

## Expanded View Figures

### Figure EV1. The silencing activity of AGOs depends on the integrity of the W-binding pockets.

- A Localization of HA-AGO2 (wild type or P1+2 mutant) in HeLa cells expressing GFP-DDX6. The merged images show the GFP signal in green and HA signal in red. Percentages indicate the fraction of cells exhibiting a staining identical to that shown in the representative panel (> 100 cells were counted per experiment). Scale bar, 10  $\mu$ m.
- B–E Tethering assay using the indicated luciferase reporters lacking tethering sites (5BoxB or 6xMS2 sites) in *Dm* and human cells expressing  $\lambda$ N-HA- or MS2-tagged proteins as indicated. The assays with the corresponding reporters containing 5BoxB or 6xMS2 sites are shown in Figs 2A–D and 3B–G. Samples were analyzed as described in Fig 2.
- F Complementation assay. Control S2 cells (treated with  $\beta$ -Gal siRNA) or cells depleted of endogenous AGO1 were transfected with a mixture of three plasmids: one expressing the F-Luc-*cg5281* reporter; another expressing miR-12 primary transcript or the corresponding empty vector; and a third expressing *Renilla* luciferase (R-Luc). Plasmids encoding HA-AGO1 (wild type or the indicated pocket mutants) or HA-MBP (as negative control) were included in the transfection mixtures as indicated. For each condition, firefly luciferase activities and mRNA levels were normalized to those of the *Renilla* luciferase transfection control and set at 100% in the absence of the miR-12. Normalized firefly luciferase activities and mRNA levels are shown.
- G A complementation assay was carried out using the F-Luc-*nerfin-1* reporter and miR-9b or miR-279. F-Luc activity was analyzed as described in (F). Plasmids expressing miR-9b or miR-279 primary transcript or the corresponding empty vector were included in the transfection mixtures as indicated.
- H Western blot showing AGO1 knockdown efficiency. Dilutions of control cell lysates were loaded in lanes 1–4 to estimate the efficacy of the depletion. Tubulin served as a loading control.
- I Western blots showing the efficiency of the AGO1 knockdown and the expression levels of endogenous and recombinant AGO1.  $\alpha$ -tubulin served as a loading control.
- J, K Western blots showing the expression of the tethered proteins in the experiments shown in Fig 2M and N, respectively. Tubulin served as a loading control. The asterisk in (K) indicates a crossreactivity of the anti-HA antibody with an endogenous human protein.

Data information: In panels (B–G), bars represent mean values and error bars represent standard deviations from at least three independent experiments. Source data are available online for this figure.

