1   **No evidence for genome editing in mouse zygotes and HEK293T human cell line**

2   **using the DNA-guided *Natronobacterium gregoryi* Argonaute (NgAgo).**

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4   **Short title:** No evidence for genome editing using NgAgo

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24   **Abstract:** A recently published research article reported that the extreme halophile

25   archaebacterium *Natronobacterium gregoryi* Argonaute enzyme (NgAgo) could cleave

26   the cellular DNA under physiological temperature conditions in cell line and be

27   implemented as an alternative to CRISPR/Cas9 genome editing technology. We assessed

28   this claim in mouse zygotes for four loci (*Sptb*, *Tet-1*, *Tet-2* and *Tet-3*) and in the human

29   HEK293T cell line for the EMX1 locus. Over 100 zygotes were microinjected with nls-

30   NgAgo-GK plasmid provided from Addgene and various concentrations of 5'-

31   phosphorylated guide DNA (gDNA) from 2.5 ng/µl to 50 ng/µl and cultured to blastocyst

32   stage of development. The presence of indels was verified using T7 endonuclease 1

33   assay (T7E1) and Sanger sequencing. We reported no evidence of successful editing of

34   the mouse genome. We then assessed the lack of editing efficiency in HEK293T cell line

35   for the EMX1 endogenous locus by monitoring the NgAgo protein expression level and

36   the editing efficiency by T7E1 assay and Sanger sequencing. We reported that the NgAgo

37   protein was expressed from 8 hours to a maximum expression at 48 hours post-

38   transfection, confirming the efficient delivery of the plasmid and the gDNA but no

39   evidence of successful editing of EMX1 target in all transfected samples. Together our

40   findings indicate that we failed to edit using NgAgo.

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48   **Introduction**

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50   Type II CRISPR/Cas9 genome editing system offers the ability to efficiently and

51   precisely edit DNA using a combination of the Cas9 endonuclease enzyme and a single

52   guide RNA gRNA [1]. However the requirement of a specific protospacer adjacent motif

53   (PAM) sequence limits the ability of the Cas9 enzyme to edit any nucleotide of a

54   genome. Recently a report described a novel genome editing technology based on the

55   archaebacterium *Natrnobacterium gregoryi* Argonaute (NgAgo) enzyme. Gao et al.

56   described an endonuclease activity for NgAgo, which has the ability to create a site-

57   specific double strand break in the DNA under the guidance of the 24 nucleotide- 5'-

58   phosphorylated single stranded DNA (gDNA) which binds to the endogenous DNA [2].

59   Gao et al. demonstrated the high efficiency of NgAgo to edit the genome under

60   physiological temperature condition without the requirement of a PAM [2]. Interestingly,

61   Gao et al. demonstrated the intolerance of NgAgo to guide-target mismatches leading to

62   negligible off-target effects. With such ability to target any nucleotide in the genome and

63   with an equivalent efficiency to CRISPR/Cas9 genome editing technology, NgAgo has an

64   undeniably important therapeutic potential [3]. We sought to assess the efficiency of

65   NgAgo in two different systems: mouse zygotes and the HEK293T human cell line to

66   determine the suitability of NgAgo as an alternative to the CRISPR/Cas9 genome editing

67   tool. Here we report our attempts to edit the mouse and human genomes using NgAgo.

68   We synthetized the gDNA and co-microinjected in mouse zygotes with the nls-NgAgo-

69   GK plasmid vector provided by Gao et al. We also co-transfected the gDNA with a

70   modified Flag-nls-NgAgo-GK plasmid into HEK293T cells and assessed the DNA

71   editing using T7 endonuclease assay and Sanger sequencing. We monitored the

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72   expression of the protein for the first 48 hours post-transfection in HEK293T cells. We

73   found no evidence for a double strand break and editing of the DNA under various

74   conditions and optimizations. We concluded that we failed to edit the genome using

75   NgAgo.

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77   **Results and discussion**

78   To assess NgAgo efficiency to create a double strand break under the guidance of a

79   single gDNA as described in Gao et al. [2] we used mouse zygotes as a system model.

