DNA-guided DNA cleavage at moderate temperatures by *Clostridium butyricum* Argonaute

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Abstract

Prokaryotic Argonaute proteins (pAgos) constitute a diverse group of endonucleases of which some mediate host defense by utilizing small interfering DNA guides (siDNA) to cleave complementary invading DNA. This activity has been repurposed for programmable DNA cleavage *in vitro*. However, currently characterized DNA-cleaving pAgos require elevated temperatures (≥65°C) for their activity, making them less suitable for *in vivo* applications at moderate temperatures. Here, using biochemistry, X-ray crystallography, and single-molecule fluorescence methods, we report the functional and structural characterization of the pAgo from the mesophilic bacterium *Clostridium butyricum* (*Cb*Ago). *Cb*Ago can be reprogrammed with siDNAs to cleave complementary DNA, but not RNA. *Cb*Ago displays a preference for siDNAs that have a deoxyadenosine at the 5'-end and thymidines in the sub-seed segment (siDNA nucleotides 2-4). Furthermore, *Cb*Ago mediates DNA-guided DNA cleavage of AT-rich double stranded DNA at moderate temperatures (37°C). Taken together, this study provides an important step towards the development of pAgos for genetic engineering applications.

Introduction

Eukaryotic Argonaute proteins (eAgos) play a key role in RNA interference (RNAi) processes[1–3]. As the core of the multiprotein RNA-induced silencing complex (RISC), eAgos bind small non-coding RNA molecules as guides to direct the RISC complex towards complementary RNA targets[3–5]. Reflecting their physiological function, variation among eAgos is observed with respect to the presence or absence of a catalytic site, and to their potential to interact with other proteins[6]. Depending on the eAgo and on the sequence complementarity between guide and target RNA, eAgo-guide complexes either catalyze endonucleolytic cleavage of the target RNA[7] or indirectly silence the target RNA by repressing its translation and promoting its degradation through recruitment of additional silencing factors[8]. Independent of the mechanism, eAgo-mediated RNA binding generally results in sequence-specific silencing of gene expression. As such, eAgos can coordinate various cellular processes by regulating intracellular RNA levels.

Prokaryotes also encode Argonaute proteins (pAgos)[9,10]. Various pAgos share a high degree of structural homology with canonical eAgos as both pAgos and eAgos adopt the same four domain (N-PAZ-MID-PIWI) architecture[9–12]. Despite their structural homology, several recently characterized pAgos have distinct functional roles and preferences for their guide and/or target type compared to eAgos; certain pAgos have been implicated in host defense by directly targeting DNA instead of RNA[13–16]. One of the best characterized mechanisms that pAgos utilize is DNA-guided DNA interference, which is mediated by pAgos from *Thermus thermophilus* (*Tt*Ago), *Pyrococcus furiosus* (*Pf*Ago), and *Methanocaldococcus jannaschii* (*Mj*Ago)[13–15,17–20]. These pAgos use 5'-end phosphorylated small interfering DNAs (siDNAs) for recognition and successive cleavage of complementary DNA targets. This mechanism enables both *Tt*Ago and *Pf*Ago to mediate host defence against invading nucleic acids. Prokaryotes lack homologs of eukaryotic enzymes that are involved in guide biogenesis[21]. Instead, both *Tt*Ago and *Mj*Ago - besides the canonical siDNA-dependent target cleavage termed 'slicing' - exhibit an alternative nuclease activity termed 'chopping'[14,17]. Chopping facilitates autonomous generation of small DNA fragments from dsDNA substrates. Subsequently, these DNA fragments can serve as siDNAs for canonical slicing[14,17].

*Tt*Ago and *Pf*Ago can be programmed with short synthetic siDNA which allows them to target and cleave dsDNA sequences of choice *in vitro*[13,15]. This activity has enabled the repurposing of *Pf*Ago as an universal restriction endonuclease for *in vitro* molecular cloning[22]. In analogy with the now

commonly used CRISPR-Cas9 and CRISPR-Cas12a enzymes[23–25], it has been suggested that pAgos could also be repurposed as next-generation genome editing tools[26]. However, due to the thermophilic nature (optimum activity temperature ≥65°C) and low levels of endonuclease activity at the relevant temperatures (20-37°C), it is unlikely that *Tt*Ago, *Pf*Ago and *Mj*Ago are suitable for genome editing. The quest for a pAgo that can cleave dsDNA at moderate temperatures has resulted in the characterization of the Argonaute protein from *Natronobacterium gregory* (*Ng*Ago). *Ng*Ago was claimed to be the first pAgo suitable for genome editing purposes[27], but the study reporting its characterization was retracted after a series of reproducibility issues[27–29]. Instead, it has been shown that *Ng*Ago might target RNA rather than DNA[30].

Although considerable efforts have been made to elucidate the mechanisms and biological roles of pAgos, efforts have mainly focused on pAgo variants from (hyper)thermophiles. This has left a large group of mesophilic pAgos unexplored. We here report the characterization the Argonaute protein from the mesophilic bacterium *Clostridium butyricum* (*Cb*Ago). We demonstrate that *Cb*Ago can utilize siDNA guides to cleave both ssDNA and dsDNA targets at moderate temperatures (37°C). In addition, we have elucidated the macromolecular structure of *Cb*Ago in complex with a siDNA guide and complementary ssDNA target in a catalytically competent state. *Cb*Ago displays an unusual preference for siDNAs with a deoxyadenosine at the 5'-end and thymidines in the sub-seed segment (siDNA nt 2-4). The programmable DNA endonuclease activity of *Cb*Ago described here provides the basis for the development of pAgo proteins as genome editing tools.

Results

*Cb*Ago mediates siDNA-guided ssDNA cleavage

*Cb*Ago was successfully expressed in *E. coli* from a codon-optimized gene using a T7-based pET expression system and purified (Supplementary Fig. 1a). To determine the guide and target binding characteristics of *Cb*Ago, we performed single-molecule experiments using Förster resonance energy transfer (FRET). We immobilized either Cy5-labeled single stranded RNA or DNA targets (FRET acceptor) on a polymer-coated quartz surface (Fig. 1a). Next we introduced *Cb*Ago in complex with either a Cy3-labeled siRNA or siDNA guide (FRET donor) and recorded the interactions. Strikingly, *Cb*Ago could utilize both siRNAs and siDNAs to bind DNA or RNA targets (Fig. 1b). To test which guide is preferentially bound by *Cb*Ago we performed a competition assay in which *Cb*Ago was immobilized into the microfluidic chamber, and an equimolar mixture of siDNA and siRNAs was introduced. While only short-lived interactions were observed for siRNA, siDNA was strongly bound by *Cb*Ago (Fig. 1c). This results suggests that *Cb*Ago utilizes siDNA rather than siRNA as a guide.

*Cb*Ago is phylogenetically closest related to the clade of halobacterial pAgos, among which also pAgo from *Natronobacterium gregoryi* (*Ng*Ago) can be found (Fig. 1d and Supplementary Fig. 2). A multiple sequence alignment of *Cb*Ago with other pAgos (Supplementary Fig. 1b) suggests that *Cb*Ago contains the conserved DEDX catalytic residues (where X can be an D, H or N) which are essential for nuclease activity in 'slicing' Agos[31]. In the case of *Cb*Ago, this concerns residues D541, E577, D611 and D722.

To confirm whether *Cb*Ago indeed is an active nuclease, we performed *in vitro* activity assays in which *Cb*Ago was loaded with either synthetic siDNAs or siRNAs (21 nucleotides in length). Next the complexes were incubated at 37°C with 45-nucleotide complementary single stranded RNA or DNA target oligonucleotides. While no activity was found in any of the combinations in which siRNAs or target RNAs were used, *Cb*Ago was able to cleave target DNAs in a siDNA-dependent manner (Fig. 1e). In agreement with the predicted DEDD catalytic site (Supplementary Fig. 1b), alanine substitutions of two of aspartic acids (D541A, D611A) in the expected catalytic tetrad abolished the nuclease activity, demonstrating that the observed siDNA-guided ssDNA endonucleolytic activity was indeed catalyzed by the DEDD catalytic site. To further investigate the full temperature range at which *Cb*Ago is active, we performed additional cleavage assays at temperatures ranging from 10-95°C. While *Cb*Ago

displayed the highest activity at its physiologically relevant temperature (37°C), *Cb*Ago also catalyzed siDNA-guided target DNA cleavage at temperatures as low as 10°C and as high as 50°C (Fig. 1f).

