutagenesis kit from Stratagene. The protein GW182-PAM2^{6B} consist of DmGW182 protein sequences in which the PAM2 motif (residues T952–Q971) were replaced with the PAM2 motif of human TNRC6B (residues S1361–Q1380), the corresponding PAM2-motif sequences are shown in Figure 2A). In GW182-SD^{6B} protein sequences downstream of DmGW182 residue N937 were replaced with TNRC6B sequences downstream of residue S1361, which contain the PAM2 motif, the M2 and C-term regions and the RRM.

Complementation and luciferase assays in S2 cells

Complementation assays were performed as described before (Eulalio *et al*, 2008, 2009b). Transfections of S2 cells were performed in 24-well plates, using Effectene transfection reagent (Qiagen). For miRNA-mediated silencing assays, the transfection mixtures contained 0.02 μg of firefly luciferase reporter plasmid, 0.08 μg of the *Renilla* transfection control and 0.04 μg of plasmids expressing miRNA primary transcripts or the corresponding vector without insert. When indicated, 10–200 ng of plasmids expressing recombinant proteins were cotransfected. Firefly and *Renilla* luciferase activities were measured 3 days after transfection using the Dual-Luciferase Reporter Assay System (Promega). Total RNA was isolated using TriFast (Peqlab Biotechnologies) and analysed as described before (Eulalio *et al*, 2007).

Coimmunoprecipitations and western blots in S2 cells

For coimmunoprecipitation assays, S2 cells ($10–12 \times 10^6$ cells) were collected 3 days after transfection, washed with PBS and lysed in 0.5 ml of NET buffer (50 mM Tris at pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% Triton) supplemented with protease inhibitors. Immunoprecipitations were performed as described by Zekri *et al* (2009). When indicated, cell lysates were supplemented with 2.5 mM $CaCl_2$, treated with micrococcal nuclease (NEB, M0247S) for 30 min and spun at 18 000 g for 15 min at 4°C prior to immunoprecipitation. Antibodies to AGO1 (dilution 1:1000) were purchased from Abcam (catalogue number ab5070). Endogenous GW182 and GFP-tagged proteins were detected with polyclonal antibodies raised in rabbits. V5-tagged proteins were detected with anti-V5 antibodies (Invitrogen, dilution 1:5000). All western blot experiments were developed with the ECL Western blotting detection system (GE Healthcare) as recommended by the manufacturer.

Coimmunoprecipitations and western blots in human cells

Plasmids driving the expression of full-length TNRC6A–C or the corresponding SDs in human cells were described by Lazzaretti *et al* (2009). For coimmunoprecipitation assays, HEK-293 cells were grown in 10 cm dishes and transfected using the calcium phosphate method. The transfection mixtures contained 25 μg of plasmid for expression of HA-tagged TNRC6A–C SDs. Two days after transfection, cells were washed with PBS and lysed for 15 min on ice in NET buffer supplemented with protease inhibitors and 10% glycerol (1 ml NET buffer/plate). Cell lysates were treated with RNase A for 30 min and spun at 18 000 g for 15 min at 4°C. Alternatively, cell lysates were supplemented with 2.5 mM $CaCl_2$ and treated with micrococcal nuclease for 30 min. Monoclonal anti-HA antibodies (Covance) were added to the supernatants (dilution 1:200). Samples were incubated for 1 h at 4°C. Then, 25 μl of GammaBind G Sepharose (GE Healthcare) were added and the mixtures were rotated for an additional hour at 4°C. Beads were washed three times with NET buffer. Bound proteins were eluted with 100 μl of protein sample buffer and analysed by western blotting. Endogenous PABP was detected with a polyclonal anti-PABPC1 antibody (Abcam ab21060; dilution 1:3000).

Luciferase assays in human cells

Renilla and firefly luciferase reporters were described before (Pillai *et al*, 2005; Lazzaretti *et al*, 2009). For overexpression assays, human HeLa cells were seeded in six-well plates and transfected using the calcium phosphate method. The transfection mixtures contained 0.05 μg of R-Luc-3xlet-7 reporter plasmid or the corresponding reporter carrying mutations in the let-7-binding sites (R-Luc-Mut), 0.5 μg of the pEGFP-N3-F-Luc transfection control and 3 μg of plasmids expressing GFP or GFP-protein fusions. R-Luc and F-Luc activities were measured 48 h after transfection using the Dual-Luciferase Reporter Assay System (Promega).