80   We firstly co-injected mouse zygotes with the nls-NgAgo-GK plasmid and the gDNA

81   targeting four different genes (*Sptb*, *Tet1*, *Tet2* and *Tet3*). The gDNA selected were

82   previously shown to edit efficiently (over 60% efficiency) as an sgRNA with

83   CRISPR/Cas9 mediated genome editing [4]. Initially, we targeted exon 26 of *Sptb* in

84   mouse zygotes (Figure 1A). We titrated the gDNA at various concentrations (2.5, 25 or

85   50 ng/µl) and co-injected with 5, 10 or 15 ng/µl of nls-NgAgo-GK plasmid, provided by

86   Gao et al. and available at Addgene, into the pronucleus of the fertilized zygotes. The

87   zygotes were cultured for 4 days to the blastocyst stage of development. No abnormality

88   in the development was found in these embryos. From the 49 bastocysts that were

89   genotyped (Table 1), we found the amplification of a band corresponding to the expected

90   amplicon length for *Sptb* (Figure 1B presented on NgAgo 5 ng/µl and 50 ng/µl of

91   gDNA). We performed a T7 endonuclease assay (T7E1) and Sanger sequencing to

92   identify indels. We could not identify any indels from the T7E1 assay (Figure 1C) and the

93   Sanger sequencing (Figure 1D) on all blastocysts. We then hypothesized the lack of

94   editing could be gene specific, hence we decided to assess three other genes; exon 5 of

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95   *Tet-1* (Suppl Figure 1A), exon 3 of *Tet-2* (Suppl Figure 1B) and exon 5 of *Tet-3* (Suppl

96   Figure 1C) as described previously [4]. The zygotes were co-injected with 5ng/µl of nls-

97   NgAgo-GK plasmid and 2.5 ng/µl of gDNA. We constantly found one band using gel

98   electrophoresis corresponding to the expected amplicon size for *Tet-1* (10 blastocysts),

99   *Tet-2* (14 blastocysts) and *Tet-3* (13 blastocysts). We performed a T7E1 assay and

100   genotyped the blastocysts by Sanger sequencing. We found again, no evidence for

101   developmental phenotype and presence of indels (data not shown). We further assessed

102   the NgAgo editing efficiency for *Sptb* and *Tet-2* on pups born from the microinjection

103   sessions. All pups displayed a normal phenotype and developed normally to adulthood.

104   We performed a T7E1 assay and Sanger sequencing to assess the editing efficiency and

105   we did not find any indels, suggesting no successful editing for *Sptb* and for *Tet-2* (Table

106   2). Together, this suggests that we failed to generate indels for the four analyzed mouse

107   loci (*Sptb*, *Tet-1*, *Tet-2* and *Tet-3*) in over 86 mouse blastocysts and 25 mouse pups using

108   NgAgo, suggesting NgAgo does not create a double strand break nor that it is capable of

109   editing the mouse genome. We therefore speculated this lack of editing activity could be

110   due to the degradation of the protein within the cell. To address this hypothesis, we

111   tagged the protein with a flag tag upstream of the nls signal (Flag-nls-NgAgo-GK, Figure

112   1E) and monitored the expression of the protein and whether NgAgo edited the DNA in

113   the HEK293T cell line at various time points from 8 to 48 hours post lipofection with or

114   without the gDNA for exon 3 of the EMX1 gene (Figure 1F). We used an anti-flag

115   antibody to probe for NgAgo expression, and utilized GAPDH as a loading control. We

116   first conducted a PCR and a T7E1 assay on the NgAgo-EMX1-lipofectamine treated cells

117   to determine whether the DNA was edited. We found no evidence for editing in any

118   NgAgo-lipofection samples at 8 and 12 hours post-transfection (Figure 1G). Similarly,

119   there was no editing for the additional time points of 24 and 48 hours post transfection

120   (Supplementary Figure 2 A and B). We therefore speculated that the NgAgo protein

