Expanded View Figures

Figure EV1. Vret is a well-conserved Tudor domain-containing protein.

A The Western blot shows the specificity of the anti-Vret monoclonal antibody raised in this study. β-Tub was used as a loading control.
B *Bombyx* expresses two *Vret* isoforms, which have different 5′ terminal regions.
C Domain structures of BmVret-L, BmVret-S, mTdrd1, zTdrd1, and DmVret.
D Western blotting analysis showed that His-tagged Vret-L and Vret-S co-migrate with endogenous Vret P150 and P130, respectively.
E Immunoprecipitation and Western blotting results show that Flag-Vret-L and Flag-Vret-S associate with Ago3 in BmN4 cells.
F Ago3 was immunoprecipitated from BmN4 cells using the anti-Ago3 antibody. The cell lysate was prepared in Empigen-containing cell lysis buffer. n.i.: non-immune IgG.
G Tandem immunoprecipitation of Siwi from the Vret complex. The top three panels show Western blots performed using anti-Flag, anti-Siwi, and anti-Ago3 antibodies. The bottom panel shows the abundance of piRNAs loaded onto Siwi.
H Scatter plots of normalized Flag-Siwi-piRNA abundance in two replicates from Control, Ago3-depleted, and Vret-depleted BmN4 (Spearman's rho = 0.94, 0.90 and 0.93, respectively). Each dot represents a piRNA sequence.
I Boxplot showing log2 fold-change of Ago3-KD (RPM)/Control (RPM). piRNAs whose ratio was ≥ 2, $0.5 < \text{ratio} < 2$ or ≤ 0.5 were categorized as increased, unchanged, and decreased, respectively. Boxplot represents median, first and third quartile, and maximum and minimum values of the $\log_2$ fold-change of piRNAs ($N = 2$). Outliers are represented as open circles. Scatterplots for $\log_2$ fold-change of piRNAs between Ago3-KD (*x* axis) and Vret KD (*y* axis). Each dot represents a unique piRNA. Red dots indicate piRNAs in the increased group and blue dots indicate piRNAs from the decreased group.
J Immunoprecipitation and subsequent Western blotting using anti-sDMA and anti-Flag and anti-Vret antibodies show that both Siwi and Ago3 RK mutants defective in sDMA modification failed to co-purify with Vret.
K Flag-EGFP and Flag-Vret-L WT and mutants were immunoprecipitated using the anti-Flag antibody and then Western blotting was performed using anti-Flag and anti-Myc antibodies. Myc-Vret-S was co-expressed in the cells prior to immunoprecipitation.

Source data are available online for this figure.

