# Supporting Information

## Koh et al. 10.1073/pnas.1212917110

### SI Materials and Methods

**Protein Purification.** N-terminal $His_6$-Dicer was purified as previously described with several modifications (1). After the protein was eluted from the Ni-nitrilotriacetic acid (NTA) resin (Qiagen), the N-terminal $His_6$-tag protein was not cleaved, and instead the protein was dialyzed overnight into a low salt buffer [100 mM KCl, 10% (vol/vol) glycerol, 1 mM Tris(2-carboxyethyl) phosphine (TCEP), and 20 mM Hepes, pH 7.5]. The concentrated protein was applied to HiTrap 5 mL Q HP column (GE Healthcare) with a 0–800 mM KCl gradient. The peak material, which eluted at about 400 mM KCl, was concentrated to <1 mL and applied to a Superdex 200 16/60 column (Amersham Pharmacia) equilibrated in gel filtration buffer [150 mM KCl, 10% (vol/vol) glycerol, 1 mM TCEP, and 20 mM Hepes, pH 7.5]. Fractions containing nonaggregated protein were pooled, concentrated to ∼3 mg/mL, and used in subsequent reconstitution experiments. All purification steps were carried out at 4 °C. All protein concentrations were determined by the Nanodrop (Bio-Rad).

TRBP, PACT, and their truncations were cloned as cleavable N-terminal $His_6$-MBP fusions. Each was purified separately from bacterial overexpression using methods previously described (1). One truncation, TRBP dsRBD2 and -3, would stick to the Ni-NTA resin. Therefore, this protein was applied to the Ni-NTA column with a 100–800 mM Imidazole gradient, with peak material eluting at about 500 mM Imidazole.

R3D1 was cloned as a cleavable N-terminal $His_6$-GST fusion and overexpressed in bacteria. The protein was purified as previously described (1) with the NTA column being replaced by a 1 mL GSTrap Column (GE Healthcare).

**RNA Labeling and Annealing.** Cy3/Cy7 NHS ester and Alexa 647 NHS ester were purchased from GE Healthcare and Invitrogen, respectively. Excess dyes were mixed together with RNA containing amino modifier C6 dT in 100 mM $NaHCO_3$, pH 8.5, and incubated overnight for the RNA labeling. Residual dyes were removed by Biorad P-6 column or ethanol precipitation. Labeling efficiency was higher than 90% for all labeling reactions. For generating dsRNA from each labeled or nonlabeled ssRNA, each strand of dsRNA was mixed with its complementary one in an annealing buffer (10 mM Tris, pH 8, and 100 mM NaCl), heated at 90 °C for 2 min, and slowly cooled down to room temperature.

**Protein Labeling.** Protein and Cy3 NHS ester or Alexa 647 NHS ester were mixed together in 100 mM $NaHCO_3$, pH 8.5, buffer and incubated for 30 min at room temperature. The ratio between protein and dye was adjusted to achieve a 1:1 ratio of dye–protein by trial and error. N-terminal–specific labeling was done by an enzymatic reaction of Acp Synthase (New England Biolabs) using A1- tagged TRBP and CoA-modified fluorophores. Biorad P-6 column was used to remove the excess dyes, and then the labeling efficiency was calculated by measuring UV-VIS absorbance.

**smFRET Assays.** Single-molecule FRET detection was achieved by a homemade wide-field TIRF microscopy. Single fluorophores immobilized on a PEG-coated quartz surface emit light by an excitation of a solid-state 532 nm laser (Spectra physics). The fluorescent emission was collected through a water immersion Olympus objective (60×, Numerical Aperture = 1.2), passed through 555 nm long pass filter for rejection of laser's Rayleigh scattering, separated by two, a green and red emission, at a dichroic mirror (cutoff, 630 nm), and detected by an EMCCD camera (Andor). For three-color FRET measurement, two sets of dichroic mirrors (cutoff, 630 nm and 725 nm) were used to separate the emission by three, the one from Cy3, Cy5, and Cy7, respectively. The exposure time was 30 ms, and the detected fluorescent signals were analyzed with a custom-written IDL and MATLAB program.