121   could be not expressed post transfection or rapidly degraded after transfection and

122   required a specific timing to edit the DNA. To verify this hypothesis, we followed the

123   kinetics of the NgAgo protein production and degradation from 8 to 48 hours post

124   transfection. We noticed the NgAgo protein expression started at 8 hours and persisted

125   over 48 hours post transfection (Figure 1H), with maximum expression being observed at

126   48 hours post transfection compared to 8 hours (p = 0.02) (Figure 1H and Supplementary

127   Figure 3) suggesting an efficient delivery of NgAgo and the gDNA. Interestingly, we

128   noticed the presence of small-Flag-tagged fragments by 8 hours post transfection,

129   suggesting the protein started degrading, with the fragment intensity reaching its peak at

130   48 hours post-transfection associated with the peak protein expression mentioned above

131   with and without the co-transfection of the gDNA (Figure 1H). We noted no difference in

132   protein expression and degradation with or without the gDNA (p = 0.0631 and p = 0.25

133   respectively) (Figure 1H and Supplementary Figure 3). Therefore, since degradation of

134   the protein is the same between NgAgo treated samples with and without gDNA, the

135   difference in protein expression is unlikely to be due to this rapid degradation.

136

137   Gao et al. reported that NgAgo creates a double strand break in the DNA using a single

138   DNA guide [2], with a reported efficiency equivalent to Cas9. Importantly, and in

139   agreement with recently published reports [5-7], we found no evidence of the mouse and

140   human DNA editing with NgAgo despite an efficient delivery of NgAgo and the gDNA.

141   We did not observe an editing event in over 100 mouse embryos injected with NgAgo,

142   giving an editing efficiency of less than 1%, contradicting the results reported in Gao et

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143   al. Interestingly, we found in the mouse embryos, mouse pups and in the HEK293T

144   human cell line no evidence of a single indel using a T7E1 assay, and confirmed by

145   Sanger sequencing. Qi et al reported recently a silencing role of NgAgo that may affects

146   the phenotype of the Zebrafish embryos [6]. We have not noted such a change in

147   phenotype in our mouse embryos. The plausible explanation for this lack of phenotype is

148   the genes targeted were not expressed during early embryonic development [8].

149   Although, we did not isolate mRNA from these mouse blastocysts and we did not assess

150   the expression of the genes.

151   In summary, in contradiction with Gao et al's study, and in agreement with recently

152   published reports, we found that NgAgo does not edit endogenous genomic DNA under

153   physiological temperature conditions.

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155   **Material and Methods:**

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157   **Design and preparation of NgAgo, 5'-phosphorylated guide DNA (gDNA):**

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159   Four genes were targeted to design the primers and the 5'-phosphorylated

160   oligonucleotides. These genes were the exon 26 of *Beta-Spectrin1* (*Sptb*), exon 5 of *Tet-*

161   *1*, exon 3 of *Tet-2* and exon 5 of *Tet-3*. We choose gDNA previously published to be

162   highly efficient using the CRISPR/Cas9 genome editing system. The NgAgo plasmid

163   containing a Nuclear Localisation Signal was obtained from the Addgene repository [2].

164   The plasmid was cultured as per protocol and the DNA extracted using a PureLink Quick

165   Plasmid Miniprep Kit (Invitrogen, K210010) according to the manufacturer's

166   instructions. 22 and 24 bp 5' phosphorylated oligonucleotides and the amplification

167   primers were synthetized from Integrated DNA Technologies. The sequences of these

168   oligonucleotides are listed below (Table 3).

