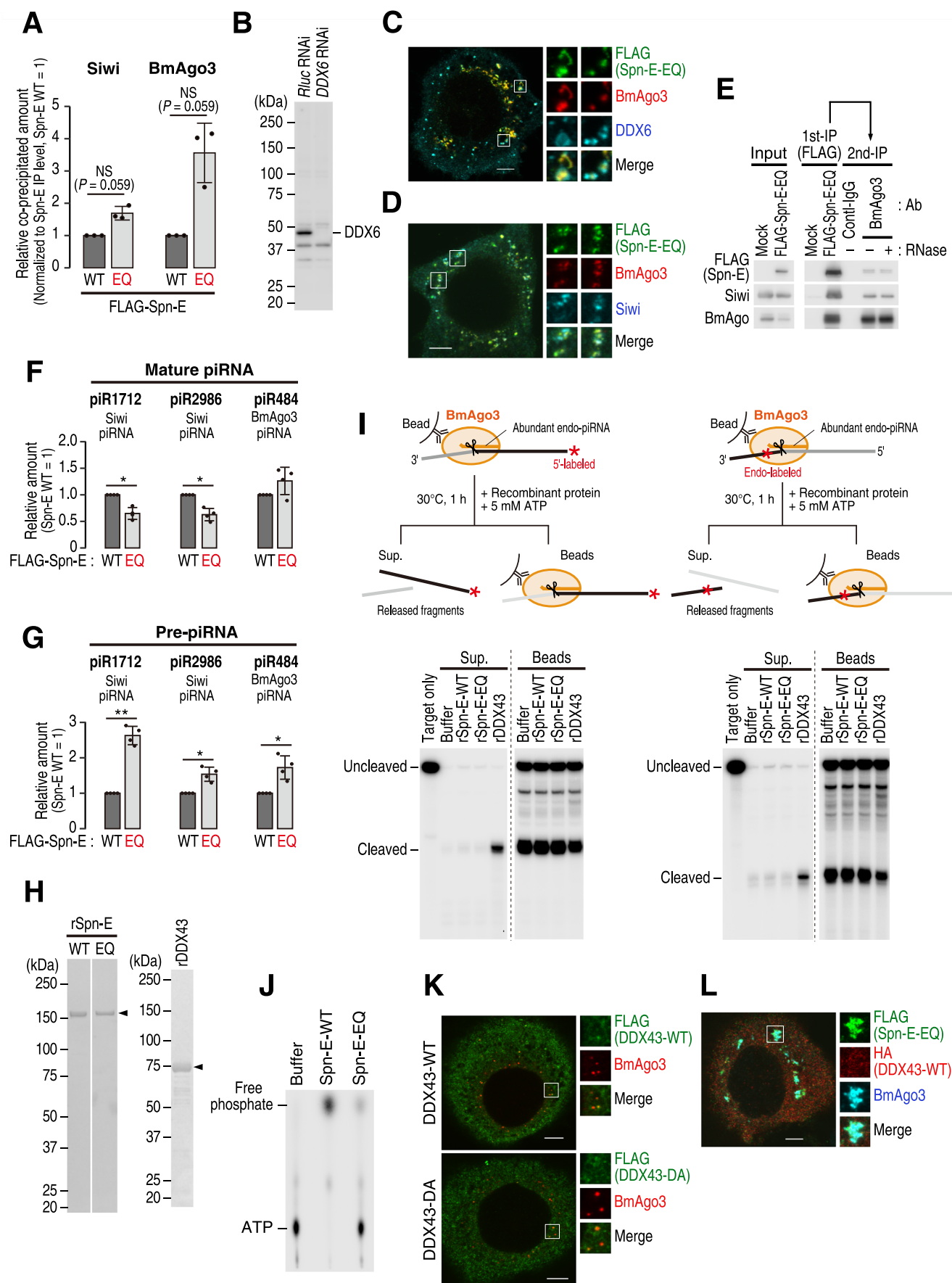


Expanded View Figures

Figure EV1. Characterization of Spn-E-EQ aggregates and BmAgo3-cleavage fragment release assay.

