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DNA-guided genome editing using the *Natronobacterium gregoryi* Argonaute

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The RNA-guided endonuclease Cas9 has made genome editing a widely accessible technique. Similar to Cas9, endonucleases from the Argonaute protein family also use oligonucleotides as guides to degrade invasive genomes. Here we report that the *Natronobacterium gregoryi* Argonaute (NgAgo) is a DNA-guided endonuclease suitable for genome editing in human cells. NgAgo binds 5′ phosphorylated single-stranded guide DNA (gDNA) of ~24 nucleotides, efficiently creates site-specific DNA double-strand breaks when loaded with the gDNA. The NgAgo–gDNA system does not require a protospacer-adjacent motif (PAM), as does Cas9, and preliminary characterization suggests a low tolerance to guide–target mismatches and high efficiency in editing (G+C)-rich genomic targets.

The RNA-guided endonuclease Cas9 is the most commonly used genome editing tool and employs RNA–DNA hybridization to specifically cleave genomic sequences[1–5]. Although many modifications have been introduced to the Cas9 system to improve its efficiency and specificity, its practical utility is still limited by its tolerance to guide–target mismatches and the relatively easy formation of secondary structure by its RNA guides[6].

Argonautes are a family of endonucleases that use 5′ phosphorylated short single-stranded nucleic acids as guides to cleave targets[7]. Similar to Cas9, Argonautes have key roles in gene expression repression and defense against foreign nucleic acids[7–12]. However, Argonautes differ from Cas9 in many ways[7]. Cas9 only exist in prokaryotes, whereas Argonautes are preserved through evolution and exist in virtually all organisms; although most Argonautes associate with single-stranded (ss)RNAs and have a central role in RNA silencing, some Argonautes bind ssDNAs and cleave target DNAs[11,13]; guide RNAs must have a 3′ RNA-RNA hybridization structure for correct Cas9 binding, whereas no specific consensus secondary structure of guides is required for Argonaute binding; whereas Cas9 can only cleave a target upstream of a PAM, there is no specific sequence on targets required for Argonaute. Once Argonaute and guides bind, they affect the physicochemical characteristics of each other and work as a whole with kinetic properties more typical of nucleic-acid-binding proteins[14]. Probably owing to these characteristics, Argonautes have evolved into a large family of proteins that fulfill many different tasks. Here we show that the Argonaute of *N. gregoryi* can be programmed with ssDNA guides, and is a precise and efficient tool for genome editing in mammalian cells.

RESULTS

Argonaute derived from *N. gregoryi* uses DNA guides and cleaves DNA

Argonaute from *Thermus thermophilus* (TtAgo) or *Pyrococcus furiosus* (PfAgo) catalyzes cleavage of DNA targets *in vitro* when supplied with complementary 5′ phosphorylated ssDNA[11,13]. However, both of them require a reaction temperature >65 °C, precluding them from acting in mammalian cells. To find an Argonaute suitable for editing mammalian genome, we performed a search using Position-Specific Iterative Basic Local Alignment Search Tool (PSI-BLAST) against the US National Center for Biotechnology Information (NCBI) nonredundant protein sequence database using the amino acid sequences of TtAgo and PfAgo and identified an Argonaute derived from haloalkaliphilic archaebacterium *N. gregoryi* SP2 (NgAgo, protein identifier AFZ73749.1) as a potential candidate.

To confirm that NgAgo uses ssDNA guides, we analyzed the nucleic acids purified from NgAgo expressed in *Escherichia coli*, as those bacteria produce both 5′-phosphorylated ssRNAs and ssDNAs, and the nucleic acids of proper length (13–25 nt) naturally bind to prokaryotic Argonautes[11,12]. Nuclease digestion confirmed that DNA but not RNA binds NgAgo (**Fig. 1a**). To test whether the NgAgo expressed by *E. coli* can cleave target DNA at 37 °C *in vitro*, we performed an *in vitro* plasmid cleavage assay. We designed three 5′ phosphorylated 24-nucleotide (nt) ssDNA guides, among which two guides were complementary to each other ('FW' and 'RV' guides) and corresponded to a target site in plasmid pACYCDuet-eGFP, which had no homologous sequence to the NgAgo-encoding plasmid pGEX6P-1 (**Fig. 1b**). The other guide had a random sequence without overlap with pACYCDuet-eGFP (noncomplementary ('NC') guide). We also designed a pair of 5′-phosphorylated RNA guides corresponding to the same target site. Before the cleavage assay, we replaced the native nucleic acids bound to the purified) NgAgo with our designed guides by incubating NgAgo with the guides at 55 °C for 1 h. NgAgo could not catalyze cleavage without guide or with the NC guide (**Fig. 1b**). When supplied with either of the complementary FW or RV guides, NgAgo could nick the negatively supercoiled plasmid at 37 °C; when supplied with both FW and RV guides, NgAgo linearized the plasmid. However, neither ssDNA guides without 5′ phosphorylation nor 5′-phosphorylated ssRNA guides led to plasmid

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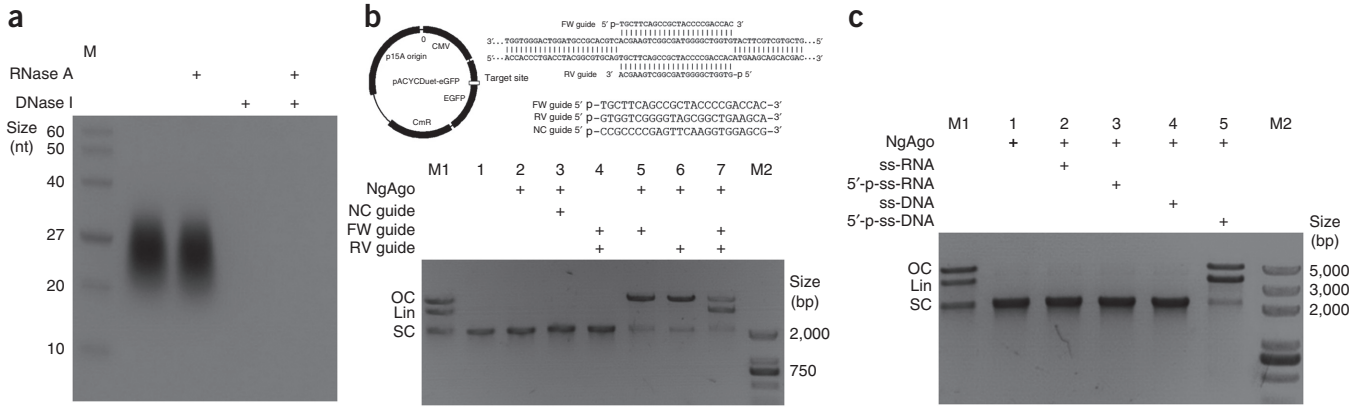

