Supplementary Table S1.

Crystallographic data collection and refinement statistics

	Tt-Ago ternary complex
Data collection	
Space group	P21
Cell dimensions	
a, *b*, *c* (Å)	61.2, 120.5, 109.1
α, β, γ(°)	90.0, 105.3, 90.0
Resolution (Å)	50-3.0 (3.1-3.0) *
R_{sym} or R_{merge}	0.081 (0.397)
$I/\sigma I$	15.8 (2.1)
Completeness (%)	98.6 (99.4)
Redundancy	4.1 (3.9)
Refinement	
Resolution (Å)	30-3.0
No. reflections	30610
R_{work}/ R_{free}	0.225/0.282
No. atoms	
Protein	10,077
Ligand/ion	1,038
Water	18
B-factors	
Protein	67.8
Ligand/ion	87.4
Water	59.4
R.m.s deviations	
Bond lengths (Å)	0.0089
Bond angles (º)	1.79

*Highest resolution shell is shown in parenthesis.

Supplementary Table 2.
Sequences of the DNA guides and RNA target described in Fig.4.

Guide strands were oligodeoxynucleotides and the 5'-phosphate is indicated by 'p'. (b) Positions of 2'-O-methylated nucleotide substitutions are indicated as bold letters. The target RNA was an *in vitro* transcript.
All guides (Fig. 4a,b,c,d,e) were DNA oligonucleotides. Presence of a 5'-phosphate is indicated by 'p'. (a,b) Alterations to the fully complementary sequence (21-nt) are indicated as bold letters; the target sequence within the target RNA is underlined. (c) '__' indicates the deletion of two nucleotides; bold letters indicate unpaired nucleotides to the target sequence resulting in bulges within the guide oligo. (d) Alterations within the seed or non-seed region of the target RNA itself (Fig. 4d) are indicated in bold also. Only the target segment of the target RNA is shown.

Fig. 4a
Mutated residues are indicated in bold.

21-nt	p-TCGAAGTATTCCGCGTACGTG
T1G	p-**G**CGAAGTATTCCGCGTACGTG
C2A	p-T**A**GAAGTATTCCGCGTACGTG
G3T	p-TC**T**AAGTATTCCGCGTACGTG
A4C	p-TCG**C**AGTATTCCGCGTACGTG
A5C	p-TCGA**C**GTATTCCGCGTACGTG
G6C	p-TCGAA**C**TATTCCGCGTACGTG
T7G	p-TCGAAG**G**ATTCCGCGTACGTG
A8C	p-TCGAAGT**C**TTCCGCGTACGTG
T9G	p-TCGAAGTA**G**TACGCGTACGTG
T10G	p-TCGAAGTAT**G**ACGCGTACGTG
C11A	p-TCGAAGTATT**A**CGCGTACGTG
C12A	p-TCGAAGTATTC**A**GCGTACGTG
G13C	p-TCGAAGTATTCC**C**CGTACGTG
C14A	p-TCGAAGTATTCCG**A**GTACGTG
G15C	p-TCGAAGTATTCCGC**C**TACGTG
T16G	p-TCGAAGTATTCCGCG**G**ACGTG
A17C	p-TCGAAGTATTCCGCGT**C**CGTG
C18A	p-TCGAAGTATTCCGCGTA**A**GTG
G19A	p-TCGAAGTATTCCGCGTAC**A**TG

Fig. 4b
Mutated residues are indicated in bold.

21-nt	p-TCGAAGTATTCCGCGTACGTG
15-16	p-TCGAAGTATTCCGC**TG**ACGTG
14-17	p-TCGAAGTATTCCG**ATGC**CGTG
13-18	p-TCGAAGTATTCC**TATGCA**GTG
12-19	p-TCGAAGTATTC**ATATGCAT**TG

Fig. 4c
Deleted residues are indicated by '__'; bold letters indicate nucleotide insertions leading to bulges.

21-nt	p-TCGAAGTATTCCGCGTACGTG

10,11del	p-TCGAAGTAT__CGCGTACGTG
G5bulge	p-TCGA**G**AGTATTCCGCGTACGTG
T5bulge	p-TCGA**T**AGTATTCCGCGTACGTG
G11bulge	p-TCGAAGTATT**G**CCGCGTACGTG
A14bulge	p-TCGAAGTATTCCG**A**CGTACGTG
T14bulge	p-TCGAAGTATTCCG**T**CGTACGTG

Fig. 4d

Bold letters indicate nucleotide insertions leading to bulges. The symbols '//' indicate constant flanking sequences of the cleavage RNA substrate.

21-nt	p-TCGAAGTATTCCGCGTACGTG
Unmodified	5'-//CACGUACGCGGAAUACUUCGA//
A5 bulge	5'-//CACGUACGCGGAAUACU**A**UCGA//
A5,6 bulge	5'-//CACGUACGCGGAAUACU**AA**UCGA//
A6 bulge	5'-//CACGUACGCGGAAUAC**A**UUCGA//
A6,7 bulge	5'-//CACGUACGCGGAAUAC**AA**UUCGA//
A15,16 bulge	5'-//CACGUAC**AA**GCGGAAUACUUCGA//
A19,20 bulge	5'-//CAC**AA**GUACGCGGAAUACUUCGA//

Sequence of the cleavage RNA substrate.
The region underlined is complementary to the standard 21mer guide DNA.

5'pGAACAAUUGCUUUUACAGAUGCACAUAUCGAGGUGAACAUCACGUACGCGGA
AUACUUCGAAAUGUCCGUUCGGUUGGCAGAAGCUAUGAAACGAUAUGGGCUGAA
UACAAAUCACAGAAUCGUCGUAUGCAGUGAAAACUCUCUUCAAUUCUUUAUGCC
UAUAGUGUCACCUAAAU

Supplementary Table 3. Sequences of the DNA guides described in Fig. 4e and Supplementary Fig. 13.