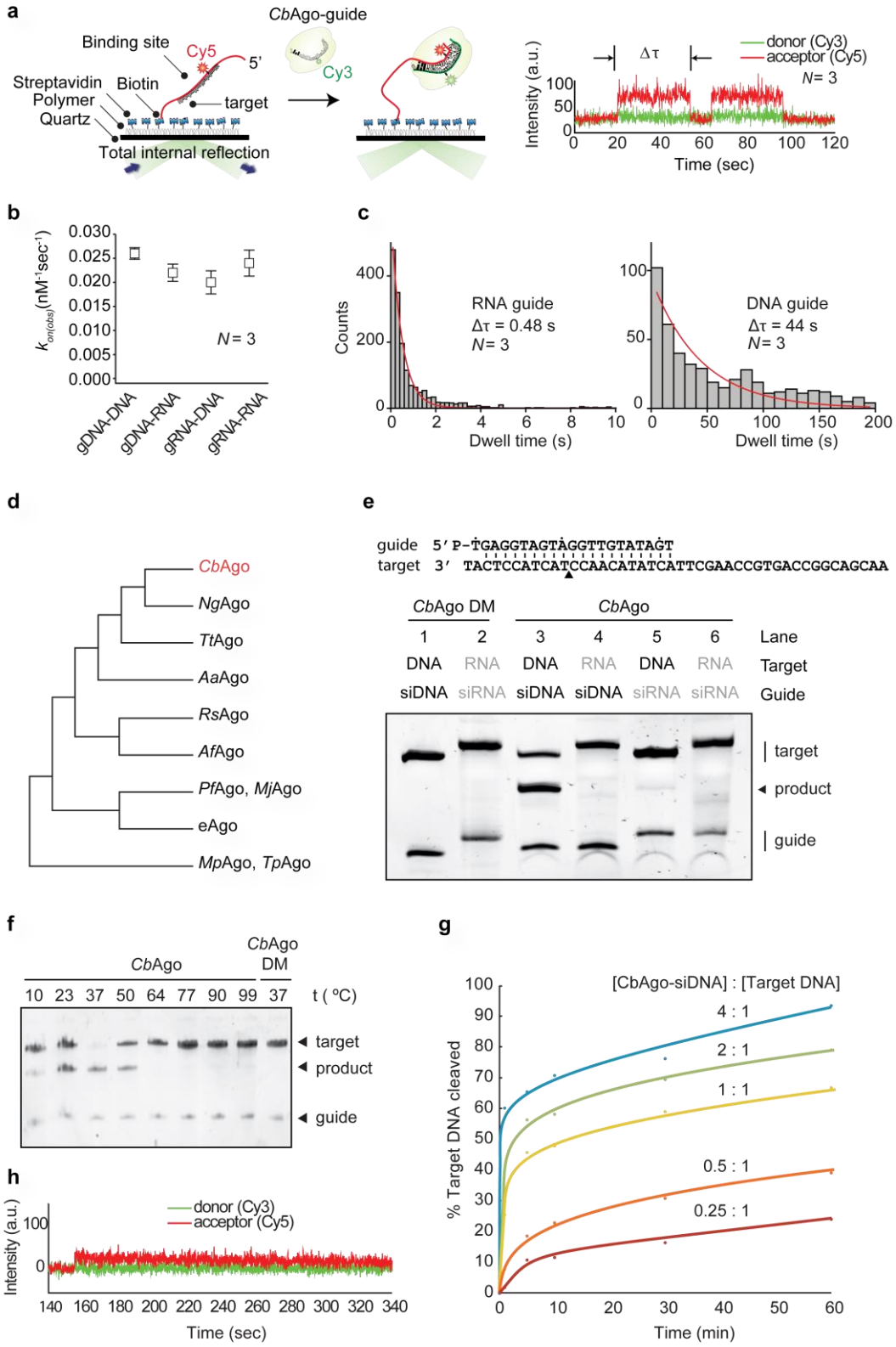

Figure 1 | *Cb*Ago exhibits DNA-guided DNA endonuclease activity at 37°C. a, Left: Overview of the single molecule assay to determine the binding characteristics of *Cb*Ago. Right: FRET diagram of a *Cb*Ago-siDNA complex that has 3 complementary base pairs (2-4nt) to the DNA target. Indicated is the dwell time (Δτ)**. b,** Comparison of the binding rates (k_{on}) of *Cb*Ago in complex with siDNA or siRNA to bind DNA or RNA targets. The rates are similar for each nucleic acid type guide and target. *N* is the number of basepaired nucleotides. **c,** Dwell time histograms showing CbAgo preferentially binds siDNAs in siDNA-siRNA competition experiments. **d,** Schematic phylogenetic tree of characterized pAgos. **e,** *Cb*Ago exhibits DNA-guided DNA endonuclease activity. Upper panel: Sequence of the synthetic let7 miRNA-based siDNA guide and target DNA sequences that were used for the *in vitro* assays. Lower panel: *Cb*Ago, guides and targets were mixed in a 1:1:1 molar ratio and incubated for 1 h at 37°C. Catalytic mutant CbAgoDM was used as a control. Cleavage products were analysed by denaturing polyacrylamide electrophoresis. **f,** *Cb*Ago displays highest activity at 37°C. *Cb*Ago and siDNA were mixed and pre-incubated at various temperatures for 10 minutes. Next, target DNA was added and the sample was incubated for 1 h at the same temperature. *Cb*AgoDM was used as a control. Cleavage products were analysed by denaturing polyacrylamide electrophoresis. **g,** Quantified data of a *Cb*Ago-mediated siDNA-guided ssDNA cleavage turnover experiment using 5 pmol target DNA and increasing concentrations of *Cb*Ago-siDNA (1.25-20 pmol). **h,** FRET diagram showing that a cleavage compatible *Cb*Ago-siDNA remains bound to a fully complementary target DNA (*N*=21) during the entire the measurement (340 seconds).

When *Cb*Ago-siDNA complexes and target ssDNA substrates (45nt) were mixed in equimolar amounts, cleavage of the target DNA was not complete after 1 hour incubation (Fig. 1e). Therefore, we investigated the substrate turnover kinetics of *Cb*Ago by monitoring the cleavage assays in a time course using variable *Cb*Ago:siDNA:target DNA ratios (Fig. 1g). A rapid burst of activity was observed during the first minute, likely indicating the first target binding and cleavage event. This stage was followed by a slow steady state, suggesting that under these conditions the *Cb*Ago-siDNA complex slowly dissociates from the cleaved target DNA product before being able to bind and cleave a new target DNA strand. The cleavage kinetics were confirmed using single-molecule assays which demonstrated that the *Cb*Ago-siDNA complex remains bound to the DNA target (*N*=21) for several minutes (Fig. 1h), which prevents *Cb*Ago-siDNA complexes from binding and limits substrate turnover. Thus, while CbAgo functions as a multi-turnover nuclease enzyme, its steady-state rate is limited by product release.

Structure of *Cb*Ago in the cleavage-competent conformation

To investigate the molecular architecture of *Cb*Ago in light of its biochemical activity, we crystalized *Cb*AgoDM in complex with both a 21-nt siDNA and a 19-nt DNA target, and solved the structure of the complex at 3.54 Å resolution (Fig. 2 and Table 1). Like other Agos, *Cb*Ago adopts a bilobed conformation in which one of the lobes comprises the N-terminal, linker L1, and PAZ domains, which are linked by linker L2 to the other lobe comprising the MID and PIWI domains. Nucleotides 2-16 of the siDNA constitute a 15 base-pairs, A-form-like duplex with the target DNA (Fig. 2a). The 5'-terminal nucleotide of the siDNA is anchored in the MID domain pocket, where the 5'-phosphate group of the siDNA makes numerous interactions with MID domain residues and the C-terminal carboxyl group of *Cb*Ago (Supplementary Fig. 3). However, there are no base-specific interactions with the 5'-terminal cytosine base. To test whether these interactions are important for *Cb*Ago activity, we performed target DNA cleavage assays in which we used siDNAs with a 5' phosphate or a 5' hydroxyl group (Supplementary Fig. 4). As for other pAgos, *Cb*Ago was able to utilize both siDNAs for target DNA cleavage, but it cleaved target DNA much more efficiently when the siDNA contained a 5' phosphate group, in agreement with the observed mode of siDNA binding in the structure. Furthermore, the backbone phosphates of the siDNA seed segment form hydrogen-bonding and ionic interactions with specific residues in the MID, PIWI and L1 domains (Supplementary Fig. 3). At the distal end of the siDNA-target DNA duplex, the N-domain residue His35 caps the duplex by stacking onto the last base pair. After this point, the remaining 3'-terminal nucleotides of the siDNA are unordered, while the target DNA bends away from the duplex and enters the cleft between the N-terminal and PAZ domains. In agreement with other ternary pAgo complexes[18,32,33], the PAZ domain pocket, which normally binds the 3' end of the guide in a binary Ago-guide complex, is empty.

*Cb*Ago is phylogenetically closely related to *Tt*Ago (Fig. 1c). However, *Cb*Ago is 63 amino acids (9.2%) longer than *Tt*Ago (748 amino acids vs. 685 amino acids) and *Cb*Ago and *Tt*Ago share only 23% sequence identity. Superposition of the *Cb*Ago complex structure with the structure of *Tt*Ago bound to a siDNA and DNA target (PDB: 4NCB) (Fig. 2c) reveals that the macromolecular architecture and conformation of these *Tt*Ago and *Cb*Ago structures are highly similar (Core root mean square deviation of 3.0 Å over 563 residues), with differences found mostly in the loop regions. This agrees with the fact that loops of thermostable proteins are generally more compact and shorter[34,35]. In the *Tt*Ago structure, which is thought to represent a catalytically competent state, a 'glutamate finger' side chain (Glu512^{TtAgo})

is inserted into the catalytic site completing the catalytic DDED tetrad[32]. Similarly, the corresponding residue in *Cb*Ago (Glu577) is located within a flexible loop and is positioned near the other catalytic residues (Fig. 2c; Asp541, Asp611, and Asp722). This observation implies that this *Cb*Ago complex structure also represents the cleavage competent conformation. Since only 15 siDNA-target DNA base pairs are formed in the complex, this suggests that additional siDNA-target DNA binding is not essential for target DNA cleavage.

To determine the minimum siDNA length that *Cb*Ago requires for target binding, we performed single-molecule fluorescence assays. First, *Cb*Ago was immobilized on a surface and next it was incubated with 5'-phosphorylated Cy3-labelled siDNAs (Fig. 2e). These assays demonstrate that *Cb*Ago can bind siDNAs with a minimal length of 12 nucleotides. Next, we determined the minimum siDNA length for CbAgo-siDNA mediated target DNA cleavage (Fig. 2f). In line with the observation that the *Cb*Ago complex structure adopts a catalytically active confirmation, *Cb*Ago was active with siDNAs as short as 14 nt under the tested conditions. This resembles *Pf*Ago, *Mj*Ago, and *Mp*Ago, all of which require siDNAs with a minimal length of 15 nt to catalyze target DNA cleavage[14,15,36]. Only *Tt*Ago has been reported to mediate target DNA cleavage with siDNAs as short as 9 nt[12].

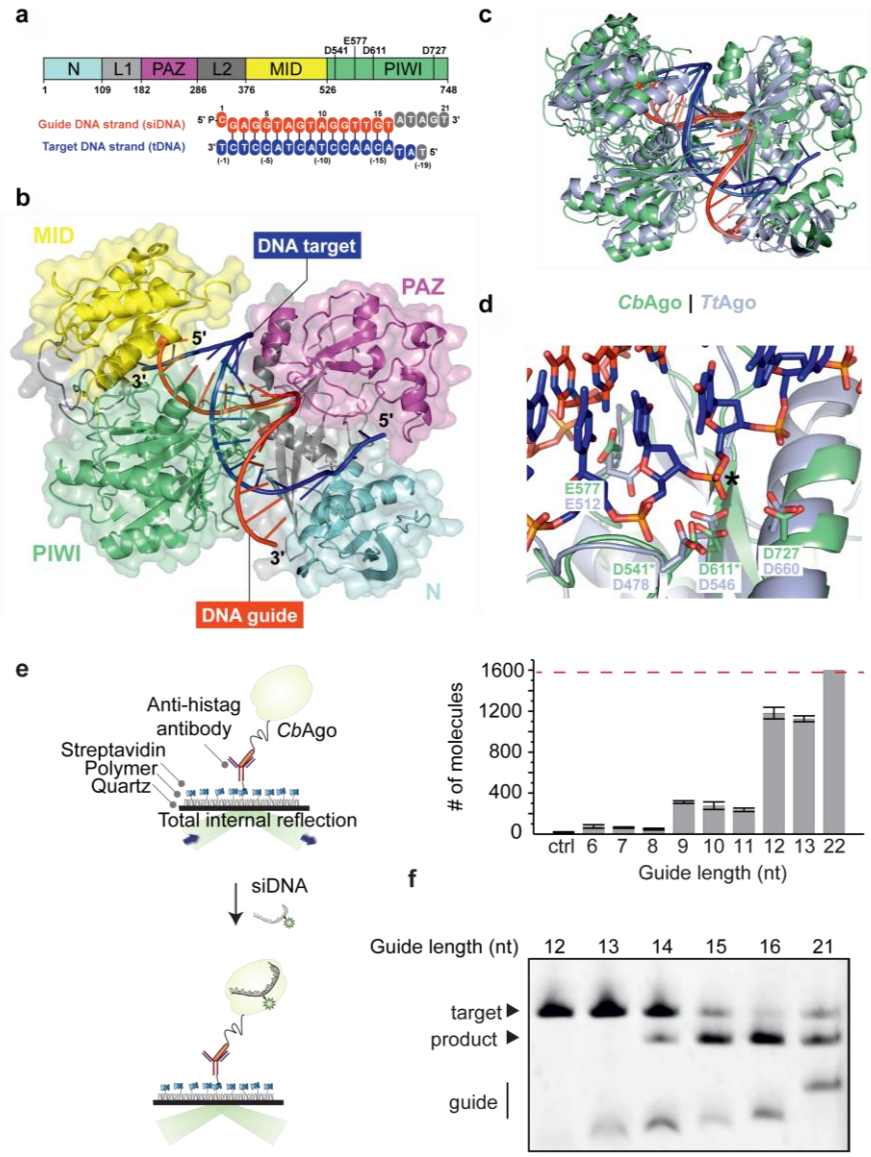

Figure 2 | Structure of *Cb*Ago in complex with a siDNA and a DNA target. a, Upper panel: Schematic diagram of the domain organization of *Cb*Ago. L1 and L2 are linker domains. Lower panel: Sequences of the siDNA (red) and target DNA (blue). Nucleotides that are unordered in the structure are coloured grey. See also Table S1. **b**, Overall structure of the *Cb*Ago-siDNA-target DNA complex. Domains are coloured according to the colour scheme in panel a. **c**, Structural alignment of *Cb*Ago (green) and *Tt*Ago (light purple; PDB: 4NCB). Core Root Mean Square Deviation of 3.0 Å over 563 residues. **d**, Close-up view of the aligned DDED catalytic sites of *Cb*Ago (green) and *Tt*Ago (light purple; PDB: 4NCB). Modelled side chains of D541 and D611 in *Cb*Ago are indicated with green asterisks. The glutamate finger of both pAgos (E512 in *Tt*Ago or E577 in *Cb*Ago) are inserted into the catalytic site. The scissile phosphate in the target DNA (blue) is indicated with a black asterisk. **e**, Total internal reflection microscopy (TIRM) was used to determine the minimal length for siDNA to be bound by *Cb*Ago. Left panel:

Graphical overview of the TIRM method. Right panel: Histogram with TIRM results demonstrated that synthetic siDNAs of at least 12nt in length are efficiently bound by *Cb*Ago. The red line indicates the total number of countable molecules within the microscope image. The raw microscope images are given in Supplementary Fig. 5. **f**, *Cb*Ago mediates target DNA cleavage with siDNAs as short as 14 nucleotides. *Cb*Ago was incubated with siDNA and target DNA in a 1:1:1 ratio. Cleavage products were analysed by denaturing polyacrylamide electrophoresis.