An oxygen scavenger system [0.5% (wt/vol) glucose, 100 mg/mL glucose oxidase (Sigma), and 8.8 kU/mL catalase (Calbiochem)] was used together with 5–10 mM trolox (Sigma) to stabilize fluorophore during a single-molecule data acquisition. TRBP sliding assay was performed in 20 mM Tris, pH 7.5, 25 mM NaCl, 1 mM DTT, and 0.1 mg/mL BSA in combination with the oxygen scavenger system. A total of 2 mM $MgCl_2$ was added to initiate an RNA cleavage by Dicer–TRBP.

1. MacRae IJ, Ma E, Zhou M, Robinson CV, Doudna JA (2008) In vitro reconstitution of the human RISC-loading complex. *Proc Natl Acad Sci USA* 105(2):512–517.

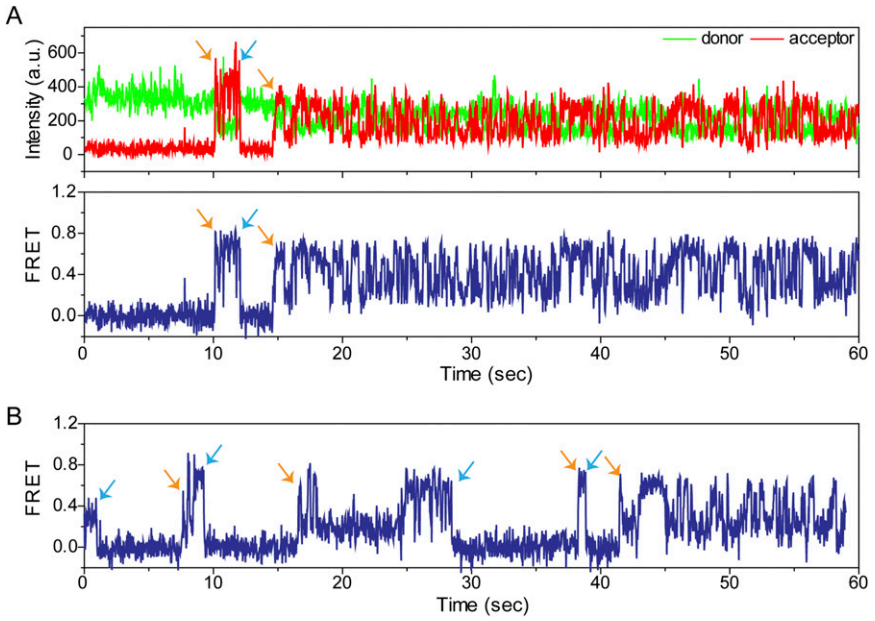


**Fig. S1.** Multiple binding and dissociation of TRBP while slidng on dsRNA. (*A* and *B*) Single-molecule time traces show TRBP's binding (displayed by an orange arrow) and dissociation (blue arrow) as well as its repetitive FRET change at its dsRNA-bound state, demonstrating that the observed FRET changes aren't caused by rapid binding and dissociation of TRBP.

