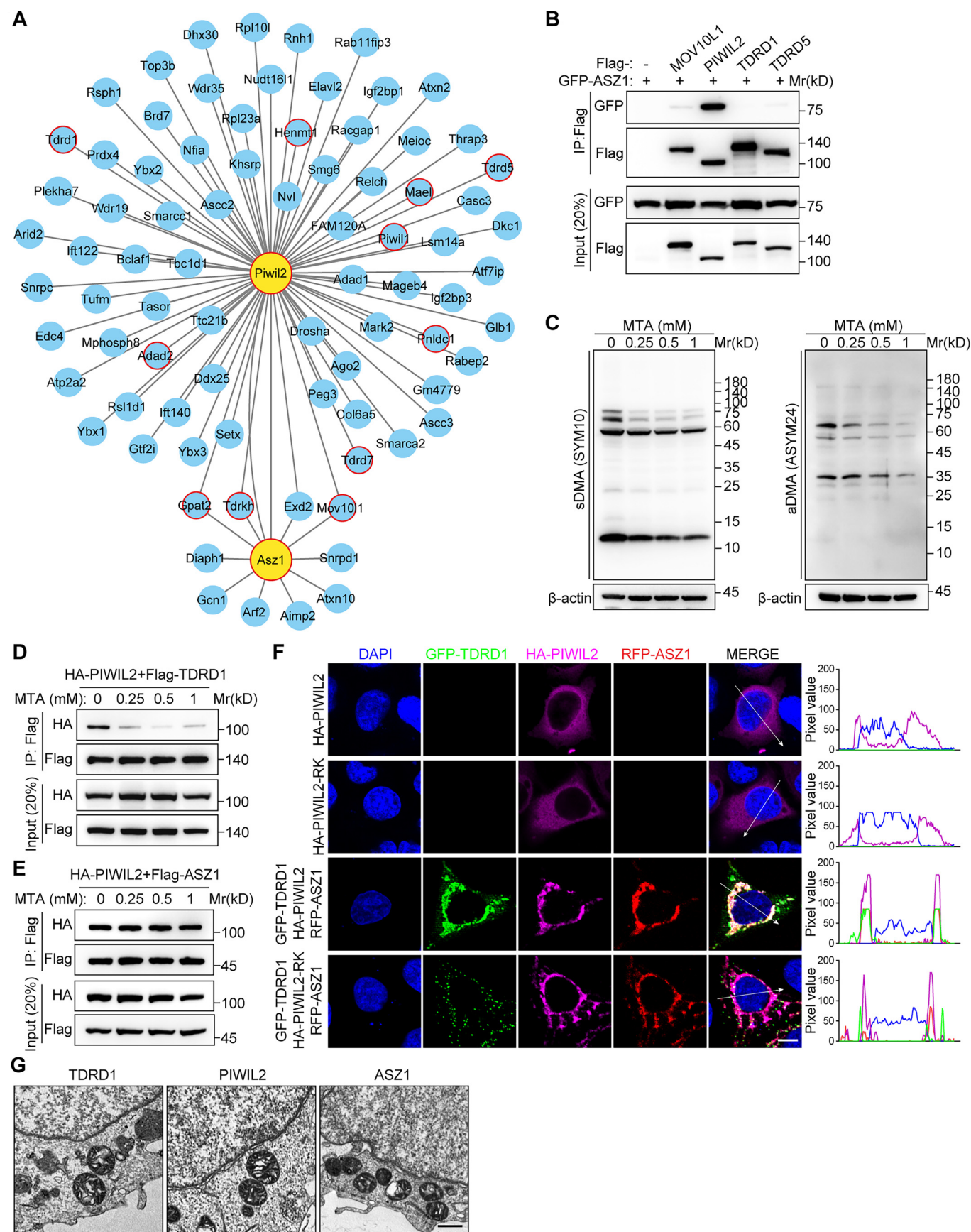


Expanded View Figures

Figure EV1. PIWIL2 serves as an adapter to form the ASZ1-PIWIL2-TDRD1 complex.