Cb*Ago associates with plasmid-derived siDNAs *in vivo

It has previously been demonstrated that certain pAgos co-purify with their guides and/or targets during heterologous expression in *Escherichia coli*[13,16]. To determine whether *Cb*Ago also acquires siDNAs during expression, we isolated and analyzed the nucleic acid fraction that co-purified with *Cb*Ago. Denaturing polyacrylamide gel electrophoresis revealed that *Cb*Ago co-purified with small nucleotides with a length of ~12-19 nucleotides (Fig. 3a). These nucleic acids were susceptible to DNase I but not to RNase A treatment, indicating that *Cb*Ago acquires 12-19 nucleotide long siDNAs *in vivo*, which fits with its observed binding and cleavage activities *in vitro* (Fig. 1 and 2).

We cloned and sequenced the siDNAs that co-purified with *Cb*Ago to determine their exact length and sequence. The majority of the siDNAs had a length of 16 nucleotides and are complementary to the plasmid used for expression of *Cb*Ago (Fig. 3b and 3c). Likewise the siRNAs and siDNAs that co-purify with respectively *Rhodobacter sphaeroides* (*Rs*Ago) and *Tt*Ago are also mostly complementary to their expression plasmids[13,16]. As both *Tt*Ago and *Rs*Ago have been demonstrated to interfere with plasmid DNA, this suggests that also *Cb*Ago might play a role in protecting its host against invading DNA. However, no significant reduction of plasmid content could be detected during or upon expression of *Cb*Ago in *E. coli* (Supplementary Fig. 6). We also investigated whether *Cb*Ago co-purified with nucleic acids that were enriched for certain motifs. Sequence analysis revealed that most siDNAs co-purified with *Cb*Ago contain a deoxyadenosine at their 5' ends (Fig. 3d). In addition, we observed an enrichment of thymidine nucleotides in the three positions directly downstream of the siDNA 5' end (nt 2-4) (Fig. 3d).

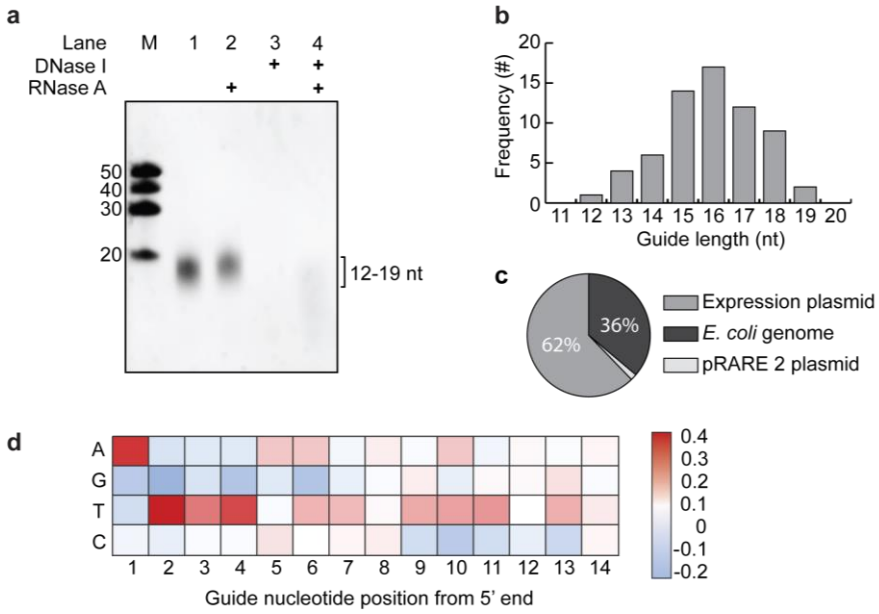

Figure 3 | *Cb*Ago associates with small plasmid derived siDNA *in vivo*. a, Nucleic acids that co-purified with *Cb*Ago were treated with either RNAse A, DNAse I or both, and were analyzed by denaturing polyacrylamide gel electrophoresis. **b**, Histogram displaying the length of DNA co-purified with *Cb*Ago as determined by sequencing. **c**, Sequenced nucleic acids that co-purified with *Cb*Ago are mostly complementary to the *Cb*Ago expression plasmid. **d,** Heat map showing the base preference of the co-purified nucleic acids at each position. The red squares indicate bases that were more often found compared to a random distribution (25%); blue squares indicate bases that were less frequently found.

The sequence of the siDNA affects *Cb*Ago activity

To investigate if the 5'-terminal nucleotide of the siDNA affects the activity of *Cb*Ago, we performed cleavage assays. *Cb*Ago was loaded with siDNA guides with varied nucleotides at position 1 (g1N) and incubated with complementary target DNAs (Fig 4a). Surprisingly, the highest cleavage rates were observed with *Cb*Ago loaded with siDNAs containing a 5'-T, followed by siDNAs containing 5'-A. *Cb*Ago bound 5'-G or 5'-C siDNAs displayed slightly lower initial cleavage rates. Also for other pAgos the g1N preference is not reflected in the *in vitro* activities; *Tt*Ago (which preferentially co-purifies with g1C siDNAs) as well as *Pf*Ago and *Mp*Ago (of which the *in vivo* g1N preferences are unknown) demonstrate no clear preference for a specific g1N during *in vitro* cleavage reactions[13,17,36]. Instead, the preference of *Tt*Ago for 5'-C siDNAs is determined by specific recognition of a guanosine nucleotide in the

corresponding position (t1) in the target DNA[17]. Indeed, *Tt*Ago structures and models have revealed base-specific interactions with target strand guanine, while base-specific interactions with the 5'-terminal cytidine in the siDNA are less obvious[17]. In the structure of the *Cb*Ago-siDNA-target DNA complex, we observe no base-specific interactions with the 5'-terminal (Supplementary Fig. 7). Strikingly, the thymine base is not placed in the t1 binding pocket as has been observed in *Tt*Ago, *Rs*Ago and hAGO2[17,37,38]. Instead, the thymine bases is flipped and stacks on Phe557 that also caps the siDNA-target DNA duplex (Supplementary Fig. 7). At present, we are unable to rationalize the preferentially co-purification of 5'-A siDNAs with *Cb*Ago.

In order to characterize the seed segment of *Cb*Ago, and test whether the seed length changes depending on the nature of the guide and the target (*i.e.* DNA vs. RNA), we performed additional single-molecule binding assays. The length of seed was determined based on the minimal number of complementary nucleotide pairs between guide and target that were required to achieve a stable binding event. We first tested the sub-seed (nt 2-4), a 3-nt motif involved in initial target recognition in hAgo2[39,40]. When only the sub-seed segment of the siDNA was complementary to the DNA and RNA targets, CbAgo-siDNA complexes bound to the DNA target with an average dwell time 58-fold longer than for the RNA target (Fig. 4b). When nt 2-7 of the guide were complementary to the target, the *Cb*Ago-siDNA complex stably bound to both to target DNA and RNA beyond our observation time of 300 s. This suggests *Cb*Ago prefers DNA above RNA targets and that the seed segment of the siDNAs bound by *Cb*Ago comprises nucleotides 2-7.

Next, we set out to investigate whether *Cb*Ago displays a preference for siDNAs with a TTT sub-seed (nt 2-4) *in vitro*, similar to the observed sequence preference for siDNAs that co-purified with *Cb*Ago *in vivo*. *Cb*Ago was incubated with siDNAs in which the sub-seed was varied and complementary target DNAs were added. In agreement with the *in vivo* preference, *Cb*Ago displays the highest cleavage rates with TTT sub-seed siDNAs (Fig. 4c). To confirm these findings, we performed single-molecule assays in which we compared the target binding properties of *Cb*Ago-siDNA complexes containing siDNAs with either a TTT or an AAA sub-seed segment. These assays demonstrate that the dwell time of *Cb*Ago loaded with an TTT sub-seed siDNA on a target was 18-fold longer compared to *Cb*Ago loaded with siDNA containing a AAA sub-seed (Fig. 4d). Combined, these data indicate that *Cb*Ago displays a preference for siDNAs containing a TTT sub-seed segment.

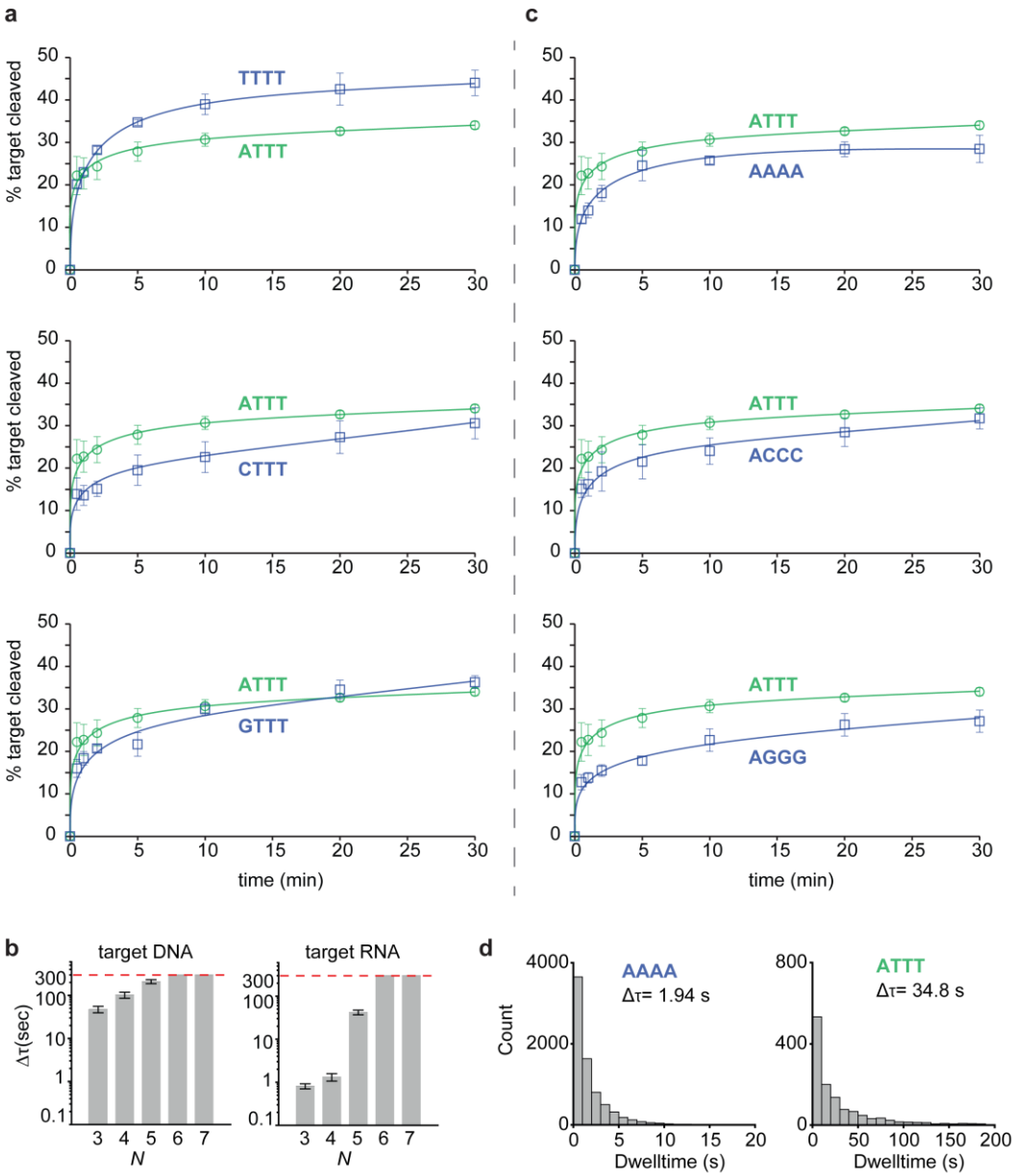

Figure 4 | The siDNA sequence affects *Cb*Ago activity. a, *Cb*Ago has no strong 5'-end nucleotide preference. *Cb*Ago was incubated with siDNA with varied 5'-end and incubated with complementary DNA targets. Cleavage products were analysed by denaturing polyacrylamide electrophoresis and quantified. Graphs display the amount of target DNA cleaved. Error bars indicate the standard variation of three independent experiments. **b**, Histrograms displaying dwell time of *Cb*AgoDM-siDNA complexes binding either DNA or RNA targets with a varied sequence complementarity (N = number of complementary nucleotides between the siDNA and the target, starting at nt2. Thus *N* 3= nt 2-4) The photobleaching limit is reached where the signal is deactivated (300s). **c**, *Cb*Ago preferentially utilizes siDNAs with a TTT sub-seed segment. *Cb*Ago was incubated with siDNA with varied sub-seed segments (nt 2-4) and incubated with complementary DNA targets. Cleavage products were analysed by denaturing polyacrylamide electrophoresis and quantified. Graphs display the amount of target DNA cleaved. Error bars

indicate the standard variation of three independent experiments. **d**, Histograms displaying dwelltime of *Cb*AgoDM in complex with a 5'-ATTT siDNA or 5'-AAAA siDNA binding to a target DNA. interactions that are on average ~18-fold longer than CbAgo in complex with siDNAs containing a 5'-AAAA motif.