Guide strands were oligodeoxynucleotides, except when substituted with 2'-O-methyl ribonucleoside residues. The presence of a 5'-phosphate is indicated by 'p'. (b) Positions of 2'-O-methylated nucleotide substitutions are indicated as bold letters.

Sequences of oligodeoxynucleotides described in Fig. 4e

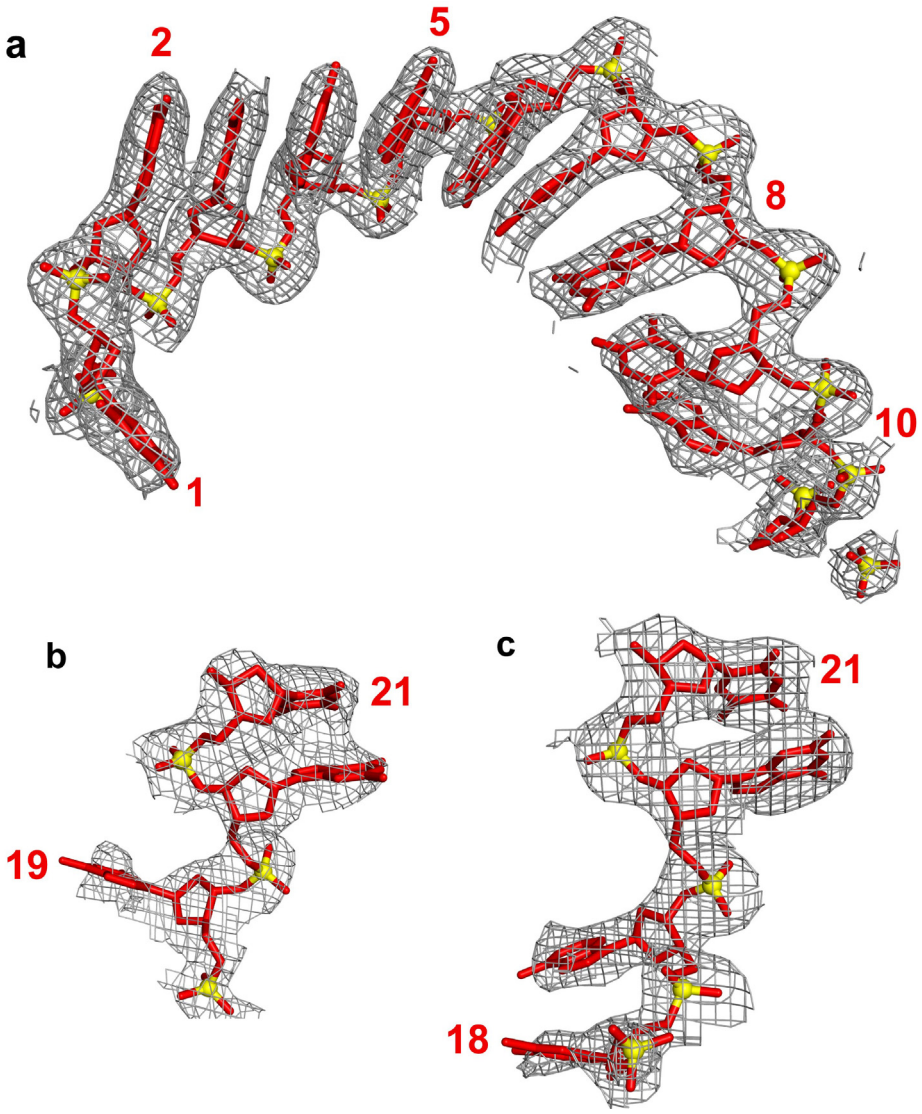
7	p-TCGAAGT
8	p-TCGAAGTA
9	p-TCGAAGTAT
10	p-TCGAAGTATT
11	p-TCGAAGTATTC
12	p-TCGAAGTATTCC
15	p-TCGAAGTATTCCGCG
18	p-TCGAAGTATTCCGCGTAC
21	p-TCGAAGTATTCCGCGTACGTG
24	p-TCGAAGTATTCCGCGTACGTGATG
30	p-TCGAAGTATTCCGCGTACGTGATGTTCACC
36	p-TCGAAGTATTCCGCGTACGTGATGTTCACCTCGATA

Sequences of oligodeoxynucleotides described in Supplementary Fig. 13.