(A) Interaction networks of ASZ1 and PIWIL2 based on IP/MS from P20 testes. The known piRNA-related proteins are highlighted by red circles. (B) Co-IP assay in 293T cells transfected with indicated constructs. Flag-tagged and GFP-tagged proteins were detected by WB. (C) 293T cells were treated with methyltransferase inhibitor MTA. WB was performed using sDMA (SYM10) or aDMA (ASYM24) antibodies. β -actin served as a control. (D, E) Co-IP assay in transfected 293T cells with MTA treatment. Flag-tagged and HA-tagged proteins were detected by WB. (F) HeLa cells were transfected with indicated constructs. Immunostaining was performed using the HA antibody. Scale bars, 10 μ m. Fluorescence intensity through positions denoted by the white lines are shown in right. (G) Image of TEM on HeLa cells transfected with GFP-TDRD1, HA-PIWIL2, or RFP-ASZ1, respectively. Scale bars, 1 μ m.



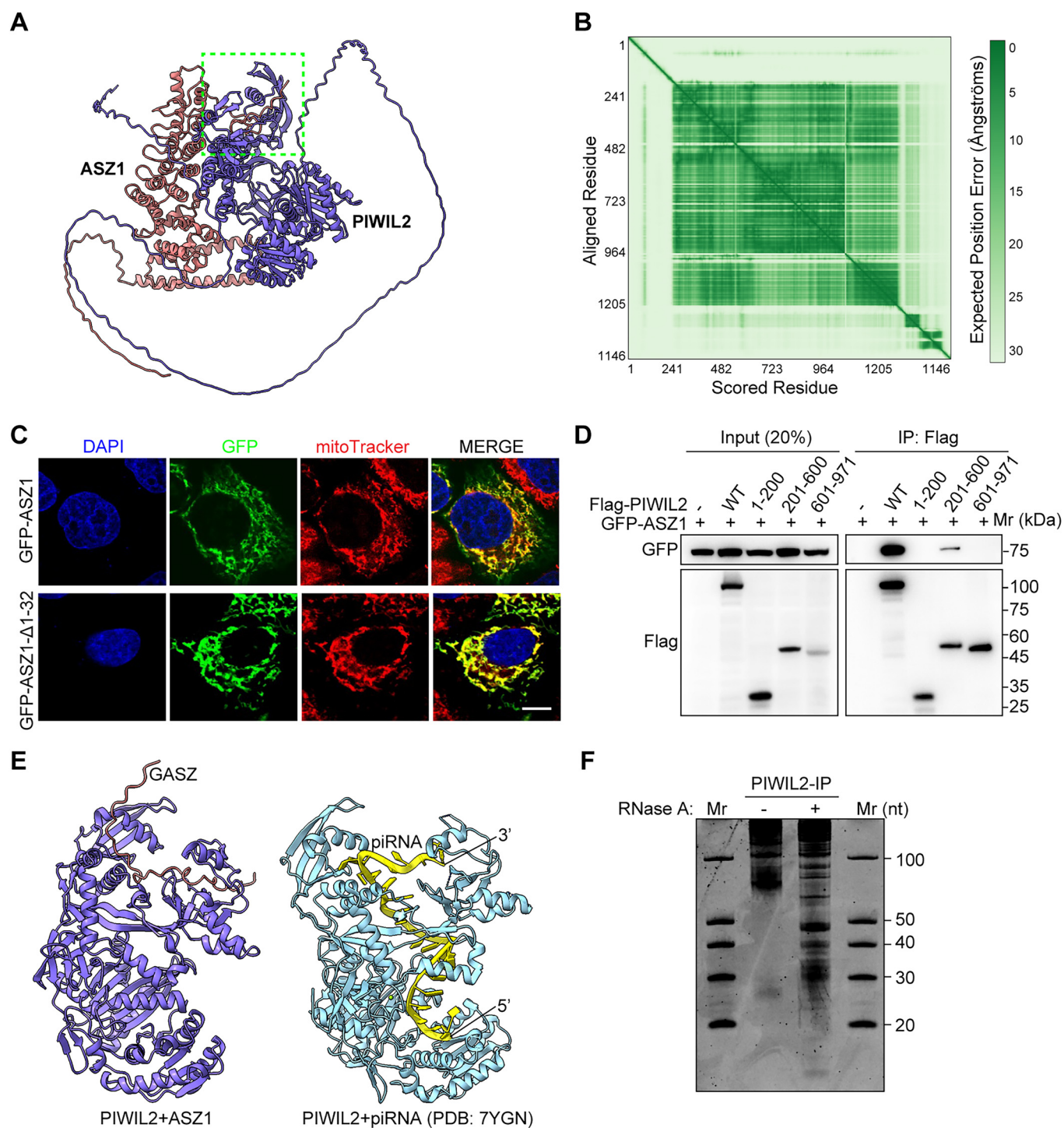


Figure EV2. piRNA loading onto PIWIL2 disrupts ASZ1-PIWIL2 interaction.

(A) Interaction structure model of the PIWIL2-ASZ1 complex predicted by AlphaFold. The green box highlights the interaction interface. (B) PAE (predicted aligned error) plot showing regions of high confidence (dark green) and low confidence (pale green) for the predicted structure of PIWIL2 and ASZ1 complex. (C) HeLa cells were transfected with indicated constructs. Transfected cells were stained with Mitotracker to label mitochondria. Scale bars, 10 μ m. (D) Co-IP assay in 293T cells transfected with indicated constructs. Flag-tagged and GFP-tagged proteins were detected by WB. (E) Left, PIWIL2-ASZ1 structure predicted by AlphaFold; right, PIWIL2-piRNA structure from PDB (7YGN). (F) PIWIL2 bound RNAs from adult testes with or without RNase A treatment were detected by Urea-PAGE gel.