(A) Quantification of Siwi and BmAgo3 signals co-immunoprecipitated with FLAG-Spn-E (wild type or EQ) in Fig. 1D. Relative co-immunoprecipitated levels normalized to the Spn-E IP level are shown. Data are mean $\pm$ s.d. of three independent experiments (biological replicates). Statistical analysis was performed using a two-sided Student's paired *t*-test, with *p*-values adjusted using the Holm method. NS not significant. (B) Western blot analysis of whole cell lysates of BmN4 cells treated with dsRNA for *Rluc* (control) or *DDX6*. The anti-DDX6 antibody successfully detected endogenous DDX6. (C) Subcellular localization of FLAG-Spn-E-EQ, DDX6, a P-body marker protein, and BmAgo3 in BmN4 cells. Scale bar, 5 μ m. (D) Subcellular localization of FLAG-Spn-E-EQ, BmAgo3 and Siwi in BmN4 cells. Scale bar, 5 μ m. (E) Tandem IP experiment on BmN4 cells co-transfected with the FLAG-Spn-E-EQ expression plasmid and dsRNA targeting the *Spn-E* 3' UTR. FLAG-Spn-E-EQ was first immunoprecipitated with the FLAG tag, and the resulting immunopurified complex was then subjected to a second IP with normal rabbit IgG (Contl-IgG) or an anti-BmAgo3 antibody. Siwi in the second immunoprecipitate was detected by western blotting with or without RNase A treatment. (F) Quantification of mature piRNA signals in Fig. 1E. Relative expression levels normalized to those under Spn-E-WT expression are shown. Data are mean $\pm$ s.d. of four independent experiments (biological replicates). Statistical analysis was performed using a two-sided Student's paired *t*-test, with *p*-values adjusted using the Holm method (**p* = 0.023). (G) Quantification of pre-piRNA signals in Fig. 1E. Relative expression levels normalized to those under Spn-E-WT expression are shown. Data are mean $\pm$ s.d. of four independent experiments (biological replicates). Statistical analysis was performed using a two-sided Student's paired *t*-test, with *p*-values adjusted using the Holm method (**p* = 0.025, ***p* = 0.003). (H) CBB staining of purified recombinant Spn-E and DDX43 proteins (rSpn-E and rDDX43, indicated by arrowheads) used for the in vitro cleavage fragment release assay. (I) Top: Schematic representation of the in vitro cleavage fragment release assay. To detect cleaved 5' and 3' fragments separately, the target RNA was radiolabeled at different positions (*). The 5' or internally radiolabeled target RNAs were subjected to a cleavage assay using BmAgo3 immunoprecipitates. After the reaction, the bead fraction was incubated with rSpn-E or rDDX43 in the presence of ATP. Bottom: The cleaved fragments in the supernatant and bead fractions were detected by autoradiography. (J) Thin-layer chromatography for the detection of ATPase activity of the recombinant Spn-E protein. (K) Subcellular localization of FLAG-DDX43 (wild type or DA, an ATPase-deficient mutant) and BmAgo3 in BmN4 cells. Scale bar, 5 μ m. (L) Subcellular localization of FLAG-Spn-E-EQ, HA-DDX43, and BmAgo3 in BmN4 cells. Scale bar, 5 μ m.