Figure 1 NgAgo uses 5′ phosphorylated ssDNA guides and cleaves DNA targets *in vitro*. (**a**) Electrophoresis of GST-NgAgo expressed in *E. coli*, purified by Sephrose-4B, digested by protein kinase K, and treated with the indicated exonucleases. M, Single Strand molecular weight marker (nt). (**b**) Schematic of the target plasmid (top left) and sequences of the 5′-phosphorylated ssDNA guides (top right). *In vitro* plasmid cleavage assay, performed by reloading NgAgo expressed in *E. coli* with the indicated guide ssDNAs at 55 °C for 1 h, followed by incubation with the target plasmid at 37 °C for 12 h, and analysis of the target plasmid by electrophoresis (bottom). M1: super-coiled, open circular and linearized pACYCDuet-eGFP plasmids. SC, supercoiled; Lin, linearized; OC, open circular; M2, double strands molecular weight marker D2000 (GenStar Co. Ltd) (bp). (**c**) Plasmid cleavage assay performed by using the indicated nucleic acids with or without 5′ phosphorylation. Results are representative of three independent experiments. Full-length gels are presented in **Supplementary Figure 9**.

cleavage (**Fig. 1c**). Thus, NgAgo can cleave DNA double helix targets at 37 °C with 5′ phosphorylated ssDNA guide.

NgAgo binds ssDNA guide and makes double-strand break on targets

To evaluate whether NgAgo can be used as a genome editing tool, we first expressed NgAgo in 293T cells and examined whether it associates with endogenous nucleic acids in human cells. There were no detectable nucleic acids associated with NgAgo purified from 293T cells (**Fig. 2a**), which implies that there is very limited 5′ phosphorylated ssDNA present in human cells, suggesting that endogenous ssDNAs will not mislead NgAgo to off-target sites. Next, we supplied 24-nt ssDNA or ssRNAs with or without 5′ phosphorylation to NgAgo by transfecting 293T cells with NgAgo-encoding plasmid and the synthetic oligos. NgAgo could only bind the 5′-phosphorylated ssDNA but not ssRNAs or ssDNA without 5′-phosphorylation (**Fig. 2a**). When we postponed the delivery of 5′-phosphorylated ssDNAs into the cells by 24 h after transfection of NgAgo-encoding plasmid, NgAgo-ssDNA binding decreased substantially (**Fig. 2b**), which suggests that ssDNA loading occurs in a short time window when NgAgo is expressed. Consistent with this notion, no ssDNA could be loaded to NgAgo *in vitro* at 37 °C even when nonloaded NgAgo purified from 293T cells was incubated with ssDNAs for up to 8 h (**Fig. 2c**). Again, ssDNAs could only be loaded at 55 °C, a nonphysiological, high temperature (**Fig. 2c**). Only the NgAgo purified from the cells that had been also transfected with ssDNAs, but not the NgAgo incubated with ssDNAs at 37 °C *in vitro* could cleave DNA (**Supplementary Fig. 1**). Moreover, NgAgo derived from the cells with an NC guide could not cleave target