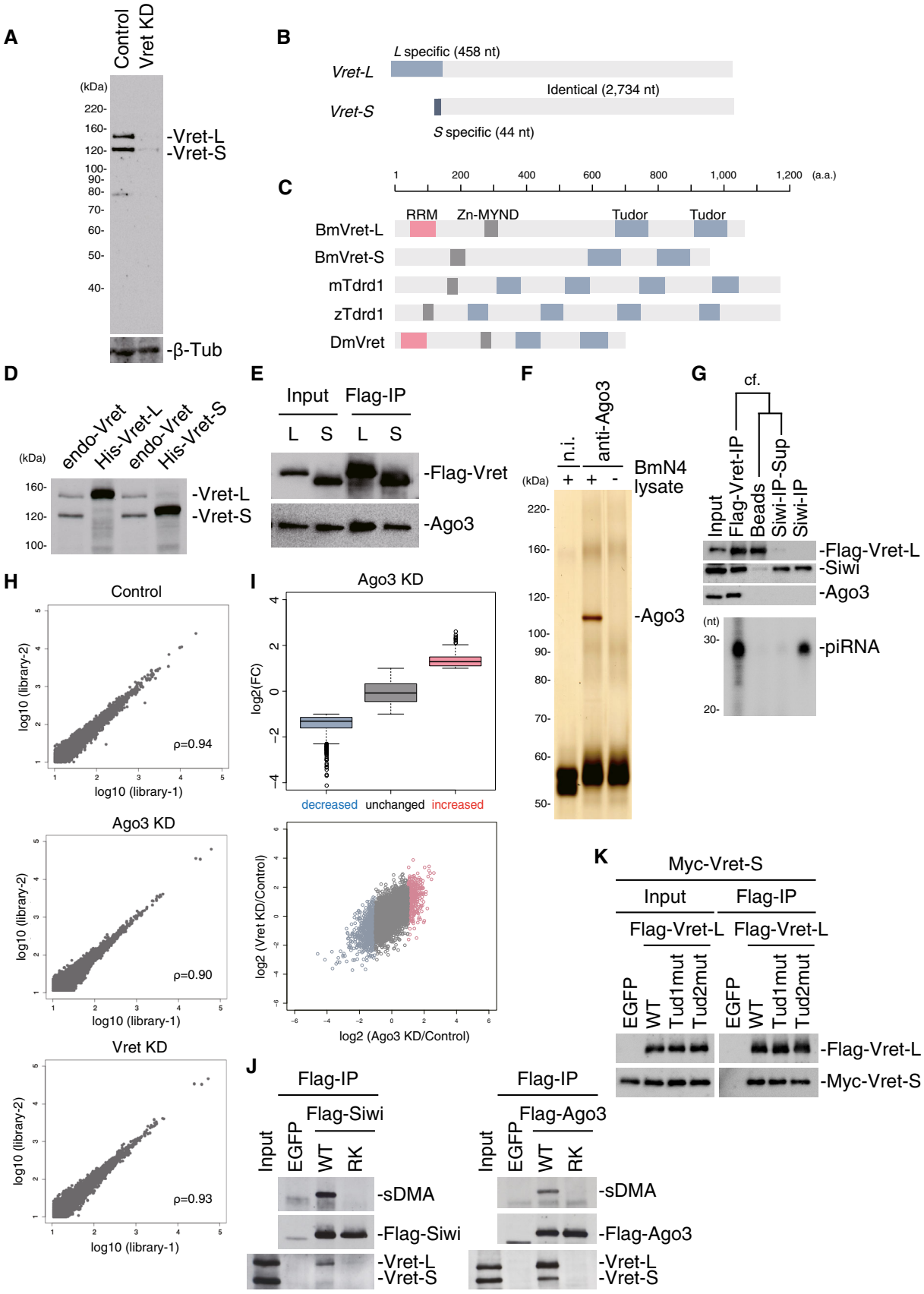
A
Control
Vret KD
(kDa)
220-
160-
120-
100-
90-
80-
70-
60-
50-
40-
-Vret-L
-Vret-S
-β-Tub
B
L specific (458 nt)
Vret-L
Identical (2,734 nt)
Vret-S
S specific (44 nt)
C
1
200
400
600
800
1,000
1,200
(a.a.)
RRM
Zn-MYND
Tudor
Tudor
BmVret-L
BmVret-S
mTdrd1
zTdrd1
DmVret
D
(kDa)
endo-Vret
His-Vret-L
endo-Vret
His-Vret-S
160-
120-
100-
-Vret-L
-Vret-S
E
Input
Flag-IP
L
S
L
S
-Flag-Vret
-Ago3
F
n.i.
anti-Ago3
(kDa)
+
+
-
BmN4 lysate
220-
160-
120-
100-
90-
80-
70-
60-
50-
-Ago3
G
cf.
Input
Flag-Vret-IP
Beads
Siwi-IP-Sup
Siwi-IP
-Flag-Vret-L
-Siwi
-Ago3
(nt)
30-
20-
-piRNA
H
Control
log10 (library-2)
log10 (library-1)
ρ=0.94
Ago3 KD
log10 (library-2)
log10 (library-1)
ρ=0.90
Vret KD
log10 (library-2)
log10 (library-1)
ρ=0.93
I
Ago3 KD
log2(FC)
decreased
unchanged
increased
log2 (Vret KD/Control)
log2 (Ago3 KD/Control)
J
Flag-IP
Flag-Siwi
Input
EGFP
WT
RK
-sDMA
-Flag-Siwi
-Vret-L
-Vret-S
Flag-IP
Flag-Ago3
Input
EGFP
WT
RK
-sDMA
-Flag-Ago3
-Vret-L
-Vret-S
K
Myc-Vret-S
Input
Flag-IP
Flag-Vret-L
Flag-Vret-L
EGFP
WT
Tud1mut
Tud2mut
EGFP
WT
Tud1mut
Tud2mut
-Flag-Vret-L
-Myc-Vret-S

Figure EV1.