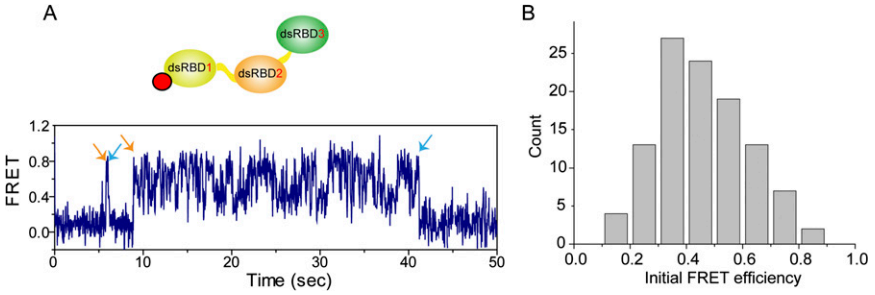


**Fig. S2.** Interaction of N-terminal–labeled TRBP with dsRNA. (*A*) N-terminal–labeled TRBP using A1-tag–engineered TRBP, CoA-647, and ACP synthase showed a similar FRET trace with the randomly labeled TRBP as shown in Fig. 1. The similar FRET change regardless of a dye position suggests that it is likely due to the movement of an entire TRBP along dsRNA rather than a specific subdomain's conformational change. (*B*) Histogram of an initial FRET when TRBP binds to dsRNA shows a wide distribution covering the whole FRET range of TRBP movement on dsRNA, suggesting that TRBP could bind to dsRNA in a nonsequence-specific manner.

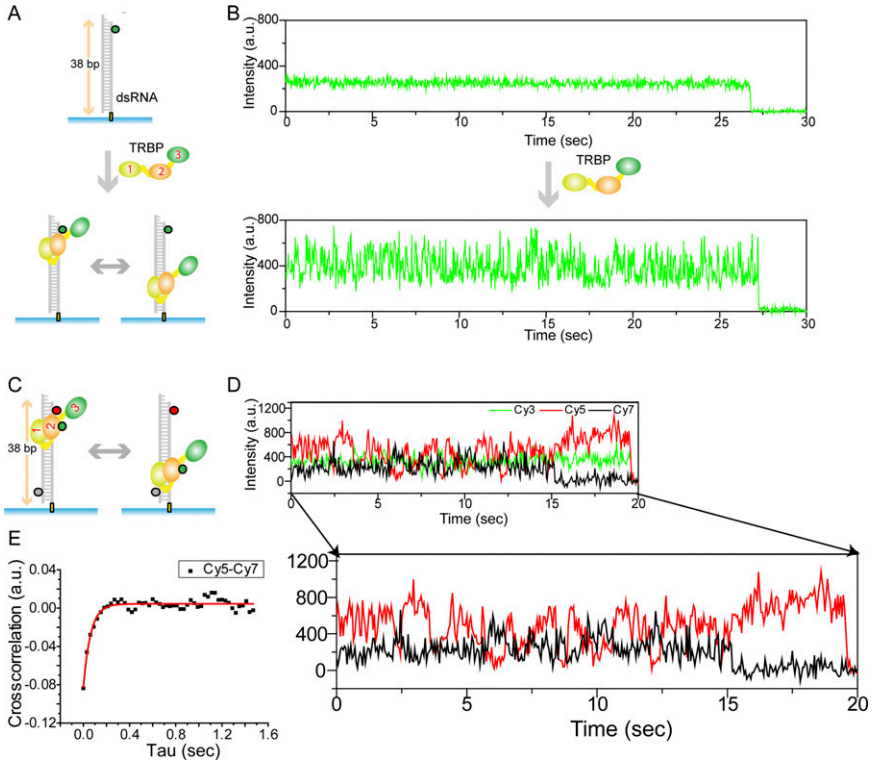


**Fig. S3.** PIFE (one-color) and three-color visualization of TRBP's diffusion on dsRNA. (*A* and *B*) Repetitive Cy3 intensity fluctuations were obtained by adding unlabeled TRBP to Cy3-labeled dsRNA. The intensity of Cy3 can increase only by a proximal protein contact, so the rapid intensity changes are consistent with the TRBP's repetitive sliding along dsRNA as seen in Fig. 1. (*C*) Cy3-labeled TRBP was added to dsRNA labeled with Cy5 and Cy7 at each end for the three-color FRET assay. (*D*) Anticorrelation between Cy5 and Cy7 intensity was observed, supporting TRBP's sliding along dsRNA. An enlarged view of Cy5 and Cy7 intensity was displayed to show the anticorrelation clearly. (*E*) Cross-correlation analysis on Cy5 and Cy7 intensity generated a curve fitted to an exponential rise, further demonstrating the anticorrelation between Cy5 and Cy7 intensity.

