Supporting Information

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SI Materials and Methods

Protein Expression and Purification. The gene encoding the eTudor domain of TDRD2 (residues 309–498) or TDRD2 [(residues 309–498)–$(GSA)_5$–PIWIL1(residues 2–13)] was cloned into the pET28-MHL vector. These plasmids were transformed into BL21(DE3) cells for overexpression. The protein expression was induced by adding 0.3 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 16 °C. The cells were collected and then disrupted by sonication in buffer A [25 mM Tris·HCl (pH 8.0), 500 mM NaCl, 5 mM β-mercaptoethanol, 1 mM PMSF]. The supernatants were loaded onto a Ni-NTA resin gravity column and eluted with buffer B [25 mM Tris·HCl (pH 8.0), 500 mM NaCl, 250 mM imidazole, 5 mM β-mercaptoethanol]. The proteins were further purified by gel-filtration chromatography using Superdex 200 (GE) equilibrated with buffer C [25 mM Tris·HCl (pH 8.0), 150 mM NaCl]. Peak fractions were concentrated by ultrafiltration to 30 mg/mL.

Other eTudor proteins and BmPAPI (residues 243–462) were expressed and purified using the same procedure.

Crystallization. Crystal screenings were performed by sitting-drop vapor-diffusion methods at 18 °C. Crystals of eTudor were grown by mixing 1 μL of protein with 1 μL of reservoir solution [0.2 M magnesium chloride, 0.1 M sodium cacodylate (pH 5.5), 15% (wt/vol) PEG8000]. The TDRD2–PIWIL1 crystals were obtained in the mixture solution of 0.2 M ammonium formate, 20% (wt/vol) PEG3350. Before data collection, the crystals were soaked in the reservoir solution supplemented with 20% glycerol before being flash-frozen.

Diffraction Experiments and Structure Determination. Diffraction data were reduced with XDS (26). The program PHASER was used for molecular replacement (27). REFMAC5 (28) and/or AutoBuster (29) were used for restrained refinement of molecular models. COOT (30) was used for interactive model rebuilding. Model geometry was analyzed with MOLPROBITY (31). The CCP4 (32), and PHENIX (33) programs were used to prepare the molecular models for publication.

Specifically, diffraction data for ligand-free eTudor were collected at CLS beamline 08-ID. Coordinates from PDB ID code 3FDR (5) and a low-resolution TDRD2 crystal structure (https://doi.org/10.5281/zenodo.1021859) were used for molecular replacement. Automatic rebuilding based on the molecular replacement solution was performed with ARP/wWARP (34). The model was further refined against diffraction data from an additional, nearly isomorphous crystal, for which statistics are shown in Table S2. Even though a modified PIWIL1 peptide had been added for crystallization, we did not find interpretable electron density for this additive.

Diffraction data for a crystal of the fusion protein were collected using a rotating copper anode (FR-E; Rigaku Corp.). The structure was solved by molecular replacement with coordinates from the apo TDRD2 crystal structure.

Fluorescence Polarization. Fluorescence polarization measurements were carried out with a Synergy 2 microplate reader (BioTek) at 25 °C. The fluorescein-labeled peptides synthesized by Tufts University Core Services were prepared in the buffer consisting of 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM DTT, and 0.01% Triton X-100. The labeled peptides (40 nM) were mixed with increasing concentrations of TDRD2 in 384-well plates. The fluorescence polarization signals were measured and processed as previously (11).

ITC Measurements. WT and mutant TDRD2 proteins or BmPAPI were expressed and purified as described above. All proteins and peptides were in buffer C [25 mM Tris (pH 8.0), 150 mM NaCl] unless otherwise indicated. The lyophilized peptides (Ontores Biotechnologies) were dissolved in the same buffer, and the pH was adjusted by adding NaOH. Peptide concentrations were estimated from the mass of lyophilized material. ITC experiments were performed using a MicroCal VP-ITC isothermal titration calorimeter at 25 °C. A typical titration experiment involved 26 injections of peptide (1 mM) solution into the cell containing protein (30 μM). The binding data were analyzed using Origin (MicroCal).

Cell Transfection, Lysate Preparation, and Immunoprecipitation. HEK293T cells were transfected with indicated plasmids using Lipofectamine 2000 (Thermo Fisher Scientific), according to the manufacturer's instructions. Forty-eight hours after transfection, HEK293T cells were collected and lysed with Nonidet P-40 lysis buffer containing 50 mM Tris·HCl (pH 7.4), 1% Nonidet P-40, 1 mM EDTA, and 150 mM NaCl. The lysates were incubated with M2 anti-FLAG resin (Sigma) overnight at 4 °C. After washing four times with Nonidet P-40 lysis buffer, the beads were boiled for 5 min, and loading buffer was added. Western blots were performed using FLAG antibody (Sigma) and GFP antibody (Abcam).

For the preparation of trimming lysate (Fig. 3*B*), HEK293T cells were transfected with TDRD2–shRNA expression vector using Lipofectamine 3000 (Thermo Fisher Scientific) twice. After reseeding the cells, mouse PNLDC1- and shRNA-insensitive TDRD2 expression vectors and TDRD2–shRNA expression vector were cotransfected using Polyethylenimine Max (Polysciences) and were harvested 2 d later. The cells were resuspended in hypotonic buffer [10 mM Hepes·KOH (pH 8.0), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 1× cOmplete EDTA-free protease inhibitor (Roche)] and sonicated. The whole-cell lysate was used as trimming lysate.