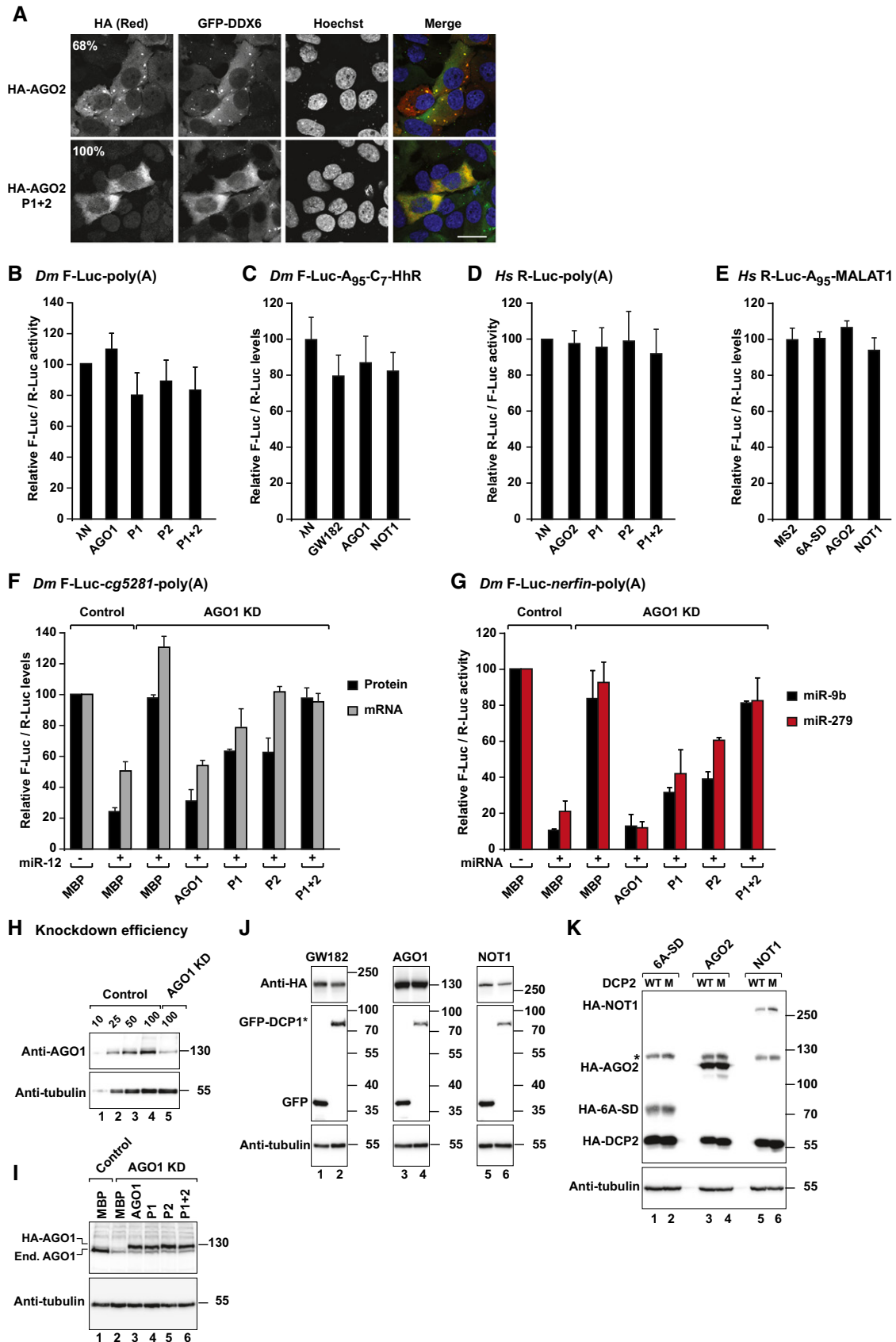


Figure EV1.

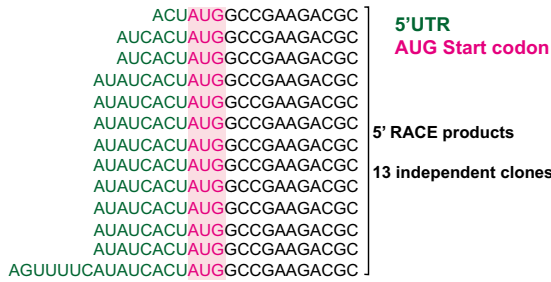
**Figure EV2. Translation efficiencies of the reporters used in Fig 3A and B.**

- A 5' RACE sequences obtained in S2 cells expressing the 8nt-F-Luc-5BoxB-A<sub>95</sub>C<sub>7</sub>-Hhr reporter after cloning the RACE products.
- B–E S2 cells were transfected with the F-Luc-5BoxB reporters shown in Fig 3A. (B) Absolute values of luciferase activity (not normalized). Background levels are indicated by a dashed red line. (C) Luciferase activity normalized to that of the R-Luc transfection control and set to 100 for the polyadenylated reporter containing a 109-nt 5'-UTR. (D) Northern blot of representative RNA samples. (E) The translation efficiency for each reporter was calculated by dividing the normalized values for F-Luc activity shown in (C) by the normalized mRNA levels, shown in (D). Translation efficiencies were set to 100% for the polyadenylated reporter containing a 109-nt 5'-UTR. Note that although the translation efficiency of the reporters lacking a poly(A) tail is reduced, the absolute F-Luc values are three orders of magnitude greater than background levels (B).
- F–H A 109-nt-F-Luc-5BoxB-poly(A) reporter containing a N-terminally truncated F-Luc ORF starting at Met31 is expressed, but no F-Luc activity is detected above background (red dashed line). These results indicate that F-Luc activity will not be detectable if ribosomes bypass the first AUG and start translation at the first in-frame Met31.
- I Western blot analysis showing the expression of HA-GST reporters containing 5'-UTRs of 109 nt (lanes 1 and 2) and 8 nt (lanes 3 and 4). Transfection mixtures contained either 100 or 300 ng of reporter plasmids. HA-MBP served as a transfection control.
- J Normalized luciferase mRNA levels corresponding to the experiment shown in Fig 3B.
- K–N Representative Western and Northern blots corresponding to the experiments shown in Fig 3D and E.