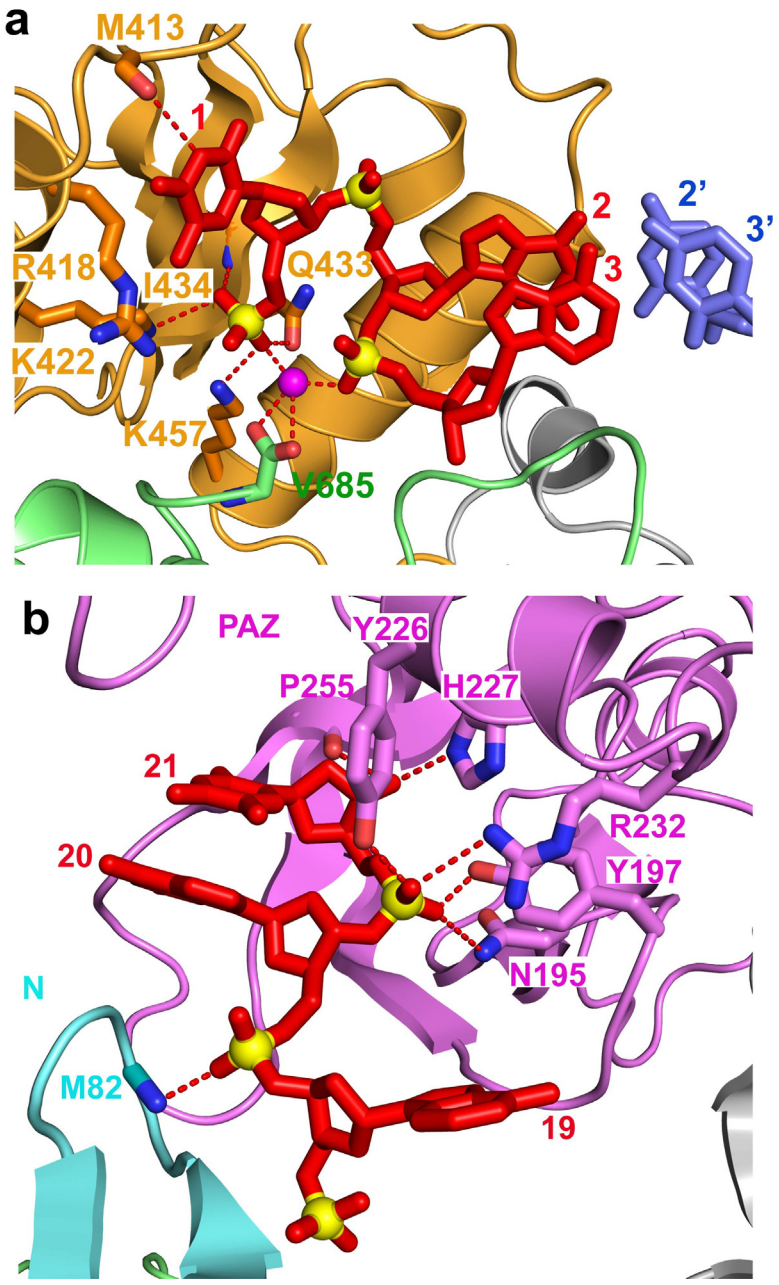
21-nt	p-TCGAAGTATTCCGCGTACGTG
full	p-**UCGAAGUAUUCCGCGUACGTG**
2+3	p-T**CG**AAGTATTCCGCGTACGTG
4+5	p-TCG**AA**GTATTCCGCGTACGTG
7+8	p-TCGAAG**UA**TTCCGCGTACGTG
10+11	p-TCGAAGTAT**UC**CGCGTACGTG
16+17	p-TCGAAGTATTCCGCG**UA**CGTG
2-9	p-T**CGAAGUAU**TCCGCGTACGTG
12-19	p-TCGAAGTATTC**CGCGUACG**TG

Sequences of oligodeoxynucleotides described in Supplementary Fig. 9 were the same as for Fig. 5a, but without 5'-phosphate when indicated.