For the preparation of trimming lysate (Fig. 3*D*), *Drosophila* S2 cells were cotransfected with Trimmer and BmPAPI expression vectors using X-tremeGene HP DNA Transfection Reagent (Sigma) and were harvested 3 d later. The cells were resuspended in hypotonic buffer [10 mM Hepes·KOH (pH 8.0), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 1× cOmplete EDTA-free protease inhibitor (Roche)] and were incubated on ice for 30 min. After vortexing, the lysate was centrifuged at 1,000 × *g* for 20 min, and the pellet fraction resuspended in hypotonic buffer was used as trimming lysate.

For immunoprecipitation of BmPapi mutants (Fig. 3*C*), BmN4 cells were cotransfected with BmPapi and SIWI expression vectors using X-tremeGene HP DNA Transfection Reagent (Sigma) and were harvested 4 d later. The cells were resuspended in lysis buffer [20 mM Tris·HCl (pH 7.4), 150 mM NaCl, 1.5 mM $MgCl_2$, 8% glycerol, 0.15% Triton X-100, 1 mM DTT, 1× cOmplete EDTA-free protease inhibitor (Roche)] and were incubated on ice for 30 min. The soluble fraction was obtained by centrifugation at 17,000 × *g* for 30 min at 4 °C. After incubation with anti-FLAG M2 antibody (Sigma) conjugated on Dynabeads protein G (Thermo Fisher Scientific) at 4 °C for 2 h, the immunoprecipitates were washed with lysis buffer containing 0.5% Triton X-100 and were eluted with 3× FLAG peptides (Sigma).

Western blots were performed using anti-FLAG M2 (Sigma) and anti-SBP antibodies (Santa Cruz Biotechnology).

Phage Display Selections. Protein immobilization and phage selections were carried out as previously described (35). Briefly, biotin-labeled TDRD2 proteins (0.5 μg/mL in PBS buffer) were coated on 96-well MaxiSorp plates (Nalgene Nunc International) and were incubated overnight at 4 °C. The plates were blocked with 0.5% BSA for 1 h, followed by incubation with 100 μL of library phage solution for 1 h with gentle shaking. Afterward, the plates were washed 10 times using the PT buffer (PBS, 0.05% Tween-20) to remove the unbound phage. The bound phage was eluted by adding actively growing *E. coli* XL1-blue culture. After overnight growth the phage pellet was collected and used for the additional rounds of selection. Three rounds of panning were performed against immobilized TDRD2 proteins. Phages from individual clones were tested by phage ELISA, and the positive phage clones were sequenced.

Phage ELISA. Phage ELISA screens were performed as previously described (36). In brief, biotin-labeled proteins were immobilized on NeutrAvidin plates and incubated with selected phage supernatants for 2 h at 4 °C. The plate was washed with PT buffer, incubated with horseradish peroxidase/anti-M13 antibody conjugate (GE Healthcare) for 30 min, washed with PBS including 2% BSA, and developed with 3,3′,5,5′-tetramethylbenzidine substrate. Absorbance was measured at 450 nm.

In Vitro Trimming Assay. ssRNA loading and trimming were performed as previously described (20). In Fig. 3*B*, FLAG-MIWI expressing BmN4 cell lysate and 5′-radiolabeled ssRNA (UCGAAGUAUUCCGCGUACGUUAUGCUAGCUGAUCCUGACCGCUGAGUCGU) were used.

Thermal Shift Assay. The protein thermal shift assays were carried out using static light scattering (StarGazer). TDRD2 proteins [50 mM Hepes (pH 7.4), 150 mM NaCl] were mixed with the R10me0 or R10me2s peptides in a clear-bottomed 384-well plate. Thermal denaturation from 20 °C to 85 °C was monitored using the sensitive dye SYPRO Orange (Life Technologies) in increments of 1 °C/min. The melting temperature (Tm) is the middle point of the transition phage.