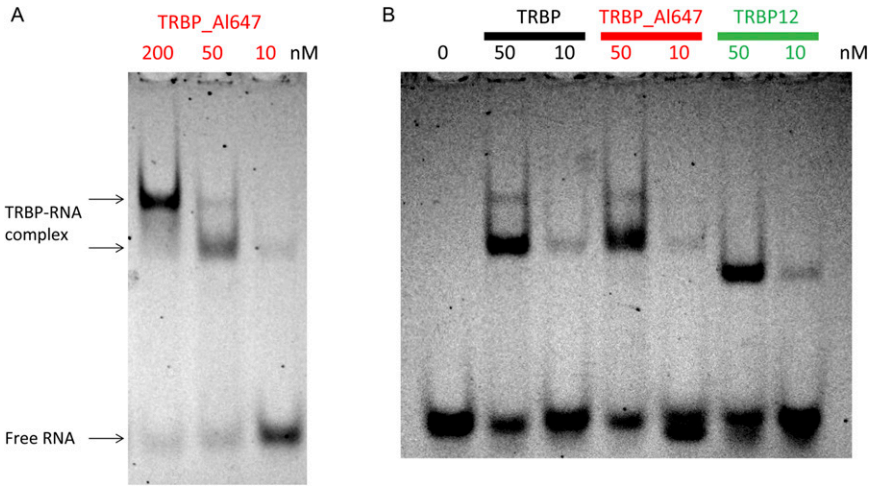


**Fig. S4.** TRBP binds to dsRNA by a single event at 10 nM TRBP concentration. (*A*) An electrophoretic mobility shift assay using Alexa 647–labeled TRBP (named as TRBP_Al647) and 10 nM Cy3-labeled 19 bp dsRNA exhibited one TRBP–RNA complex at 10 nM TRBP concentration and two TRBP–RNA complexes at higher TRBP concentration. Even at 50 nM TRBP concentration, ~90% of TRBP binds to dsRNA as a single complex. (*B*) We compared nonlabeled TRBP, TRBP_Al647, and dsRBD3-truncated TRBP mutant (named as TRBP12). Alexa 647 labeling of TRBP didn't affect its binding affinity to dsRNA, and dsRBD-truncated TRBP mutant showed a single TRBP–RNA complex.

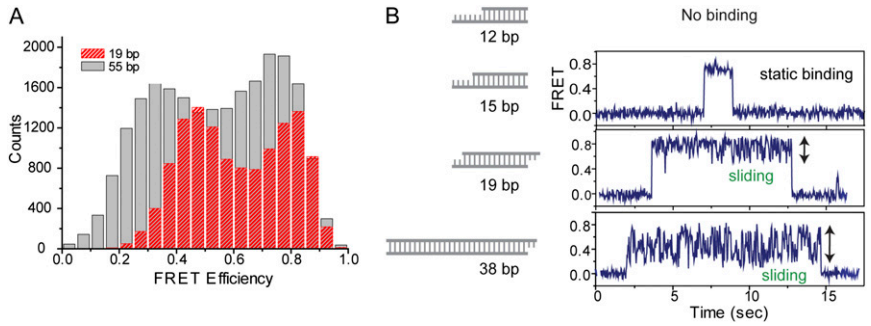


**Fig. S5.** dsRNA length dependence of TRBP diffusion. (*A*) FRET histogram of *siRNA* (19 bp) and 55 bp dsRNA shows a larger FRET dynamic range with longer dsRNA. Between 60–80 molecules were collected to generate the FRET histograms. The *y*-axis displays counts per 30 ms for selected FRET traces among the collected molecules. (*B*) TRBP didn't bind dsRNA as short as 12 bp, did bind but rarely slide on 15 bp dsRNA, and did slide on longer dsRNA, suggesting that a minimal length of dsRNA is required for both TRBP's binding and sliding, and the one for binding is not long enough for sliding.

