Figure S1

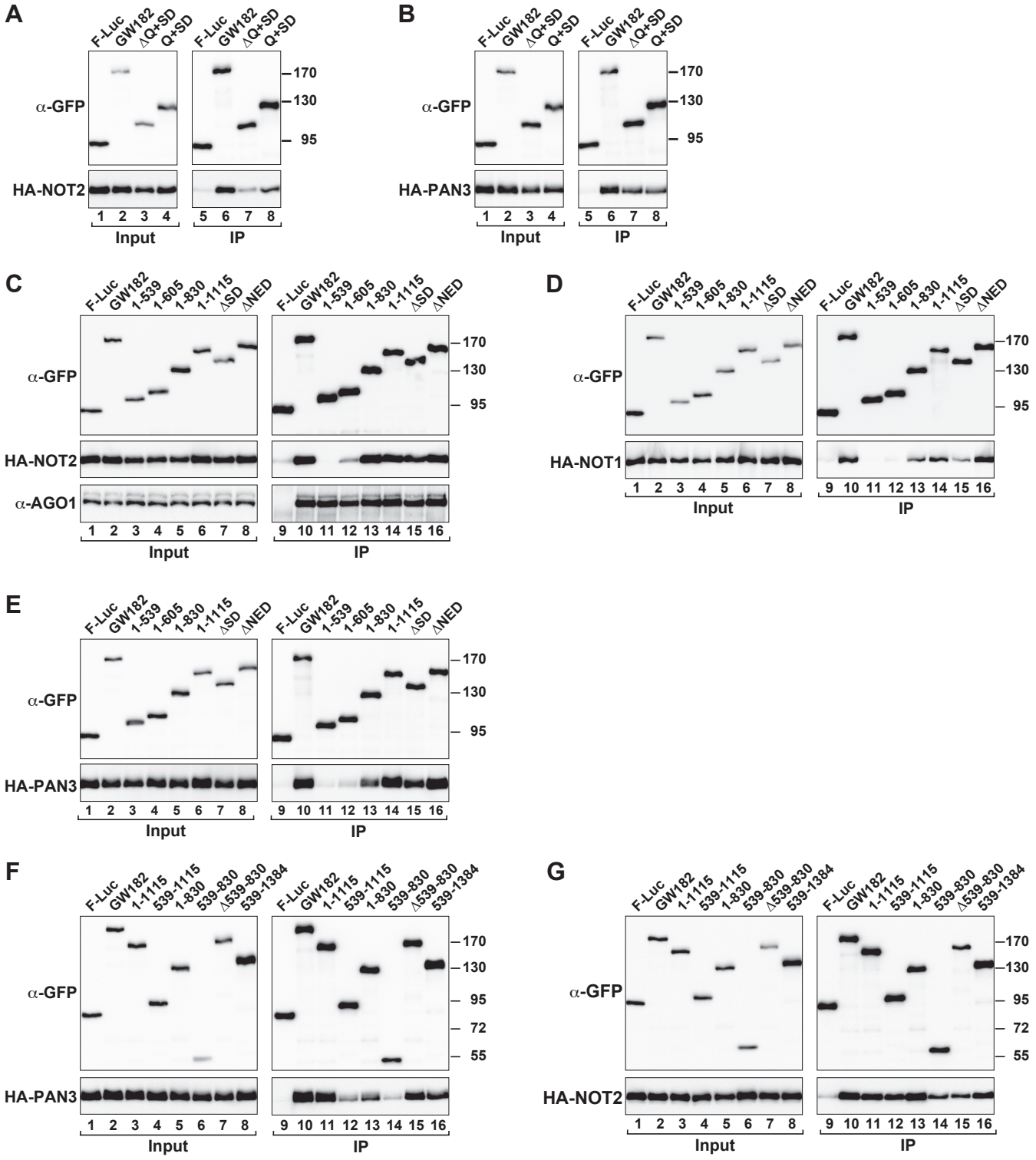


Figure S1. Interaction of *Dm* GW182 N-term fragments with NOT1, NOT2 and PAN3. (**A**–**G**) The interactions between GFP-tagged wild-type *Dm* GW182 or the indicated protein fragments and HA-tagged deadenylation factors were analyzed as described in Figure 2. In panel C, the presence of endogenous *Dm* AGO1 in the immunoprecipitates was analyzed using a specific anti-AGO1 antibody.