Figure EV2. Ago3 bodies are required for the concentration of the Ago3-Vret-Siwi complex.

A Left: An enlarged view of the full cell image in Fig 2A. Right: A magnification image of the part indicated by the white dashed line box in the image of the left-hand side.

B Left: An enlarged view of the full cell image in Fig 2B. Right: A magnification image of the part indicated by the white dashed line box in the image of the left-hand side.

C Immunofluorescence shows that the Ago3 signals (green) co-localize with the Siwi signals (red). DAPI shows the nuclei (blue). Scale bar: 10 μm. The three panels on the right-hand side show high magnification images of the part indicated by the white dashed line box in the image on the left-hand side. The bar chart shows the percentage of Ago3-positive nuage in Siwi-positive nuage.

D Immunoprecipitation and subsequent Western blotting show that Flag-Vret-L only weakly associates with Siwi upon Ago3 depletion.

E Immunoprecipitation and subsequent Western blotting show that Ago3 associates with Siwi much less upon Vret depletion.

F ^{32}P-labeling showing the levels of piRNAs loaded onto Flag-Ago3 WT and DDH and KA mutants. Lys637 was mutated to alanine in the KA mutant. Asp697, Asp767, and His901 were mutated to alanine in the DDH mutant.

G Immunofluorescence showing Flag-Ago3 WT, KA mutant, and DDH mutant signals (green) expressed in naïve BmN4 cells (Control). Vret is shown in red. DAPI shows the nuclei (blue). Scale bar: 10 μm.

H Immunofluorescence showing Flag-Ago3 WT, KA mutant, and DDH mutant signals (green) expressed in Ago3-depleted BmN4 cells (Ago3 KD). Vret is shown in red. DAPI shows the nuclei (blue). Scale bar: 10 μm.

I Immunoprecipitation and subsequent Western blotting show that Flag-Ago3 WT and DDH and KA mutants associate with Vret.

Source data are available online for this figure.