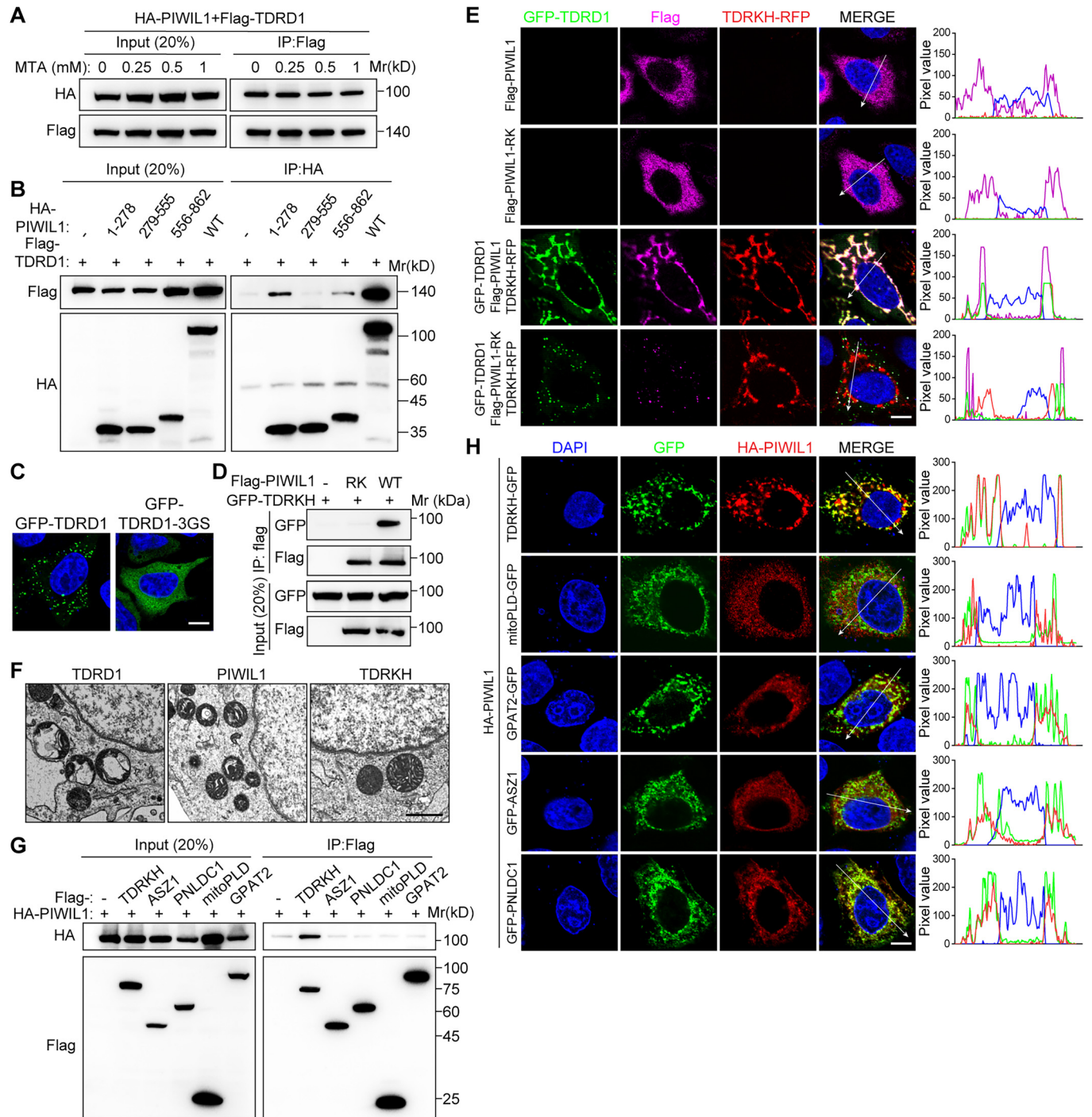


Figure EV3. TDRKH-PIWIL1 complex cooperates with ASZ1-PIWIL2 complex to recruit TDRD1 to mitochondria.

(A) Co-IP assay in transfected 293T cells with MTA treatment. Flag-tagged and HA-tagged proteins were detected by WB. (B) Co-IP assay in 293T cells transfected with indicated constructs. Flag-tagged and HA-tagged proteins were detected by WB. (C) Images of HeLa cells transfected with the indicated plasmids. Scale bars, 10 μ m. (D) Co-IP assay in 293T cells transfected with indicated constructs. Flag-tagged and GFP-tagged proteins were detected by WB. (E) HeLa cells were transfected with indicated constructs. Immunostaining was performed using the Flag antibody. Scale bars, 10 μ m. Fluorescence intensity through positions denoted by the white lines are shown in right. (F) Image of TEM on HeLa cells transfected with GFP-TDRD1, Flag-PIWIL1, or TDRKH-RFP, respectively. Scale bars, 1 μ m. (G) Co-IP assay in 293T cells transfected with indicated constructs. Flag-tagged and HA-tagged proteins were detected by WB. (H) HeLa cells were transfected with indicated constructs. Immunostaining was performed using the HA antibody. Scale bars, 10 μ m. Fluorescence intensity through positions denoted by the white lines are shown in right.