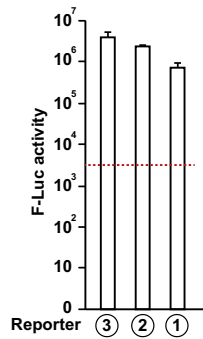
Data information: Error bars represent standard deviations from at least three independent experiments.

Source data are available online for this figure.

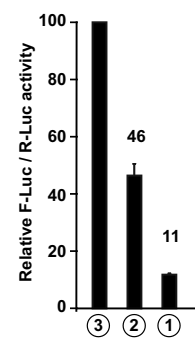
**A** *Dm* 5' RACE of 8nt-F-Luc-5BoxB-A<sub>95</sub>-C<sub>7</sub>-HhR



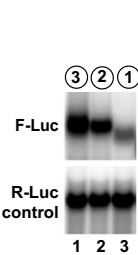
**B** F-Luc activity



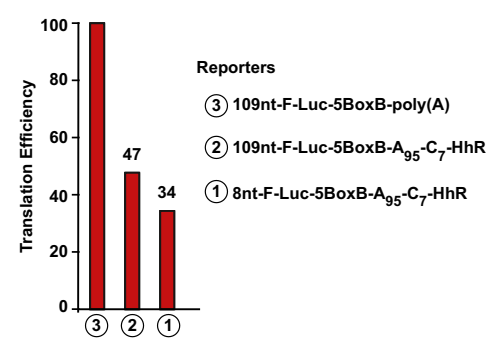
**C** F-Luc / R-Luc



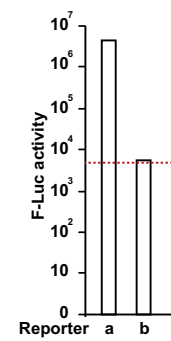
**D** Northern



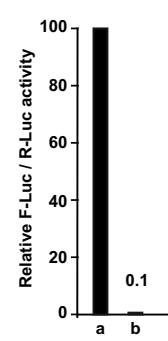
**E** Translation efficiency



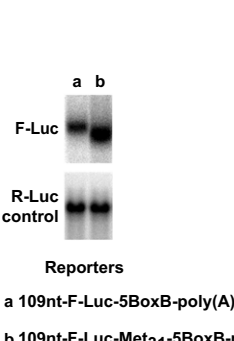
**F** F-Luc activity



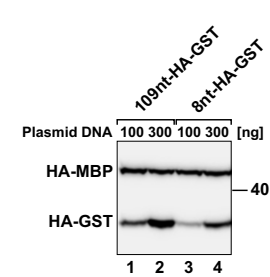
**G** F-Luc / R-Luc



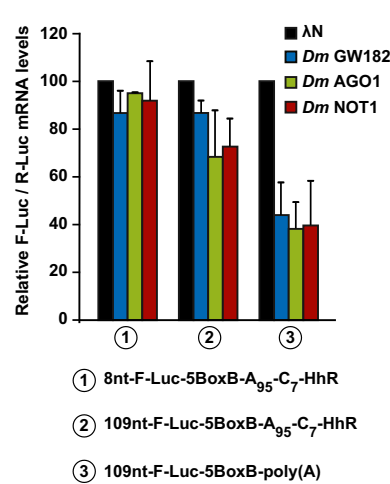
**H** Northern



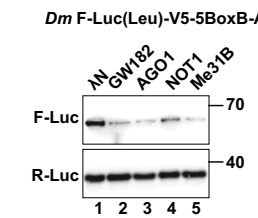
**I** *Dm* 8nt-HA-GST-poly(A)



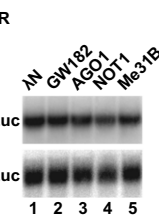
**J** *Dm* reporters



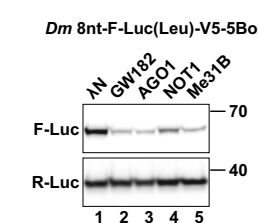
**K** Western



**L** Northern



**M** Western



**N** Northern

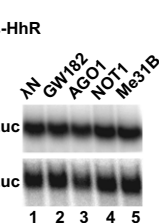


Figure EV2.