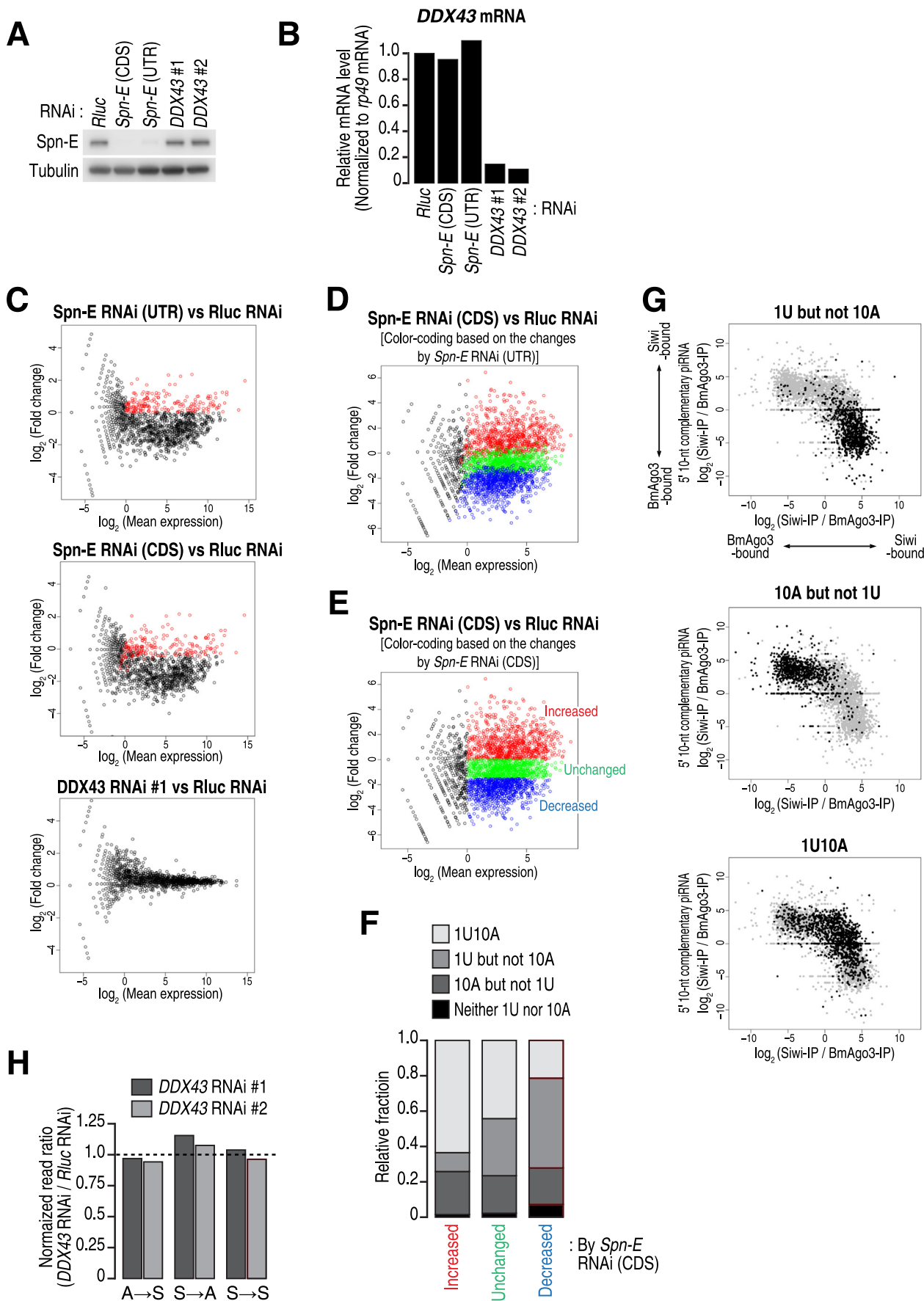


Figure EV2. Changes in the expression of TE-mapped piRNAs upon KD of *Spn-E* or *DDX43*.