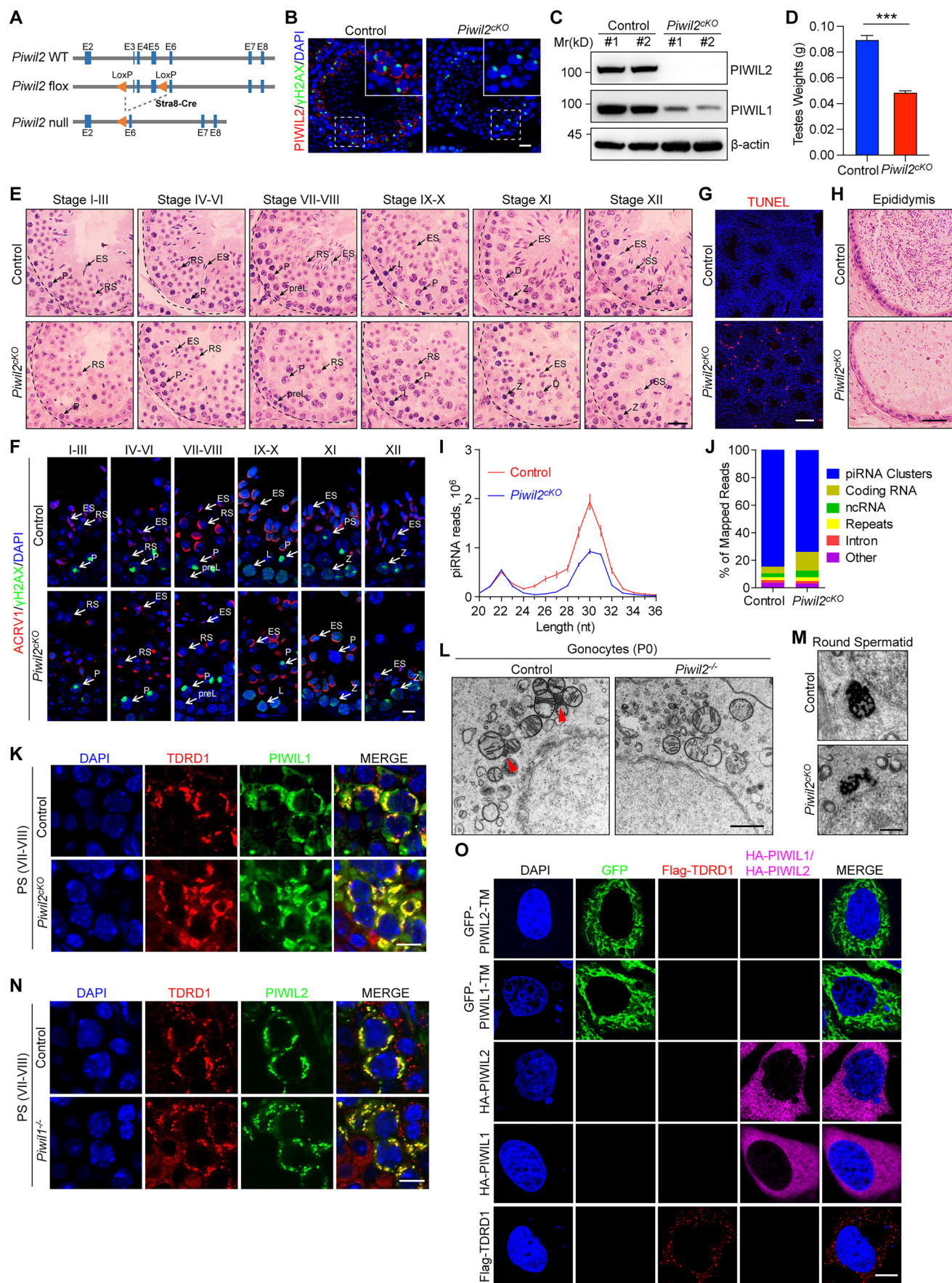