A pair of *Cb*Ago-siDNA complexes can cleave double stranded DNA

Thermophilic pAgos have successfully been used to generate double stranded DNA breaks in plasmid DNA[13,15]. As each pAgo-siDNA complex targets and cleaves a single strand of DNA only, two individual pAgo-siDNA complexes are required for dsDNA cleavage, each targeting another strand of the target dsDNA. Although all pAgos characterized so far appear to lack the ability to actively unwind or displace a dsDNA duplex substrate, it has been proposed that, at least *in vitro*, thermophilic pAgos rely on elevated temperatures (>65 °C) to facilitate local melting of the dsDNA targets to target each strand of the DNA individually. However, *Cb*Ago is derived from a mesophilic organism and we therefore hypothesize that is also able to mediate protection against invading DNA at moderate temperatures (37°C). To test if *Cb*Ago can indeed cleave dsDNA targets at 37°C, we incubated apo-*Cb*Ago and pre-assembled *Cb*Ago-siDNA complexes with a target plasmid. Previous studies showed that the 'chopping' activity of siDNA-free apo-*Tt*Ago and apo-*Mj*Ago can result in plasmid linearization or degradation, respectively[14,17]. We observe that apo-*Cb*Ago converted the plasmid substrate from a supercoiled to open-circular state, possibly by nicking one of the strands, but did not observe significant linearization or degradation of the plasmid DNA (Fig. 5a). When the plasmid was targeted by *Cb*Ago loaded with a single siDNA, we also observed loss of supercoiling (Fig. 5a). As this activity was not observed with nuclease-deficient *Cb*AgoDM, we conclude that apo-*Cb*Ago and *Cb*Ago-siDNA complexes are able to generate nicks in dsDNA plasmid targets. When using two *Cb*Ago-siDNA complexes, each targeting one strand of the plasmid, we observed that a large fraction of the plasmid DNA was linearized (Fig. 5a). This implies that *Cb*Ago-siDNA complex-mediated nicking of each of the plasmid DNA strands generated a double stranded DNA break. Next, we investigated if the spacing between the two siDNAs affects the ability of *Cb*Ago to cleave the plasmid. The most efficient plasmid linearization was achieved when the siDNAs were orientated exactly opposite each other (Fig. 5a).

Finally, we investigated whether the GC-content of the target DNA plays a role during DNA targeting by *Cb*Ago. For *Tt*Ago, it was previously observed that AT-rich DNA is cleaved more efficiently that GC-rich DNA[17]. To test this for *Cb*Ago, we designed a target plasmid containing 16 gene fragments of 100 base pairs complementary to sequences from the human genome, with an increasing GC content

(Fig. 5b). *Cb*Ago-siDNA complexes were only able to generate dsDNA in gene fragments with a GC-content of up to 31% (Fig. 5c). This indicates that, at least *in vitro*, the GC-content is an important factor that determines target DNA binding by *Cb*Ago.

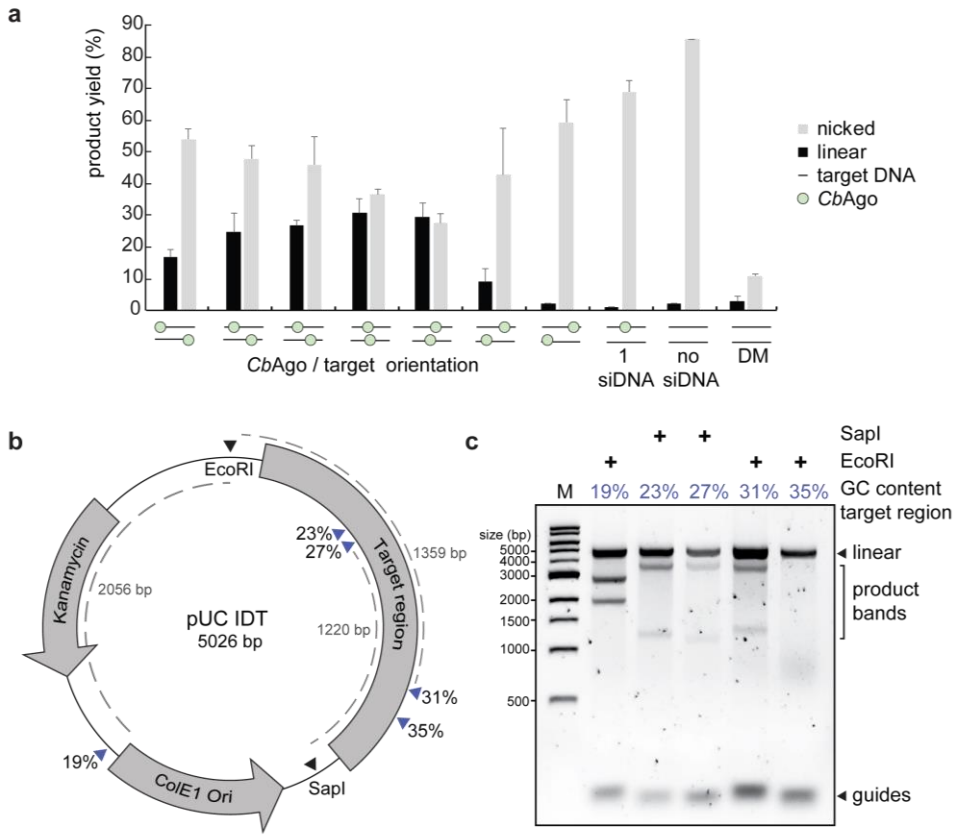

Figure 5 | Double stranded plasmid DNA cleavage by *Cb*Ago. **a**, Two *Cb*Ago-siDNA complexes can generate double stranded DNA breaks in plasmid DNA. CbAgo-siDNA complexes were pre-assembled and incubated with target plasmid DNA. Cleavage products were analysed by agarose gel electrophoresis and quantified. The spacing between both *Cb*Ago-siDNA target sites affects the linearization efficiency (nucleotide spacing between the predicted cleavage sites: +15 nt, +10 nt, +5 nt, 0 nt, -5 nt, -10 nt, -15 nt, a single siDNA, no siDNA). With 0 nt spacing, both *Cb*Ago-siDNA complexes are exactly on top of each other. **b**, Schematic overview of the pUC IDT target plasmid. Blue arrows indicate target sites while percentages indicate the GC-content of the 100 bp segments in which these target sites are located. **c**, Pre-assembled *Cb*Ago-siDNA complexes targeting various pUC IDT segments were incubated with pUC IDT. Cleavage products were incubated with EcoRI or SapI and were further analysed by agarose gel electrophoresis. The GC-content of the segments in which the target sites were located are indicated by the percentage (in blue),

Discussion

Several prokaryotic Argonaute proteins have been demonstrated to protect their host against invading nucleic acids, such as plasmid DNA[13,15,16]. Similar to *Tt*Ago and *Rs*Ago, *Cb*Ago co-purifies with guides which are preferentially acquired from the plasmid used for its heterologous expression in *E. coli*. In addition, *Cb*Ago mediates programmable DNA-guided DNA cleavage *in vitro*. This suggests that, similar to the phylogenetically related *Tt*Ago, also *Cb*Ago provides host defense via DNA-guided DNA interference.

Sequencing of the nucleic acids that co-purified with *Cb*Ago revealed that *Cb*Ago preferentially associates with DNA with a 5'-ATTT-3' sequence at the 5' end. It was previously shown that the guide RNA utilized by eAgos can be divided into functional segments. These segments are (from 5' to 3') the anchor nucleotide (nt 1), the seed (nt 2-8) and sub-seed segments (nt 2-4), and the central (nt 9-12), 3' supplementary (nt 13-16) and tail (nt 17-21) segments[39,41]. Extending this to the siDNAs that co-purified with *Cb*Ago, *Cb*Ago preferentially associates with siDNAs that have a 5'-terminal A anchor and a T-rich sub-seed. In RNAi pathways, the preference for a specific 5'-terminal nucleotide is important for guide RNA loading into a subset of eAgos[42–44] Similarly, several pAgos including RsAgo, *Tt*Ago, and now *Cb*Ago also preferentially associate with specific 5'-terminal nucleotides *in vivo*[13,16]. However, for both *Cb*Ago and *Tt*Ago, there is no clear preference for the same 5'-base during cleavage assays *in vitro*. Rather than having a functional importance, the 5' end preference of pAgos might be a consequence of the way siDNAs are being generated and/or loaded, as has been demonstrated for *Tt*Ago[17]. Several studies on human Ago2 have described the importance of the sub-seed segment (nt 2-4) in its RNA guides[39,40,45]. For hAgo2, a complete match between the guide RNA sub-seed segment and the target RNA triggers a conformational change that first exposes the remainder of the seed (nt 5-8), and eventually the rest of the guide. This facilitates progressive base paring between the guide RNA and the target[46]. However, a specific nucleotide preference in the sub-seed segment, as we have observed for *Cb*Ago, has not been described for any other Argonaute protein. The preference for the T-rich sub-seed is not only observed in the *in vivo* acquired siDNAs, but also plays a clear role during target binding and cleavage assays *in vitro.* This may reflect a structural preference for these thymidines in the cleft of the PIWI domain. We have not been able to obtain diffracting crystals of *Cb*Ago in complex with siDNAs that have a 5'-ATTT-3' sequence at the 5'-end. Future research will thus be necessary to determine the structural basis the apparent preference for these nucleotides at these positions. We hypothesize that

this bias might reflect the mesophilic nature of *Cb*Ago, which might have better access to AT-rich dsDNA fragments, both for siDNA acquisition and for target cleavage.

The potential of pAgos for genome editing applications has previously been discussed[26]. However, the previously characterized DNA-cleaving pAgos originate from thermophilic prokaryotes and therefore require temperatures for their activity that are too high for genome editing at moderate temperatures. The biochemical characterization of *Cb*Ago reporter herein is the first example of a pAgo that catalyzes efficient DNA-guided DNA cleavage at 37°C. Yet, we were unable to demonstrate CbAgo-mediated genome editing in human HEK293 cells (unpublished work). It is conceivable that our experiments were not performed under the optimal conditions for efficient editing. If *Cb*Ago or other pAgos could be harnessed for genome editing, they will have certain advantages over the currently well-established genome editing tools CRISPR-Cas9 and CRISPR-Cas12a. While CRISPR-based genome editing tools can be programmed with a guide RNA to target DNA sequences of choice, target DNA cleavage additionally requires the presence of a protospacer adjacent motif (PAM) next to the targeted sequence (5'-NGG-3' for Cas9 and 5'-TTTV-3' for Cas12a)[47]. This limits the possible target sites of Cas9 and Cas12a. In contrast, pAgos do not require a PAM for DNA targeting, which would make them much more versatile tools compared to CRISPR-associated nucleases. However, PAM binding by Cas9 and Cas12a also promotes unwinding of dsDNA targets[48–50] which subsequently facilitates strand displacement by the RNA guide, and eventually R-Loop formation. The absence of such mechanism in pAgos might explain their limited nuclease activity on dsDNA targets.