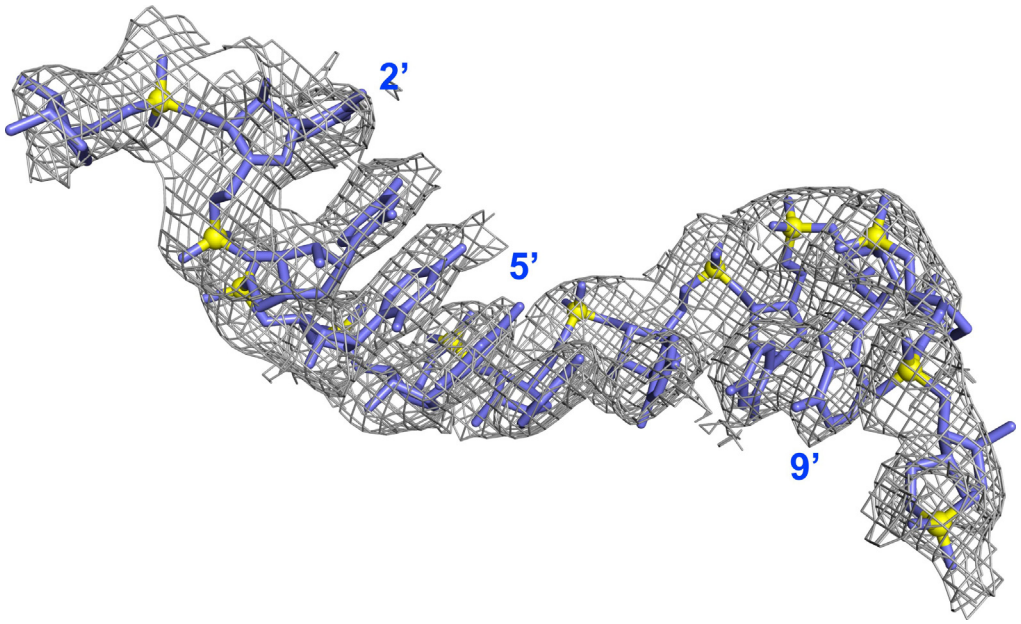
Supplementary Figures



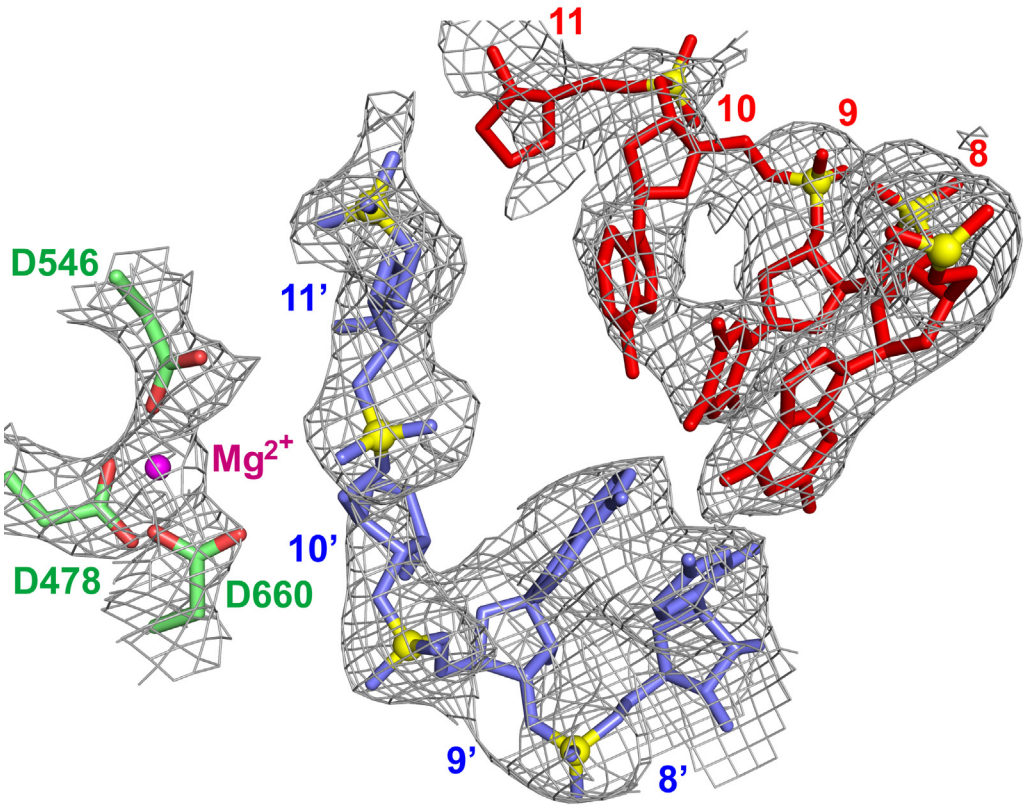
Supplementary Figure 1. Fitting the guide DNA into the electron density in the *T. thermophilus* Ago ternary complex. 2Fo-Fc electron density maps (1σ cut-off) of the bound DNA guide strand spanning **a,** bases 1 to 10 in one of the two molecules in the asymmetric unit, and **b,c,** bases 18/19 to 21 in each of individual molecules in the asymmetric unit of the complex.



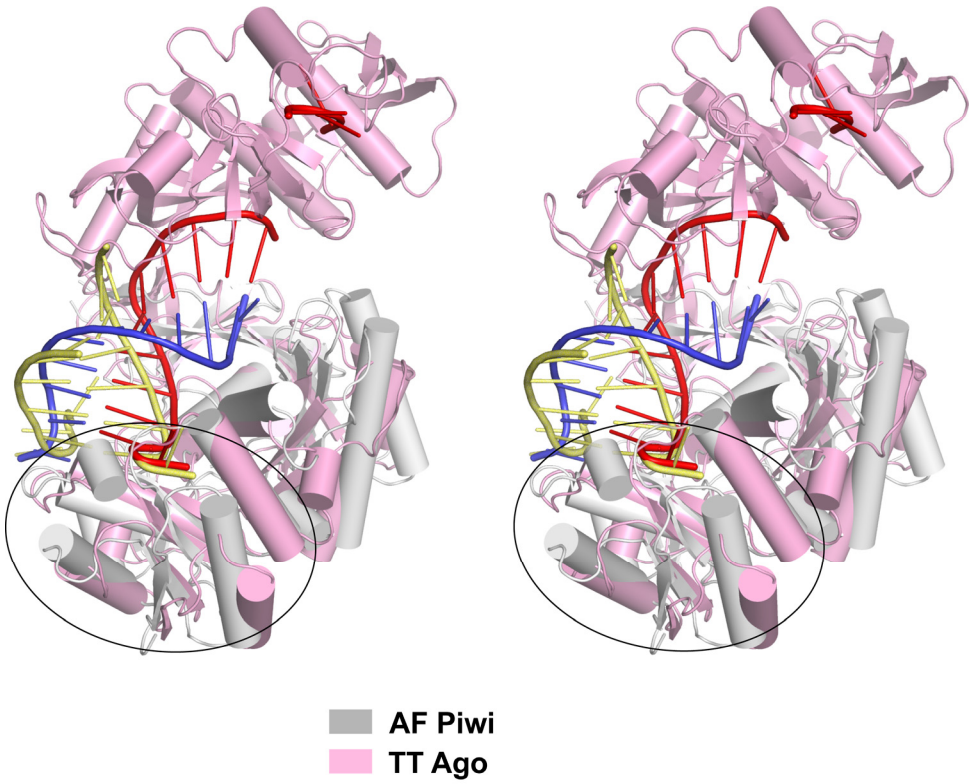
Supplementary Figure 2. Insertion of the 5'- and 3'- ends of the guide DNA strand into recognition pockets in the Ago ternary complex. a, Insertion of the 5'-phosphate of the DNA guide strand into the binding pocket in the Mid domain. **b,** Positioning of stacked 3'-end residues 20 and 21 of the DNA guide strand into the binding pocket in the PAZ domain.