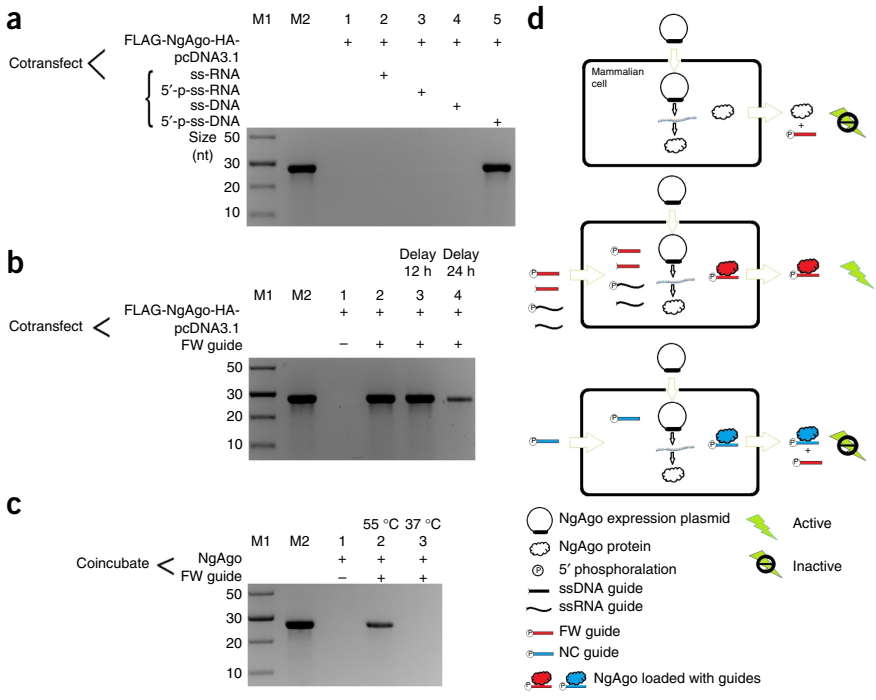

Figure 2 NgAgo binds ssDNA guide in a one-guide-faithful manner. (**a**) Electrophoresis of nucleic acids bound to NgAgo that had been expressed in 293T cells and extracted after 48 h growth with or without the indicated 24-nt nucleic acids. M1, ssDNA ladder; M2, ssDNA guide. (**b**) Electrophoresis of nucleic acids bound to NgAgo that had been expressed in 293T cells with transfection of a 5′ phosphorylated ssDNA guide (FW) with delays as indicated, and extracted 48 h after initial transfection of NgAgo-encoding plasmid. (**c**) Electrophoresis of nucleic acids bound to NgAgo that had been expressed in 293T cells, extracted after 48 h, and incubated with a 5′ phosphorylated ssDNA guide at 55 °C for 1 h or at 37 °C for 8 h. (**d**) Schematic of the one-guide-faithful rule abided by NgAgo. Results are representatives of at least three independent experiments. Full-length gels are presented in **Supplementary Figure 9**.

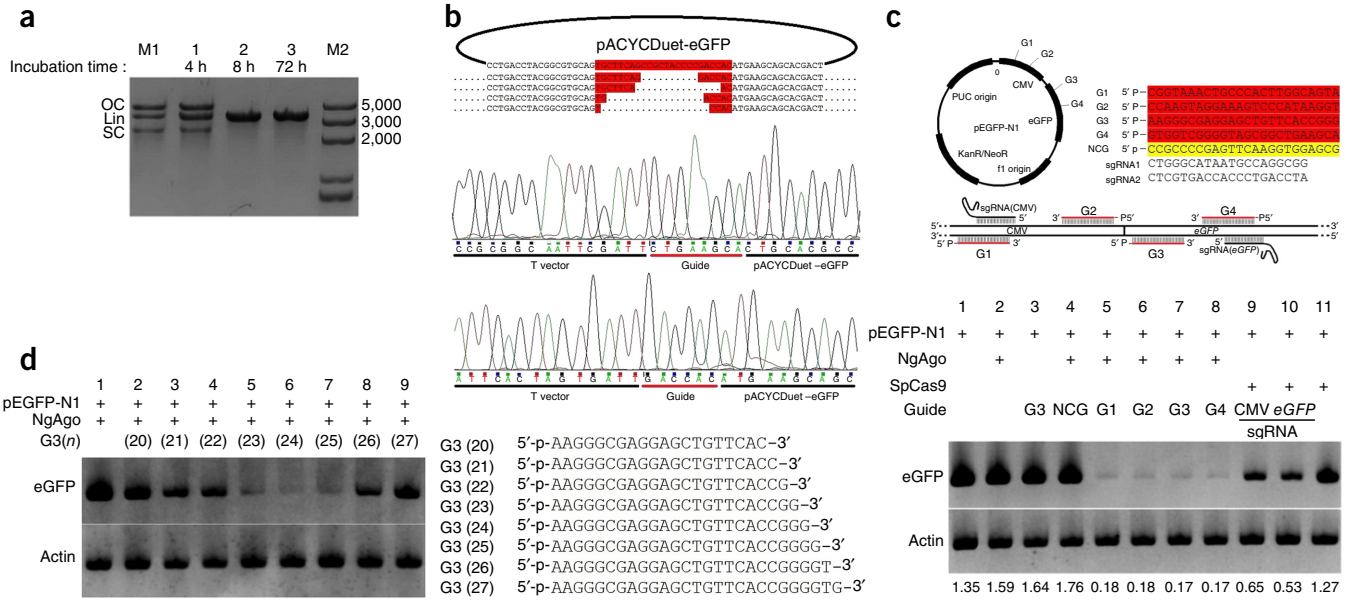

Figure 3 NgAgo works as an endonuclease and can cleave DNA targets *in vivo*. (**a**) Electrophoresis of target pACYCDuet-eGFP plasmid after it was incubated with purified NgAgo, pre-loaded with FW guide in 293T cells for 4 h, 8 h or 72 h. Representative results from three independent experiments. (**b**) Examples of sequencing results from the plasmid cleavage products of NgAgo after 72-h incubation, which shows that 1–20 nucleotides within the guide/target region were randomly removed. Representative sequencing results from 20 independent experiments. (**c**) Schematic (top) shows the positions of ssDNA guides for NgAgo and sgRNA for Cas9 on the target plasmid pEGFP-N1. Western blot analysis (below) of eGFP expression in HeLa cells transfected with pEGFP-N1 target plasmid together with either the NgAgo-expressing plasmid and the indicated ssDNA guides, or Cas9-expressing plasmid and the indicated sgRNA transcription vectors. Densitometric ratios between eGFP and actin bands are shown below the blot. Representative of three independent experiments. (**d**) Western blot analysis of eGFP expression in HeLa cells transfected with pEGFP-N1 target plasmid, NgAgo-encoding plasmid and the G3 guide of various lengths (*n*). Blot is representative of three independent experiments. Full-length gels and blots are presented in **Supplementary Figure 9**.

DNA even if it was later incubated *in vitro* with the FW guide for 8 h (**Supplementary Fig. 1**). These data indicate that NgAgo is faithful to its original guide and does not allow DNA swapping at 37 °C (**Fig. 2d**), similar to mammalian Ago2, which cannot exchange its bound oligos with free oligos at 37 °C[15,16]. This feature of NgAgo minimizes the possibility that it will be loaded with unexpected 'guides'.

Unlike the guide-reloaded NgAgo, the NgAgo loaded with one guide *in vivo* could efficiently linearize a target plasmid (**Supplementary Fig. 1**), indicating that NgAgo makes a double-strand break when loaded with single guide at 37 °C but not when reloaded with a guide at 55 °C. We speculated that the guide-reloading process at 55 °C may denature NgAgo and partially suppress its activity. Indeed, a prolonged incubation at 55 °C led to a complete loss of its nuclease activity (**Supplementary Fig. 2**).