(A) Western blot analysis of whole cell lysates from BmN4 cells treated with dsRNA for *Rluc* (control), *Spn-E*, or *DDX43*. Two different dsRNAs (*Spn-E*: CDS and 3' UTR; *DDX43*: two different regions of the CDS, #1 and #2) were used for RNAi. *Rluc*; *Renilla luciferase*. (B) Quantitative real-time PCR analysis of the expression of *DDX43* in BmN4 cells treated with dsRNA for *Rluc* (control), *Spn-E*, or *DDX43*. Relative mRNA expression levels normalized to those of *rp49* are shown. *Rluc*; *Renilla luciferase*. (C) MA plots showing piRNA expression changes for each TE between the control KD (*Rluc* RNAi) and *Spn-E* or *DDX43* KD. Two different dsRNAs (CDS and 3' UTR) were used for *Spn-E* RNAi. Each dot represents one TE. TEs with increased piRNA production in the *Spn-E* RNAi (UTR) are colored red. (D) MA plot showing piRNA expression changes between the control KD (*Rluc* RNAi) and *Spn-E* KD using dsRNA targeting the *Spn-E* CDS. Each dot represents one piRNA. Based on the three groups defined in Fig. 2A, piRNAs were color-coded as follows: "increased" (red, $n = 825$), "unchanged" (green, $n = 824$), and "decreased" (blue, $n = 825$). (E) MA plot showing piRNA expression changes between control KD (*Rluc* RNAi) and *Spn-E* KD using dsRNA targeting the *Spn-E* CDS. Each dot represents one piRNA. Based on the changes in expression, piRNAs were divided into three groups: "increased" (red, $n = 805$), "unchanged" (green, $n = 805$), and "decreased" (blue, $n = 805$). (F) Relative fractions of 1U10A, 1U but not 10A, 10A but not 1U, and neither 1U nor 10A piRNAs of each group in (E). (G) Scatter plots showing the PIWI binding bias of 1U but not 10A, 10A but not 1U, or 1U10A piRNAs (x-axis) and that of their putative partner piRNAs in the ping-pong cycle (y axis). (H) Changes in the expression of A $\rightarrow$ S, S $\rightarrow$ A, and S $\rightarrow$ S piRNAs in *DDX43* KD relative to control KD (*Rluc* RNAi). Two different dsRNAs targeting the *DDX43* CDS were used for RNAi. *Rluc*; *Renilla luciferase*.