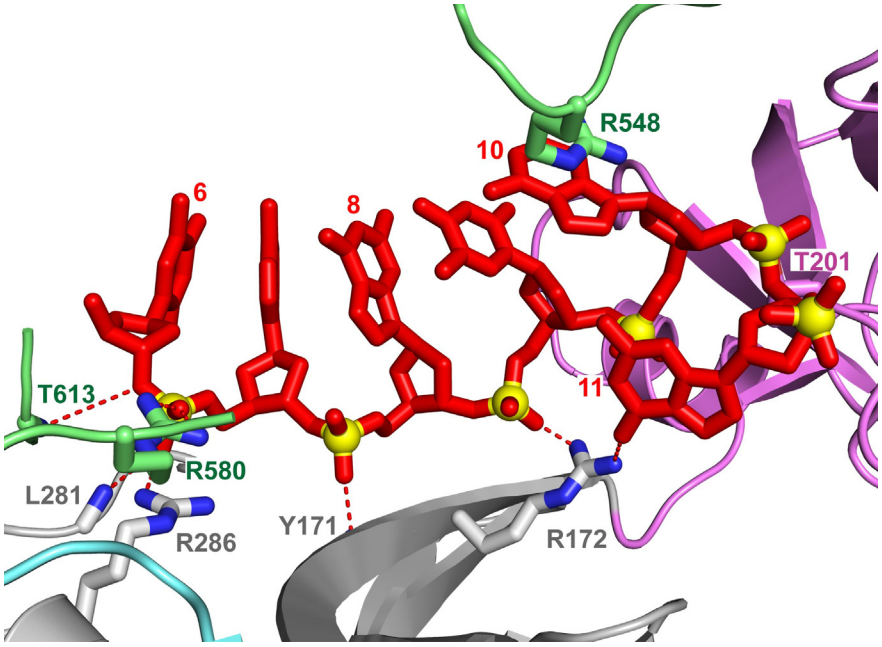
Supplementary Figure 3. Fitting the target RNA into the electron density in the *T. thermophilus* Ago ternary complex. 2Fo-Fc electron density maps (1σ cut-off) of the bound RNA target strand (spanning bases 1’ to 9’) in one of the two molecules in the asymmetric unit.



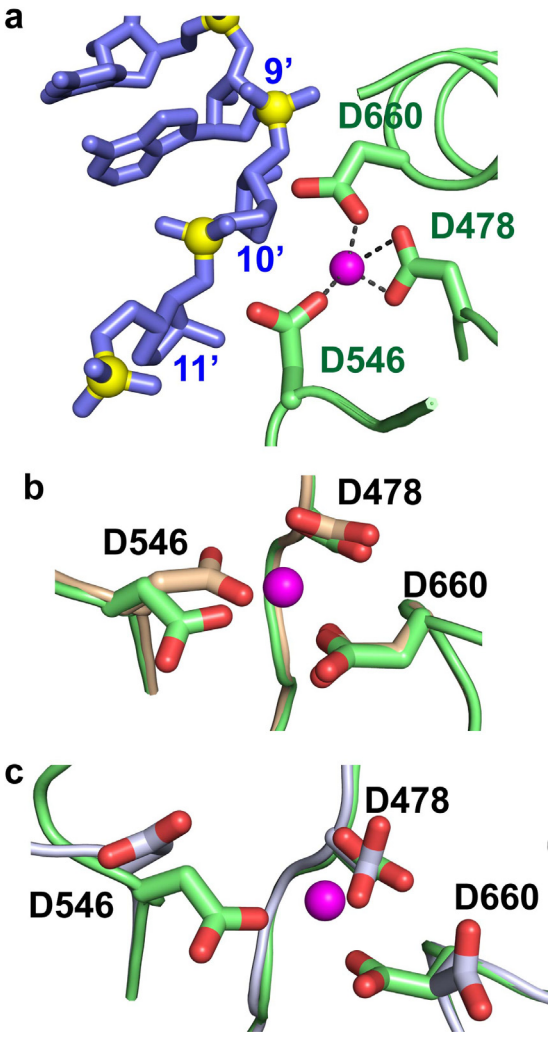
Supplementary Figure 4. Fitting the guide DNA and target RNA for the 8 to 11 steps into the electron density in the *T. thermophilus* Ago ternary complex. 2Fo-Fc electron density maps (1σ cut-off) spanning guide DNA 8-11 and target RNA 8'-11' steps, together with divalent cation-coordinated catalytic Asp residues. The electron density is better defined for the guide DNA strand that interacts with the protein through its sugar-phosphate backbone than for the target RNA strand that lacks such intermolecular contacts.



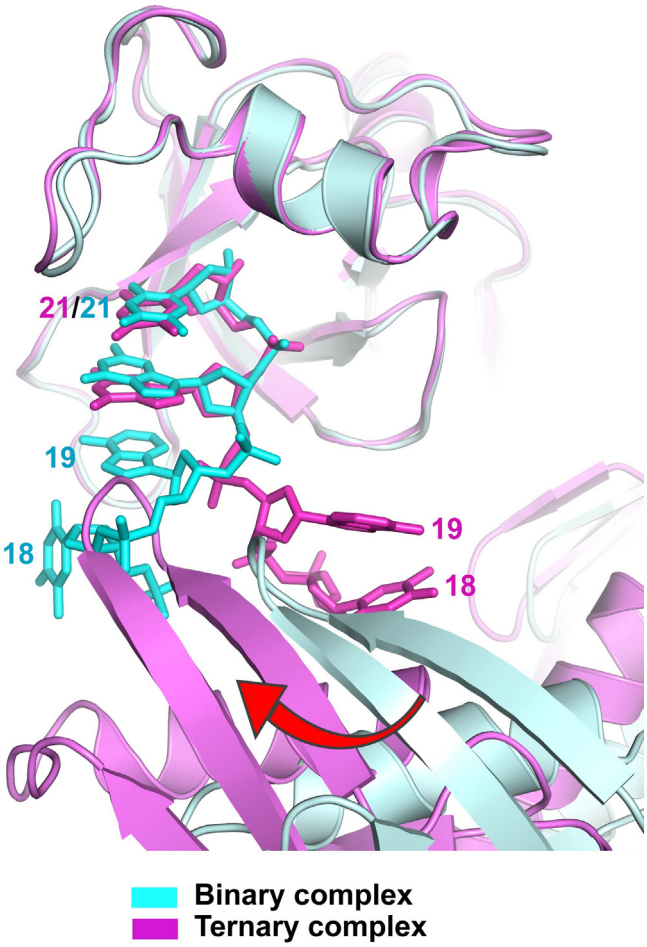
Supplementary Figure 5. Comparison of the trajectory of the guide-message duplex in the *A. fulgidus* Piwi[21,22] and *T. thermophilus* Ago ternary complexes. Stereo view of *A. fulgidus* Piwi[21] (Ago protein in silver; guide and target RNA strands in gold) and *T. thermophilus* Ago (Ago protein in magenta; guide DNA in red and target RNA in blue) ternary complexes, following superposition of their Mid domains.