171   **Ethics statement :**

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173   All animal experiments were approved by The Australian National University Animal

174   Experimentation Ethics Committee under the permit A2014/058 and the institutional

175   Biosafety Committee NLRD 15.10 in accordance with the National Health and

176   Medical Research Council (NHMRC) code of practice.

177

178   **Mouse husbandry and microinjection:**

179   C57BL/6 and recipient ICR females were purchased from Charles River laboratory

180   and maintained in a specific pathogen-free environment at the Australian Phenomics

181   Facility, the Australian National University. Mice were maintained on a 12h light/12h

182   dark cycle and had ad libitum access to food and water *ad libitum*. Female C57BL/6

183   (3-4 weeks old, >10g) were superovulated by intraperitoneal injection of 5IU of

184   pregnant mare serum gonadotropin (PMSG), followed by 5IU of human chorionic

185   gonadotropin hormone (hCG) 46-48 hours later. Following injection with hCG,

186   superovulated females were mated with stud C57BL/6 males (10-20 weeks old). The

187   embryos were collected from oviducts approximately 45 hours after the last injection

188   and held in M16 medium (Sigma M7292) overlaid with mineral oil at 37ºC and 5%

189   CO_2 until injection.

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191   Microinjection was performed in M2 medium (Sigma, M7167) under mineral oil using

192   an inverted microscope (Leica DMi8) and micromanipulators. Pronuclear injection of

193   fertilized zygotes was performed with the following mixes: circular plasmid DNA at 5

194   ng/µl and 5'-P oligonucleotide at 2.5, 25 and 50 ng/µl. For Sptb CRISPR injection, the

195   gRNA was cloned into px330 vector [9] obtained from Addgene (ID 42330) using the

196   following oligonucleotides 5'- CACCGTGACATGTGGGCGGACCTGC - 3' and 5' –

197   AAACGCAGGTCCCCACATGTCAC – 3'. 5 ng/µl of px330 circular plasmid was

198   injected into the fertilized zygotes by pronuclear injection to obtained live mice. For

199   blastocysts culture, fertilized zygotes were microinjected using the following mix: 50

200   ng/µl of Cas9 purified protein from PNA BIO (Thoussand Oaks, CA), 0.6 pMol of

201   CrRNA 5' – UGACAUGUGGGCGGACCUGGUUUAGAGCUAUGCUGUUUUG -

202   3' and 0.6 pMol of TracRNA. The CrRNA and TracRNA were complexed with the

203   Cas9 protein by incubating at 37ºC for 10 minutes. Microinjected zygotes were

204   cultured overnight in M16. Resulting two-cell embryos were surgically transferred into

205   the ampulla of pseudo-plugged ICR female recipients (8-12 weeks old) or cultured in

206   M16 media for 4 days at 37ºC.

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208   **Genotyping:**

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210   A subset of the microinjected zygotes was cultured for 4 days to the blastocyst stage in

211   M16 medium overlaid with mineral oil at 37ºC and 5% CO_2. The other zygotes were

212   cultured for 24 hours and surgically transferred into the surrogate mouse ampulla. The

213   mice were maintained and the resulting pups were maintained and genotyped 15 days

214   after birth. DNA was extracted from the blastocysts at day 5 or live mouse pups over

215   15 days old using a crude DNA extraction protocol. In short, the blastocysts were

216 lysed in Tris-EDTA-Tween lysis buffer (50mMTris HCl, pH8.0, 0.125mM EDTA, 2%

217 Tween 20) with 1µl of proteinase K (20 mg/ml in 10mM Tris chlorate, 0.1 mM

218 ethylenediaminetretaacetic acid (EDTA) pH 8.0) and incubated at 56ºC for an hour

219 before being denatured at 95ºC for 10 minutes. We amplified regions encompassing

220 the gDNA with 2x MyTaq HS mix (Bioline, cat no. BIO-25045) under the following

221 PCR conditions: 95ºC for 3 minutes followed by 35 cycles (95ºC for 15", 58ºC for 15"

222 and 72ºC for 20") and 72ºC for 3 minute. The PCR products were checked on a 1.5%

223 electrophoresis gel. The PCR products were purified with ExoSAP-IT® (affymetrix,

224 Cat no. 78202), or cut from the gel and purified using the Wizard® SV Gel and PCR

225 Clean-Up System (Promega, Cat no. A9282) kit according to the manufacturer's

226 instructions. The Sanger sequencing was conducted at the Biomedical Resource

227 Facility at the John Curtin School for Medical Research, The Australian National

228 University.

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230 **T7 endonuclease assay.**

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232 After PCR amplification, the PCR fragments were hybridized and digested with a T7

233 endonuclease (NEB, Cat no. M0302S) for 15-30 minutes at 37ºC. After digestion, the

234 enzymatic reaction was stopped using 1 µl of 0.25 M EDTA and the digested product

235 run on a 1.5% agarose gel alongside the undigested PCR product as a control.