**Figure EV3. AGO, GW182/TNRC6, and NOT1 silence mRNA reporters translated via a scanning-independent mechanism.**

- A 5' RACE sequences obtained in HEK293 cells expressing the TISU-R-Luc-6xMS2-A<sub>95</sub>-MALAT reporter after cloning the RACE products.
- B–E The translation efficiency of the MS2 reporters shown in Figs 3F and 4 was analyzed as described in Fig EV2B–E. (B) Absolute values of *Renilla* luciferase activity (not normalized). Background levels are indicated by a dashed red line. (C) R-Luc activity normalized to that of the F-Luc transfection control and set to 100% for the polyadenylated reporter containing a 216-nt 5'-UTR. (D) Upper panel: Northern blot of representative RNA samples. Lower panel: RNase H assay for reporters a and d. (E) The translation efficiency for each reporter was calculated by dividing the normalized values for F-Luc activity shown in (C) by the normalized mRNA levels, shown in (D). Translation efficiencies were set to 100% for the polyadenylated reporter containing a 109-nt 5'-UTR. Note that although the translation efficiency of the reporters lacking a poly(A) tail is reduced, the absolute F-Luc values are three orders of magnitude greater than background levels (B). Note that although the translation efficiency of the TISU reporters is very low, the absolute R-Luc values are 2–3 orders of magnitude greater than background levels (B). In all panels, bars represent mean values, which are indicated above the bars.
- F Normalized luciferase mRNA levels corresponding to the experiment shown in Fig 3G.
- G Western blot analysis showing the expression of TISU-HA-R-Luc-6xMS2-MALAT1. F-Luc-GFP served as a transfection control.
- H Luciferase activity for a bicistronic GFP-IRES<sub>HCV</sub>-F-Luc reporter, which served as a transfection control in the experiment shown in Fig 4A. Note that when cap-dependent translation is inhibited by silvestrol treatment, IRES-mediated translation increases, as reported before (Cope *et al*, 2014), which precludes the use of IRES reporters as normalization controls. Therefore, R-Luc and F-Luc activities measured in silvestrol-treated cells were normalized to the respective values obtained in control cells treated with DMSO.
- I, J Northern blot analysis of representative RNA samples corresponding to the experiment shown in Fig 4A. R-Luc mRNA levels were normalized to those of the F-Luc transfection control and set to 100% in control cells treated with DMSO. Error bars represent standard deviations from four independent experiments.
- K–M HeLa cells were transfected with plasmids expressing R-Luc-*let-7*-A<sub>95</sub>-MALAT1 reporter or the corresponding reporter carrying mutations in the *let-7*-binding sites. HeLa cells were treated with silvestrol (300 nM) or DMSO for 16 h. Panel (K) shows the inhibitory effect of silvestrol on the translation of R-Luc reporter. *Renilla* luciferase activities were measured and set to 100 in cells transfected with the reporter carrying mutations in the *let-7*-binding sites (L). A representative Northern blot is shown in panel (M). Note the effect of silvestrol on mRNA levels. Although the panels are separated, the source data for Fig EV3M clearly shows that the samples were analyzed on the same Northern blot.

Data information: Bars represent mean values and error bars represent standard deviations from at least three independent experiments.

Source data are available online for this figure.