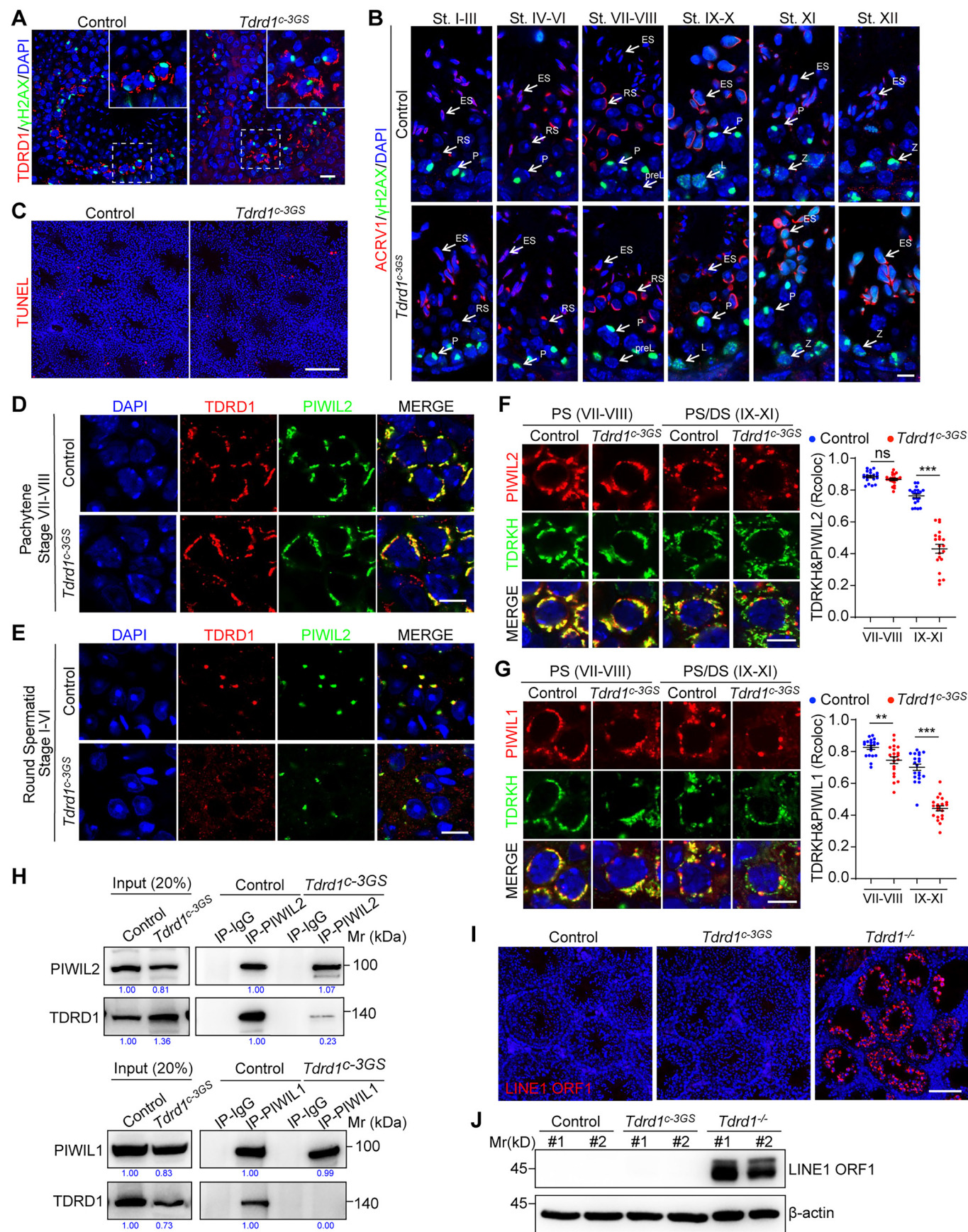