Here, we have demonstrated that *Cb*Ago does not strictly rely on other proteins when targeting AT-rich DNA sequences *in vitro*. As such, this study provides a foundation for future efforts to improve double stranded DNA target accessibility of pAgos and to facilitate the further development of pAgos as genome editing tools.

Materials and methods

Plasmid construction

The *Cb*Ago gene was codon harmonized for E.coli Bl21 (DE3) and inserted into a pET-His6 MBP TEV cloning vector (obtained from the UC Berkeley MacroLab, Addgene #29656) using ligation independent cloning (LIC) using oligonucleotides oDS067 and oDS068 (Table S1) to generate a protein expression construct that encodes the *Cb*Ago polypeptide sequence fused to an N-terminal tag comprising a hexahistidine sequence, a maltose binding protein (MBP) and a Tobacco Etch Virus (TEV) protease cleavage site.

Generation of the Double mutant

*Cb*Ago double mutant (D541A, D611A) was generated using an adapted Quick Directed Mutagenesis Kit instruction manual (Stratagene). The primers were designed using the web-based program primerX (http://bioinformatics.org/primerx).

*Cb*Ago expression and purification

The *Cb*Ago WT and DM proteins were expressed in E.coli Bl21(DE3) Rosetta™ 2 (Novagen). Cultures were grown at 37°C in LB medium containing 50 µg ml-1 kanamycin and 34 µg ml-1 chloramphenicol until an OD600nm of 0.7 was reached. *Cb*Ago expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. During the expression cells were incubated at 18°C for 16 hours with continues shaking. Cells were harvested by centrifugation and lysed by sonication (Bandelin, Sonopuls. 30% power, 1s on/2s off for 5min) in lysis buffer containing 20 mM Tris-HCl pH 7.5, 250 mM NaCl, 5 mM imidazole, supplemented with a EDTA free protease inhibitor cocktail tablet (Roche). The soluble fraction of the lysate was loaded on a nickel column (HisTrap Hp, GE healthcare). The column was extensively washed with wash buffer containing 20 mM Tris-HCl pH 7.5, 250 mM NaCl and 30 mM imidazole. Bound protein was eluted by increasing the concentration of imidazole in the wash buffer to 250 mM. The eluted protein was dialysed at 4°C overnight against 20 mM HEPES pH 7.5, 250 mM KCl, and 1mM dithiothreitol (DTT) in the presence of 1mg TEV protease (expressed and purified according to Tropea et al. 2009[51]) to cleave of the His6-MBP tag. Next the cleaved protein was diluted in 20mM HEPES pH 7.5 to lower the final salt concentration to 125 mM KCl. The diluted protein was applied to a heparin column (HiTrap Heparin HP, GE Healthcare), washed with

20 mM HEPES pH 7.5, 125 mM KCl and eluted with a linear gradient of 0.125-2 M KCl. Next, the eluted protein was loaded onto a size exclusion column (Superdex 200 16/600 column, GE Healthcare) and eluted with 20 mM HEPES pH 7.5, 500mM KCl and 1 mM DTT. Purified *Cb*Ago protein was diluted in size exclusion buffer to a final concentration of 5 uM. Aliquots were flash frozen in liquid nitrogen and stored at -80°C.

Co-purification nucleic acids:

To 500 pmoles of purified *Cb*Ago in SEC buffer CaCl2 and proteinase K (Ambion) were added to final concentrations of 5 mM CaCl2 and 250 µg/mL proteinase K. The sample was incubated for 4 hours at 65°C. The nucleic acids were separated from the organic fraction by adding Roti phenol/chloroform/isoamyl alcohol pH 7.5-8.0 in a 1:1 ratio. The top layer was isolated and nucleic acids were precipitated using ethanol precipitation by adding 99% ethanol in a 1:2 ratio supplied with 0.5% Linear polymerized acrylamide as a carrier. This mixture was incubated overnight at -20°C and centrifuged in a table centrifuge at 16,000 g for 30 min. Next, the nucleic acids pellet was washed with 70% ethanol and solved in 50 µL MilliQ water. The purified nucleic acids were treated with either 100 µg/mL RNase A (Thermo), 2 units DNase I (NEB) or both for 1 hour at 37°C and resolved on a denaturing urea polyacrylamide gel (15%) and stained with SYBR gold.

Single stranded Activity assays:

Unless stated otherwise 5 pmoles of each *Cb*Ago, siDNA and target were mixed in a ratio of 1:1:1, in 2x reaction buffer containing 20 mM Tris-HCl (pH 7.5) supplemented with 500 uM $MnCl^{2+}$. The target was added after the *Cb*Ago and siDNA had been incubation for 15 min at 37°C. Then the complete reaction mixture was incubated for 1 hour at 37°C. The reaction was terminated by adding 2x RNA loading dye (95% Formamide, 0.025% bromophenol blue, 5 mM ETDA) and heating it for 5 minutes at 95°C. After this the samples were resolved on a 20% denaturing (7 M Urea) polyacrylamide gel. The gel was stained with SYBR gold nucleic acid stain (Invitrogen) and imaged using a G:BOX Chemi imager (Syngene).

Double stranded Activity assay

In two half reactions 12.5 pmoles of *Cb*Ago was loaded with either 12.5pmoles of forward or reverse siDNA in reaction buffer containing 10 mM Tris-HCl, 10 µg/ml BSA, 250 µM $MnCl_2$. The half reactions

were incubated for 15 min at 37°C. Next, both half reactions were mixed together and 120 ng target plasmid was added after which the mixture was incubated for 1 hour of 37°C. After the incubation the target plasmid was purified from the mixture using a DNA clean and concentrate kit (DNA Clean & Concentrator™-5, Zymogen) via the supplied protocol. The purified plasmid was subsequently cut using either EcoRI-HF (NEB) or SapI-HF (NEB) in Cutsmart buffer (NEB) for 30 min at 37°C. A 6x DNA loading dye (NEB) was added to the plasmid sample prior to resolving it on a 0.7% agarose gel stained with SYBR gold (invitrogen).

Crystallization

To reconstitute the *Cb*Ago DM-siDNA-target DNA complex, siDNA and target DNA were pre-mixed at a 1:1 ratio, heated to 95°C, and slowly cooled to room temperature. The formed dsDNA duplex (0.5M) was mixed with *Cb*Ago DM in SEC buffer at a 1:1:4 ratio (*Cb*Ago DM:duplex DNA), and $MgCl_2$ was added to a final concentration of 5 mM. The sample was incubated for 15 minutes at 20°C to allow complex formation. The complex was crystallized at 20°C using the hanging drop vapour diffusion method by mixing equal volumes of complex and reservoir solution. Initial crystals were obtained at a *Cb*Ago DM concentration of 5 mg/ml with a reservoir solution consisting of 4 M Sodium Formate. Data was collected from crystals grown obtained using a complex concentration of 4.3 mg/ml and reservoir solution containing 3.8 M Sodium Formate and 5 mM $NiCl_2$ at 20°C. For cryoprotection, crystals were transferred to a drop of reservoir solution and flash-cooled in liquid nitrogen.

X-ray diffraction data were measured at beamline X06DA (PXIII) of the Swiss Light Source (Paul Scherrer Institute, Villigen, Switzerland). Data were indexed, integrated, and scaled using AutoPROC (Vonrhein et al (2011)). Crystals of the *Cb*Ago-siDNA-target DNA complex diffracted to a resolution of 3.55 Å and belonged to space group $P6_3\,2\,2$, with one copy of the complex in the asymmetric unit. The structure was solved by molecular replacement using Phaser-MR (McCoy et al., 2007). As search model, the structure of *Tt*Ago in complex with guide and target DNA strands (PDB: 5GQ9) was used after removing loops and truncating amino acid side chains. Phases obtained using the initial molecular replacement solution were improved by density modification using phenix.resolve (Terwilliger, 2004) and phenix.morph_model (Terwilliger et al., 2013). The atomic model was built manually in Coot (Emsley et al., 2010) and refined using phenix.refine (Afonine et al., 2012). The final binary complex model

contains *Cb*Ago residues 1-463 and 466-748, guide DNA residues 1–16, and target DNA residues (-18)–(-1).

Structure analysis

Core Root Means Square Deviations (rmsd) of structure alignments were calculated using Coot SSM superpose (Krissinel et al 2004). Intramolecular interactions were analysed using PDBePISA (Krissinel and Henrick, 2007). Figures were generated using PyMOL (Schrödinger).

Single-Molecule Experimental Set-Up

Single-molecule fluorescence FRET measurements were performed with a prism-type total internal reflection fluorescence microscope. Cy3 and Cy5 molecules were excited with 532 nm and 637 nm wavelength, respectively. Resulting Cy3 and Cy5 fluorescence signal was collected through a 60X water immersion objective (UplanSApo, Olympus) with an inverted microscope (IX73, Olympus) and split by a dichroic mirror (635dcxr, Chroma). Scattered laser light was blocked out by a triple notch filter (NF01-488/532/635, Semrock). The Cy3 and Cy5 signals were recorded using a EM-CCD camera (iXon Ultra, DU-897U-CS0-#BV, Andor Technology) with exposure time 0.1 s. All single-molecule experiments were done at room temperature (22 ± 2C).

Fluorescent DNA and RNA preparation

The RNAs with amine-modification (amino-modifier C6-U phosphoramidite, 10-3039, Glen Research) were purchased from STPharm (South Korea) and DNAs with amine-modification (internal amino modifier iAmMC6T) Ella biotech (Germany). The guide and target strands were labeled with donor (Cy3) and acceptor (Cy5), respectively, using the NHS-ester form of Cy dyes (GE Healthcare). 2012).1 uL of 1 mM of DNA/RNA dissolved in MilliQ H20 is added to 5 uL labeling buffer of (freshly prepared) sodiumtetraborate (380 mg/10mL, pH 8.5). 1 uL of 20 mM dye (1 mg in 56 uL DMSO) is added and incubated overnight at room temperature in the dark, followed by washing and ethanol precipitation. The labeling efficiency was ~100%.

Single-molecule sample preparation

A microfluidic chamber was incubated with 20 µL Streptavidin (0.1 mg/mL, Sigma) for 30 sec. Unbound Streptavidin was washed with 100 µL of buffer T50 (10 mM Tris-HCl [pH8.0], 50 mM NaCl buffer). The fifty microliters of 50 pM acceptor-labelled target construct were introduced into the chamber and

incubated for 1 min. Unbound labeled constructs were washed with 100 µL of buffer T50. The CbAgo binary complex was formed by incubating 10 nM purified CbAgo with 1 nM of donor-labeled guide in a buffer containing 50 mM Tris-HCl [pH 8.0] (Ambion), 1mM MnCl2, and 100 mM NaCl (Ambion) at 37°C for 20 min. For binding rate (kon) measurements, the binary complex was introduced into the fluidics chamber using syringe during the measurement. The experiments were performed at the room temperature (23 ± 1°C).

For fluorescence Guide Loading Experiments before immobilizing CbAgo on the single-molecule surface, 1 mL of 5 mM His-tagged apo-CbAgo was incubated with 1 mL of 1 mg/ml biotinylated anti-6x His antibody (Abcam) for 10 min. Afterward, the mixture was diluted 500x in T50 and 50 mL were loaded in the microfluidic channel for 30 s incubation, followed by washing with 100 mL of T50 buffer. Cy3-labeled ssDNA (0.1) was applied to the microfluidic chamber in imaging buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM MnCl2, 1 mM Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), supplemented with an oxygen-scavenging system (0.5 mg/mL glucose oxidase (Sigma), 85 mg/mL catalase (Merck), and 0.8% (v/v) glucose (Sigma)).

Single-molecule data acquisition and analysis

CCD images of time resolution 0.1 or 0.3 sec were recorded, and time traces were extracted from the CCD image series using IDL (ITT Visual Information Solution). Co-localization between Cy3 and Cy5 signals was carried out with a custom-made mapping algorithm written in IDL. The extracted time traces were processed using Matlab (MathWorks) and Origin (Origin Lab).

The binding rate (k_{on}) was determined by first measuring the time between when CbAgo binary complex was introduced to a microfluidic chamber and when the first CbAgo- guide docked to a target; and then fitting the time distribution with a single-exponential growth curve, $A(1 - e^{-k_{on}t})$. The dissociation rate was estimated by measuring the dwell time of a binding event. A dwell time distribution was fitted by single-exponential decay curve ($Ae^{-t/\Delta\tau}$).