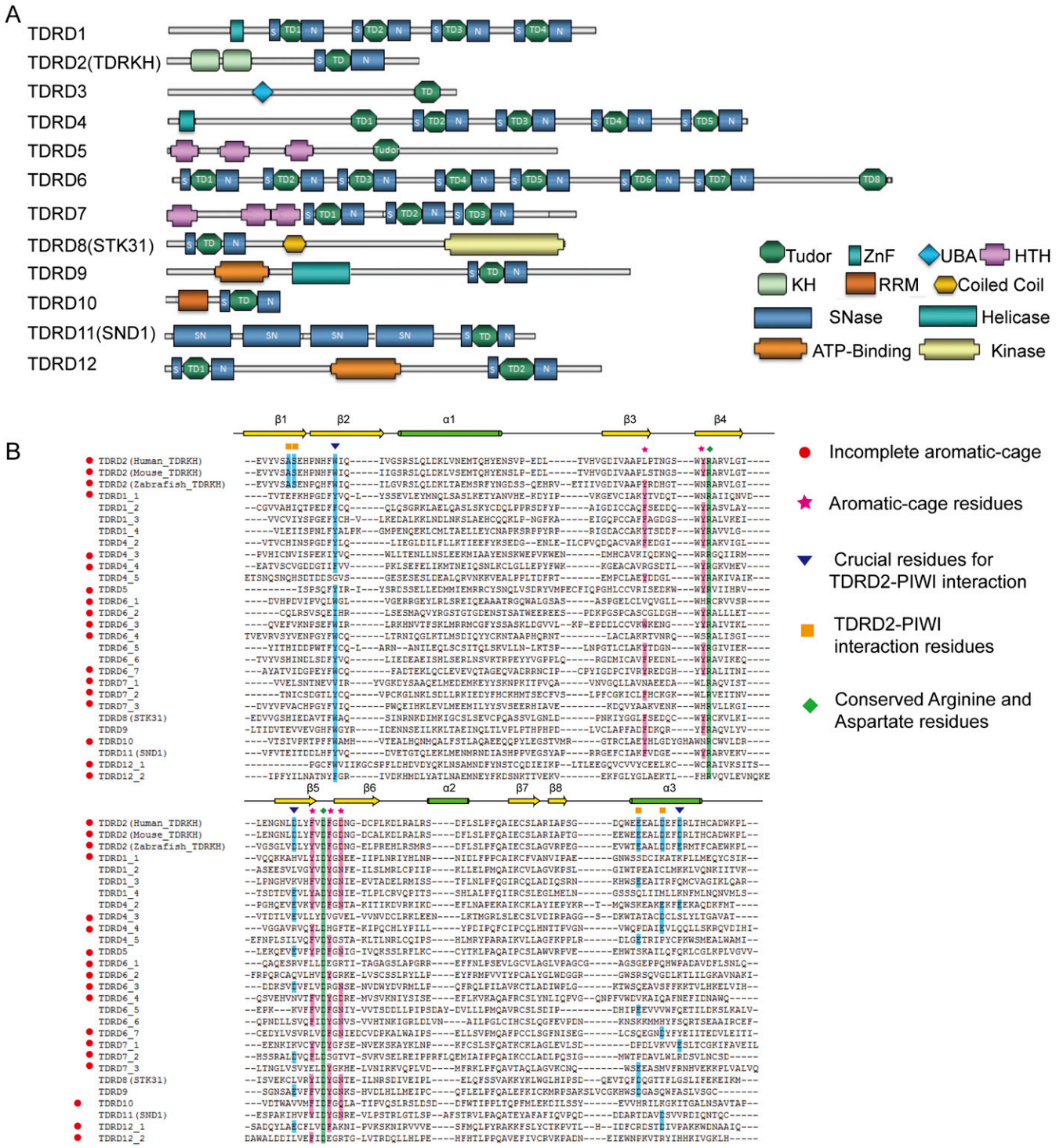


Fig. S1. Schematic diagrams and protein sequence alignment of human TDRD members. (*A*) Schematic diagrams of human TDRD members. (*B*) Sequence alignment of human TDRD members. Secondary structural elements of TDRD2 are indicated above the sequences. The strictly conserved residues arginine and aspartic acid are shaded green. The residues comprising the aromatic cage are indicated by pink stars. eTudor domains harboring an incomplete aromatic cage are indicated by red dots. The residues mediating interactions between TDRD2 and PIWIL1 are indicated by orange cubes. The residues critical for the TDRD2–PIWIL1 interactions are identified by blue triangles. The conserved arginine–aspartate motif, the signature of eTudor domains, is indicated by green diamonds.

A

Peptide Sequence	Kd (μM)
TGRARARARGRARGQY	0.18±0.03 *
TGRARARARme2sGRARGQY	0.44±0.06 *
TGRARARARGRARGQE	0.18±0.01
TGRARARARme2sGRARGQE	0.34±0.02
TGRme2sARARARGRARme2sGQE	13.23±1.76
TGRARARA	12.72±1.88
TGRme2sARARA	350±30
RGRARGQE	56±8
RGRARme2sGQE	300±23