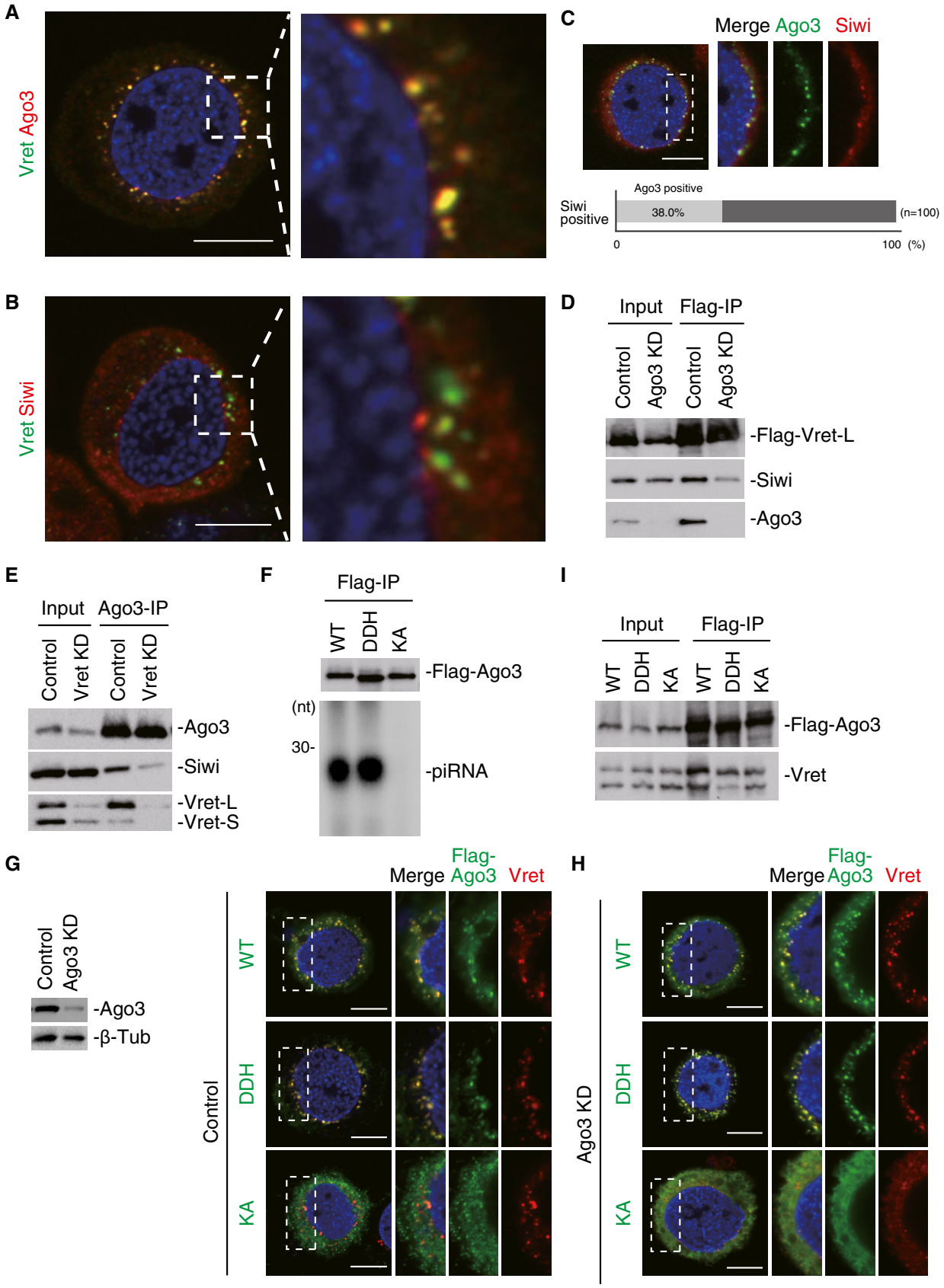

Figure EV2.

Figure EV3. Ago3 phosphorylation in Siwi-depleted cells.

A Boxplot represents median, first and third quartile, and maximum and minimum values of the size of Ago3 particle (left panel; Control; $n = 32$, Siwi KD; $n = 37$) and Vret particle (right panel; Control; $n = 54$, Siwi KD; $n = 26$). Outliers are represented as filled circles. To calculate the size of nuage, we subtracted the background from the original images and generated binary images. After separating the adjacent object by the watershed algorithm and reducing noise, the area of nuage was obtained by *Analyze Particles* in ImageJ. *P*-values were calculated by Brunner-Munzel test using Scipy. $*P < 0.05$.

B Immunofluorescence showing the Ago3 signals (green) and the Vret signals (red) in control and Siwi-depleted BmN4 cells (Siwi KD). DAPI shows the nuclei (blue). Scale bar: 10 μm. The three panels on the right-hand side show high magnification images of the part indicated by the white dashed line box in the left-hand side images.

C Silver staining showing unphosphorylated and phosphorylated Ago3 immunoisolated from Siwi-depleted BmN4 cells.

D Representative ETD tandem mass spectra for Ago3 peptides, which include serine modifications. Mascot scores are shown on the top right of each spectrum.

E ^{32}P-labeling shows the levels of piRNAs loaded onto Flag-Ago3 WT and 8SA.

F ^{32}P-labeling shows the level of piRNAs loaded onto Flag-Ago3 is not affected by Vret depletion. Vret and β-Tub as a loading control were also detected by Western blotting.

G Bar chart indicates dot sizes of > 100 Flag-Ago3 WT particles under control and Siwi-depleted conditions (Large: *IntDen* value > 10^5; Small: *IntDen* value < 10^5).