Fluorescence competition experiments

MBP-tagged CbAgo was immobilized on the quartz surface using an anti-MBP antibody. An equimolar mixture of let7 DNA guide (Cy3 labeled) and let7 RNA guide (Cy5 labeled) in imaging buffer was introduced to the microfluidic chamber. After 5 minutes, 10 snapshots of independent fields of view with simultaneous illumination were collected to estimate the amount of guide molecules bound to

protein. Movies were taken for 200 s (2000 frames) at continuous illumination of Cy3 and Cy5 molecules to determine the dwell times of the binding events. Dwell times were binned in a histogram and fitted with a single exponential decay curve.

FRET targeting experiments of ATTT and AAAA guide target combinations

100 pM of target construct annealed with biotin handle were flushed in the microfluidic chamber. After incubation of 1 min, the microfluidic chamber was rinsed with 100 uL T50 buffer. 10 nM of apo-CbAgo was loaded with 1 nM of ATTT seed DNA guide or with AAAA seed DNA guide at 37°C for 30 minutes in imaging buffer after which the mixture is introduced inside the microfluidic chamber. Movies of 200 s were taken at continuous illumination of the Cy3 signal. Site specific protein target interactions were identified as FRET signals and were further analysed.

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Author contributions

J.W.H. and J.v.d.O. conceived the project and designed the biochemical experiments, which were performed by J.H and J.K. Single-molecule experiments were designed by S.C., T.J.C and C.J. and performed by S.C and T.J.C. X-ray crystallographic analysis was performed by D.C.S. under the supervision of M.J.. J.W.H., D.C.S., C.H., M.J., C.J. and J.v.d.O. wrote the manuscript. All authors read and approved the manuscript.

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Supplementary tables:

Table S1. Data collection and structure refinement statistics.

Dataset	
*Cb*Ago	D541A, D611A
siDNA	oDS313 (Let7a-based siDNA)
target DNA	oDS314 (Let7a-based target DNA)
X-ray source	SLS PXIII
Space group	$P6_3$ 2 2
Cell dimensions	
a, b, c (Å)	181.45, 181.45, 142.99
α, β, γ, (°)	90, 90, 120
Wavelength (Å)	1.00
Resolution (Å)*	68.86-3.548 (3.675-3.548)
R_{merge}* (%)	28 (187.6)
CC1/2 (%)*	99.9 (86.1)
I/σ*I**	17.05 (2.47)
Completeness (%)*	99.89 (99.94)
Redundancy*	39 (38)
Refinement	
Resolution (Å)	68.86-3.548
No. reflections	17344
R_{work} / R_{free}	0.247/0.276
No. Atoms	
Macromolecules	6,730
Ligands	16
Solvent	3
B Factors	
Mean (Å^2)	110.40
Macromolecules (Å^2)	110.41
Ligands (Å^2)	111.11
Water (Å^2)	83.70
RMSDs	
Bond lengths (Å)	0.0044
Bond angles (°)	0.720
Ramachandran plot	
Favoured (%)	95.69%
Allowed (%)	4.31%
Outliers (%)	0%
Molprobity	
Clashscore	16.8

Table S2. Target sequences used during the *in vitro* cleavage assays

oligo	Sequence (5'- 3')	description
BG13030	TCGACTTTATATTTAAATAATTTAATATACTATACAACCTACTACAAATTATAAATTTTTAAATAAA TATTGCATTCAAGCTTTTAATTTAATTAAAT	ATTT DNA target
BG13032	TCGACTTTATATTTAAATAATTTAATATACTATACAACCTACTACAAAATATAAATTTTTAAATAAA TATTGCATTCAAGCTTTTAATTTAATTAAAT	TTTT DNA target
BG13034	TCGACTTTATATTTAAATAATTTAATATACTATACAACCTACTACAAAGTATAAATTTTTAAATAAA TATTGCATTCAAGCTTTTAATTTAATTAAAT	CTTT DNA target
BG13036	TCGACTTTATATTTAAATAATTTAATATACTATACAACCTACTACAAACTATAAATTTTTAAATAAA TATTGCATTCAAGCTTTTAATTTAATTAAAT	GTTT DNA target
BG13038	TCGACTTTATATTTAAATAATTTAATATACTATACAACCTACTACTTTTTATAAATTTTTAAATAAAT ATTGCATTCAAGCTTTTAATTTAATTAAAT	AAAA DNA target
BG13040	TCGACTTTATATTTAAATAATTTAATATACTATACAACCTACTACGGGTTATAAATTTTTAAATAAA TATTGCATTCAAGCTTTTAATTTAATTAAAT	ACCC DNA target
BG13042	TCGACTTTATATTTAAATAATTTAATATACTATACAACCTACTACCCCTTATAAATTTTTAAATAAA TATTGCATTCAAGCTTTTAATTTAATTAAAT	AGGG DNA target
BG7023	AAACGACGGCCAGTGCCAAGCTTACTATACAACCTACTACCTCAT	Let-7 based DNA target
BG8385	AAACGACGGCCAGUGCCAAGCUUACUAUACAACCUACUACCUCAU	Let-7 based RNA target
oDS314	TATACAACCTACTACCTCT	Let-7 based target crystallization
SM021	TTTTTTTTTTTTTTTTTTTTTTAACTATACAACCTACTACCTCTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTGGCGACGGCAGCGAGGC	Single perfect target
SM022	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACTACCTCTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTGGCGACGGCAGCGAGGC	Single target 8nt
SM023	[BiotinTEG]GCCTCGCTGCCGTCGCCA	Biotin handle
SM024	UUUUUUUUUUUUUUUUUUUUUUUUU*UUUUUUUACUACCUC	let-7 single target 8nt
SM025	[phos]UUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUU[biotin]	U30 + biotin
SM029	CCCCCCCCCCTCCCCCCCCTACTTTTCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCTGGCGA CGGCAGCGAGGC	AAAA target seed
SM030	CCCCCCCCCCTCCCCCCCCTACAAATCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCTGGCGA CGGCAGCGAGGC	ATTT target seed

Table S3. Guide sequences used during the *in vitro* cleavage assays

oligo	sequence (5'- 3')	description
BG13031	ATTTGTAGTAGGTTGTATAGT	DNA guide ATTT
BG13033	TTTTGTAGTAGGTTGTATAGT	DNA guide TTTT
BG13035	CTTTGTAGTAGGTTGTATAGT	DNA guide CTTT
BG13037	GTTTGTAGTAGGTTGTATAGT	DNA guide GTTT
BG13039	AAAAGTAGTAGGTTGTATAGT	DNA guide AAAA
BG13041	ACCCGTAGTAGGTTGTATAGT	DNA guide ACCC
BG13043	AGGGGTAGTAGGTTGTATAGT	DNA guide AGGG
BG11523	ATACTGAATTTGTATGTTTTG	DNA (FW) guide pUCDRB 23%
BG11524	TATTAATATATGCTGGCAAAT	DNA (FW) guide pUCDRB 27%
BG11525	GTATGTATTAGTGTGCTAGAT	DNA (FW) guide pUCDRB 31%
BG11526	AAAGATTGTTTCCTGTGCTTT	DNA (FW) guide pUCDRB 35%
BG11539	CAAAACATACAAATTCAGTAT	DNA (RV) guide pUCIDT 23%
BG11540	ATTTGCCAGCATATATTAATA	DNA (RV) guide pUCIDT 27%
BG11541	ATCTAGCACACTAATACATAC	DNA (RV) guide pUCIDT 31%
BG11542	AAAGCACAGGAAACAATCTTT	DNA (RV) guide pUCIDT 35%
BG10631	TTACTCATATATACTTTAGAT	DNA (FW) guide pUC19 -15
BG10632	CATATATACTTTAGATTGATT	DNA (FW) guide pUC19 -10
BG10633	ATACTTTAGATTGATTTAAAA	DNA (FW) guide pUC19 -5
BG10634	TTAGATTGATTTAAAACTTCA	DNA (FW) guide pUC19 0

BG10635	TTGATTTAAAACTTCATTTTT	DNA (FW) guide pUC19 5
BG10636	TTAAAACTTCATTTTTAATTT	DNA (FW) guide pUC19 10
BG10637	ACTTCATTTTTAATTTAAAAG	DNA (FW) guide pUC19 15
BG10646	ATCTAAAGTATATATGAGTAA	DNA (RV) guide pUC19 -15
BG10647	AATCAATCTAAAGTATATATG	DNA (RV) guide pUC19 -10
BG10648	TTTTAAATCAATCTAAAGTAT	DNA (RV) guide pUC19 -5
BG10649	TGAAGTTTTAAATCAATCTAA	DNA (RV) guide pUC19 0
BG10650	AAAAATGAAGTTTTAAATCAA	DNA (RV) guide pUC19 5
BG10651	AAATTAAAAATGAAGTTTTAA	DNA (RV) guide pUC19 10
BG10652	CTTTTAAATTAAAAATGAAGT	DNA (RV) guide pUC19 15
BG8050	[phos]UGAGGUAGUAGGUUGUAUAGU	RNA guide based on let-7 miRNA
BG6790	[phos]TGAGGTAGTAGGTTGTATAGT	DNA guide based on let-7 miRNA
BG5640	[phos]TGAGGTAGTAGGTTGT	DNA guide based on BG6790
BG5713	[phos]TGAGGTAGTAGGTTG	DNA guide based on BG6790
BG5714	[phos]TGAGGTAGTAGGTT	DNA guide based on BG6790
BG5715	[phos]TGAGGTAGTAGGT	DNA guide based on BG6790
BG5641	[phos]TGAGGTAGTAGG	DNA guide based on BG6790
oDS313	[phos]CGAGGTAGTAGGTTGTATAGT	Let-7 miRNA based DNA guide crystallization
SM001	[phos]CGAGGTAGTAGGTTGTATAGTT	Perfect guide
SM002	[phos]CGAGTATTTTTTTTTTTTTTTT	3nt match (nt 2-4) (nt 6, T changed to A)
SM003	[phos]CGAGGATTTTTTTTTTTTTTTT	4nt match (nt 2-5) (nt 6, T changed to A)
SM004	[phos]CGAGGTTTTTTTTTTTTTTTTT	5nt match (nt 2-6)
SM005	[phos]CGAGGTATTTTTTTTTTTTTTT	6nt match (nt 2-7)
SM006	[phos]CGAGGTAGATTTTTTTTTTTTTT	7nt match (nt 2-8) (nt 9, T changed to A)
SM007	[phos]CCATCT	6nt
SM008	[phos]CCATCTG	7nt
SM009	[phos]CCATCTGT	8nt
SM010	[phos]CCATCTGTC	9nt
SM011	[phos]CCATCTGTCT	10nt
SM012	[phos]CCATCTGTCTT	11nt
SM013	[phos]CCATCTGTCTTA	12nt
SM014	[phos]CCATCTGTCTTAG	13nt
SM015	[phos]GAGGUAGU*AGGUUGUAUAGUU	Mature let-7a perfect guide
SM016	[phos]GAGUAUUU*UUUUUUUUUUUUU	Mature let-7a 3nt match (nt 2-4) (nt 6, U changed to A)
SM017	[phos]GAGGAUUU*UUUUUUUUUUUUU	Mature let-7a 4nt match (nt 6, U changed to A)
SM018	[phos]GAGGUUUU*UUUUUUUUUUUUU	Mature let-7a 5nt match
SM019	[phos]GAGGUAUU*UUUUUUUUUUUUU	Mature let-7a 6nt match
SM020	[phos]UGAGGUAGAU*UUUUUUUUUUUU	Mature let-7a 7nt match (nt 9, U changed to A; U* is at nt 10)
SM027	[phos]AAAAGTAGTTTTTTTTTTTTT	AAAA guide seed
SM028	[phos]ATTTGTAGTTTTTTTTTTTTT	ATTT guide seed

U* in italic is the uridine that is conjugated with amino-modifier C6-U phosphoramidite.

