Supplementary Data

GW182 proteins cause PABP dissociation from silenced miRNA targets in the absence of deadenylation

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Supplemental Materials and Methods

Plasmids

Luciferase reporters and plasmids for the expression of miRNAs and HA-tagged proteins in *D. melanogaster* cells (GST, AGO1, GW182, GW182-SD, PABP, NOT1, POP2 and PAN3) were described elsewhere (Zekri et al, 2009; Huntzinger et al, 2010; Braun et al, 2011). Plasmids for the expression of HA-tagged *Dm* eIF4E and eIF4G were obtained by inserting the eIF4E (CG4035) and eIF4G (CG10811) ORFs into the *EcoR*I-*Not*I and *Not*I-*Xba*I sites, respectively, of the pAc5.1-λN-HA vector. GST was cloned into the *EcoR*I-*Xho*I sites of the pAc5.1-λN-HA-V5 vector. The F-Luc-5BoxB-$(A)_{95}$-HhR was generated by site-directed mutagenesis using the Stratagene Quickchange mutagenesis kit and the F-Luc-5BoxB-HhR reporter as template (Eulalio et al, 2008). The internal poly(A) stretch of 95 nucleotides and a poly(C) stretch of 7 nucleotides were consecutively inserted immediately upstream of the hammerhead ribozyme cleavage. To generate the F-Luc-$(A)_{93}$-5BoxB-HhR reporter, an internal poly(A) stretch of 93 nucleotides was inserted between the *Avr*II and *Fse*I restrictions sites of the F-Luc-5BoxB-HhR reporter upstream of the Box B hairpins. A poly(C) stretch of 7 nucleotides was inserted by site-directed mutagenesis immediately upstream of the hammerhead ribozyme cleavage site. To generate the F-Luc-5BoxB-6MS2-HhR reporter, six MS2 binding sites instead of the poly(A) stretch were inserted by site directed mutagenesis into the F-Luc-5BoxB-$(A)_{95}$-HhR. Plasmids for the expression of MS2-GST and MS2-PABP were derived from pAc5.1-λN-HA constructs in which the λN-HA tag was replaced with the MS2 protein. Plasmids for the expression of HA-tagged human TNRC6C silencing domain were obtained by inserting the TNRC6C-SD cDNA into the *Not*I-*Xba*I sites of the pAc5.1-λN-HA vector. Mutations in the PAM2, CIM-1 and CIM-2 motifs were introduced by

site-directed mutagenesis using the Stratagene Quickchange mutagenesis kit. Mutations in the CIM-1 and CIM-2 motifs of TNRC6C-SD were as previously described (Fabian et al, 2011). The PAM2 mutant carries an alanine substitution of F1389 in the TNRC6C-SD PAM2 motif.

Antibodies and western blotting

Polyclonal anti-eIF4E, eIF4G and PABP antibodies were generated by immunizing of rabbits with purified recombinant *Dm* eIF4E (full-length), eIF4G (amino acids 942–1208) and PABP (amino acids 501–634). HA-tagged proteins were immunoprecipitated using monoclonal anti-HA antibodies (Covance, 1:200 dilution and detected by western blotting using an HRP-conjugated monoclonal anti-HA antibody from Roche (3F10, dilution 1:5,000). GFP-tagged proteins were immunoprecipitated using polyclonal antibodies raised in rabbits and detected by western blotting using monoclonal HRP-conjugated anti-GFP antibodies (Roche 11814460001; 1:2,000). For western blotting antibodies were used at the following dilutions: eIF4E (1:3,000), eIF4G (1:3,000), PABP (1:10,000), DCP1 (1:2,000), EDC4 [1:500; (Eulalio et al, 2007)] and GW182 [1:2,000; (Behm-Ansmant et al, 2006)]. All western blotting experiments were developed with the ECL western blotting detection system (GE Healthcare) as recommended by the manufacturer.