Figure S2

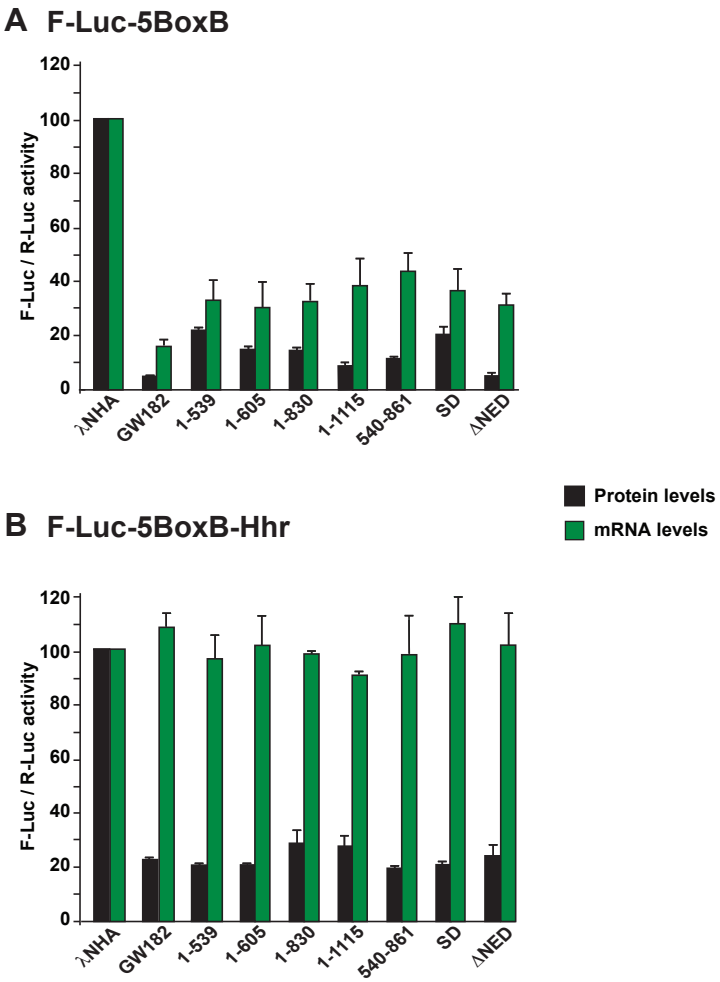


Figure S2. Activity of GW182 protein fragments in tethering assays. (**A**) S2 cells were transfected with a mixture of three plasmids: one expressing the F-Luc-5BoxB reporter; another expressing the λN-HA peptide or λN-HA-tagged *Dm* GW182 (full-length or fragments); and a third expressing *Renilla* luciferase (R-Luc). Firefly luciferase activity and mRNA levels were normalized to those of the *Renilla* luciferase control. For each condition, the normalized values of F-Luc activity and mRNA levels were set at 100% in cells expressing the λN-HA peptide. Mean values ± standard deviations from three independent experiments are shown. (**B**) The experiment shown in panel A was repeated using the F-Luc-5BoxB-Hhr reporter in which the polyadenylation signal was substituted by a self-cleavable hammerhead ribozyme (Hhr). A western blot showing equivalent expression levels for all protein fragments is shown in Figure 3I.