B

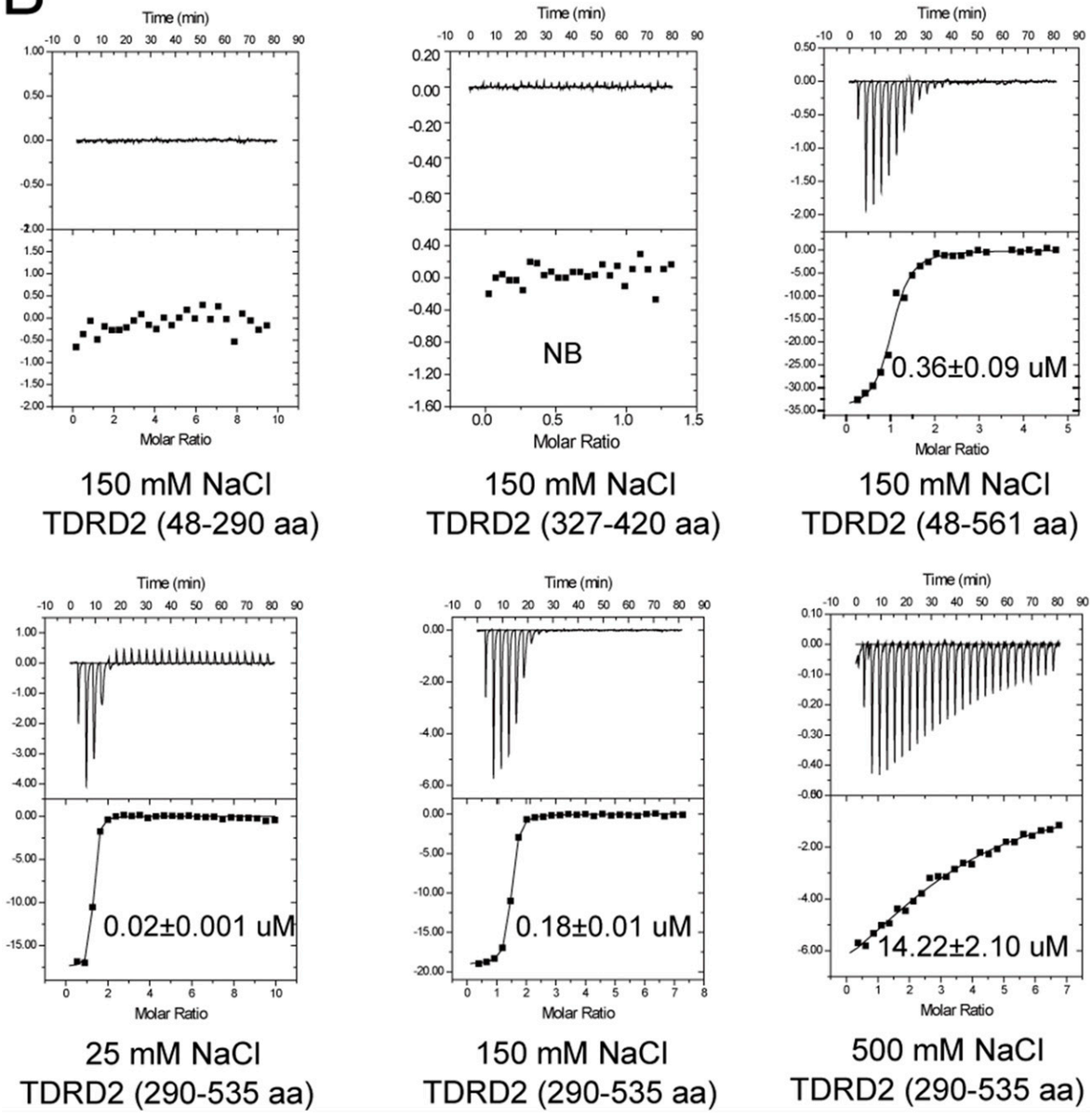


Fig. S2. ITC results of TDRD2 binding to various PIWIL1 peptides. (*A*) Binding affinities of different PIWIL1 peptides to the eTudor domain of TDRD2. Asterisks indicate that a tyrosine residue was added to the peptide for quantifying the concentration of the peptide. (*B*, *Upper Row*) ITC binding data of different TDRD2 truncation mutants to the PIWIL1 peptide (N-TGRARARARGRARGQE): (*Left*) KH domain: amino acids 48–290; (*Center*) single Tudor domain: amino acids 327–420; (*Right*) the N-terminal transmembrane-deleted region: amino acids 48–561). (*Lower Row*) ITC data for the interaction of TDRD2 with the PIWIL1 peptide (N-TGRARARARGRARGQE) at different salt concentrations.

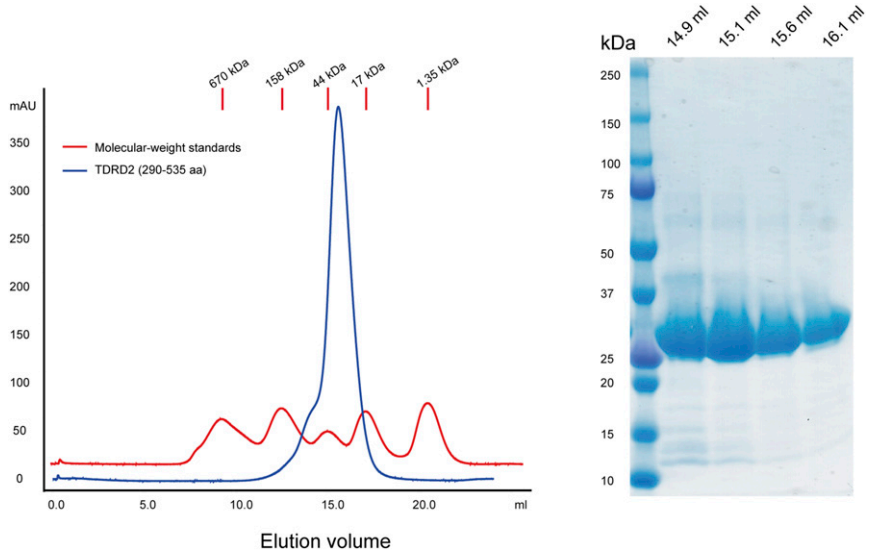


Fig. S3. TDRD2 behaves as a monomer in solution. (*Left*) Overlaid size-exclusion chromatograms (SEC) of TDRD2 (amino acids 290–535) and the protein molecular mass standard mixture solution. (*Right*) SDS/PAGE analysis of the SEC elution fractions corresponding to TDRD2 is shown with the standard protein marker.

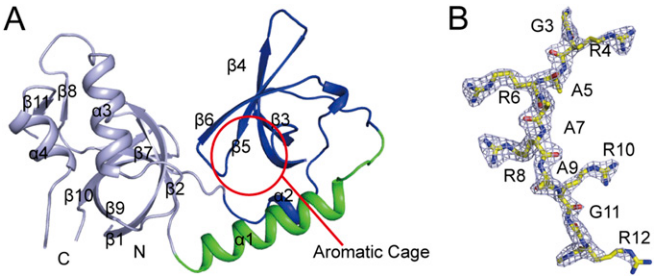


Fig. S4. Crystal structures of the eTudor domain of TDRD2. (*A*) Overall structure of the apo eTudor domain of TDRD2. The color scheme is the same as in Fig. 1*A*. (*B*) The *2Fo-Fc* electron density maps contoured at 2.0 σ for the PIWIL1 peptide.