H Bar chart indicates dot sizes of > 100 Flag-Ago3 8SA/8SE particles under control and Siwi-depleted conditions (Large: *IntDen* value > 10^5; Small: *IntDen* value < 10^5).

I Immunofluorescence showing the Flag-Ago3 8SA and 8SE mutant signals (red) in normal (Control) and Siwi-depleted BmN4 cells (Siwi KD). DAPI shows the nuclei (blue). Scale bar: 10 μm.

Source data are available online for this figure.

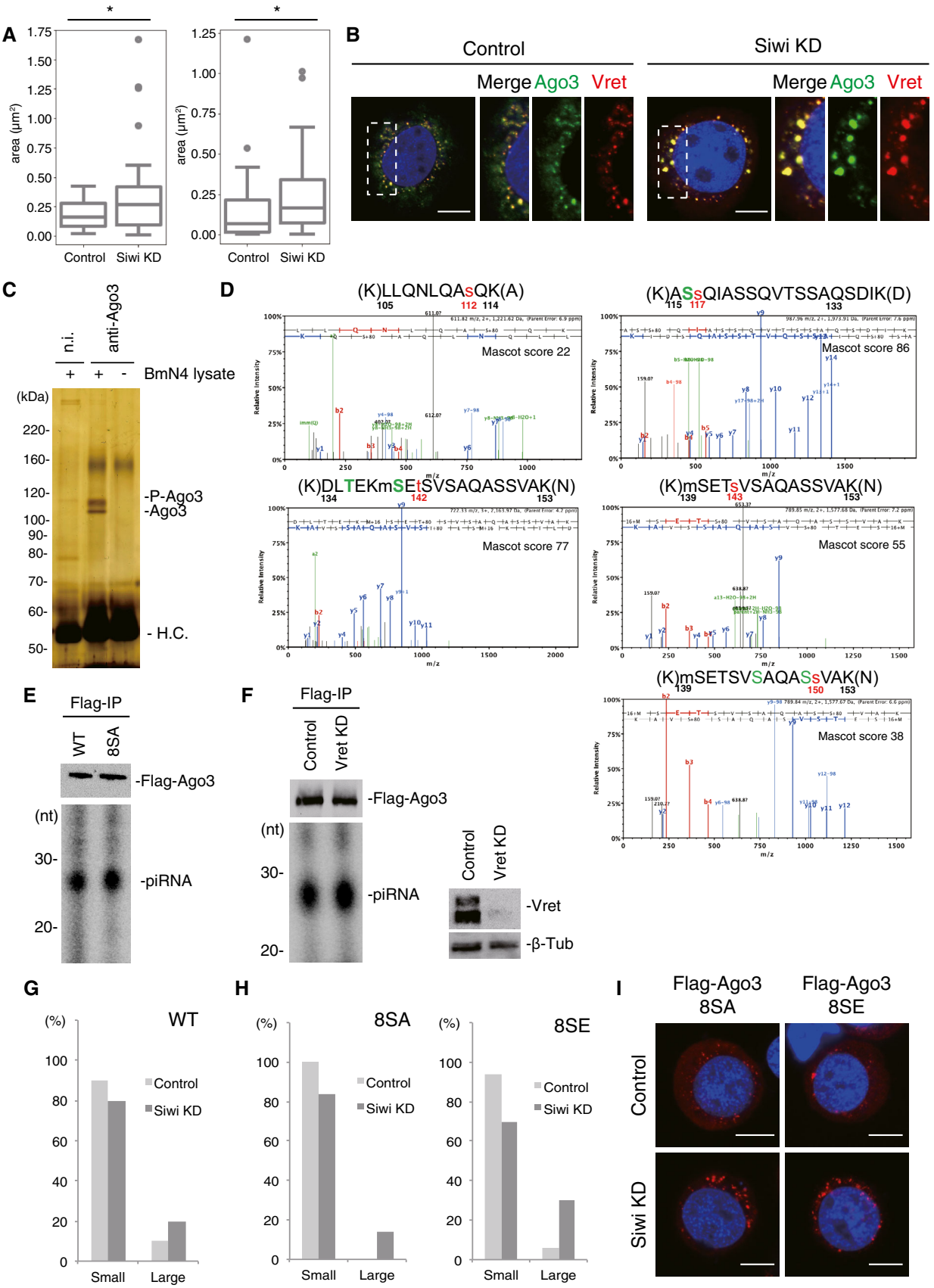

Figure EV3.