Transfections and luciferase assays

Transfections were performed in 6-well plates using the Effectene transfection reagent (Qiagen). For the miRNA reporter assays, the transfection mixtures contained 0.3 μg of a Firefly luciferase reporter plasmid, 0.4 μg of a *Renilla* luciferase transfection control and 0.3 μg of a plasmid expressing the miRNA primary transcript or the

corresponding vector without an insert. When indicated, plasmids expressing HA tagged proteins were cotransfected (0.3 µg for GST, eIF4E and PABP; 0.5 µg for AGO1 and eIF4G). In the experiment shown in Figure 2, 1 µg of plasmid encoding PABP-V5 was included. A plasmid encoding GST-V5 served as a negative control. For the λN-tethering assay, S2 cells were co-transfected with the following plasmids: 25 ng of F-Luc-5BoxB reporter or 0.1 µg of F-Luc-BoxB-$(A)_{95}$-HhR, 0.4 µg pAc5.1C-R-Luc-V5, and plasmids expressing various λN-HA-tagged proteins. When necessary, the total amount of transfected DNA was adjusted to 1.5 µg using the pAc5.1A plasmid lacking an insert. For the experiment shown in Figure 8A–F, S2 cells were co-transfected with the following plasmids: 1 µg of F-Luc-5BoxB-HhR reporter or 75 ng of F-Luc-5BoxB-$(A)_{95}$-HhR, 0.4 µg pAc5.1C-R-Luc-V5, 10 ng of plasmids expressing either λN-HA or λN-HA-TNRC6C-SD. For the tethering assay with the MS2 reporter, S2 cells were co-transfected with the following plasmids: 200 ng of F-Luc-5BoxB-6MS2-HhR reporter or 75 ng of F-Luc-5BoxB-$(A)_{95}$-HhR, 0.4 µg pAc5.1C-R-Luc-V5, 5 ng of plasmids expressing either λN-HA or λN-HA-GW182 and 5 ng of plasmids expressing either MS2-GST or MS2-PABP-V5. For the experiment shown in Figure 9, 0.5 µg of F-Luc-$(A)_{93}$-5BoxB-HhR were transfected. The amounts of plasmids expressing λN-HA-tagged proteins were as follows: 2 to 5 ng for the λN-HA or λN-HA-GW182, 10 ng for AGO1-F2V2, NOT1, POP2 and TNRC6C-SD, and 50 ng for PAN3. For the tethering assay with the MS2 reporter, S2 cells were co-transfected with the following plasmids: 200 ng of F-Luc-5BoxB-6MS2-HhR reporter or 75 ng of F-Luc-5BoxB-$(A)_{95}$-HhR, 0.4 µg pAc5.1C-R-Luc-V5, 5 ng of plasmids expressing either λN-HA or λN-HA-GW182, and 5 ng of plasmids expressing either MS2-GST or MS2-PABP-V5.

Supplementary Figure legends

Supplementary Figure S1 (**A**) Western blots showing the expression levels of HA-tagged proteins (marked with asterisks) relative to the corresponding endogenous proteins. HA-eIF4G migrates faster than endogenous eIF4G. (**B**) The efficiency of protein immunoprecipitation in the absence or presence of miR-279 was analyzed using western blotting. (**C, D** and **E**) Western blot showing similar immunoprecipitation of endogenous eIF4E, eIF4G and PABP in the absence or presence of miR-9b. The samples correspond to the experiment shown in Figures 1G–I.

Supplementary Figure S2 GW182 depletion restores the association of eIF4E, eIF4G and PABP with the miRNA reporter. (**A–E**) S2 cells depleted of GW182 (KD) or treated with GFP dsRNA (control cells) were cotransfected with a mixture of four plasmids: one expressing a F-Luc-Nerfin-1 reporter, another expressing the miR-9b primary transcript or the corresponding empty vector (-), a third expressing various HA-tagged proteins, and a fourth expressing *Renilla* luciferase (R-Luc). (A) Firefly luciferase activity was normalized to that of the *Renilla* luciferase. For each condition (control or knockdown cells), the normalized values of F-Luc activity were set at 100 in the absence of miR-9b (white bars). (B) Northern blot of representative RNA samples. Numbers below the panels indicate F-Luc-Nerfin-1 mRNA levels normalized to the R-Luc transfection control and set at 100 in the absence of miR-9b. (C) Western blot showing the efficiency of GW182 knockdown. (D and E) The association of the F-Luc-Nerfin-1 reporter with the indicated HA-tagged proteins was analyzed by coimmunoprecipitation as described in Figure 1.