To determine the position of the cleavage site of NgAgo, we polyadenylated the linearized plasmids and cloned them into pGEM-T vectors (Promega) for sequencing. Instead of just breaking a single phosphodiester bond, NgAgo removed several nucleotides within the guide/target region. This finding is consistent with the TtAgo reaction, which also removes nucleotides in its targets[17]. To test the possibility that the nucleotide removal is due to an exonuclease activity of NgAgo, we prolonged the enzymatic reaction to 72 h even though complete linearization was achieved after 8 h under the same conditions. We observed no degradation of the target after 72 h (**Fig. 3a**). Sequencing of the 72-h cleavage products showed that 1–20 nt within the guide/target region were randomly removed (**Fig. 3b**). Moreover, NgAgo did not cleave linearized plasmid or ssDNA (**Supplementary Fig. 3**). These data suggest that NgAgo is not an exonuclease.

To investigate whether the NgAgo-gDNA system is an efficient tool for targeted DNA cleavage in mammalian cells, we performed an *in vivo* plasmid cleavage assay. We transfected HeLa cells with an pEGFP-N1 plasmid together with the NgAgo-encoding plasmid and ssDNA guides against the CMV promoter or the eGFP reading frame. We measured eGFP expression as an inverse readout of cleavage efficiency (**Fig. 3c**). To compare this system to the Cas9-sgRNA system, we also transfected cells with pEGFP-N1 target plasmid together with a Cas9-encoding plasmid and sgRNA transcription vectors. When providing cells with the same dose of NgAgo and Cas9 plasmid, we observed that the NgAgo-gDNA system was as efficient in inactivating eGFP expression as the Cas9-sgRNA system (**Fig. 3c**). Using this approach, we confirmed that the optimal length of gDNA for NgAgo is about 24 nt (**Fig. 3d**).

Broad targeting range and low tolerance to mismatches

Next, we tested the ability of NgAgo to edit endogenous sites in the human genome. We designed a modified NgAgo by attaching a nuclear localization signal (NLS) to its N terminus to ensure nuclear compartmentalization (**Supplementary Fig. 4**). We designed five gDNAs against exon 11 of human *DYRK1A* gene (**Fig. 4a**). Using the T7 endonuclease I (T7EI) assay to detect endogenous cleavages[1–5,18], we found that all the guides could lead a highly efficient target cleavage by NgAgo, which we confirmed by sequencing.

We also tested 47 guides targeting eight different genes. The efficiency of genome editing was comparable for all guides (21.3–41.3%; **Fig. 4b**, **Supplementary Fig. 5** and **Supplementary Table 1**), and we did not observe obvious preferences of NgAgo to sequences with specific properties. We also examined the NgAgo-gDNA system in