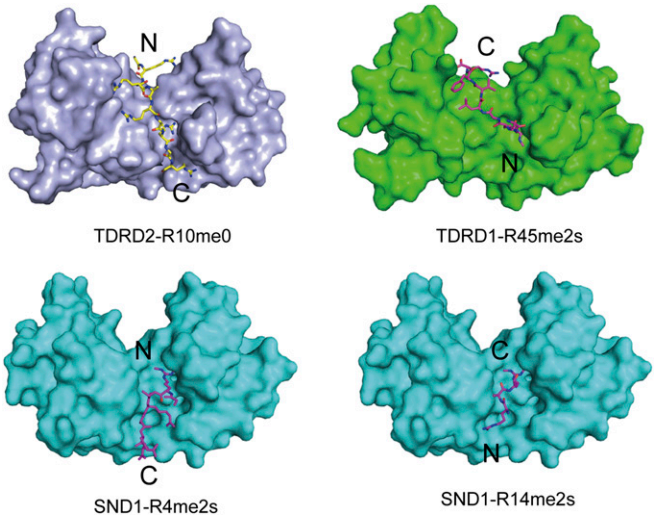


Fig. S5. Structural comparison of the PIWIL1 peptide complexes of TDRD2, SND1, and TDRD1. The eTudor-bound PIWIL1 peptides adopt different directions. The eTudor proteins are shown in surface representations. The bound ligands are shown in stick representations. (*Left*) R10me0 binds to TDRD2 (light blue) in the same direction as R4me2s binds to SND1 (cyan, PDB ID code 3OMC). (*Right*) R45me2s binds to murine TDRD1 (green, PDB ID code 4B9W) in the same direction as R14me2 binds to SND1 (cyan, PDB ID 3OMG).

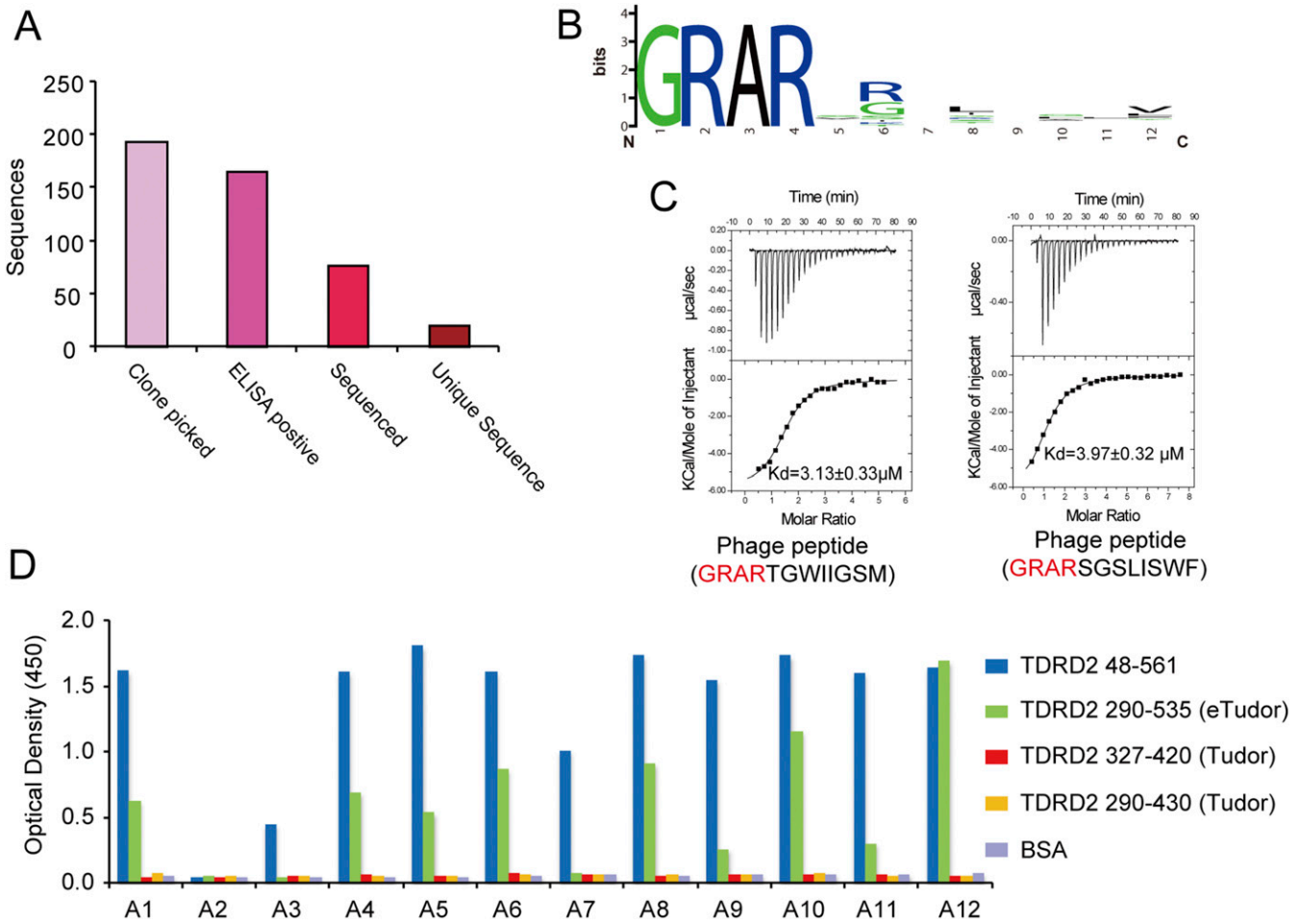


Fig. S6. Phage display selection of 12-mer peptide binding to TDRD2. (*A*) Histogram of the sequencing data of the phage pools after third round of panning. (*B*) A selective binding motif of TDRD2 generated by the phage display selection assay. (*C*) TDRD2-binding studies of the selected peptides by ITC. (*D*) Binding of selected phage clones to TDRD2 (amino acids 48–561), TDRD2 (amino acids 290–535), TDRD2 (amino acids 327–420), and TDRD2 (amino acids 290–430) as measured by ELISA.