Supplementary Figure S3 eIF4E binding to a silenced miRNA target is restored in cells depleted of decapping factors. (**A**) The efficiency of DCP1 and EDC4 knockdowns shown in Figure 3 were confirmed using western blotting. (**B**) Western blots showing that the expression of endogenous eIF4G, eIF4E and PABP were not affected in depleted cells. Endogenous tubulin served as a loading control. (**C** and **D**) The association of the F-Luc-Nerfin-1 reporter with the indicated HA-tagged proteins in the absence of miRNA (white bars) or in the presence of miR-9b (blue bars) was analyzed in control cells as described in Figure 3. The samples correspond to the control cells from the experiment described in Figure 3 and Supplementary Figure S4.

Supplementary Figure S4 eIF4E binding to a silenced miRNA target is restored in cells depleted of Me31B and EDC4. (**A–D**) Control S2 cells (treated with GFP dsRNA) or cells codepleted of Me31B and EDC4 were cotransfected with a mixture of four plasmids as described in Figure S2. (A) Firefly luciferase activity was normalized to that of the *Renilla* luciferase. For each condition (control and knockdown cells, KD), the normalized values of F-Luc activity were set at 100 in the absence of miR-9b (white bars). (B) Northern blot of representative RNA samples. Note that the Me31B and EDC4 knockdowns shown in this figure were performed in parallel with the DCP1 and EDC4 knockdowns shown in Figure 3 and Supplementary Figure S3. Therefore, the control samples (cells treated with GFP dsRNA) are common to both experiments and are shown in Supplementary Figure S3. (C and D) The association of the F-Luc-Nerfin-1 reporter with the indicated HA-tagged proteins in the absence of miRNA (white bars) or in the presence of miR-9b (blue bars) was analyzed in knockdown cells as described in Figure 3.

Supplementary Figure S5 GW182 tethering causes PABP dissociation. (**A–C**) S2 cells were transfected with a mixture of three plasmids: one expressing the F-Luc-5BoxB-$(A)_{95}$-HhR reporter, another expressing the indicated λN-HA-tagged proteins, and a third plasmid expressing *Renilla* luciferase (R-Luc). (A) Firefly luciferase activity was normalized to that of the *Renilla* luciferase and set at 100 in cells expressing the λN-HA peptide (white bar). (B) Northern blot of representative RNA samples. Numbers below the panel indicate the levels of the F-Luc-5BoxB-$(A)_{95}$-HhR reporter normalized to that of R-Luc mRNA and set at 100 in cells expressing the λN-HA peptide. (C) The association of the F-Luc-5BoxB-$(A)_{95}$-HhR reporter with endogenous PABP in cells expressing the λN-HA peptide (white bars) or the indicated proteins (colored bars) was analyzed as described in Figure 1.

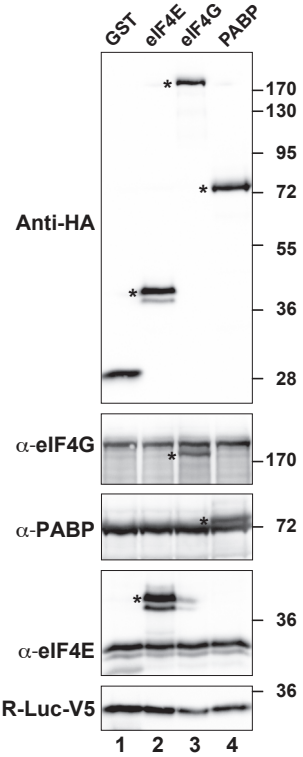
Supplementary Figure S6 Contribution of PABP dissociation to silencing. (**A, B**) S2 cells were transfected with a mixture of two plasmids: one expressing either the F-Luc-5BoxB-$(A)_{95}$-HhR reporter [BoxB-$(A)_{95}$, 100 ng] or the F-Luc-5BoxB-HhR reporter (BoxB-$(A)_0$, 100 and 600 ng), and another expressing *Renilla* luciferase (R-Luc). (A) Relative luciferase activity for each reporter. Firefly luciferase activities were normalized to those of the *Renilla* luciferase for each reporter and set at 100 for the F-Luc-5BoxB-$(A)_{95}$-HhR reporter. (B) Northern blot of the corresponding RNA samples. Numbers below the panel indicate the levels of the F-Luc reporters normalized to those of R-Luc mRNA and set at 100 for the F-Luc-5BoxB-A_{95}-HhR reporter. Note that there is a very good correlation between F-Luc activity and mRNA levels. (**C, D**) S2 cells were transfected with a mixture of three plasmids: one expressing either the F-Luc-5BoxB-$(A)_{95}$-HhR reporter (200 ng) or the F-Luc-5BoxB-