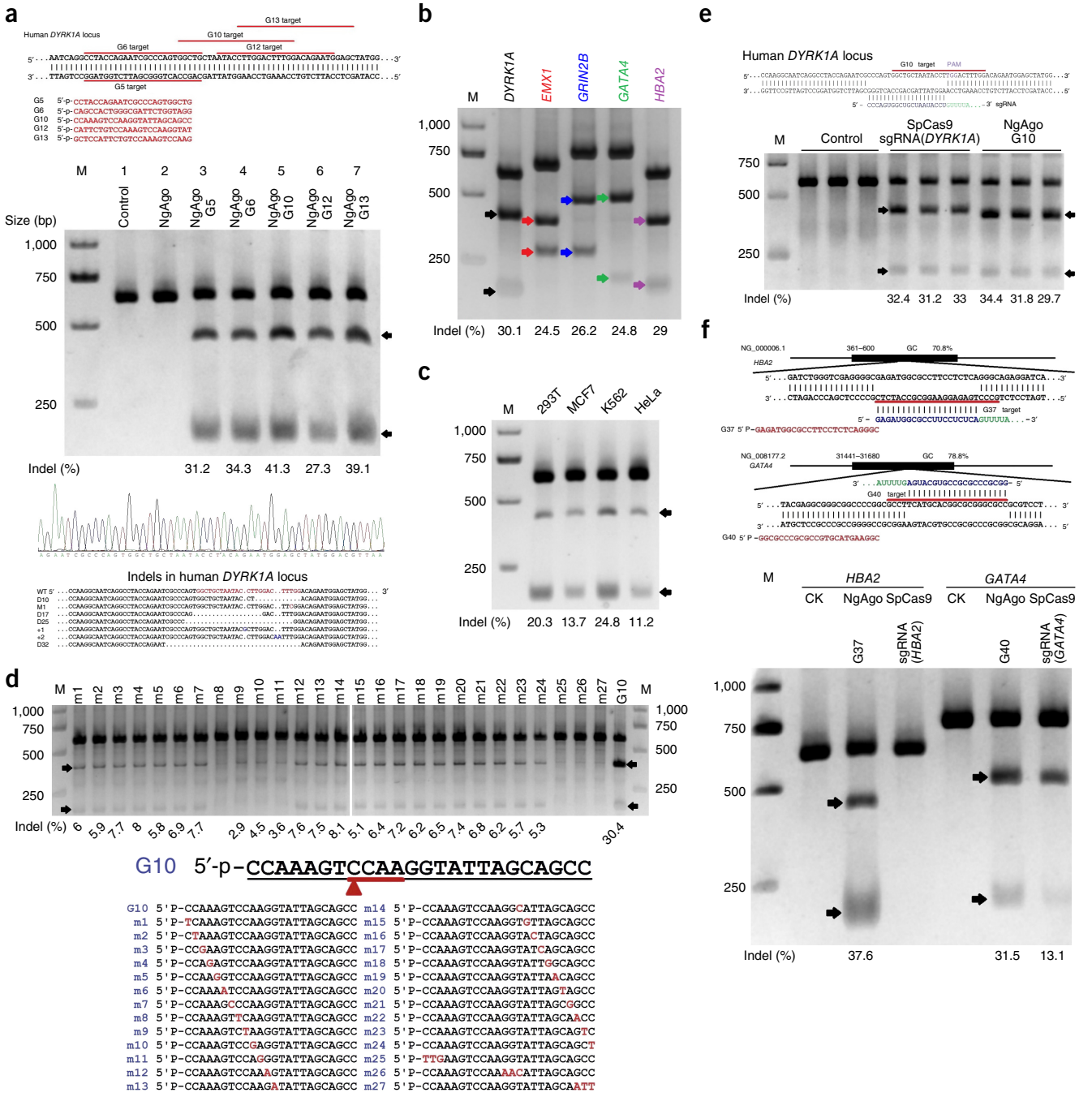

Figure 4 NgAgo can make targeted double-strand breaks in mammalian genome. (**a**) Schematic of the guides corresponding to the loci of the exon 11 of human *DYRK1A* gene (top). T7E1 assay (middle) for NgAgo-mediated insertion-deletions (indels). Arrows show the anticipated positions of T7E1 products. The control is normal 293T cell genomic DNA. Example chromatogram showing microdeletion as well as representative sequences of mutated alleles identified from clonal amplicons by using G10 guide (bottom). WT, wild type; D, deletions; M, mutations; +, insertions. (**b**,**c**) T7E1 assays of NgAgo-mediated indels at the indicated targets in human genome (**b**) and at the *DYRK1A* gene in the indicated cell lines (**c**). (**d**) T7E1 assay of G10 guide with indicated nucleotide mismatches (marked in red). (**e**,**f**) Comparison of the efficiency between NgAgo-gDNA system and Cas9-sgRNA system in cleaving the human *DYRK1A* gene (**e**), and the (G+C)-rich sequences at *HBA2* and *GATA4* genes (**f**). The controls were normal 293T cell genomic DNA. Data in **b**–**f** are representative of three independent experiments. Full-length gels are presented in **Supplementary Figure 9**.

a variety of mammalian cell lines including breast cancer cell line MCF-7, human myeloid cell line K562 and human epithelial cell line HeLa. In all of these cells, NgAgo could efficiently induce targeted double-strand breaks at the *DYRK1A* gene (**Fig. 4c**).

To investigate the effects of nucleotide mismatch between gDNA and target sequence on NgAgo activity, we introduced single mismatch at every position of a 24-nt gDNA for a *DYRK1A* locus (**Fig. 4d**). NgAgo-mediated target cleavage was sensitive to a single-nucleotide mismatch at every position of its guide (cleavage efficacy reduced by 73–100%) with the largest reduction in cleavage efficacy observed at P8 to P11 (by 85~100%). Moreover, mismatches at three consecutive nucleotides at any place completely abolished cleavage. To investigate

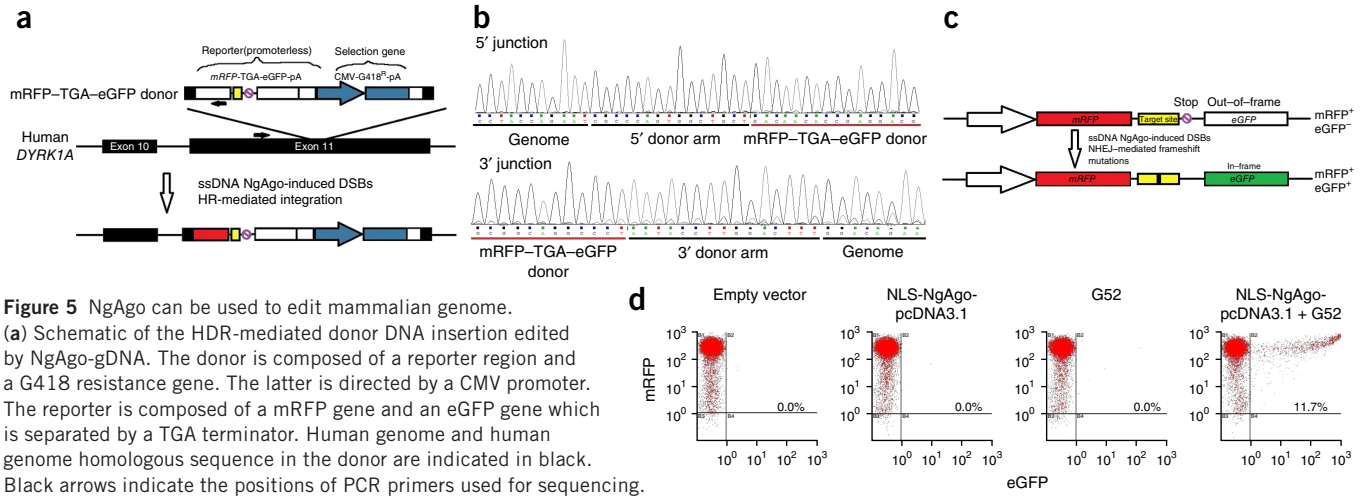

Figure 5 NgAgo can be used to edit mammalian genome. (**a**) Schematic of the HDR-mediated donor DNA insertion edited by NgAgo-gDNA. The donor is composed of a reporter region and a G418 resistance gene. The latter is directed by a CMV promoter. The reporter is composed of a mRFP gene and an eGFP gene which is separated by a TGA terminator. Human genome and human genome homologous sequence in the donor are indicated in black. Black arrows indicate the positions of PCR primers used for sequencing. (**b**) HDR-mediated donor DNA insertion into the targeted genome locus, confirmed by Sanger sequencing of genomic PCR amplicon. (**c**) Schematic of NHEJ-mediated mutation of the terminator edited by NgAgo-gDNA. eGFP expression will be turned on afterward. (**d**) Flow cytometry analysis, showing the efficiency of turning on eGFP expression in the cells. Results in **b** and **d** are representative of three independent experiments.

whether NgAgo is still sensitive to single nucleotide mismatches when guide length is varied, we adopted the same approach to study a shorter guide (21 nt). Single-nucleotide mismatch at any position of the 21-nt guide could still considerably impair NgAgo activity. Although guide length varied, P8–P11 were still the key sensitive positions for NgAgo activity (**Supplementary Fig. 6**). Considering that the Cas9-sgRNA system can tolerate even five mismatches[6,19], these data, although preliminary, support high fidelity of the NgAgo-gDNA system. The phosphodiester bonds on targets corresponding to P8 are possibly where NgAgo initiates endonuclease activity, because nucleotide mismatch at P8 alone could completely block target cleavage.