Figure EV4. PIWIL2 is required for pachytene piRNA biogenesis.

(A) A schematic diagram showing the gene targeting strategy for the generation of a *Piwi2* conditional allele. Cre-mediated deletion removed the exon 3–5 of *Piwi2* and generated a protein null allele. (B) Co-immunostaining of PIWIL2 and γ H2AX on control and *Piwi2*^{CKO} adult testes. Scale bars, 20 μ m. (C) WB of PIWIL2 and PIWIL1 expression in control and *Piwi2*^{CKO} adult testes. β -actin served as a control. (D) The average weight of adult testes ($n = 6$; *** $p < 0.001$). (E) H&E staining on adult testes. preL preleptotene, L leptotene, Z zygotene, P pachytene, D diplotene, RS round spermatid, ES elongated spermatid, SS secondary spermatocytes. Scale bars, 20 μ m. (F) Co-immunostaining of ACRV1 and γ H2AX on adult mouse testes. preL preleptotene, L leptotene, Z zygotene, P pachytene, D diplotene, M metaphase, RS round spermatid, ES elongated spermatid; Scale bars, 10 μ m. (G) TUNEL assays on adult testes. Scale bars, 100 μ m. (H) H&E staining of the mouse adult epididymis. Scale bars, 50 μ m. (I) The length distribution of PIWIL1-piRNAs from adult testes. Data were normalized by total reads. $n = 2$. (J) Genomic annotation of PIWIL1-piRNAs from control and *Piwi2*^{CKO} adult testes. Data were representative of two biological replicates. (K) Co-immunostaining of TDRD1 and PIWIL1 on control and *Piwi2*^{CKO} adult testes. Stage VII–VIII seminiferous tubules were distinguished according to DAPI staining. PS pachytene spermatocytes. Scale bars, 10 μ m. (L) Images of TEM on gonocytes from mouse PO testes. IMCs were indicated by red arrowheads. Scale bars, 1 μ m. (M) Images of TEM on round spermatids from adult testes showing CB structure. Scale bars, 1 μ m. (N) Co-immunostaining of TDRD1 and PIWIL2 on control and *Piwi1*^{-/-} adult testes. Stage VII–VIII seminiferous tubules were distinguished according to DAPI staining. PS pachytene spermatocytes. Scale bars, 10 μ m. (O) HeLa cells were transfected with indicated constructs. Immunostaining was performed using Flag, PIWIL1, and PIWIL2 antibodies. Scale bars, 10 μ m. Data information: In (D, I), data were presented as mean $\pm$ s.e.m. In (D), p values were calculated using Student's t -test (*** $p < 0.001$). $p = 3.1469\text{E-}07$ (D).



◀ **Figure EV5. TDRD1 phase separation is required for IMC assembly, piRNA biogenesis, and spermiogenesis in adult testes.**

(A) Co-immunostaining of TDRD1 and γ H2AX on control and *Tdrd1^{c-3G5}* adult testes. Scale bars, 20 μ m. (B) Co-immunostaining of ACRV1 and γ H2AX on adult mouse testes. preL preleptotene, L leptotene, Z zygotene, P pachytene, D diplotene, RS round spermatid, ES elongated spermatid. Scale bars, 10 μ m. (C) TUNEL assays on adult testes. Scale bars, 200 μ m. (D, E) Co-immunostaining of TDRD1 and PIWIL2 on pachytene spermatocytes (D) and round spermatids (E) from adult testes. Scale bars, 10 μ m. (F, G) Co-immunostaining of PIWIL2-TDRKH (F) or PIWIL1-TDRKH (G) on adult testes. PS pachytene spermatocytes, DS diplotene spermatocytes. Scale bars, 10 μ m. Quantification of the colocalization ratio is shown on the right. ($n = 20$; ** $p < 0.01$; *** $p < 0.001$; ns not significant). The developmental stages of germ cells were distinguished according to DAPI staining. (H) Co-IP assay of PIWIL2 and PIWIL1 in control and *Tdrd1^{c-3G5}* adult testes. PIWIL2, PIWIL1, and TDRD1 protein levels were detected by WB. (I) Immunostaining of LINE1 ORF1 on adult testes. Scale bars, 50 μ m. (J) WB of LINE1 ORF1 in adult testes. β -actin served as a control. Data information: In (F, G), data were presented as mean $\pm$ s.e.m and p values were calculated using Student's t -test (** $p < 0.01$; *** $p < 0.001$; ns not significant). p values from left to right (F): $p = 0.1052$, $p = 2.1886E-13$; p values from left to right (G): $p = 0.0026$, $p = 2.6065E-12$.