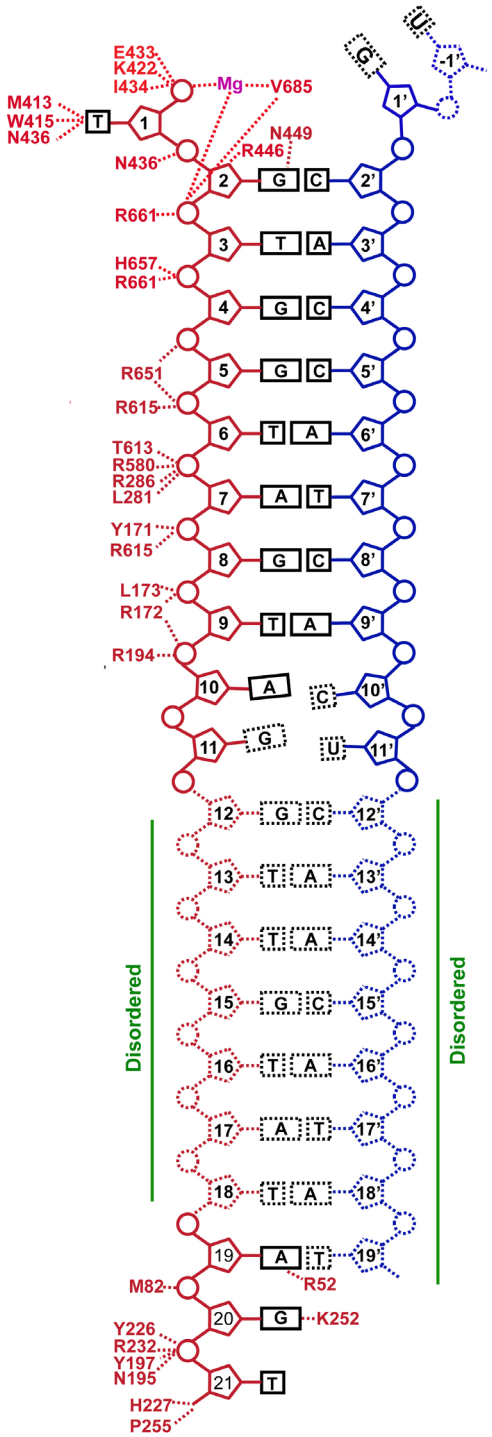
Supplementary Figure 6. Intermolecular hydrogen-bonding alignments in the *T. thermophilus* Ago binary complex with 21-nt DNA guide strand[5]. Positioning of stacked residues 6 to 11 of the DNA guide strand, with emphasis on intermolecular interactions. Note the stacking of the side chain of R548 over the purine ring of residue 10, thereby disrupting stacking at the 10-11 step.



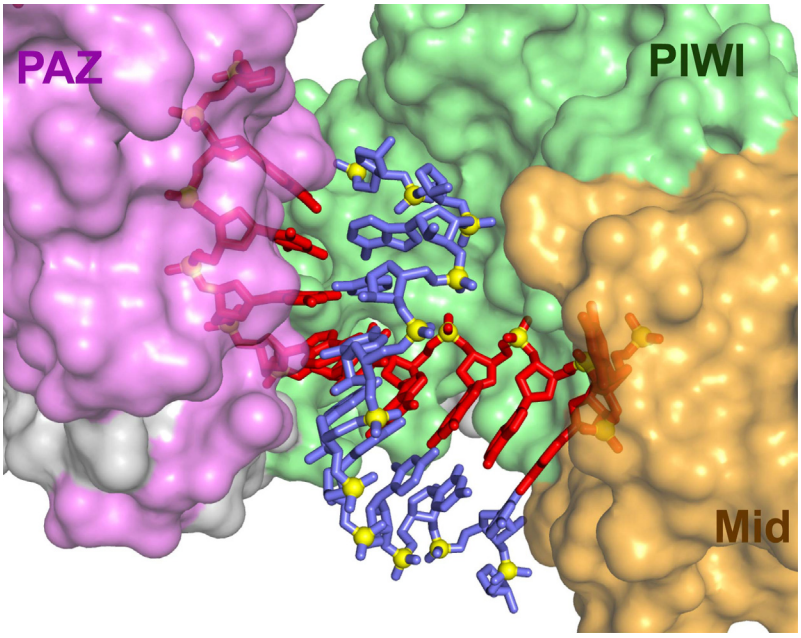
Supplementary Figure 7. Conformational change in the positioning of the catalytic D478, D546 and D660 residues between binary and ternary complexes. a, Positioning of catalytic Asp residues relative to the sugar-phosphate backbone at the 10-11 step of the target RNA in one molecule in the asymmetric unit of the ternary complex. **b,** Best-fit superposition of catalytic Asp residues in the two molecules (colored biscuit and green) in the asymmetric unit of the ternary Ago complex. A Mg^{2+} cation is coordinated to the Asp residues in one molecule (green) in the asymmetric unit. **c,** Best-fit superposition of the catalytic Asp residues between the binary (in silver) and ternary (in green) Ago complexes.