Figure S3

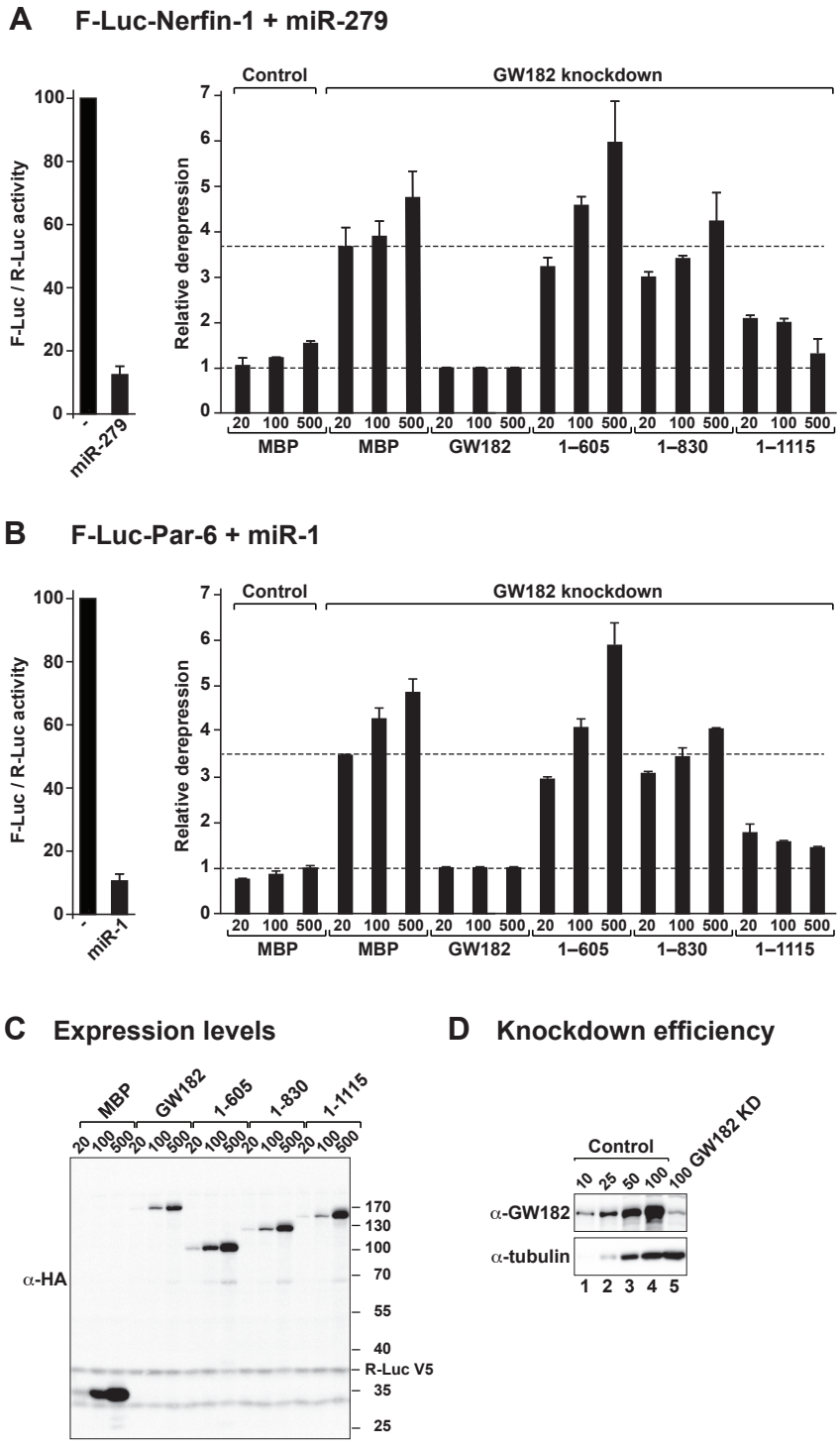


Figure S3. The *Dm* GW182 SD is generally required for silencing. Complementation assays using the indicated miRNA reporters were carried out as described in Figure 3. The graphs on the left of each panel show normalized firefly luciferase activities in the absence or presence of miRNAs in control cells (i.e., cells treated with GFP dsRNA and transfected with MBP). The graphs on the right show the relative derepression of the F-Luc reporters for each condition. Mean values ± standard deviations from three independent experiments are shown. (**A, B**) Complementation assays using the F-Luc-Nerfin-1 and F-Luc-Par-6 reporters and increasing amounts of the indicated GW182 N-term fragments. (**C**) A western blot showing the expression levels of full-length GW182 and N-term fragments. (**D**) Western blot showing the efficiency of the GW182 knockdown. Dilutions of control cell lysates were loaded in lanes 1–4 to estimate the efficacy of the depletion. α-tubulin served as a loading control.