Figure EV4. Bioinformatic analysis of RNAs bound with Ago3-piRISCs in Siwi-depleted cells.

A The size distribution of Target-S fragments.
B The size distribution of Target-L fragments.
C Heat maps show the read frequency of transposon-derived Ago3-piRNA, Target-S, and Target-L. Color intensities indicate the degree of read frequency: (orange) higher and (white) lower.
D Characteristics of Target-L and Target-S. The 5′-ends of Siwi-associated piRNAs displayed a strong overlap with the 5′-ends of Target-L fragments.

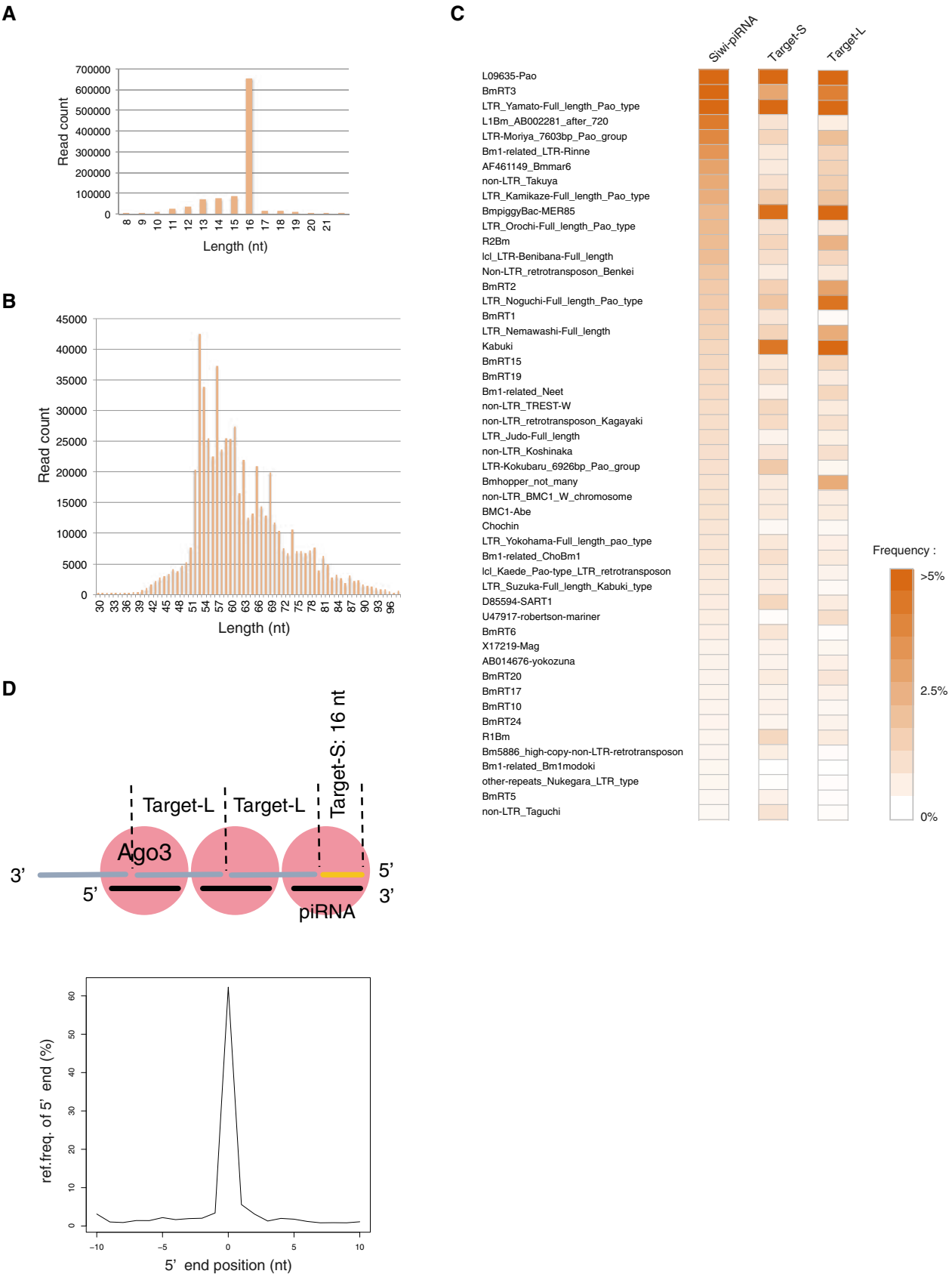
A
Read count
Length (nt)
B
Read count
Length (nt)
C
Siwi-piRNA
Target-S
Target-L
L09635-Pao
BmRT3
LTR_Yamato-Full_length_Pao_type
L1Bm_AB002281_after_720
LTR-Moriya_7603bp_Pao_group
Bm1-related_LTR-Rinne
AF461149_Bmmar6
non-LTR_Takuya
LTR_Kamikaze-Full_length_Pao_type
BmpiggyBac-MER85
LTR_Orochi-Full_length_Pao_type
R2Bm
lcl_LTR-Benibana-Full_length
Non-LTR_retrotransposon_Benkei
BmRT2
LTR_Noguchi-Full_length_Pao_type
BmRT1
LTR_Nemawashi-Full_length
Kabuki
BmRT15
BmRT19
Bm1-related_Neet
non-LTR_TREST-W
non-LTR_retrotransposon_Kagayaki
LTR_Judo-Full_length
non-LTR_Koshinaka
LTR-Kokubaru_6926bp_Pao_group
Bmhopper_not_many
non-LTR_BMC1_W_chromosome
BMC1-Abe
Chochin
LTR_Yokohama-Full_length_pao_type
Bm1-related_ChoBm1
lcl_Kaede_Pao-type_LTR_retrotransposon
LTR_Suzuka-Full_length_Kabuki_type
D85594-SART1
U47917-robertson-mariner
BmRT6
X17219-Mag
AB014676-yokozuna
BmRT20
BmRT17
BmRT10
BmRT24
R1Bm
Bm5886_high-copy-non-LTR-retrotransposon
Bm1-related_Bm1modoki
other-repeats_Nukegara_LTR_type
BmRT5
non-LTR_Taguchi
Frequency :
>5%
2.5%
0%
D
Target-L
Target-L
Target-S: 16 nt
Ago3
3'
5'
5'
3'
piRNA
ref.freq. of 5' end (%)
5' end position (nt)