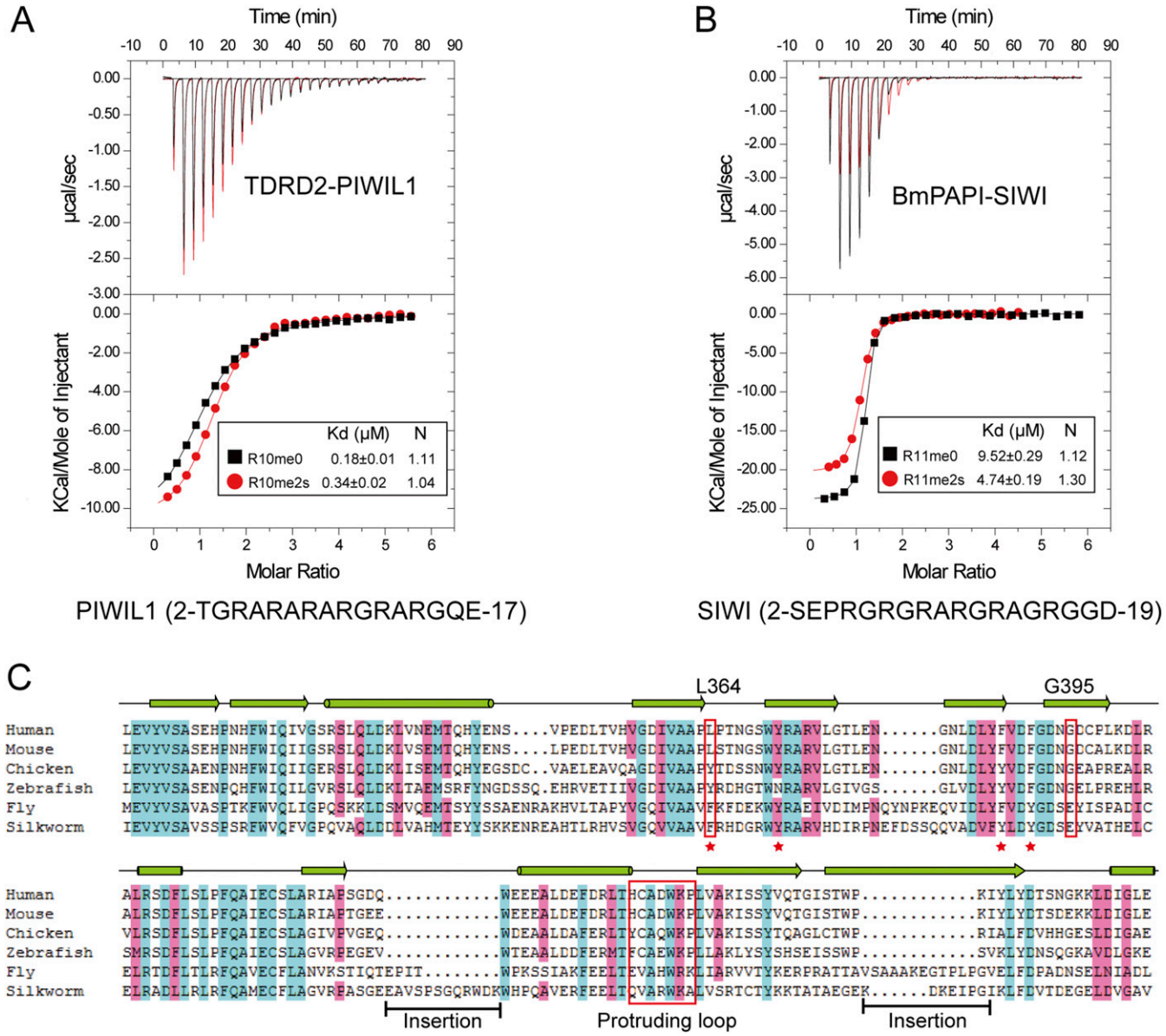
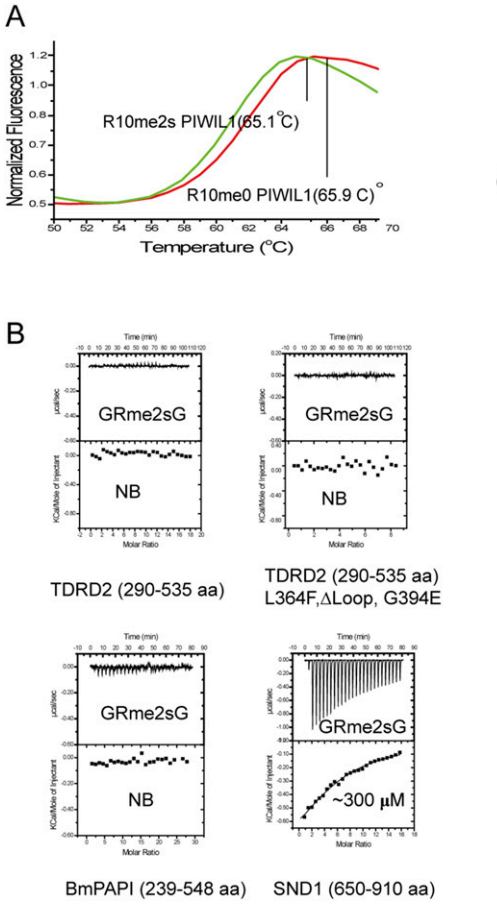