Figure S4

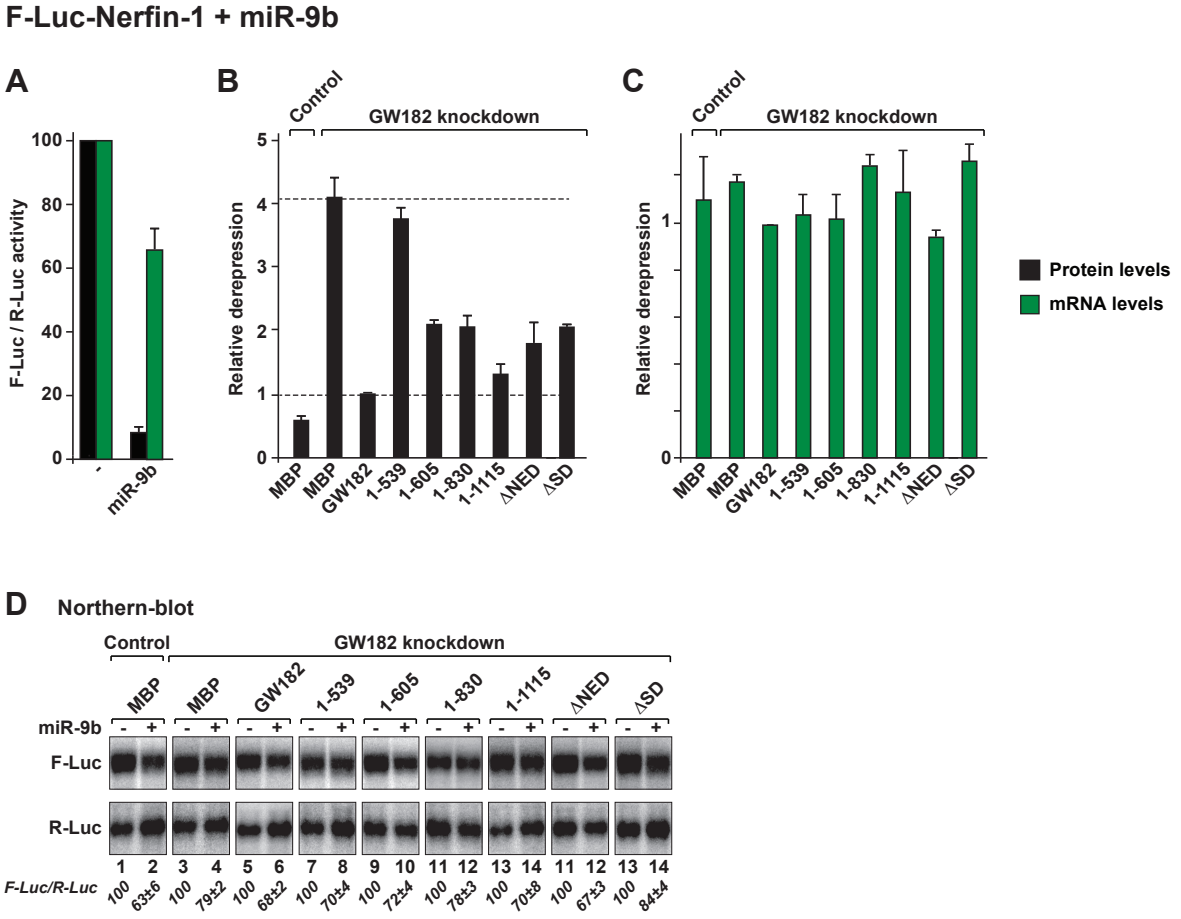


Figure S4. The *Dm* GW182 N-terminal fragments complement silencing of the F-Luc-Nerfin reporter silenced by miR-9b. (**A–D**) A complementation assay using the F-Luc-Nerfin reporter silenced by miR-9b was carried out as described in Figure 3. Numbers in italics below panel (D) indicate the levels of the F-Luc-Nerfin reporter normalized to that of R-Luc mRNA and set at 100 in the absence of miR-9b.