Figure EV4.

Figure EV5. A new model for the ping-pong cycle in silkworm germ cells.

A Immunofluorescence showing the Ago3 signals (green) in Siwi-depleted BmN4 cells (Siwi KD) and Siwi KD cells where Flag-Siwi (red) was expressed. DAPI shows the nuclei (blue). Scale bar: 10 μm. The panels on the right-hand side show high magnification images of the part indicated by the white dashed line box in the images shown on the left-hand side.

B ^{32}P-labeling showing the levels of piRNAs loaded onto the Flag-Siwi WT, DDH mutant, and KA mutant. Lys611 is mutated to alanine in the KA mutant, and Asp670, Asp740, and His874 are mutated to alanine in the DDH mutant.

C Northern blotting showing the levels of PiggyBac-piRNA and Bantam miRNA in BmN4 cells. Flag-Siwi WT and the DDH and KA mutants were individually expressed in Siwi-depleted cells. Flag-EGFP was expressed as a control.

D When RNA within secondary Siwi-piRISC has extra bases at the 3′-end, the complex relocates to mitochondria, where the piRISC precursor is docked onto Papi. Zuc processes the RNA to produce more secondary Siwi-piRISC (see * in Fig 6).

E Bar chart indicates that 70.4% of Papi-binding RNAs were identical to Target-L.