We compared the efficiencies of NgAgo-gDNA and Cas9-sgRNA systems in cleaving the mammalian genome using *DYRK1A* gene locus, which has been used extensively to study Cas9-sgRNA[20,21]. NgAgo-gDNA and Cas9-sgRNA cleaved this locus with comparable efficiencies (**Fig. 4e**, 31.97% for NgAgo vs. 32.2% for Cas9). Next, we investigated whether NgAgo-gDNA has advantages over Cas9-sgRNA for targeting (G+C)-rich loci, as RNAs but not DNAs are prone to form secondary structures, which may interrupt binding and normal conformation of Cas9-sgRNAs. Indeed, Cas9-sgRNA was substantially less efficient than NgAgo-gDNA in cleaving (G+C)-rich loci of *HBA2* gene and *GATA4* gene (**Fig. 4f**). Thus, the NgAgo-gDNA system has the potential to be applied to broader range of genomic loci than the Cas9-sgRNA system. Moreover, whereas Cas9 requires that target is present immediately upstream of a PAM, Argonaute has no target sequence restrictions based on our observation and others' reports[11,13].

Precise insertion of DNA fragments into genome by NgAgo

Having verified that NgAgo can target and cleave the mammalian genome, we next tested whether the NgAgo-gDNA system can be used to edit genome. Donor capture meditated by homology-directed repair (HDR) is a widely used strategy to generate specific modifications of genome. We designed a donor DNA fragment composed of a reporter region and a G418 resistance gene (**Fig. 5a**). The reporter region consisted of two reading frames: one encoding a mRFP and an out-of-frame sequence encoding eGFP separated by a TGA terminator (**Supplementary Fig. 7**). The reporter region had no promoter so it would not be expressed unless inserted in-frame into a gene. We targeted a locus in the last exon of *DYRK1A* gene. After transfecting 293T cells with the NgAgo-encoding plasmid, guide and the donor fragment, we were detected cells expressing the mRFP. By genomic PCR and subsequent sequencing, we confirmed that the donor was correctly inserted into the desired locus in these cells (**Fig. 5b**). After G418 selection, we isolated the clones containing the donor fragments. Next, we reused the NgAgo-gDNA system to target the TGA terminator in the genome of the positive clones. Imprecise genome repair mediated by nonhomologous end joining (NHEJ) mutated the TGA terminator and made the expression of mRFP-eGFP chimera possible (**Fig. 5c**). By flow cytometry analysis, we detected that 11.7% of the cells were eGFP$^+$, which confirmed the high efficiency of the NgAgo-gDNA system in editing the mammalian genome (**Fig. 5d**). When we introduced the same dose of Cas9 and NgAgo into 293T cells with sgRNA or gDNA, respectively, targeting the same location of the genome, Cas9 but not NgAgo caused off-target donor DNA insertion as manifested by Southern blot (**Supplementary Fig. 8**).

DISCUSSION

Argonautes are widely studied as the major component of the RNA-induced silencing complex (RISC), which mediates RNA interference. Here we report that an Argonaute derived from *N. gregoryi* SP2 is a DNA-guided DNA endonuclease that can cleave genomic DNA in mammalian cells. NgAgo uses DNA–DNA hybridization to target cleavage sites and, similar to Cas9, enables the cleavage of genomic sequence by a single protein. However, NgAgo has potential advantages over Cas9 as a tool for genome editing.

The only requirements for the NgAgo guide ssDNA are 5′ phosphorylation and a length of ~24 nt. In this study, we tested > 50 guides against ten genes and observed no other guide-sequence preference (**Supplementary Table 1**), which also holds true for mammalian and archaea NgAgo counterparts[16,21]. NgAgo sequence is 2/3 the length of Cas9 (887 amino acids vs. 1,368 amino acids). In contrast, Cas9 requires three hairpin structures at the 3′ tracrRNA extension in its sgRNAs and a PAM sequence for DNA binding. The lack of a specific consensus sequence in gDNA for NgAgo does not increase off-target activity because, consistent with other reports[22], we found a very limited number of intracellular 5′-phosphorylated ssDNAs in mammalian cells. However, further high-throughput analysis is needed to evaluate both the guide-sequence and target-sequence preferences of NgAgo. A notable property of NgAgo is that it not only cleaves double-strand DNA but also removes several nucleotides

from the target sequence. This feature appears to have an evolutionary advantage in that it makes the invading genome harder to recover.

The useful features of NgAgo for genome editing include the following. First, it has a low tolerance to guide–target mismatch. A single nucleotide mismatch at each position of the gDNA impaired the cleavage efficiency of NgAgo, and mismatches at three positions completely blocked cleavage in our experiments. Second, 5′ phosphorylated short ssDNAs are rare in mammalian cells, which minimizes the possibility of cellular oligonucleotides misguiding NgAgo. Third, NgAgo follows a 'one-guide-faithful' rule, that is, a guide can only be loaded when NgAgo protein is in the process of expression, and, once loaded, NgAgo cannot swap its gDNA with other free ssDNA at 37 °C. All of these features could minimize off-target effects. Finally, it is easy to design and synthesize ssDNAs and to adjust their concentration, which is difficult with the Cas9-sgRNA system, if the sgRNA is expressed from a plasmid and the normal dosage of an ssDNA guide is only ~1/10 of that of a sgRNA expression plasmid.