Figure S5

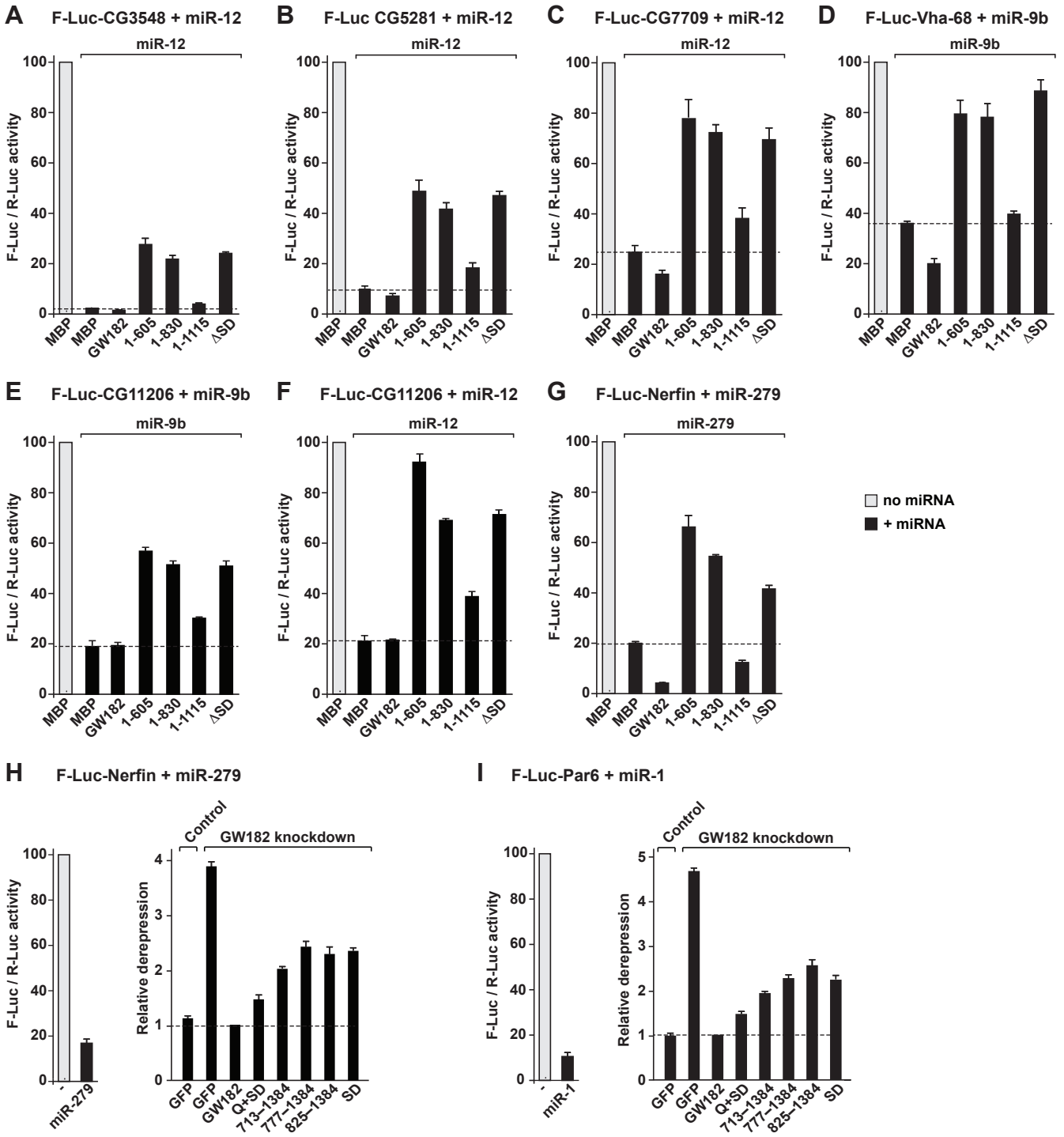
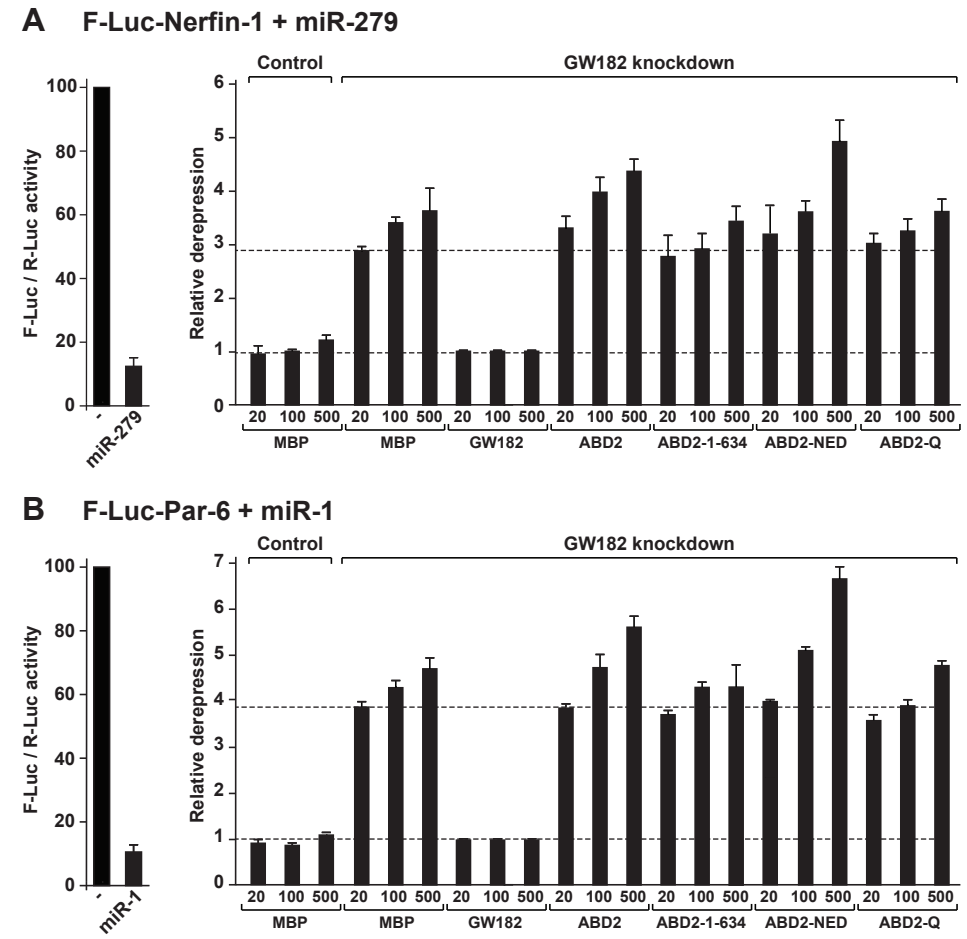