F The DISOPRED plot analyzed by PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/) predicted IDR regions in BmVret-L.

Source data are available online for this figure.

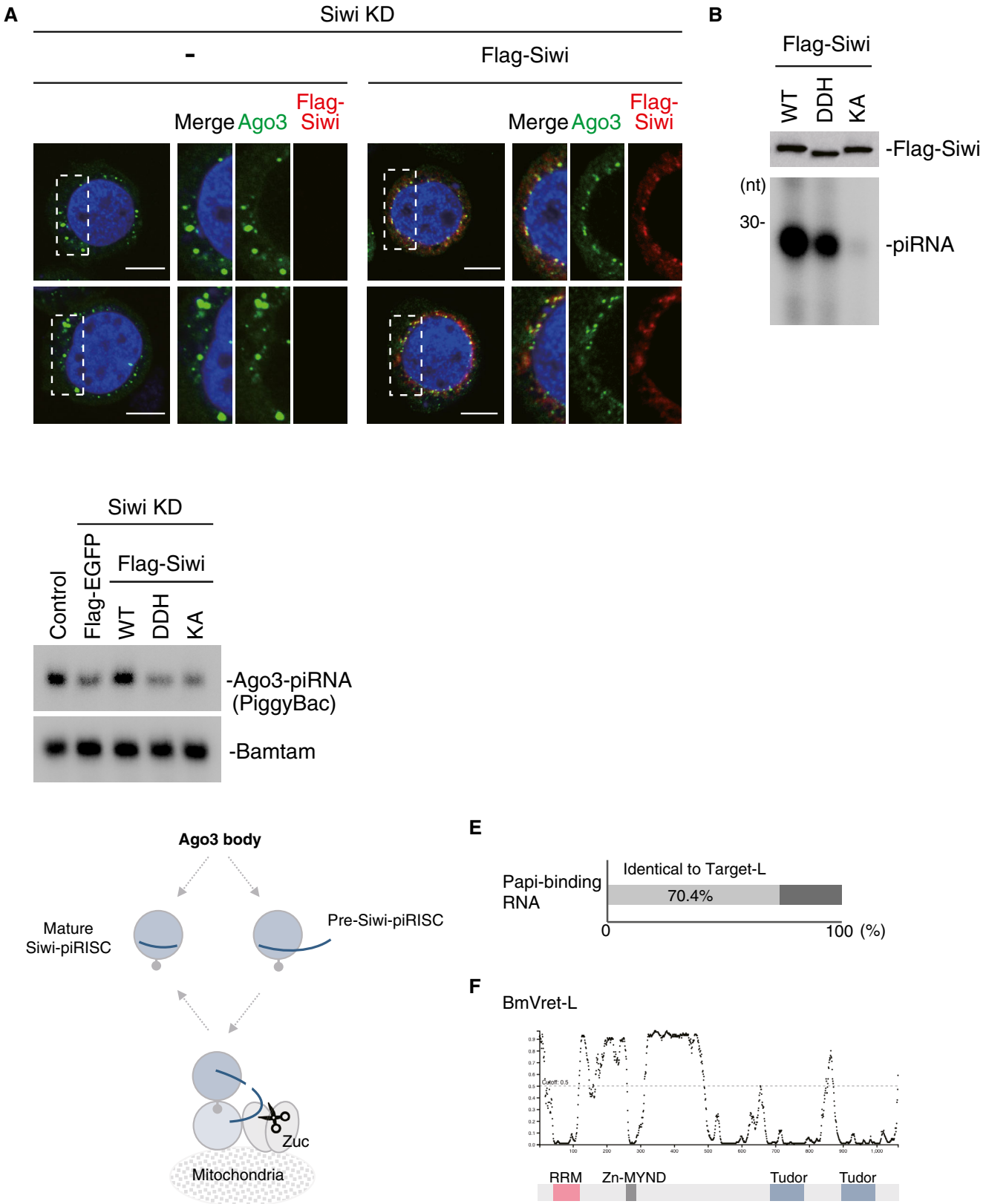

Figure EV5.