HhR reporter (1 µg), another expressing either λN-HA or λN-HA-GW182, and a third plasmid expressing *Renilla* luciferase (R-Luc). (C) Relative luciferase activity for each reporter in the absence of λN-HA-GW182. Firefly luciferase activities were normalized to those of the *Renilla* luciferase and set at 100 for the F-Luc-5BoxB-HhR reporter. (**D**) Relative luciferase activity of the reporters in the absence or presence of λN-HA-GW182. Firefly luciferase activities were normalized to those of the *Renilla* luciferase for each reporter and set at 100 in cells expressing the λN-HA peptide (white bars). (**E–G**) S2 cells were transfected with a mixture of four plasmids: one expressing the F-Luc-5BoxB-6MS2-HhR reporter, another expressing either λN-HA or λN-HA-GW182, a third plasmid expressing *Renilla* luciferase (R-Luc), and a fourth plasmid expressing either MS2-GST or MS2-PABP. (E) Relative translation efficiency of the F-Luc-5BoxB-6MS2-HhR reporter in the presence of MS2-GST or MS2-PABP in control cells (in the absence of GW182). Firefly luciferase activities were normalized to those of the *Renilla* luciferase and set at 100 in cells expressing MS2-GST. (F) Northern blot of representative RNA samples. Numbers below the panel indicate the levels of the F-Luc-5BoxB-6MS2-HhR reporter normalized to those of R-Luc mRNA and set at 100 in cells expressing the λN-HA peptide and MS2-GST. (G) Relative luciferase activity for each condition (cells expressing MS2-GST or MS2-PABP) in the absence or presence of λN-HA-GW182. Firefly luciferase activities were normalized to those of the *Renilla* luciferase for each condition and set at 100 in cells expressing the λN-HA peptide (white bars). (**H** and **I**) S2 cells were transfected with a mixture of three plasmids: one expressing either the F-Luc-5BoxB-HhR or the F-Luc-5BoxB-A_n-HhR (wherein n represents 26, 51, 74 or 95 residues) reporters, another expressing either λN-HA or λN-HA-TNRC6C-SD, a third plasmid expressing *Renilla* luciferase (R-Luc). For the F-Luc reporters the following amounts

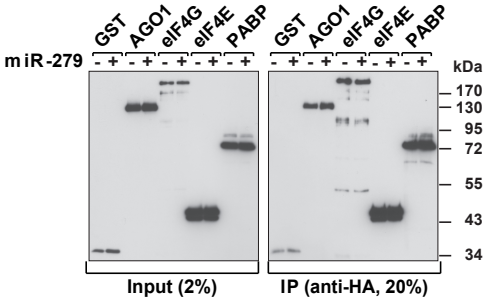
of plasmid were transfected: 1 µg of 5BoxB-$(A)_0$, 200 ng of 5BoxB-$(A)_{26}$ and 5BoxB-$(A)_{51}$, 100 ng of 5BoxB-$(A)_{74}$ and 75 ng of 5BoxB-$(A)_{95}$. (H) Relative luciferase activity of the reporters in the absence of λN-HA-GW182. (I) Relative luciferase activity of the reporters in the absence or presence of λN-HA-TNRC6C-SD. Firefly luciferase activities were normalized to those of the *Renilla* luciferase for each reporter and set at 100 in cells expressing the λN-HA peptide (white bar). (**J**) S2 cells were transfected with a mixture of two plasmids: one expressing either the F-Luc-5BoxB-HhR [5BoxB-$(A)_0$] or the F-Luc-$(A)_{93}$-5BoxB-HhR [$(A)_{93}$-5BoxB] reporters (200 ng of plasmid for each reporter), and another expressing *Renilla* luciferase (R-Luc). Firefly luciferase activities were normalized to those of the *Renilla* luciferase for each reporter and set at 100 for the reporter lacking the internal poly(A).