Figure S5. GW182 N-term fragments generally inhibit silencing in a dominant negative manner in control cells. The effects of expressing *Dm* GW182 N-term protein fragments on silencing of the indicated reporters were analyzed in control cells. The corresponding experiment, which was performed in parallel in depleted cells is shown in Figure 4. (**A–G**) Normalized F-Luc activities in cells in the absence or presence of miRNAs. Cells were co-transfected with plasmids expressing MBP or *Dm* GW182 (wild-type or mutant) as indicated. For some reporters, overexpression of wild-type GW182 enhances silencing (panels C, D and G). For all reporters, overexpression of *Dm* GW182 N-term fragments (1–605, 1–830 and ΔSD) inhibits silencing in a dominant negative manner. Mean values ± standard deviations from three independent experiments rare shown. (**H, I**) Complementation assays using the indicated miRNA reporters were carried out as described in Figure 5. The graphs on the left of each panel show normalized firefly luciferase activities in the absence or presence of miRNAs in control cells. The graphs on the right of each panel show the relative derepression of the F-Luc reporters for each condition.

Figure S6



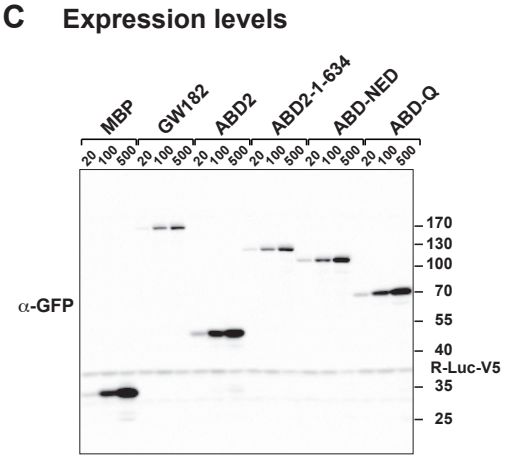
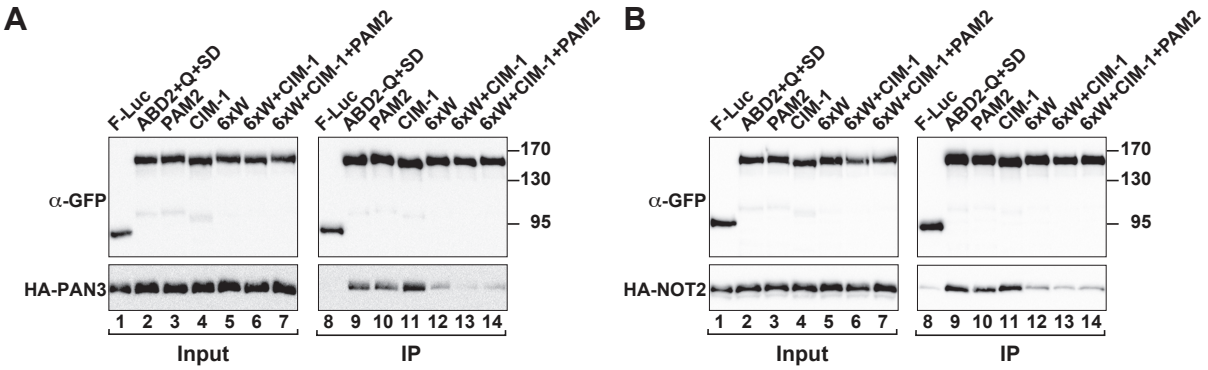


Figure S6. A chimeric GW182 protein containing the Q+SD region rescues silencing in cells depleted of *Dm* GW182. (**A, B**) Complementation assays using the F-Luc-Nerfin-1 and F-Luc-Par-6 reporters and increasing amounts of the indicated proteins were carried out as described in Figure 3. Mean values ± standard deviations from three independent experiments are shown. Labels are as described in Figure 3. (**C**) A western blot showing the expression levels of the proteins tested in panels A and B.