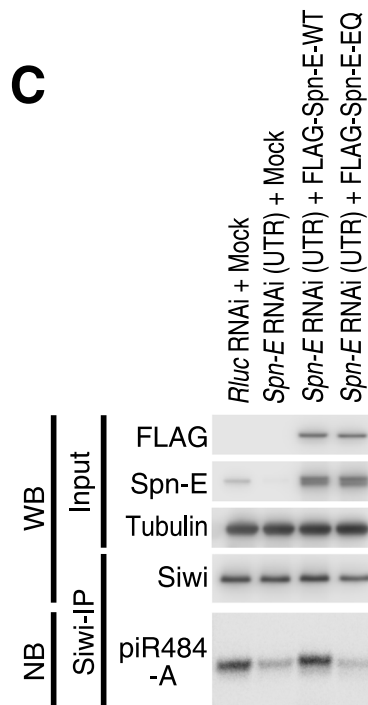
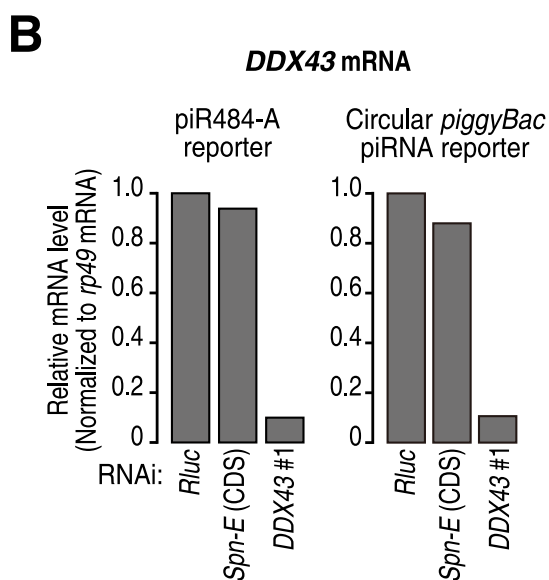
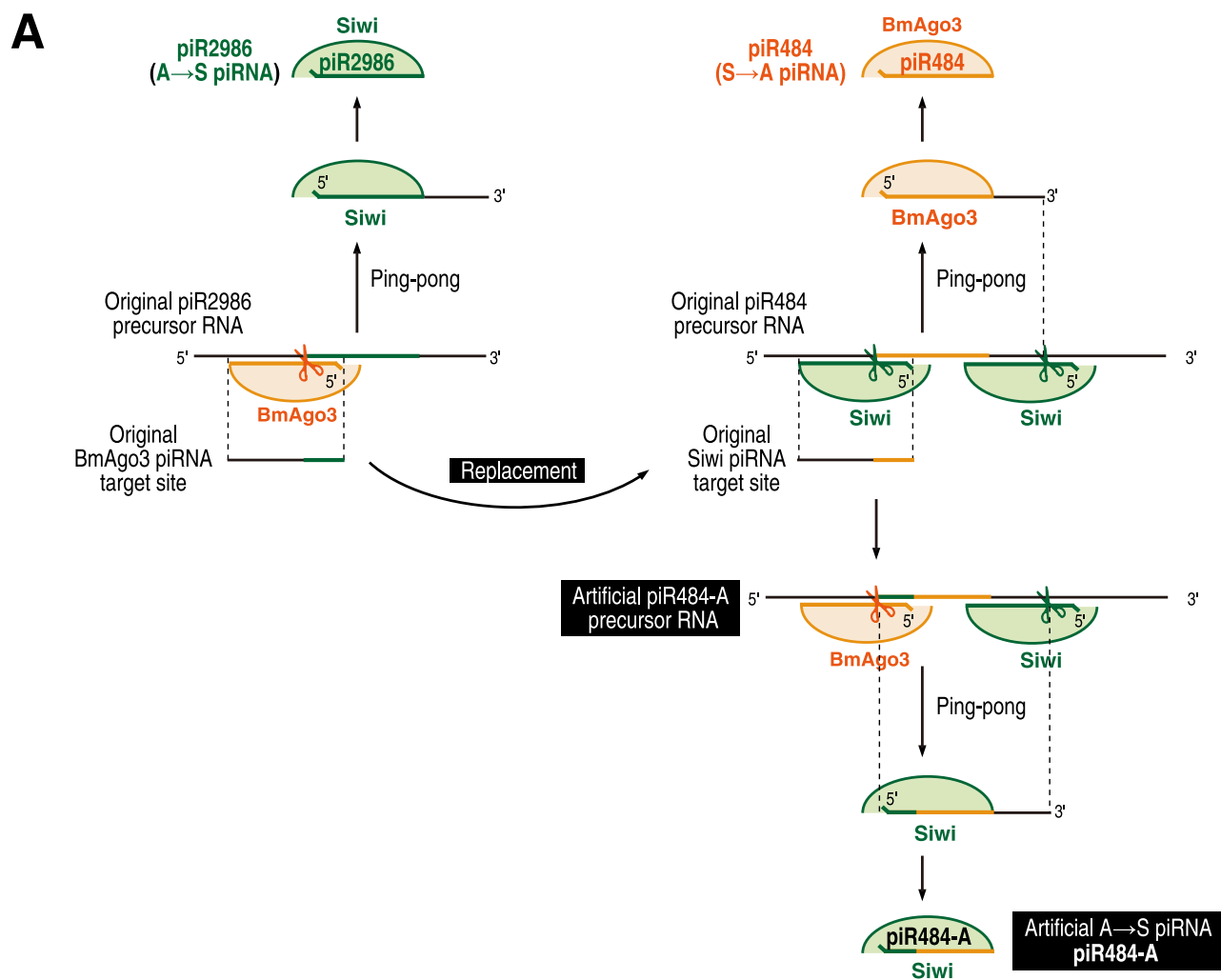


Figure EV3. Construction of the piR484-A reporter system and Spn-E KD rescue experiment using the piR484-A reporter.

(A) Schematic explanation of the construction of the piR484-A reporter. piR484 is an S → A piRNA, and the sequence including the 5' 10 nt and the upstream region of piR484 was replaced with the corresponding region of piR2986, an A → S piRNA, resulting in an artificial A → S piRNA, piR484-A. The downstream sequence of piR484 that contains a Siwi-bound piRNA target sequence was used without modification. (B) Quantitative real-time PCR analysis of the expression of *DDX43* in the reporter experiments in Fig. 3B,D. Relative mRNA expression levels normalized to those of *rp49* are shown. *Rluc*; *Renilla luciferase*, control. (C) KD rescue experiment of Spn-E using the piR484-A reporter. Siwi was immunoprecipitated from BmN4 cells co-transfected with the piR484-A reporter plasmid, the FLAG-Spn-E expression plasmid, and dsRNA targeting the *Spn-E* 3' UTR. Immunoprecipitated Siwi and Siwi-bound piR484-A were detected by western blotting and northern blotting, respectively. *Rluc*; *Renilla luciferase*, control.