Further research is needed to comprehensively assess off-target cleavage rates. Future studies will also likely reveal possible structural modification to enhance the precision and efficiency of NgAgo for genome editing.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Short Read Archive: SRP071718 and SRP071686.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

C.H. conceived the study and designed the experiments. X.Z.S. provided intellectual advice on the project and experimental design. C.H. performed mammalian genome editing. F.G. performed the BLAST search and the *in vitro* cleavage experiments. F.G., F.J. and Y.W. designed and constructed the clones under the supervision of C.H., F.J. and Y.W. performed the *in vivo* cleavage experiments. X.Z.S. and C.H. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Primers and oligos for constructs used in this study. Please refer to **Supplementary Tables 2** and **3**.

293T, HeLa, MCF-7, K-562 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China, http://www.cellbank.org.cn) where the mycoplasma detection and STR detection were performed before the cells were provided to customers.

NgAgo expression, purification and nucleic acid analysis. NgAgo-6P-1plasmid was transformed into *E. coli* strain BL21 DE3 (Clontech) to express GST-NgAgo protein. Sephrose-4B-purified GST-NgAgo was eluted with reduced glutathione buffer. For mammalian-cell NgAgo expression, the FLAG-NgAgo-HA-pcDNA3.1 plasmid was transfected into 293T cells. FLAG-NgAgo-HA protein purification was performed according to the manual of Sigma-Aldrich. Briefly, 48 h after transfection of FLAG-NgAgo-HA-pcDNA3.1 plasmid/guides, 5×10^7 293T cells were lysed with 5 ml RIPA buffer and sonicated on ice. After centrifugation, the supernatant was collected and mixed with 100 µl anti-FLAG M2 resin, followed by gentle rocking overnight at 4 °C. Then, the resin was washed with 1 ml RIPA once and 1 ml reaction buffer without BSA (*in vitro* cleavage assay) 3 times. Eluted FLAG-NgAgo-HA from the resin with 300 µl 0.2 mg/ml 3× FLAG peptide (resolved in reaction buffer without BSA). The eluates were examined by PAGE to evaluate the quality and quantity. If the FLAG-NgAgo-HA protein was above 80% purity, ultrafiltration was done to concentrate the FLAG-NgAgo-HA protein to about 1 mg/ml for subsequent enzymatic analysis; if the purity of the sample was not ideal, 100 µl anti-HA resin was used to repeat the purification steps as mentioned above. The purified protein was digested by proteinase K at 52 °C for 2 h. Nucleic acids were separated from protein by Roti phenol, chloroform, isoamyl alcohol (pH 7.5–8.0, Carl Roth GmbH) and further purified with ethanol precipitation. The air-dried precipitants from 5 ml protein solution were resuspended in 100 µl distilled water and then subjected to DNase I or RNase A digestion. The nucleic acids were then purified by Roti phenol, chloroform, isoamyl alcohol and precipitated by isopropanol. After being resuspended in 50 µl TE buffer (PH 8.0), nucleic acids were analyzed by denatured PAGE and visualized by silver staining.

***In vitro* cleavage assay and target site sequencing.** For reloading guides, 5 µg GST-NgAgo was incubated with 300 ng nucleic acid guides at 55 °C in 50 µl reaction buffer (10 mM Tris PH 8.0, 20 mM NaCl, 0.5 mM MgCl2, 0.4% glycerol, 2 mM DTT and 20 µg/ml BSA) for 1 h. 400 ng pACYCDuet-eGFP plasmid was dissolved in 20 µl reaction buffer and added into the NgAgo solution. After being co-incubated at 37 °C for 8 h, the protein was removed by proteinase K (at 52 °C for 2 h), and the plasmid was analyzed by electrophoresis in 1% agarose gel. For mammalian-cell-expressed FLAG-NgAgo-HA, 5 µg protein was incubated with 400 ng pACYCDuet-eGFP plasmid at 37 °C in reaction buffer for 8 h. Also, the reaction system was then treated with proteinase K and analyzed by electrophoresis in 1% agarose gel.

After electrophoresis, the band of the linearized pACYCDuet-eGFP fragments was excised from the agarose gel and purified by gel extraction. After PCR polyadenylation, the resultant fragments were cloned into T-Vector (Promega). Chloromycetin and ampicillin double-resistant clones were selected for plasmid extraction followed by sequencing (Sangon Biotech).

***In vivo* target plasmid cleavage analysis.** NLS-NgAgo-pcDNA3.1 plasmid and the indicated ssDNA guides were co-transfected into cells. The sequences of the guides are shown in **Supplementary Table 1**. Similarly, SpCas9-pCDNA3.1 plasmid was co-transfected with either sgRNA(CMV) or sgRNA(eGFP) vector. The target plasmid pEGFP-N1 was co-delivered with the NgAgo-gDNA system or the Cas9-sgRNA system. After 36 h of transfection, cells were collected, and lysates were subjected to western blot for measuring eGFP expression. Quantification was performed by gray scale scan (image-pro plus), and the relative scales were calculated based on β-actin expression. Anti-eGFP antibody (sc-9996) (used in 1:1,000) and anti-Actin (sc-47778) antibody (used in 1:1,000) were purchased from Santa Cruz Biotechnology, Inc.

Cell culture and transfection. 293T (ATCC CRL-3216), HeLa (ATCC CCL-2), MCF7 (ATCC HTB-22) cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM), and K562 cells (ATCC CCL-243) were maintained in PRMI-1640 medium. Media were supplemented with 10% FBS (HyClone), 2 mM GlutaMAX (Life Technologies), 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were seeded into 24-well plates (Corning) one day before transfection at a density of 200,000 cells per well. Cells were transfected using Lipofectamine 2000 (Invitrogen). Specifically, 200 ng NgAgo-expressing plasmid and 100 ng of ssDNA guides were used for transfection in each well.