Fig. S7. BmPAPI shows a preference for Rme2s over Rme0. (*A*) Dissociation constants of TDRD2 with PIWIL1 peptides by ITC. (*B*) Dissociation constants of BmPAPI with SIWI peptides by ITC. (*C*) Sequence alignment of TDRD2 eTudor domains from different species. The strictly conserved residues are shaded light blue, and the conserved residues are shown in pink. The residues comprising the aromatic cage are indicated by red stars. Compared with human TDRD2, BmPAPI (silkworm TDRD2) harbors a complete four-residue aromatic cage. The stabilizing residue glutamate is also present in BmPAPI. Finally, there are two insertions flanking the protruding loop of BmPAPI.



C

Protein	Kd (μM)
Y371D	0.06±0.001
Y371G	0.99±0.05
F388D	0.21±0.02
F388G	1.57±0.16
F391D	3.57±1.10
F391G	5.41±0.62

Fig. S8. TDRD2 is an arginine methylation-independent binder. (*A*) ThermoFluor shift melting curves of TDRD2 with the unmodified (R10me0) or R10me2s peptides. (*B*) Dissociation constants of TDRD2, BmPAPI, or SND1 with the GRme2sG peptides by ITC. BmPAPI and TDRD2, even the triple-mutant TDRD2, display no detectable binding to GRme2sG peptides. However, SND1 can bind to GRme2sG peptides. (*C*) Dissociation constants of the TDRD2 mutants with the PIWIL1 peptides by ITC. Replacement of the aromatic cage residues into aspartate or glycine had less impact on the PIWIL1 binding than the alanine substitution. NB, no detectable binding.

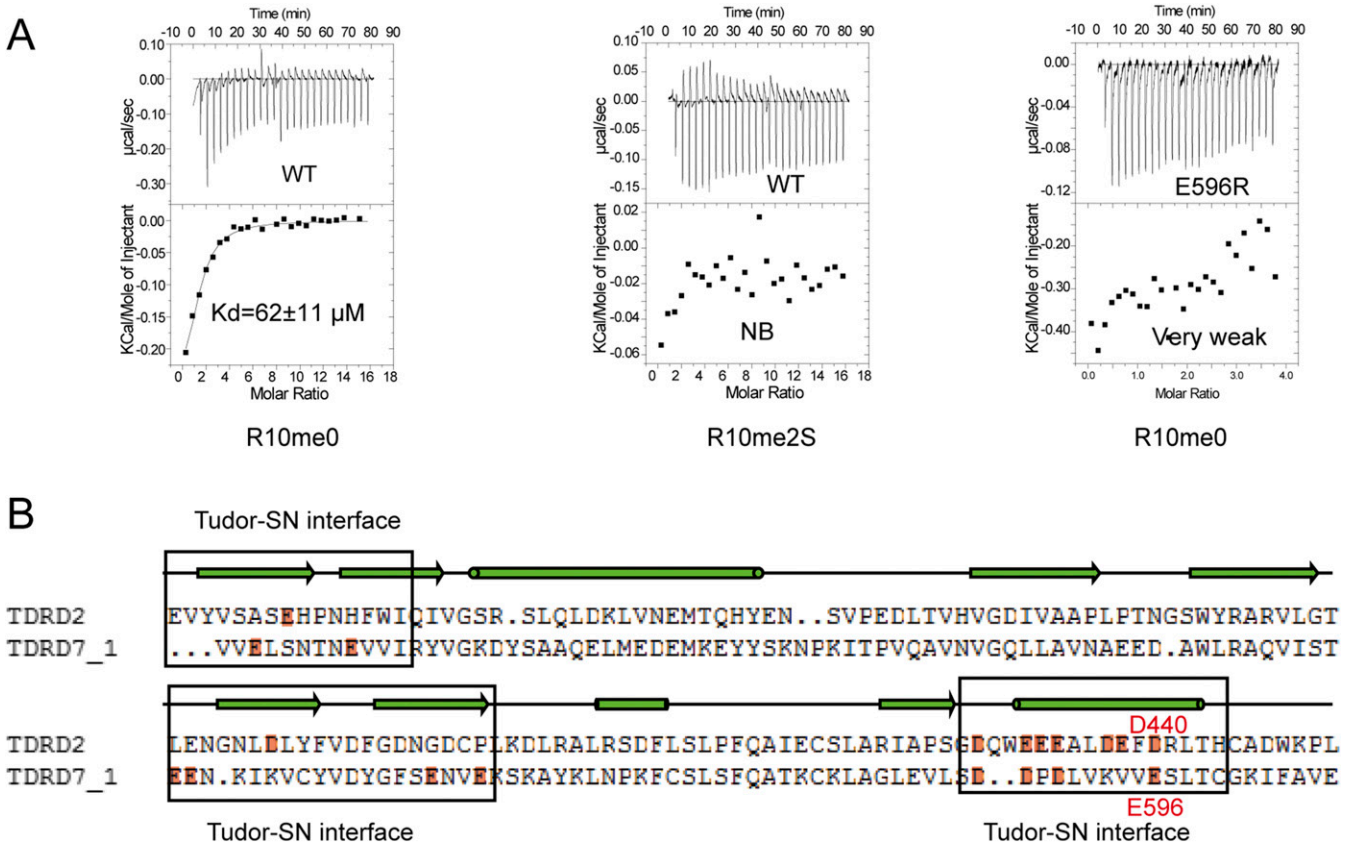


Fig. S9. The Tudor–SN interface is important for eTudor–PIWI interactions. (*A*) ITC binding curves of TDRD7-1 with PIWIL1 peptides. (*B*) Sequence alignment of eTudor domains of TDRD2 and TDRD7-1. The acidic residues in the Tudor–SN interface are shaded red.