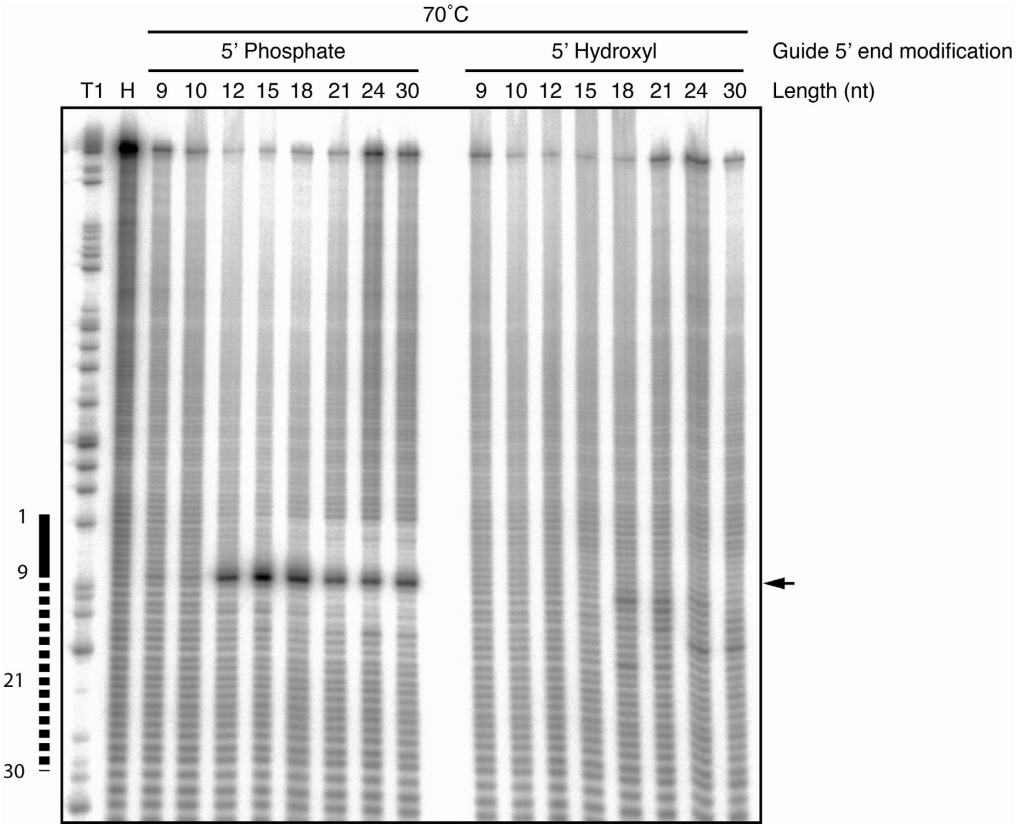
Supplementary Figure 8. Conformational changes in the vicinity of the PAZ binding pocket in *T. thermophilus* Ago on proceeding from the binary to the ternary Ago complexes. Superpositioning of the PAZ domains, together with observable 3'-ends, of the binary (in cyan) and ternary (in magenta) complexes. Note that the 18-19 segment of the DNA guide strand adopts different trajectories and there are associated conformational changes in the protein (designated by a red arrow) on proceeding from the binary to the ternary complex.



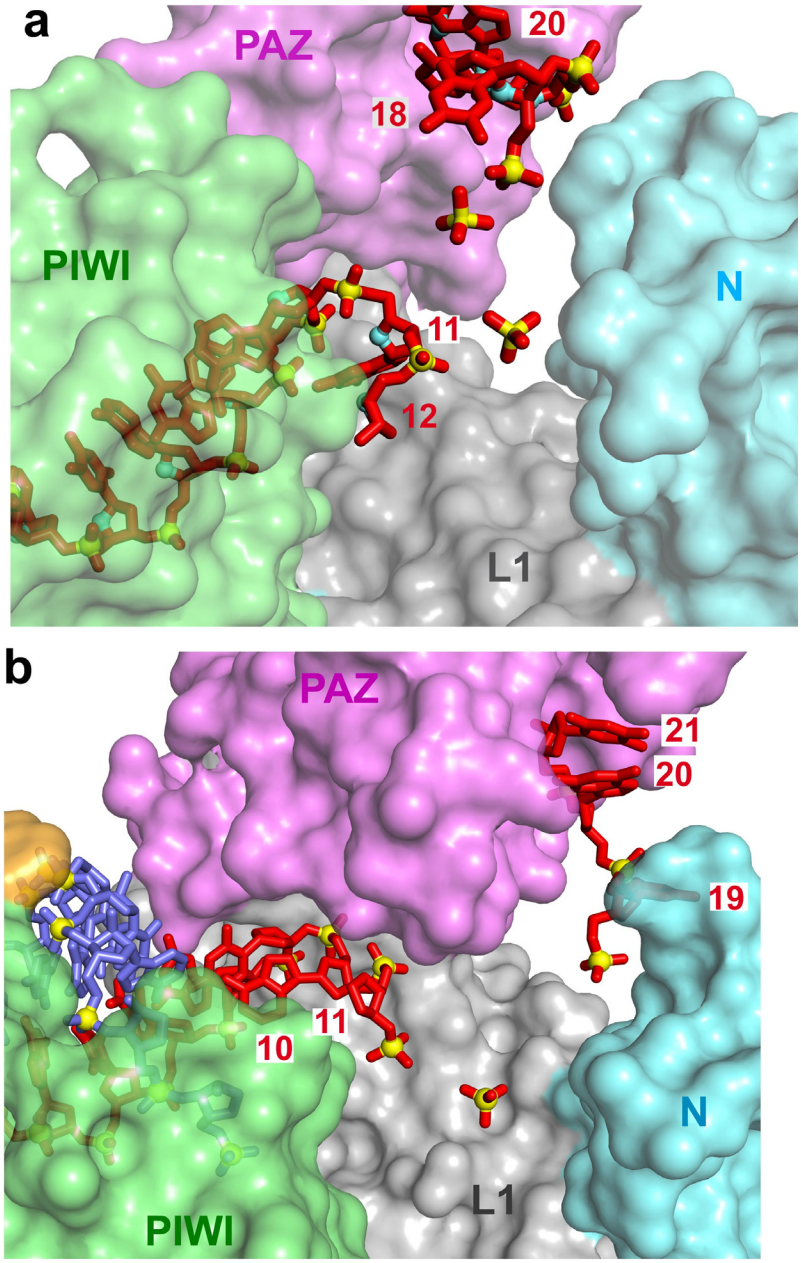
Supplementary Figure 9. Schematic of intermolecular hydrogen bonds in the *T. thermophilus* Ago ternary complex. A summary of the intermolecular hydrogen bonds shown by dashed lines between labeled protein side chains and the guide strand (in red) in the ternary complex. Note that ther are no intermolecular hydrogen bonds involving the target RNA strand.