Table S4. Primers used to construct the expression plasmids

Oligo	Sequence (5'-3')	description
BG6808	GTTTCATTGGGCTAGCTGTGGGTACCCGTG	*Cb*Ago D541A fw
BG6809	CACGGGTACCCACAGCTAGCCCAATGAAAC	*Cb*Ago D541A rv

BG6810	TTGTGATTCATCGTGCTGGGTTTTCTCGTG	*Cb*Ago D611A fw
BG6811	TCACGAGAAAACCCAGCACGATGAATCACA	*Cb*Ago D611A rv
oDS067	TACTTCCAATCCAATGCAAATAATCTGACCTTTGAGGCTTTTG	LIC cloning fw
oDS068	TTATCCACTTCCAATGTTATTACAGAAAGAATAGACGATTATCAAC	LIC cloning rv
SM026	AAAAAAAAAAAAGAGGTAGTAAAA	DNA splint 8nt match

Table S5. Plasmids

Plasmid	description	vendor
p*Cb*Ago1	Codon harmonized *C. butyricum* Ago gene in pET28 expression plasmid	GenScript (USA)
p*Cb*Ago2	p*Cb*Ago1, with substituted active site *Cb*Ago DM (D541A, D661A)	
pUC19	Standard pUC19 vector	NEB
pDRB	pUC19 with 16x 100bp Gene fragments human genome	IDT

Supplementary figures

Supplementary Figure 1 | *Cb*Ago harbors a catalytic DEDD tetrad. a. Schematic representation of the pET28 based expression vector of *Cb*Ago. *Cb*Ago consists of the typical (N-PAZ-MID-PIWI) domains. An His(6x)-MBP tag was N-terminally fused to *Cb*Ago linked with a cleavable TEV site. *Cb*Ago was under the control of a T7 promotor. **b**, Size exclusion diagram showing the elution peak of *Cb*Ago. The purity of the purified *Cb*Ago was determined using SDS-PAGE. **c**, Multiple sequence alignment of a part of the PIWI domain from *Cb*Ago with several other biochemically or structurally characterized Ago proteins.

Supplementary Figure 2 | Phylogenetic tree of prokaryotic Argonautes. Maxium-likehood phylogenetic trees were constructed by Kira Makarova (personal communication). The colored boxes indicate the Argonaute proteins that have been characterized biochemically.

Supplementary Figure 3 | Comparison of siDNA-target DNA interactions with *Cb*Ago and *Tt*Ago. Schematic representation of hydrogen bonding interactions between *Cb*Ago or *Tt*Ago and their guide-target nucleic acids using the ternary protein structures of both pAgos. The residues of *Tt*Ago and *Cb*Ago are colored according to their domains (see Fig. 2a). Nucleotides colored grey are not ordered in the structure. Base pairs are indicated with thick dashed lines, while other hydrogen bonds are indicated with thin dashed lines.

Supplementary Figure 4 | CbAgo prefers 5' phosphorylated guides over 5' hydroxylated guides. *Cb*Ago is unable to cleave a short 45nt target DNA using a 5'OH siDNA. The 45nt target DNA is efficiently cleaved into two products (34nt and 10nt; 10nt not visible on gel) using a 5'phosphorylated siDNA (either phosphorylated using PNK or ordered at IDT). *Cb*Ago cleaves a longer 120nt target DNA into two product (86 and 34nt) using a 5'OH siDNA, but less efficient than with the 5'phosphorylated siDNA.

Supplementary Figure 5 | Total internal reflection microscopy (TIRM) was used to determine the minimal length for siDNA to be bound by *Cb*Ago. Left panel: Graphical overview of the TIRM method. Right panel: Microscope snapshots of the polymer-coated quartz surface after siDNAs of different lengths were loaded. Each black dot represents a bound siDNA. The total number of bound siDNA (black dots) per microscope image are displayed in the histogram.

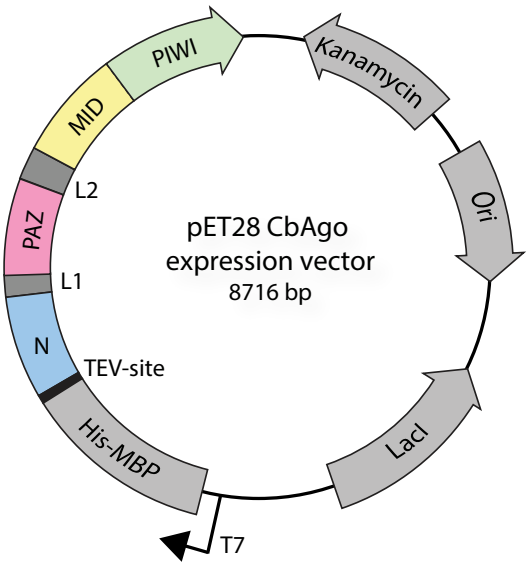
Supplementary Figure 6 | Expression of *Cb*Ago does not affect the plasmid content in *E. coli*. In biological triplicates of both strains protein expression was induced by the addition of ITPG. Four hours

after expression the plasmids were isolated and quantified. No significant differences in plasmid content could be observed.

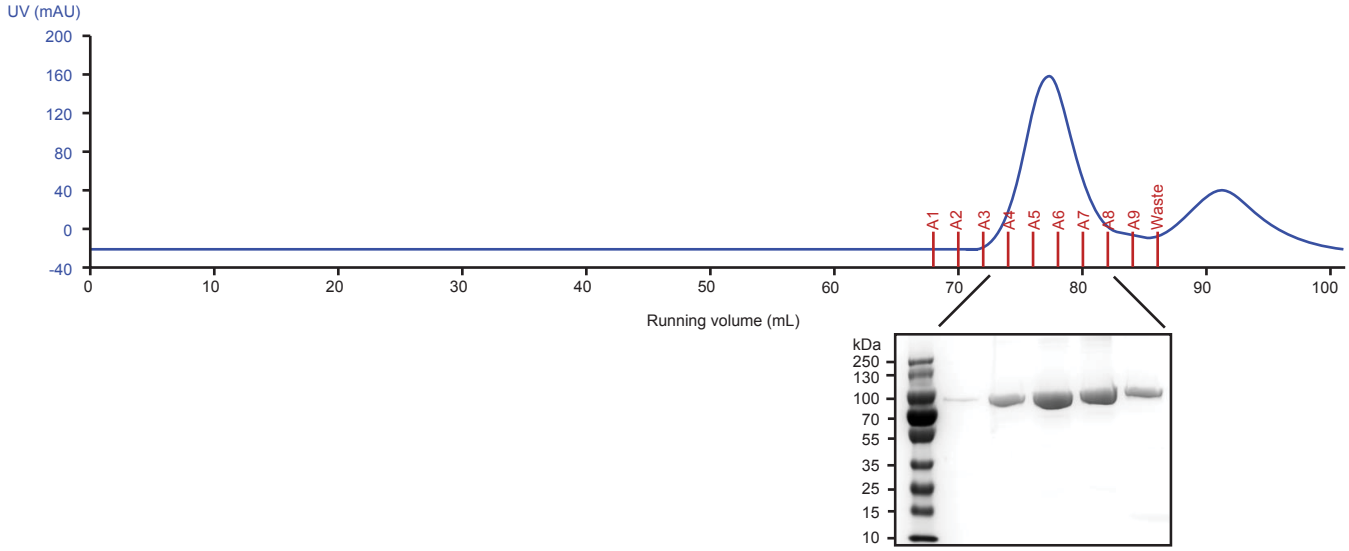
Supplementary Figure 7 | Simulations that compare the interaction between the t1N binding site of *Cb*Ago and *Tt*Ago with the t1N residues of the target strand based on molecular dynamics simulation models. In *Cb*Ago the T1 thymine base is not placed in the t1 binding pocket as has been observed in *Tt*Ago (which binding pocket is formed by D590, R640). Instead, the t1 thymine base of *Cb*Ago is flipped.