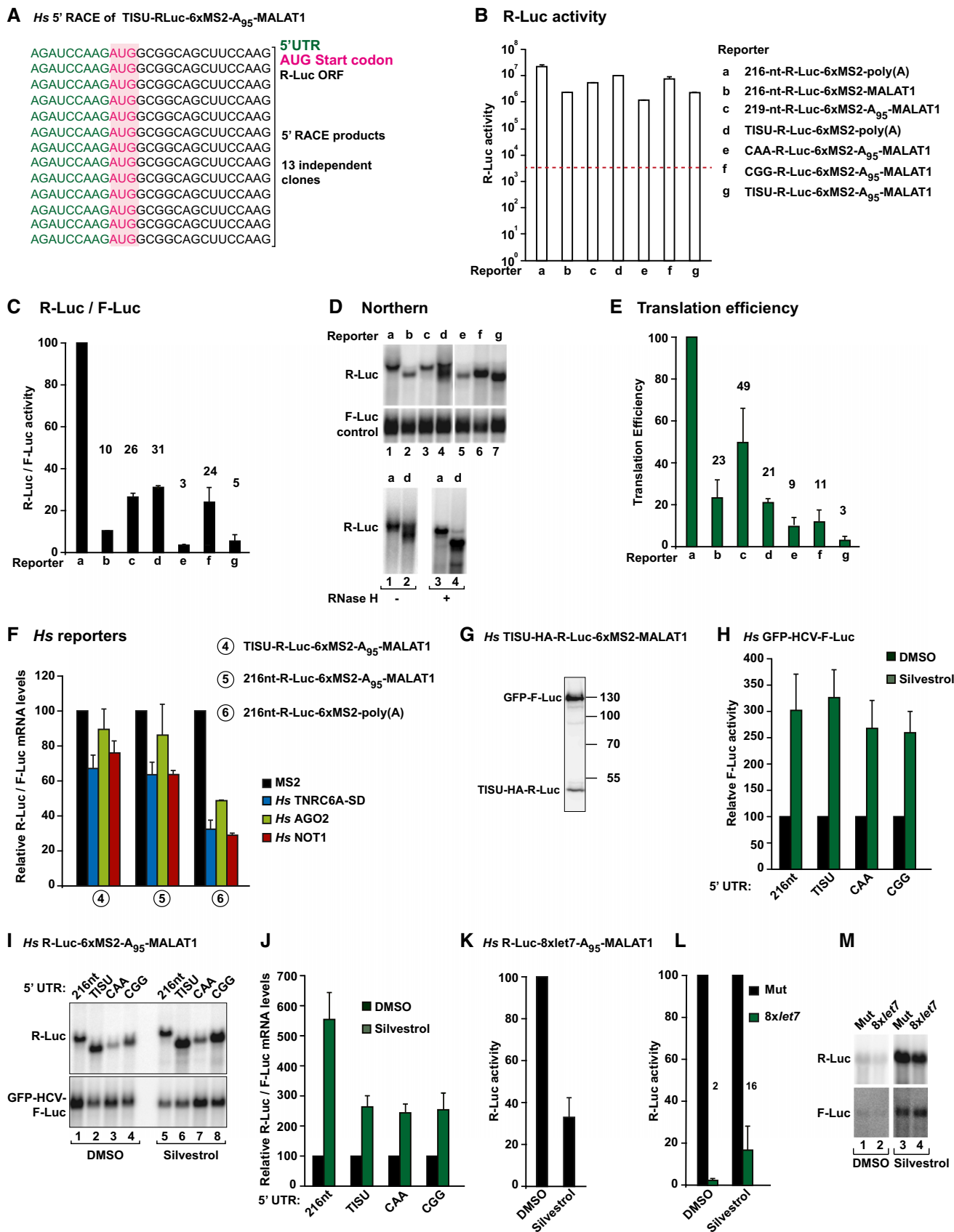
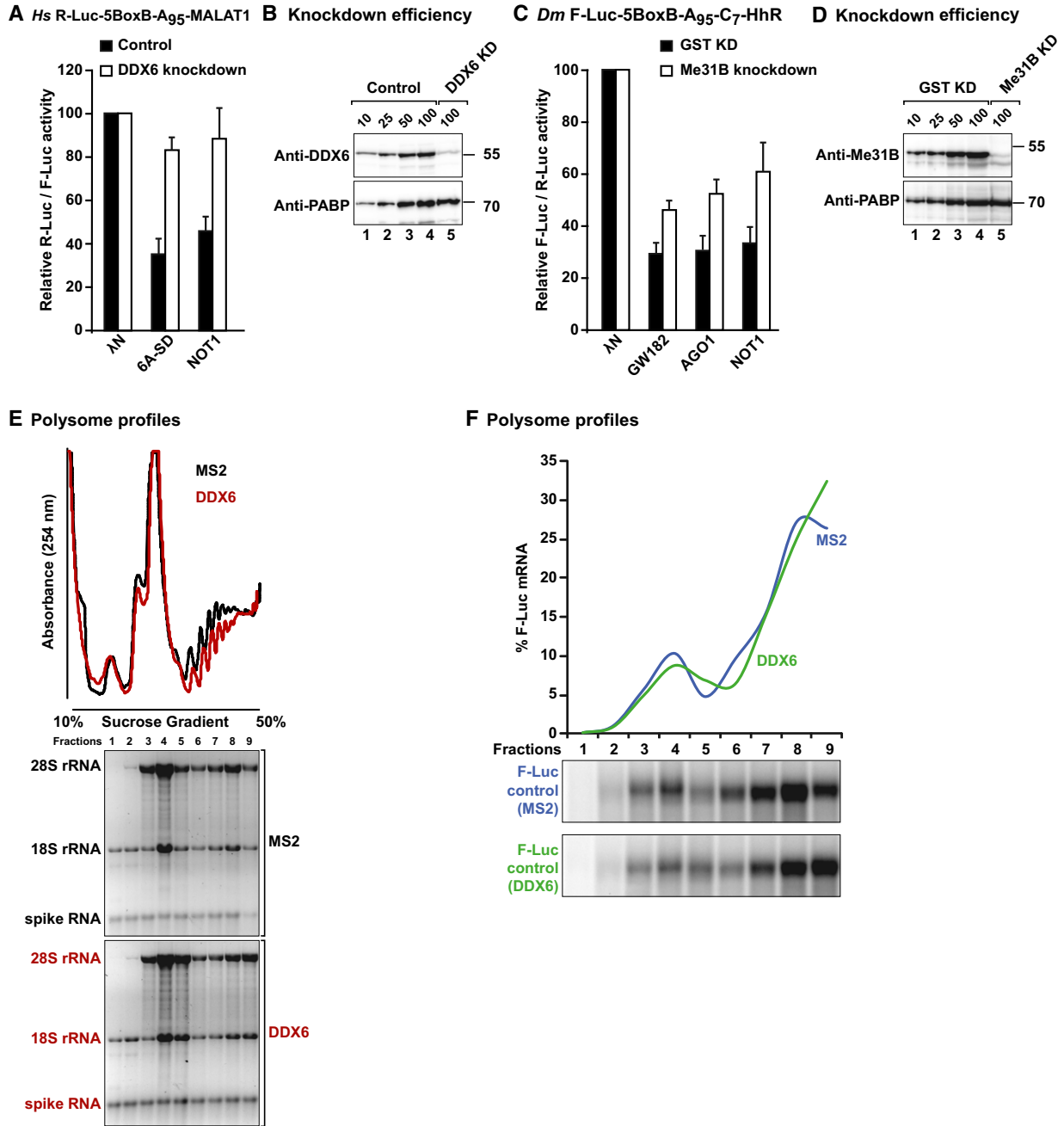