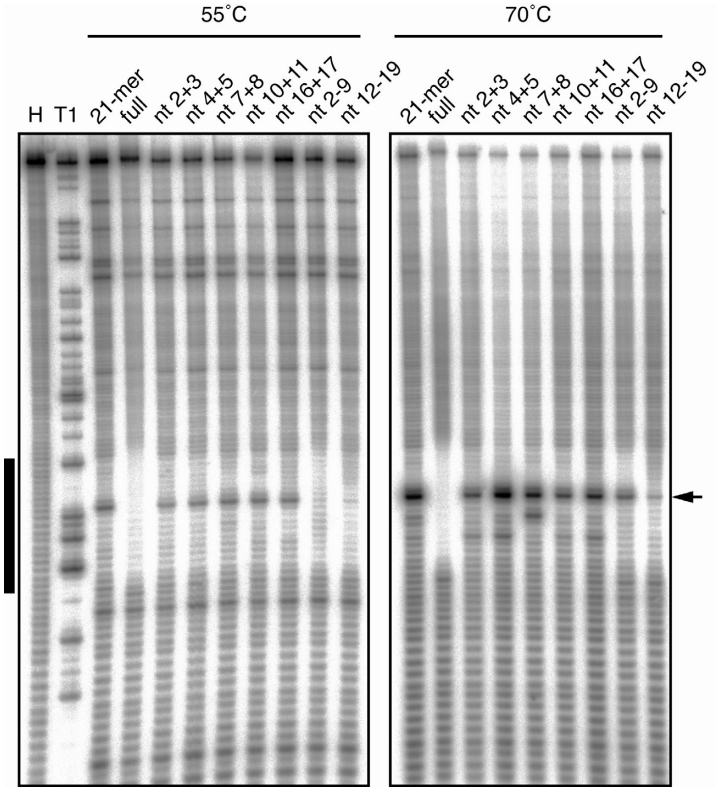
Supplementary Figure 10. Positioning of the guide-target duplex within the nucleic acid-binding channel of the *T. thermophilus* Ago complex. Positioning of the DNA guide strand in red and target RNA strand in blue between the PAZ- and PIWI-containing lobes of the bilobal Ago scaffold. The protein is shown in a surface representation with the domains and linkers color-coded as in Fig. 1.



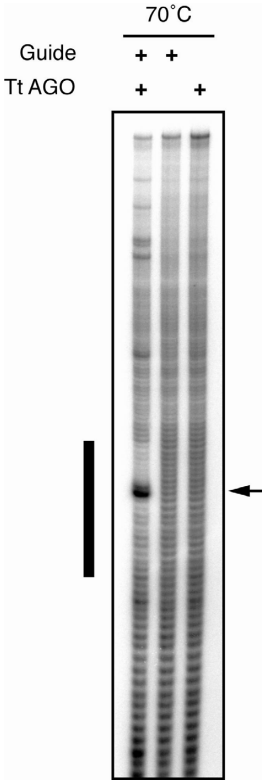
Supplementary Figure 11. The 5' phosphate of DNA guide strands is essential for target RNA cleavage. 5'-Phosphorylated or a 5'-hydroxyl-containing DNA guide strands were pre-incubated with Ago protein at 55 ºC followed by the addition of 5'-radiolabelled RNA substrate and further incubation at 70 ºC. Cleavage products were resolved on denaturing polyacrylamide gels and visualized by phosphoimaging. The black bar to the left side of the images defines the region of the cleavage substrate complementary with the guide DNA. The arrow placed right to the images indicates the cleavage site. Abbreviations: H, hydrolysis ladder of substrate RNA; T1, partial RNase T1 digest of substrate RNA. Similar results were obtained for cleavage reactions carried out at 55˚C (data not shown). The sequences of DNA guides and the RNA target are listed in Supplementary Table 3.



Supplementary Figure 12. Alternate positioning of elements of the disordered 3'-segment of the bound guide strand within the nucleic acid-binding channel in the binary and ternary Ago complexes. a, The binary Ago and **b**, the ternary Ago complexes, with residues 18-19 of the 3'-segment entering into the PAZ pocket from different directions. Note that the nucleic acid-binding channel is open to the outside in this segment of the complex.



Supplementary Figure 13. Target RNA cleavage activity of *T. thermophilus* Ago loaded with 2'-O-methyl-modified guide DNA strands. Experiments were performed according to the description in Fig. 4. The solid black bar indicates the region of the cleavage substrate covered by the 21-nt guide DNA. 2'-O-Methyl modifications were introduced into the DNA guide strand at indicates positions. The sequences of DNA guides and the RNA target are listed in Supplementary Table 3.



Supplementary Figure 14. DNA guide as well as Ago are required for target RNA cleavage. Control experiment illustrating the requirement of both Ago and guide DNA so as to mediate target RNA cleavage. Target RNA substrate was incubated with either *T. thermophilus* Ago or the fully complementary 21-nt oligodeoxynucleotide for 60 min at 70˚C. The black bar on the left side of the image defines the region of the cleavage substrate complementary to the guide DNA.