For complementation assays, human HeLa cells (0.25×10^6) were seeded in six-well plates in DMEM media without antibiotics (day 0). On day 1, cells were transfected with a mixture of two siRNAs targeting TNRC6A and TNRC6B, respectively (75 pmol of each) using Lipofectamine 2000 transfection reagent. Alternatively, cells were transfected with a control siRNA targeting β-Gal. On day 2, cells are reseeded in 12-well plates at 0.25×10^6 cells per well in DMEM without antibiotics. On day 3, cells are retransfected with the same mixture of siRNAs and three plasmids: one expressing the R-Luc-3xlet-7 reporter (20 ng) or the corresponding reporter carrying mutations in the let-7-binding sites (R-Luc-Mut), one expressing the transfection control (120 ng, pEGFP-N3-F-Luc); and a third plasmid (165 ng) expressing HA-tagged TNRC6A wild type or mutants or MBP. Cells were harvested 48 h after the second

transfection. The following siRNAs were used: TNRC6A 5′-GCCUAA UCUCCGUGCUCAATT-3′; TNRC6B 5′-GGCCUUGUAUUGCCAGCAA TT-3′; β-Gal 5′-CUACACAAAUCAGCGAUUUUU-3′; Dharmacon).

GST pull-down assays

To express the SDs of TNRC6B (amino acids 1361–1723) or DmGW182 (amino acids 937–1384) in *E. coli*, the corresponding cDNAs were cloned into the pGEX6P1 vector (GE healthcare), resulting in N-terminal GST fusions. Deletions and mutations were introduced using the QuikChange mutagenesis kit (Stratagene) and the appropriate oligos. For the MBP-tagged HsPABPC1 or DmPABPC1 constructs, the corresponding cDNAs were cloned into the pETM41 vector, resulting in N-terminal fusions with MBP.

For the GST pull-down assays shown in Figure 4E, 12 g of purified GST, GST-TNRC6B SD or the corresponding ΔPAM2 mutant were added to lysates from *E. coli* cells expressing MBP-tagged HsPABPC1, MBP-DmPABPC1 or the corresponding mutants lacking the MLLE domain in a total volume of 1 ml of binding buffer (10 mM Hepes (pH 7.5), 150 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA and 1% [v/v] Triton-X100). Samples were incubated with 40 μl GST beads (50% slurry) for 1 h at 4°C. The beads were washed three times with 1 ml of binding buffer. The proteins were eluted with 40 μl of sample buffer (50 mM Tris–HCl (pH 6.8), 2% SDS, 10% (v/v) gylcerol, 100 mM DTT and 0.05% bromophenol blue) and analysed on a 10% SDS–PAGE.

For the GST pull-down assays shown in Figure 5, lysates from *E. coli* cells expressing GST, GST-TNRC6B-SD, GST-DmGW182-SD or the indicated deletion mutants, were incubated with 40 μl GST beads (50% slurry) in lysis buffer (10 mM Hepes (pH 7.5), 300 mM NaCl, 5 mM $MgCl_2$ and 1 mM DTT) for 1 h at 4°C. The beads were washed three times with 1 ml of lysis buffer. The pre-coated beads were then incubated with ∼25 μg of recombinant MBP-HsPABPC1 or MBP-DmPABPC1 in a total volume of 1 ml of binding buffer (see above) for 1 h at 4°C. The beads were washed three times with 1 ml of binding buffer. Proteins were eluted with 40 μl of sample buffer and separated on a 10% SDS–PAGE.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

References

Albrecht M, Lengauer T (2004) Survey on the PABC recognition motif PAM2. *Biochem Biophys Res Commun* **316:** 129–138

Behm-Ansmant I, Rehwinkel J, Doerks T, Stark A, Bork P, Izaurralde E (2006) mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. *Genes Dev* **20:** 1885–1898

Behm-Ansmant I, Gatfield D, Rehwinkel J, Hilgers V, Izaurralde E (2007) A conserved role for cytoplasmic poly(A)-binding protein 1 (PABPC1) in nonsense-mediated mRNA decay. *EMBO J* **26:** 1591–1601

Derry MC, Yanagiya A, Martineau Y, Sonenberg N (2006) Regulation of poly(A)-binding protein through PABP-interacting proteins. *Cold Spring Harb Symp Quant Biol* **71:** 537–543

Ding L, Han M (2007) GW182 family proteins are crucial for microRNA-mediated gene silencing. *Trends Cell Biol* **17:** 411–416

Eulalio A, Helms S, Fritzsch C, Fauser M, Izaurralde E (2009b) A C-terminal silencing domain in GW182 is essential for miRNA function. *RNA* **15:** 1067–1077

Eulalio A, Huntzinger E, Izaurralde E (2008) GW182 interaction with Argonaute is essential for miRNA-mediated translational repression and mRNA decay. *Nat Struct Mol Biol* **15:** 346–353

Eulalio A, Rehwinkel J, Stricker M, Huntzinger E, Yang SF, Doerks T, Dorner S, Bork P, Boutros M, Izaurralde E (2007) Target-specific requirements for enhancers of decapping in miRNA-mediated gene silencing. *Genes Dev* **21:** 2558–2570

Eulalio A, Tritschler F, Izaurralde E (2009a) The GW182 protein family in animal cells: new insights into domains required for miRNA mediated gene silencing. *RNA* **15:** 1433–1442