Figure EV3.



**Figure EV4. DDX6 represses translation initiation.**

A Tethering assay using the R-Luc-5BoxB-A95-MALAT1 reporter and  $\lambda$ N-HA-tagged TNRC6A-SD and NOT1 in control HeLa cells or cells depleted of DDX6. Samples were analyzed as described in Fig 2. Error bars represent standard deviations from at least three independent experiments.

B Western blot showing DDX6 knockdown efficiency. Dilutions of control cell lysates were loaded in lanes 1–4 to estimate the efficacy of the depletion. PABP served as a loading control.

C Tethering assay using the F-Luc-5BoxB-A95-C7-HhR reporter and  $\lambda$ N-HA-tagged GW182, AGO1, and NOT1 in control S2 cells or cells depleted of Me31B. Samples were analyzed as described in Fig 2. Error bars represent standard deviations from at least three independent experiments.

D Western blot showing Me31B knockdown efficiency. Dilutions of control cell lysates were loaded in lanes 1–4 to estimate the efficacy of the depletion. PABP served as a loading control.

E Polysome profiles corresponding to Fig 7G. The absorbance at 254 nm of each fraction was quantitated and normalized to the total intensity across all fractions. The presence of 18S and 28S rRNAs in each fraction was analyzed on denaturing agarose gels. The spike RNA added to the fractions prior RNA isolation is indicated.

F The amount of F-Luc control reporter corresponding to Fig 7G was quantified in each fraction of the gradient and normalized to the total amount across all fractions. Source data are available online for this figure.