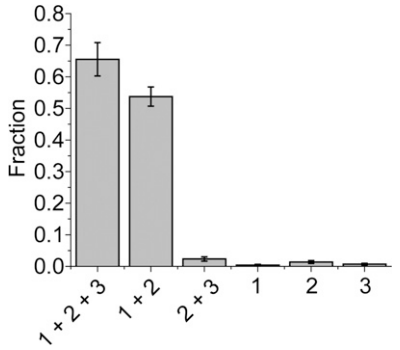


**Fig. S6.** Binding fraction of TRBP truncation mutants to dsRNA at 10 nM protein concentration. TRBP truncation mutants of dsRBD1+dsRBD2 (1+2) showed a similar binding affinity with a wild-type TRBP (1+2+3), whereas all of the other truncation mutants exhibited more than 10 times weaker dsRNA binding affinity.

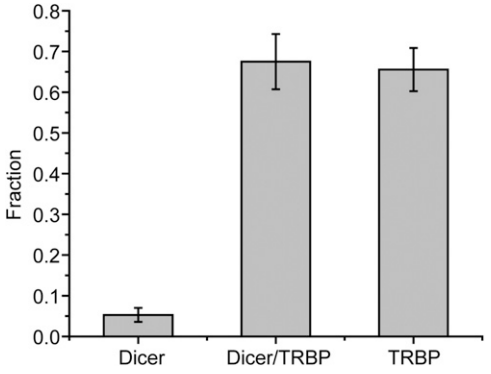


**Fig. S7.** Binding fraction of Dicer, Dicer-TRBP, and TRBP to dsRNA at 10 nM protein concentration. Dicer–TRBP and TRBP can bind to more than 60% of 38 bp dsRNA, whereas Dicer binds to less than 10% at 10 nM protein concentration, suggesting that TRBP enhances the binding affinity of Dicer to dsRNA.

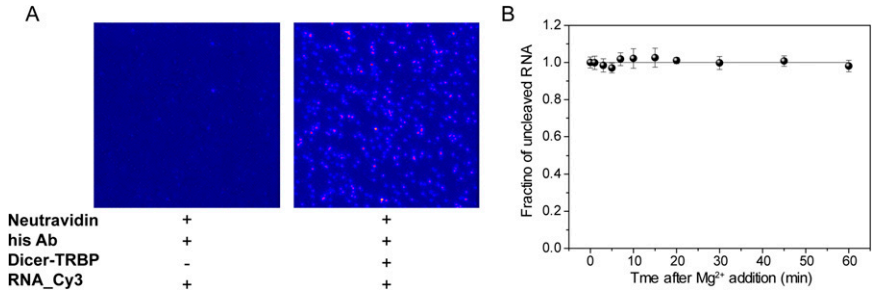


**Fig. S8.** Pull-down of dsRNA to an immobilized Dicer–TRBP complex and a cleavage assay using noncleavable RNA. (*A*) Images of fluorescence spots show that dsRNA binds specifically to Dicer–TRBP immobilized surface. (*B*) No apparent RNA cleavage by Dicer–TRBP was observed with an immobilized noncleavable RNA, which possesses three DNA bases at the cleavage site of Dicer (21st to 23rd nucleotide from 5′ end) instead of RNA bases.