T7E I assay. In-cell genome-editing efficiency was determined by T7E I assay. For each T7E I reaction, 500 ng of PCR product was denatured, reannealed and digested with T7 endonuclease I (New England BioLabs), which cleaves mismatched heteroduplex DNA. The reaction was analyzed by denatured PAGE and visualized by silver staining. Band intensities were determined using Image Lab (Bio-Rad). Editing efficiency was determined by the formula $(1 - (1 - (b + c/a + b + c))1/2) \times 100$, where a is the band intensity of DNA substrate and b and c are the band intensities of cleavage products. Quantification was based on relative band intensities. The detailed guides for NgAgo targeting and primers for amplifying target regions are listed in **Supplementary Tables 1** and **2**.

HDR-mediated mRFP-TGA-eGFP donor integration into genome target. For target integration of mRFP-TGA-eGFP construct, the 293T cells in each well of a 24-well plate were transfected using Lipofectamine with 200 ng NLS-NgAgo-pcDNA3.1 plasmid, 100 ng G10 ssDNA guide and 500 ng mRFP-TGA-eGFP donor. After 3 d of transfection, cells were treated with G418 for positive clone selection. After two weeks of G418 treatment, cell colonies with mRFP expression were isolated for further experiments. For sequencing analysis, genomic DNA was extracted using the Quick Extract DNA kit (Epicentre). The modified DYRK1A genomic loci were PCR-amplified with primer pairs: 5′ junction: DYRK1A-test-F: 5′-GGTCACTGTTGAAACTCATCC-3′ and Rm-test-R: 5′-CTTGTAGATGAAGGTGCCG -3′; 3′ Junction: PolyA-test-F: 5′-CTAACTGAAACACGGAAGGAG-3′ and DYRK1A-test-R: 5′-CTTGTAGCGGTTCAGTGTGT-3′. The PCR products were then cloned into T vector and sequenced. The detailed schematic of mRFP-TGA-eGFP is given in **Supplementary Figure 6**.

NHEJ-mediated target mutation in genome. NLS-NgAgo-pcDNA3.1 plasmid together with G52 ssDNA guide were transfected into the stable mRFP-TGA-eGFP integrated cells. Two days later, cells were harvested and flow cytometry analysis was performed to evaluate the expression of mRFP-eGFP fusion protein.

NHEJ-mediated eGFP400 donor integration into genome. The eGFP400 is a fragment derived from eGFP gene which is 400 bp in length. The eGFP400 donor was PCR-amplified from pEGFP-N1 plasmid using primers: eGFP79 5′-AAGTTCAGCGTGTCCG-3′ and eGFP483 5′-GCCGtTTCTTCTGCTTG-3′. It was co-transfected with either the NgAgo-gDNA system (NLS-NgAgo-pcDNA3.1 plasmid and G10 guide) or the Cas9-sgRNA system (SpCas9-pCDNA3.1 and sgRNA (DYRK1A) vector) into 293T cells to target a *DYRK1A* gene locus.

Southern blot analysis. The probes were generated by PCR using primers eGFP79 and eGFP483. The PCR products were then labeled with ^{32}P-dNTP by using Klenow Fragment (New England BioLabs). After 48 h of transfection, engineered 293T cells were collected and total cellular DNA was isolated using a DNeasy kit (Qiagen) according to the manufacturer's instructions. Approximately 2.5 µg DNA was digested with DNA endonucleases BglII, SalI, Sac I, Xho I, Afl II and Eco47 III (New England BioLabs). The digested fragments were separated by 0.6% agarose gel. The DNA was then transferred to a nylon membrane and UV-cross-linked. After pre-hybridization with salmon sperm DNA (about 100 base pairs), The DNA was incubated with a ^{32}P-labeled probe in hybridization buffer (7% SDS, 0.5 M sodium phosphate buffer, pH 7.4) at 55 °C for 8 h. Membrane was then washed three times in 1 × SSC with 0.1% SDS and exposed to X-ray film.

Protocol of NgAgo/gDNA-mediated genome editing and examination (T7E1 assay) and a representative experiment. Please refer to **Supplementary Note 1** and **Supplementary Figure 10**.

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Addendum

Editorial Expression of Concern: DNA-guided genome editing using the *Natronobacterium gregoryi* Argonaute

Feng Gao, Xiao Z Shen, Feng Jiang, Yongqiang Wu & Chunyu Han

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The editors of *Nature Biotechnology* are issuing an editorial expression of concern regarding this article to alert our readers to concerns regarding the reproducibility of the original results. At this time, we are publishing the results of three groups (http://dx.doi.org/10.1038/nbt.3753) that have tried to reproduce the results in the critical Figure 4 in the original paper by Han and colleagues, which demonstrates editing of endogenous genomic loci in mammalian cells. None of the groups observed any induction of mutations by NgAgo at any of the loci or under any of the conditions tested above the sensitivity of the assays used. Similar results have been recently reported by a different group of authors in *Protein & Cell* (doi:10.1007/s13238-016-0343-9).

We are in contact with the authors, who are investigating potential causes for the lack of reproducibility. The authors have been informed of this statement. While the investigations are ongoing, Chunyu Han and Xiao Z. Shen agree with this editorial expression of concern. Feng Gao, Feng Jiang and Yongqiang Wu do not feel that it is appropriate at this time.

We will update our readers once these investigations are complete.

Retraction: DNA-guided genome editing using the *Natronobacterium gregoryi* Argonaute

Feng Gao, Xiao Z Shen, Feng Jiang, Yongqiang Wu & Chunyu Han

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We are retracting our study because of the continued inability of the research community to replicate the key results in Figure 4, using the protocols provided in our paper. In this figure we report that the *Natronobacterium gregoryi* Argonaute can efficiently create double-strand breaks and edit the genome of human cells using 5′ phosphorylated single-stranded DNA as a guide. Despite the efforts of many laboratories (*Protein Cell* **7**, 913–915, 2016; *Nat. Biotechnol.* **35**, 17–18, 2017; *Cell Res.* **26**, 1349–1352, 2016; *PLOS One* **12**, e0177444, 2017), an independent replication of these results has not been reported. We are therefore retracting our initial report at this time to maintain the integrity of the scientific record. We nevertheless continue to investigate the reasons for this lack of reproducibility with the aim of providing an optimized protocol.