Zekri et al. Supplementary Figure S1

A Expression of HA-tagged proteins



B Western blot IP HA-tagged proteins



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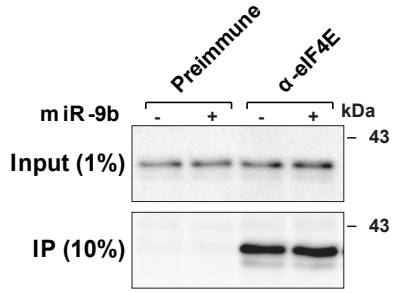

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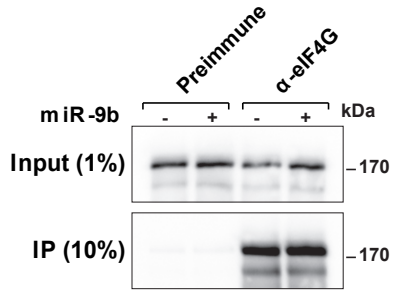
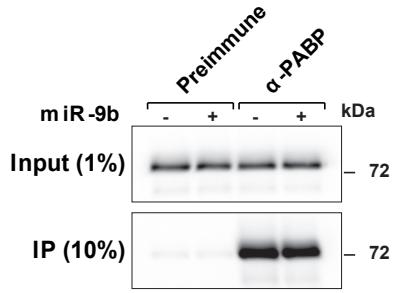
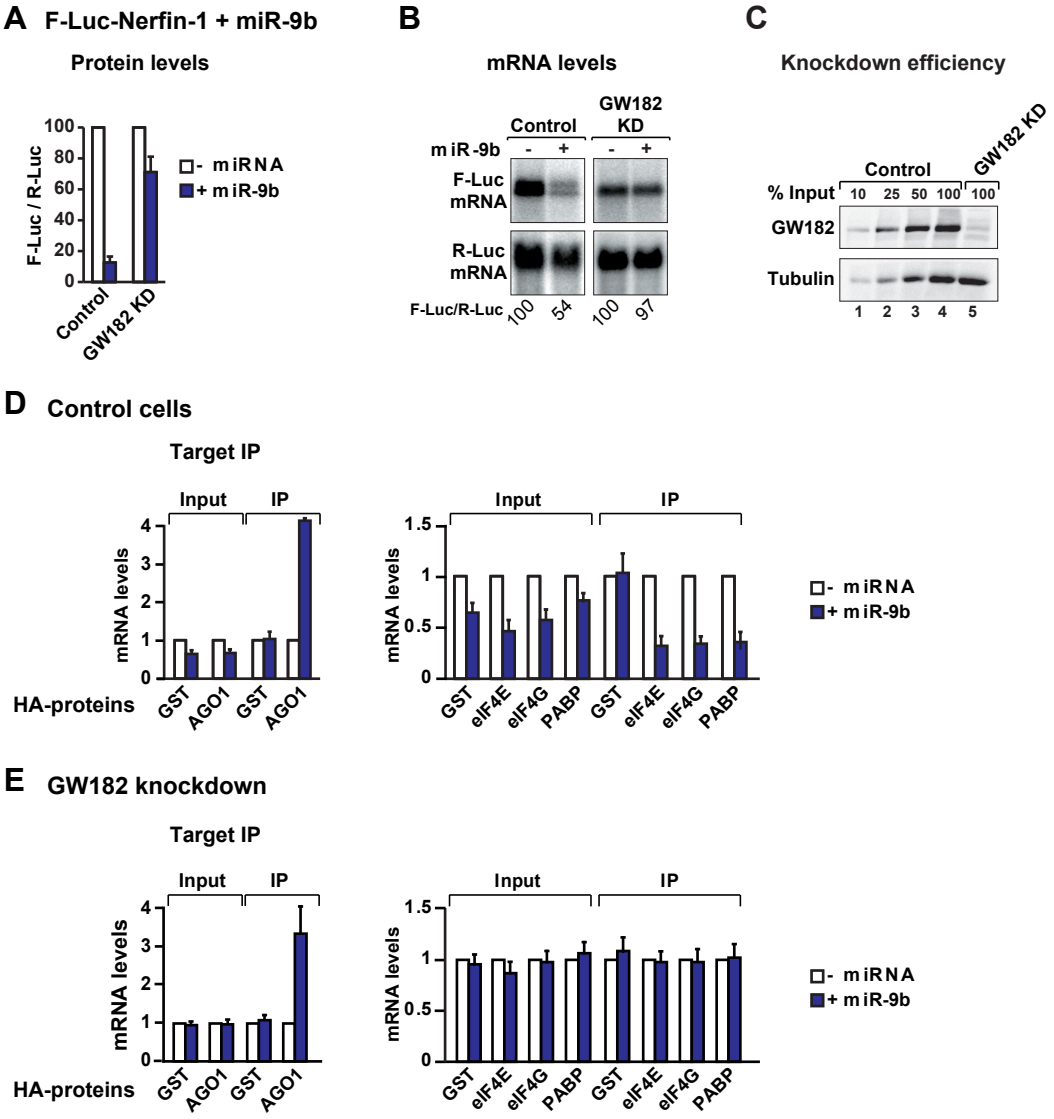