**Table S1. Sequence of oligonucleotides for TRBP sliding and Dicer–TRBP cleavage assay**

| Name | Sequence |
|---|---|
| siRNA (19RNA) | 5′-rUrAdTrArCrArArUrCrUrArCrUrGrUrCrUrUrArCrC-biotin-3′ (up)<br>5′-rUrArArGrArCrArGrUrArGrArUrUrGrUrAdTrArUrU-3′ (down) |
| 25RNA | 5′-rGrCrUrUrGrUrCrGrGrGrArGrCrGrCrCrArCrCrCrUrCrUrGrC-biotin-3′ (up)<br>5′-rGrCrArGrArGrGrGrUrGrGrCrGrCrUrCrCrCrGrArCrArArGrC-DY547-3′ (down) |
| presiRNA (38RNA) | 5′-rGrCdTrUrArArCrArArCrCrArGrArUrCrArArArGAAArArArArCrArGrArCr ArUrUrGrUrCrA-biotin-3′ (up)<br>5′-rUrGrArCrArArUrGrUrCrUrGrUrUrUrUrUrUrCrUrUrUrGrArUrCrUrGrGr UrUrGrUrUrArArGrCrGrU-DY547-3′ (down1)<br>5′-rUrGrArCrArAdTrGrUrCrUrGrUrUrUrUrUrUrCrUrUrUrGrArUrCrUrGr GrUrUrGrUrUrArArGrCrGrU-3′ (down2: three-color FRET experiment) |
| 40RNA | 5′-rArCrGrCrUrUrArArCrArArCrCrArGrArUrCrArArArGAAArArArArCrArGr ArCrArUrUrGrUrCrA-biotin-3′ (up)<br>5′-rUrGrArCrArArUrGrUrCrUrGrUrUrUrUrUrUrCrUrUrUrGrArUrCrUrGrGr UrUrGrUrUrArArGrCrGrU-DY547-3′ (down) |
| 55RNA | 5′-rArCrGrCrUrUrArArCrArArCrCrArGrArUrCrArArArGrArArArArArArCr ArGrArCrArUrUrGrUrCrArArUrUrGrCrArArArGrCrArArArArA-biotin-3′ (up)<br>5′-rUrUrUrUrUrGrCrUrUrUrGrCrArArUrUrGrArCrArArUrGrUrCrUrGrUrUrUr UrUrUrCrUrUrUrGrArUrCrUrGrGrUrUrGrUrUrArArGrCrGrU-DY547-3′ (down) |
| DNA–RNA | 5′- biotin-ATACAAACATTTTTATTTCCTTTTTAATTTAATTTAATTA-Cy3-3′ (up)<br>5′-ppprGrGrUrUrUrUrCrUrUrCrUrGrArArGrArUrArArArGrGrUrArArArGrUr GrUrCrArUrArGrCrArCrCrArArCrUrGrUrUrArArUrUrArArArUrUrArArArUr UrArArArArArGrGrArArArUrArArArArArUrGrUrUrUrGrUrArU-3′ (down) |
| 50ssRNA | 5′-ppprGrGrCrArGrCrUrUrCrGrArCrGrUrCrArUrArUrCrGrArUrCrUrGrCrUrUrGr UrCrGrGrGrArGrCrGrCrCrArCrCrCrUrCrUrGrC-biotin-3′ |
| DsrA | 5′-rArArCrArCrArUrCrArGrArUrUrUrCrCrUrGrGrUrGrUrArArCrGrArArUrUrUr UrUrUrArArG-3′ |
| rpoS1 | 5′-biotin-rArUrUrUrUrGrArArArUrUrCrGrUrUrArCrArArGrGrGrGrArArArUrCr CrGrUrArArArCrCrC-Cy3-3′ |
| rpoS2 | 5′-rCrArArGrGrGrArUrCrArCrGrGrGrUrArGrGrArGrCrCrArCrCrUrUrArUrGrArGr GrUrCrArGrArArU-3′ |
| prelet-7_n | 5′-rArCrCrArCrUrGrGrGrArGrArUrArArCrUrArUrArCrArArUrCrUrArCrUrGrUr CrArUdTrCrC-biotin-3′ (up1)<br>5′-rArCrCrArCrUrGrGrGrArGrArUrArArCrUrArUrArCrArArUrCrUrArCrUrGrUr CrArUdTrCrC-3′ (up2: cleavage assays)<br>5′-rArGdTrGrGrUrArGrUrArGrGrUrUrGrUrArUrArGrUrUrUrUrArGrGrGrUrCr ArCrArCrCrC-3′ (down) |

dT (underlined in table) is a DNA nucleotide T with an amino C6 linker, which is conjugated with a dye NHS ester at the labeling reaction.