Ezzeddine N, Chang TC, Zhu W, Yamashita A, Chen CY, Zhong Z, Yamashita Y, Zheng D, Shyu AB (2007) Human TOB, an antiproliferative transcription factor, is a poly(A)-binding protein-dependent positive regulator of cytoplasmic mRNA deadenylation. *Mol Cell Biol* **27:** 7791–7801

Fabian MR, Mathonnet G, Sundermeier T, Mathys H, Zipprich JT, Svitkin YV, Rivas F, Jinek M, Wohlschlegel J, Doudna JA, Chen CY, Shyu AB, Yates III JR, Hannon GJ, Filipowicz W, Duchaine TF, Sonenberg N (2009) Mammalian miRNA RISC recruits CAF1 and PABP to affect PABP-dependent deadenylation. *Mol Cell* **35:** 868–880

Jínek M, Fabian MR, Coyle SM, Sonenberg N, Doudna JA (2010) Structural insights into the human GW182-PABC interaction in microRNA-mediated deadenylation. *Nat Struct Mol Biol* **17:** 238–240

Kahvejian A, Roy G, Sonenberg N (2001) The mRNA closed-loop model: the function of PABP and PABP-interacting proteins in mRNA translation. *Cold Spring Harb Symp Quant Biol* **66:** 293–300

Karim MM, Svitkin YV, Kahvejian A, De Crescenzo G, Costa-Mattioli M, Sonenberg N (2006) A mechanism of translational repression by competition of Paip2 with eIF4G for poly(A) binding protein (PABP) binding. *Proc Natl Acad Sci USA* **103:** 9494–9499

Khaleghpour K, Kahvejian A, De Crescenzo G, Roy G, Svitkin YV, Imataka H, O'Connor-McCourt M, Sonenberg N (2001) Dual interactions of the translational repressor Paip2 with poly(A) binding protein. *Mol Cell Biol* **21:** 5200–5213

Kozlov G, De Crescenzo G, Lim NS, Siddiqui N, Fantus D, Kahvejian A, Trempe JF, Elias D, Ekiel I, Sonenberg N, O'Connor-McCourt M, Gehring K (2004) Structural basis of ligand recognition by PABC, a highly specific peptide-binding domain found in poly(A)-binding protein and a HECT ubiquitin ligase. *EMBO J* **23:** 272–281

Kozlov G, Ménade M, Rosenauer A, Nguyen L, Gehring K (2010a) Molecular determinants of PAM2 recognition by the MLLE domain of poly(A)-binding protein. *J Mol Biol* **397:** 397–407

Kozlov G, Safaee N, Rosenauer A, Gehring K (2010b) Structural basis of binding of P-body associated protein GW182 and Ataxin-2 by the MLLE domain of poly(A)-binding protein. *J Biol Chem* **285:** 13599–13606

Lazzaretti D, Tournier I, Izaurralde E (2009) The C-terminal domains of human TNRC6A, B and C silence bound transcripts independently of the Argonaute proteins. *RNA* **15:** 1059–1066

Pillai RS, Bhattacharyya SN, Artus CG, Zoller T, Cougot N, Basyuk E, Bertrand E, Filipowicz W (2005) Inhibition of translational initiation by Let-7 MicroRNA in human cells. *Science* **309:** 1573–1576

Roy G, De Crescenzo G, Khaleghpour K, Kahvejian A, O'Connor-McCourt M, Sonenberg N (2002) Paip1 interacts with poly(A) binding protein through two independent binding motifs. *Mol Cell Biol* **22:** 3769–3782

Takimoto K, Wakiyama M, Yokoyama S (2009) Mammalian GW182 contains multiple Argonaute binding sites and functions in microRNA-mediated translational repression. *RNA* **15:** 1078–1089

Till S, Lejeune E, Thermann R, Bortfeld M, Hothorn M, Enderle D, Heinrich C, Hentze MW, Ladurner AG (2007) A conserved motif in Argonaute-interacting proteins mediates functional interactions through the Argonaute PIWI domain. *Nat Struct Mol Biol* **14:** 897–903

Tritschler F, Huntzinger E, Izaurralde E (2010) Role of GW182 proteins and PABPC1 in the miRNA pathway: a sense of déjà vu. *Nat Rev Mol Cell Biol* **11:** 379–384

Walters RW, Bradrick SS, Gromeier M (2010) Poly(A)-binding protein modulates mRNA susceptibility to cap-dependent miRNA-mediated repression. *RNA* **16:** 239–250

Zekri L, Huntzinger E, Heimstädt S, Izaurralde E (2009) The silencing domain of GW182 interacts with PABPC1 to promote translational repression and degradation of miRNA targets and is required for target release. *Mol Cell Biol* **29:** 6220–6231

Zipprich JT, Bhattacharyya S, Mathys H, Filipowicz W (2009) Importance of the C-terminal domain of the human GW182 protein TNRC6C for translational repression. *RNA* **15:** 781–793