Figure S7



C PAM2 motif

Hs TNRC6A	1352	SVN--WPPEFRPGEPWKGYP	1369
Hs TNRC6B	1361	SSNASWPPEFQPGVPWKGIQ	1380
Hs TNRC6C	1382	SIN--WPPEFHPGVPWKGLQ	1399
Dr TNRC6A	1362	NVN--WPPEFRPGEPWKGYP	1379
Dm GW182	952	TAYTDLVQEFEPGKPWKGSQ	971

D CCR4-interacting motif 1 (CIM-1)

Hs TNRC6A	1276	KEP---QSRLRKWTTVDSIS	1292
Hs TNRC6B	1277	GKEAGTESRFKQWTSMMEGL	1296
Hs TNRC6C	1288	KDPSQSQSRLPQWTHPNSMD	1307
Dr TNRC6A	1286	KEP---QSRLKKWTAMDISV	1302
Dm GW182	863	QGASNQQSRLNQWKLPVLDK	882

E CCR4-interacting motif 2 (CIM-2)

Hs TNRC6A	1661	HGTSLWG-TPHYSTSLWGPP	1679
Hs TNRC6B	1678	AGASLWG-PPNYSSSLWGVP	1696
Hs TNRC6C	1643	SSELLWGGVPQYSSSLWGPP	1662
Dr TNRC6A	1659	HGSSLWG-VPNYSTSLWGSP	1677

F P-GL motif

Hs TNRC6A	1473	NITAPSR-PPPGLTGQKPPL	1491
Hs TNRC6B	1484	NTTPLPR-PPPGLTNPKPSS	1502
Hs TNRC6C	1467	NSTAPTR-PPPGLTNPKPSS	1485
Dr TNRC6A	1481	GISAPSR-PPPGLTGQKQPS	1499
Dm GW182	1055	LNKSSSRGPPPGLTANSNKS	1074

Figure S7. Conserved motifs in GW182 family proteins. (**A, B**) The interactions of the chimeric ABD2-Q+SD protein (wild-type or mutants) with PAN3 and NOT2 were analyzed as described in Figure 2. (**C–F**) Sequence alignment of the conserved PAM2 (C), CIM-1 (D), CIM-2 (E) and PG-L (F) motifs from *H. sapiens* (*Hs*) TNRC6A–C, *Danio rerio* (*Dr*) TNRC6A and *D. melanogaster* (*Dm*) GW182. Conserved residues are shown in red. Residues substituted with alanines in this study are indicated by asterisks. CIM-2 is absent in *Dm* GW182.