Supplementary Figure 1

a

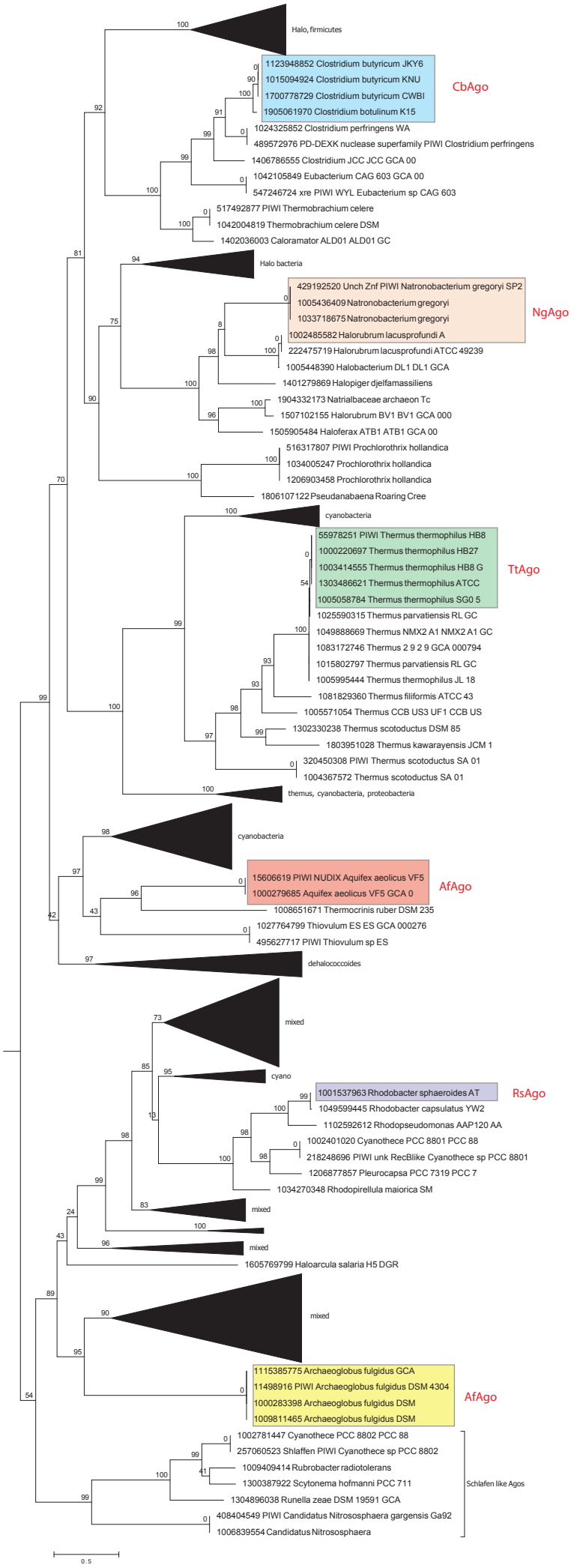


b

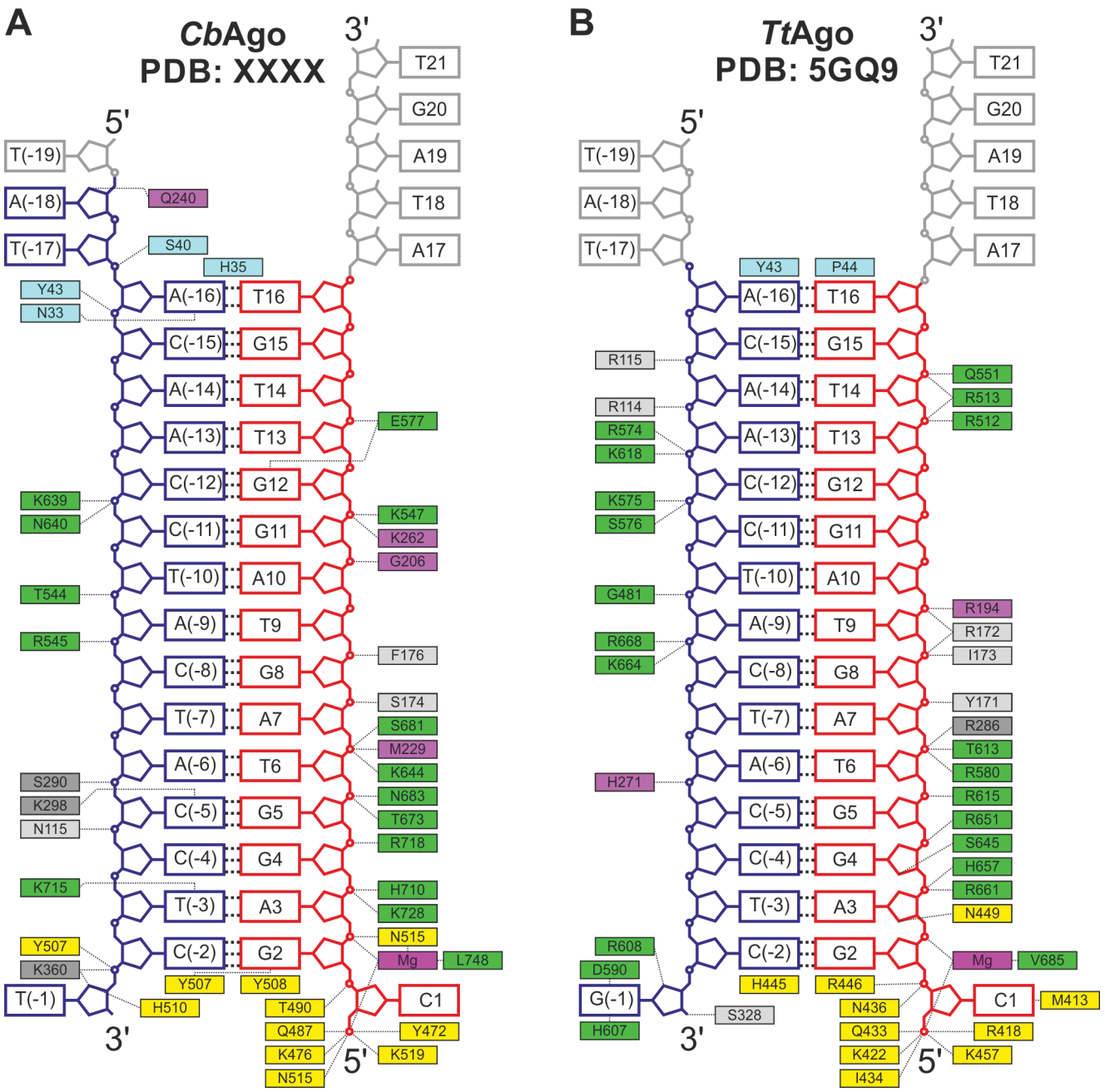


c

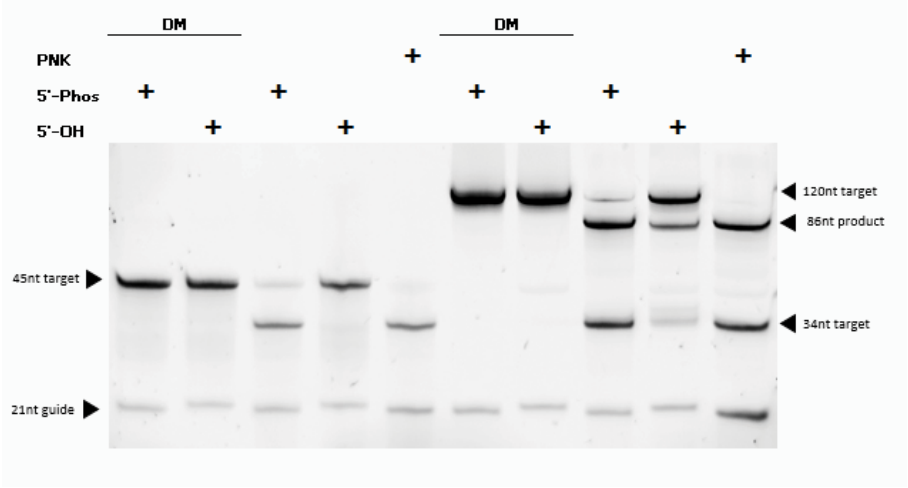
Supplementary Figure 2



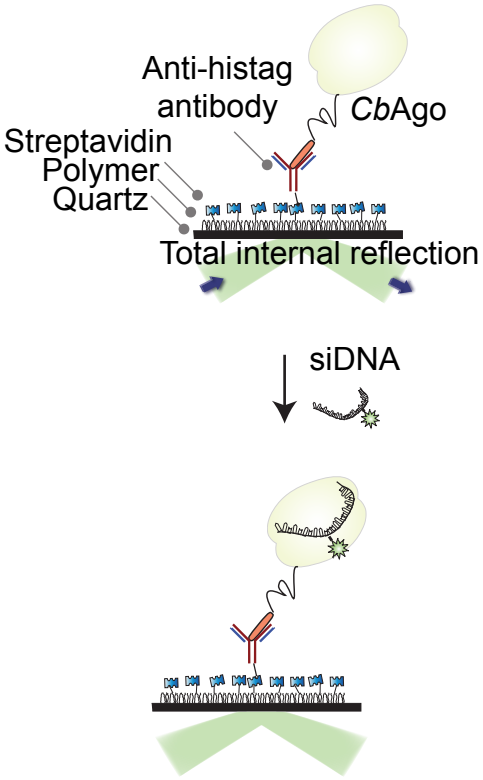
Supplementary Figure 3

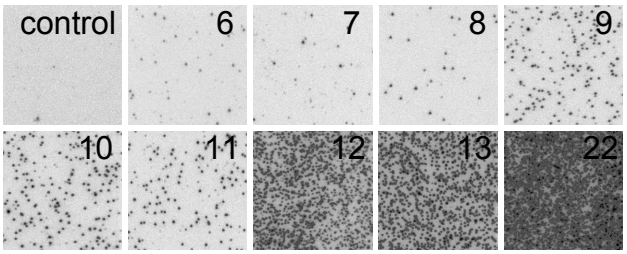


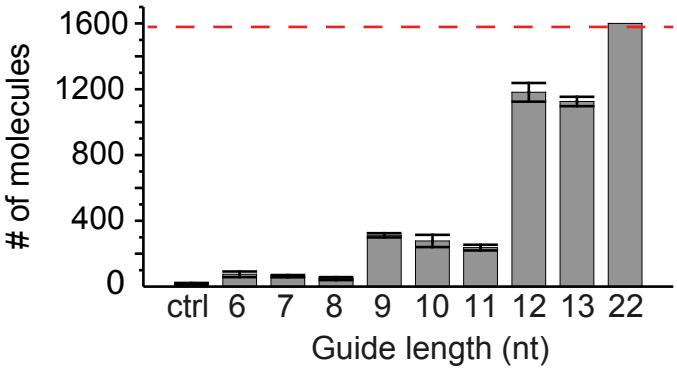
Supplementary Figure 4



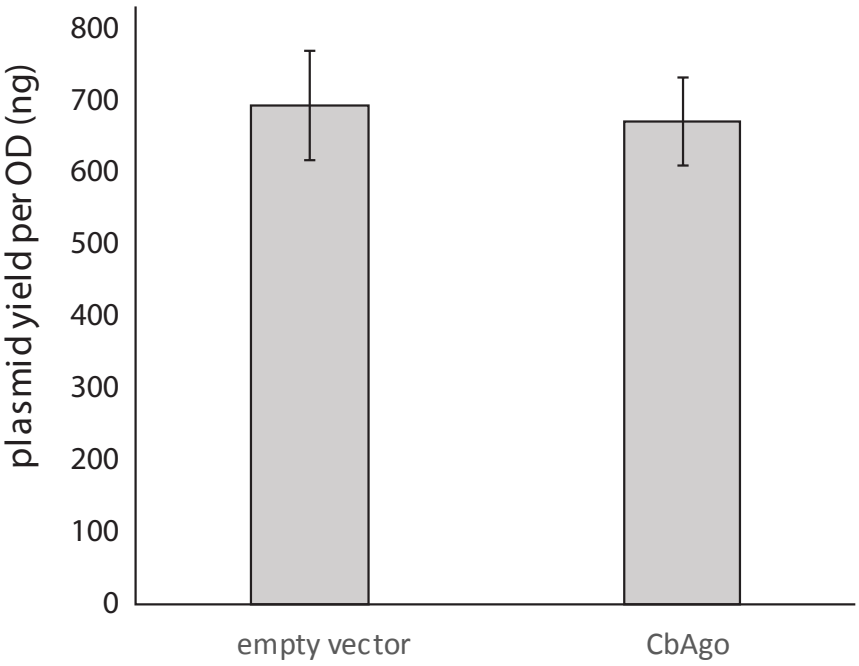
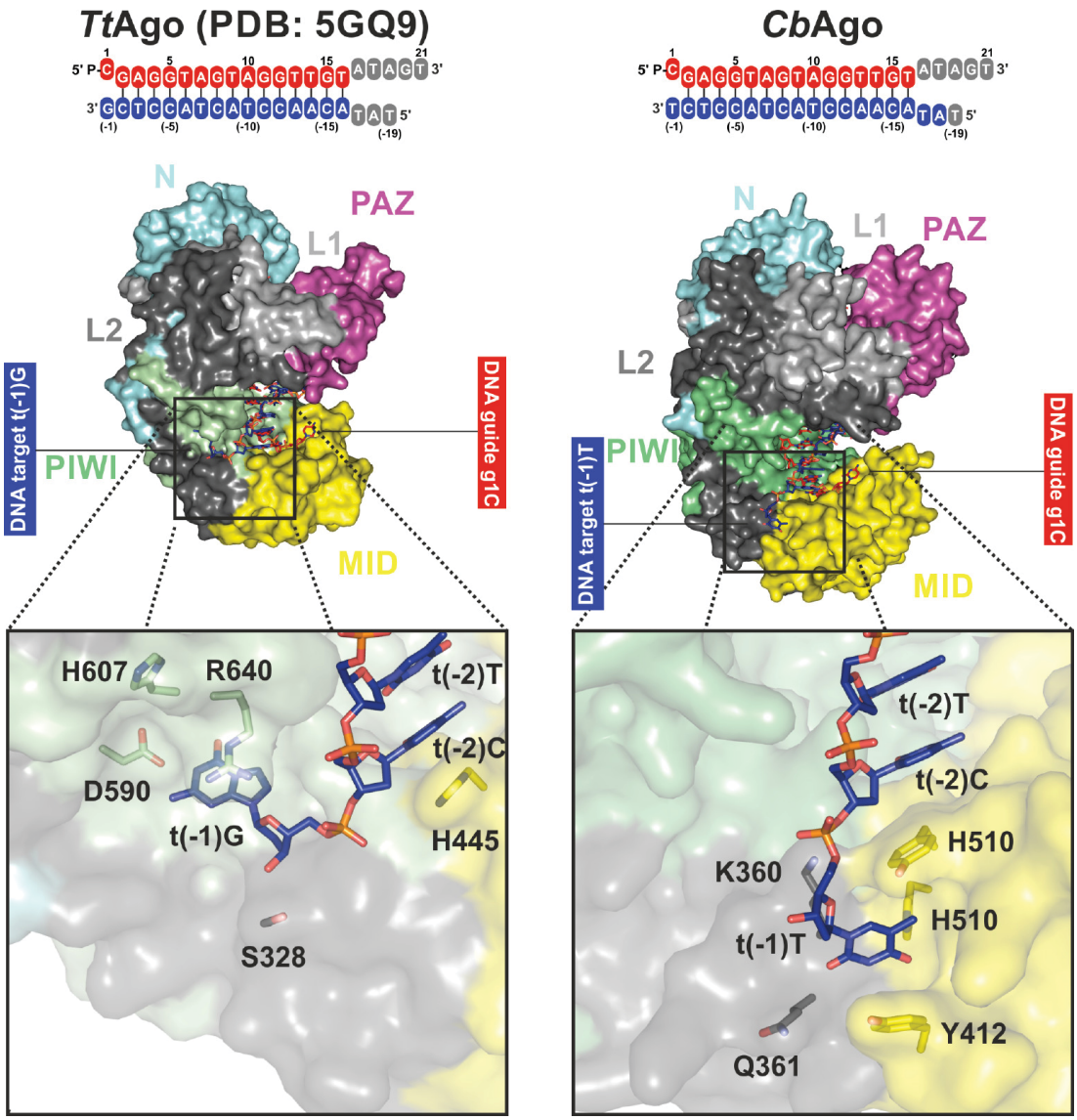
Supplementary Figure 5







Supplementary Figure 6



Supplementary Figure 7