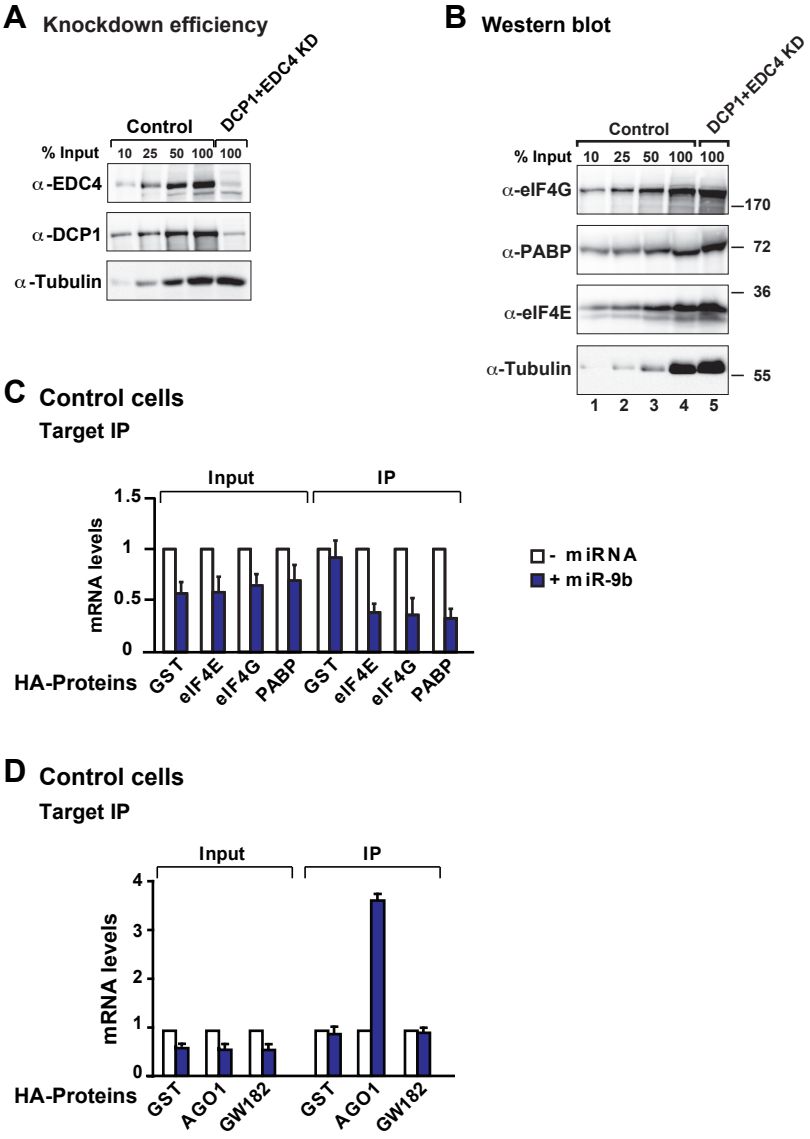
E Endogenous PABP



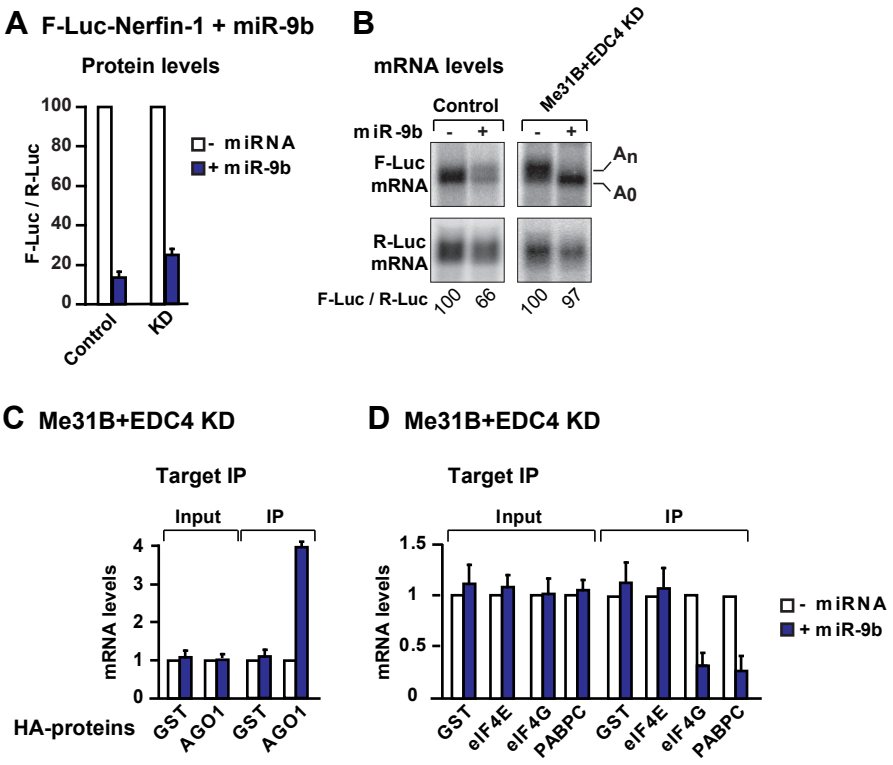
Zekri et al. Supplementary Figure S2



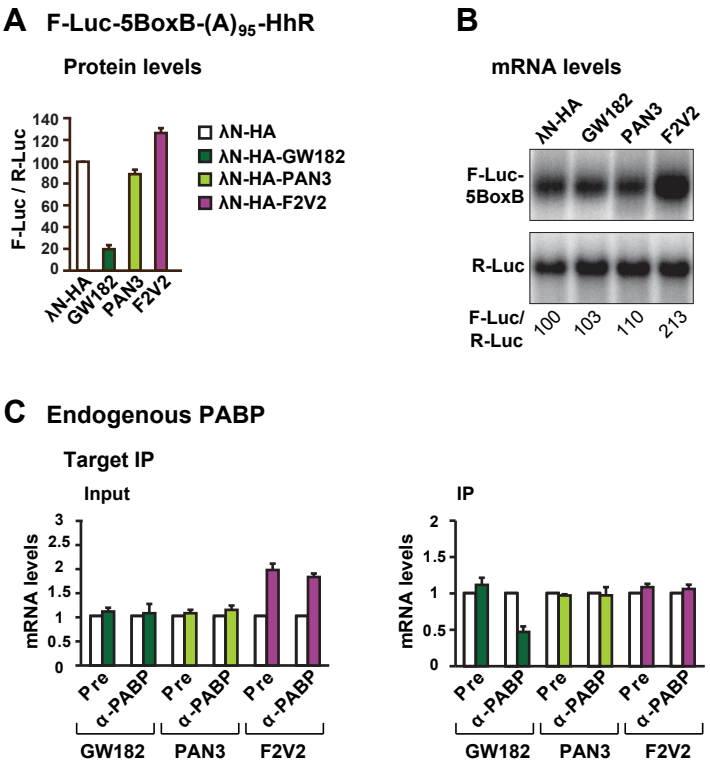
Zekri et al. Supplementary Figure S3



Zekri et al. Supplementary Figure S4



Zekri et al. Supplementary Figure S5



Zekri et al. Supplementary Figure S6