Figure S8

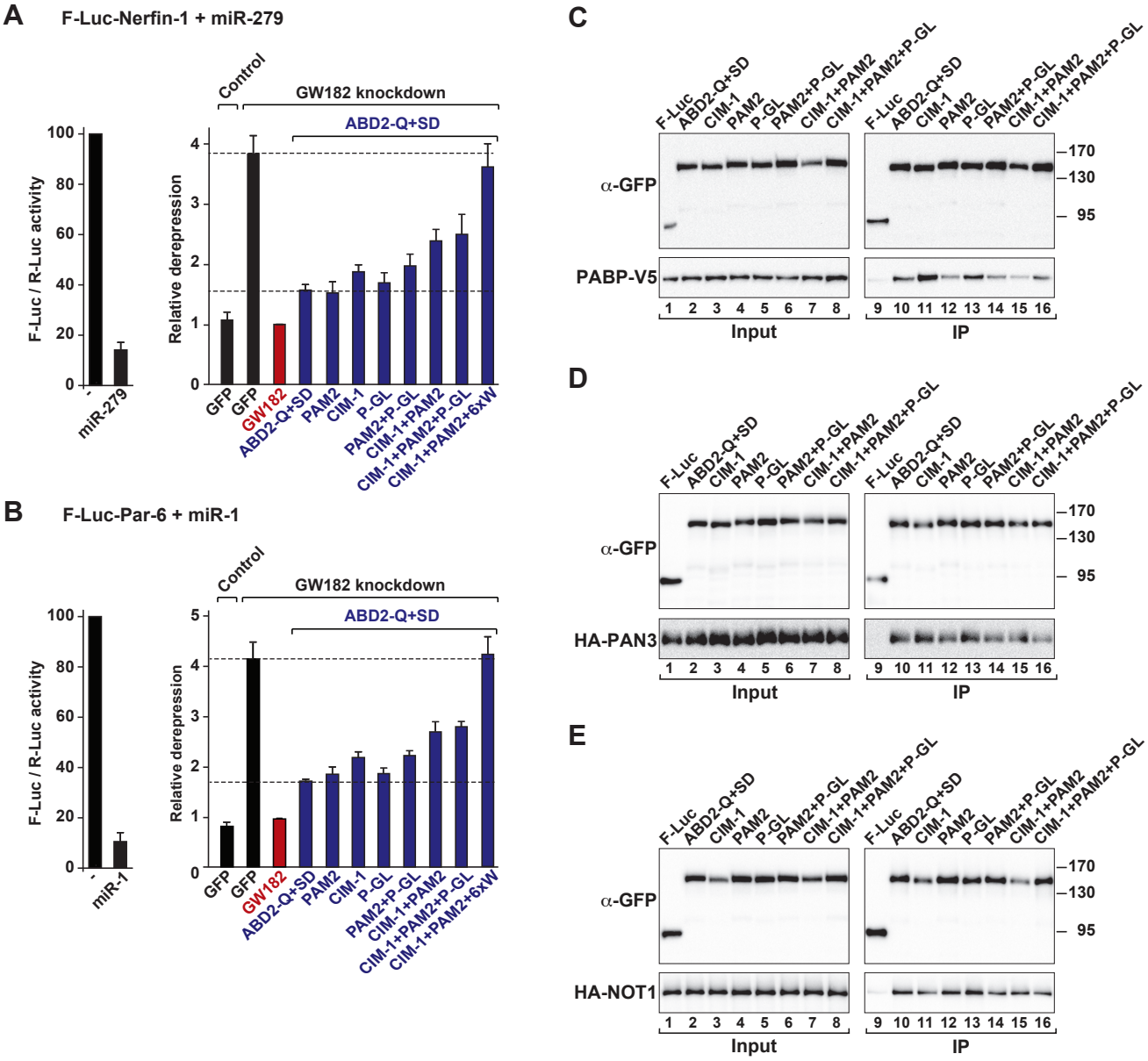


Figure S8. The PG-L motif of *Dm* GW182 does not contribute to silencing in S2 cells. Mutations in the PG-L motif were introduced in a minimal GW182 protein consisting of *Ce* AIN-2 ABD (ABD2) fused to the GW182 Q+SD region (ABD2-Q+SD). The mutations are shown in Supplementary Figure S7F. (**A, B**) The silencing activity of the ABD2-Q+SD protein (wild-type or mutants) was tested in complementation assays as described in Figure 3. (**C–F**) The interactions of ABD2-Q+SD protein (wild-type or mutants) with PABP, PAN3 and NOT1 were analyzed as described in Figure 2.

Figure S9

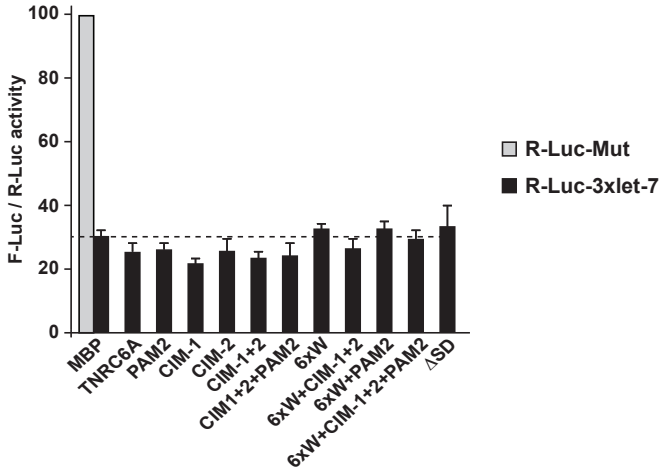


Figure S9. TNRC6A mutants do not inhibit silencing in a dominant negative manner in control cells. The effect of expressing TNRC6A mutants on silencing of the R-Luc-3xlet-7 reporter was analyzed in control cells. The corresponding experiment, which was performed in parallel in depleted cells is shown in Figure 8. HeLa cells were transfected with a mixture of three plasmids: the R-Luc-3xlet-7 or the corresponding reporter carrying mutations in let-7-binding sites (R-Luc-Mut), a plasmid expressing F-Luc as a transfection control, and a plasmid expressing GFP or siRNA-resistant versions of GFP-TNRC6A (wild-type or mutant). For each condition, *Renilla* luciferase activity was measured, normalized to that of the F-Luc transfection control and set at 100% in cells expressing R-Luc-Mut. Normalized *Renilla* luciferase activities are shown. The expression levels of the proteins tested is shown in Figure 8E.