Table S1. Dissociation constants of eTudor domains of human TDRD proteins with PIWIL1 peptides as determined by fluorescence polarization

Protein name	Protein sequence	Tudor domain no.	PIWIL1 R4me0	PIWIL1 R4me1	PIWIL1 R4me2a	PIWIL1 R4me2s	PIWIL1 R10me2s
TDRD1	215–460	TD1	WB	WB	WB	WB	WB
TDRD1	460–680	TD2	WB	65 ± 7	356 ± 76	**13 ± 2**	41 ± 6
TDRD1	680–905	TD3	NB	WB	243 ± 15	**117 ± 7**	187 ± 12
TDRD2(TDRKH)	250–515	TD	**0.2 ± 0.02**	0.6 ± 0.08	3.5 ± 0.7	4.9 ± 1	0.4 ± 0.06
TDRD4	890–1,120	TD3	WB	WB	NB	NB	NB
TDRD4	1,120–1,380	TD4	NB	NB	NB	NB	NB
TDRD4	1,380–1,623	TD 5	241 ± 39	240 ± 28	**78 ± 4**	240 ± 28	312 ± 48
TDRD7	461–644	TD1	**356 ± 47**	362 ± 54	WB	WB	WB
TDRD7	880–1,098	TD3	NB	NB	NB	NB	NB
TDRD8(STK31)	26–226	TD	NB	46 ± 3	177 ± 15	**28 ± 2**	42 ± 3
TDRD9	885–1,115	TD	177 ± 17	190 ± 20	**77 ± 6**	150 ± 15	72 ± 5
TDRD11(SND1)	650–910	TD	247 ± 19	97 ± 20	188 ± 38	**47 ± 6**	103 ± 18

The eTudor proteins that contain an incomplete aromatic cage are colored red. The highest affinity for each protein is bold. Binding affinity K_d is expressed in micromoles. NB, no detectable binding. WB, weak binding.

Table S2. Data collection and refinement statistics

	TDRD2 (amino acids 309–498)	TDRD2 [(amino acids 309–498)–(GSA)5–PIWIL1 (amino acids 2–13)]
Data collection		
Radiation source	CLS beamline 08ID	Rigaku Fr-E
Wavelength, Å	0.9796	1.5418
Space group	P1211	P212121
Cell dimensions		
a,b,c, Å	36.69, 53.80, 108.06,	47.02, 72.57, 130.55
α, β, γ, °	90.00, 84.26, 90.00	90.00, 90.00, 90.00
Resolution limits, Å	53.80–1.95 (2.00–1.95)	48.53–1.90 (1.94–1.90)
Completeness, %	99.6 (99.0)	98.0 (87.2)
R_{sym} (I), %	0.087 (1.114)	0.078 (0.965)
I/σI	12.0 (1.6)	14.2 (1.4)
Redundancy	3.8 (3.8)	6.1(4.4)
Refinement		
Refinement resolution, Å	34.11–1.95	48.53–1.90
Reflections used/free	28,758/1,872	33,532/1,724
No. of atoms		
Protein	3,053	3,057
Peptide	Not applicable	114
Water	85	111
Others	28	28
Average B-factor, $Å^2$		
Protein	42.0	42.2
Peptide	Not applicable	44.0
Water	40.0	33.4
Others	46.4	32.9
R_{work}/R_{free}	0.190/0.222	0.205/0.236
Bond lengths, Å	0.010	0.010
Bond angles, °	1.1	1.1
Ramachandran plot		
Favored	97.64	97.75
Outlier	0	0

Values in parentheses are for the respective highest-resolution shells.

Table S3. Dissociation constants of TDRD2 WT and mutants with PIWIL1 peptides by ITC

Protein	Peptide	K_d, μM	Rme2s/Rme0 binding preference
WT	TGRARARARGRARGQE	0.18 ± 0.01	0.51
	TGRARARARme2sGRARGQE	0.34 ± 0.02	
L364F	TGRARARARGRARGQE	0.64 ± 0.04	0.44
	TGRARARARme2sGRARGQE	1.45 ± 0.16	
L364F, ΔLoop	TGRARARARGRARGQE	4.18 ± 0.23	0.9
	TGRARARARme2sGRARGQE	4.63 ± 0.27	
L364F, G394E	TGRARARARGRARGQE	7.58 ± 1.05	0.85
	TGRARARARme2sGRARGQE	8.93 ± 1.99	
G394E, ΔLoop	TGRARARARGRARGQE	1.56 ± 0.21	0.87
	TGRARARARme2sGRARGQE	1.79 ± 0.07	
L364F, ΔLoop, G394E	TGRARARARGRARGQE	3.57 ± 0.21	1.24
	TGRARARARme2sGRARGQE	2.87 ± 0.55	

The symmetrical dimethylated (me2s) arginine are colored red.