236

237 **Construction of a Flag-nls-NgAgo-GK plasmid:**

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239   The nls-NgAgo-GK plasmid was a gift from Chunyu Han (Addgene plasmid 78253).

240   nls-NgAgo-GK plasmid was digested overnight with AleI (NEB, cat no. R0634S).

241   Following   the   digestion   a   forward   (5'-

242   TGGACTATAAGGACCACGACGGAGACTACAAGGATCATGATATTGATTAC

243   AAAGACGATGACGATAAGA-3')   and   a   reverse   (5'   -

244   TCTTATCGTCATCGTCTTTGTAATCAATATCATGATCCTTGTAGTCTCCGTC

245   GTGGTCCTTATAGTCCA-3') oligonucleotide sequence encoding for a flag tag were

246   annealed and ligated into the nls-NgAgo-GK plasmid upstream to the SV40 Nuclear

247   Localization Signal (nls). Sanger sequencing was used to assess the correct integration

248   of the flag-tag in frame with the start codon. The plasmid was transformed into heat-

249   chock competent BL21 *E. coli*. The plasmid is being deposited at Addgene (Plasmid

250   #73681) and will be available to the community.

251

252   **Cell culture and transfection:**

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254   HEK293T cells were obtained from ATCC (CRL-11268). The cells were maintained

255   in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, Cat. No. D6546)

256   supplemented with 10% heat-inactivated fetal bovine serum (Sigma, Cat. No.

257   12003C), 2mM L-Glutamine/1%Penicilin/Streptomycin solution (Thermofisher, Cat.

258   No. 10378016) and incubated at 37ºC with 5% CO_2. The cells were seeded at $2x10^5$

259   cells per well in 2.5 mL of medium in a 6-well plate, to reach 60-70% confluency

260   immediately prior to transfection. Per sample well, 3.0ug of plasmid DNA (Flag-nls-

261   NgAgo-GK) and/or 0.5µg of 5' phosphorylated EMX1 guide oligo was added to

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262   150uL of basal DMEM (without additives) with 3.5uL Plus Reagent (Invitrogen,

263   Thermofisher, Cat. No. 15338100). 150uL of this diluted DNA mixture was added to

264   150uL of DMEM (without additives) and 12uL of Lipofectamine LTX® Reagent

265   (Invitrogen, Thermofisher, Cat. No. 15338100). The mixture was incubated for 15

266   minutes at room temperature to form DNA-Lipofectamine LTX® Reagent complexes.

267   After incubation, 250uL of the DNA-Lipofectamine complex was added drop-wise to

268   each well, and the plate was gently rocked. Negative controls constituted a 'cell

269   growth' control, with no DNA or lipofectamine reagents, and a 'lipofectamine only'

270   control with no plasmid or gDNA. Transfected cells were incubated at 37ºC in a 5%

271   CO_2 incubator for 8, 12, 24 and 48 hours post-transfection before collecting proteins to

272   assay for transgene expression, and extracting genomic DNA for genotyping assays.

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274   **Genomic DNA extraction and protein extraction from HEK293T cells:**

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276   The genomic DNA was isolated and purified using ISOLATE II Genomic DNA Kit

277   (Bioline, BIO-52066) according to the manufacturer's instructions. To extract proteins

278   from HEK293T cells, the cells were incubated with RIPA buffer (5M NaCl, 0.5M

279   EDTA, pH 8.0, 1M Tris, pH8.0, 1% TritonX-100, 10% sodium deoxycholate, 10%

280   SDS, 1x Protease Phosphatase Inhibitor) with gentle rocking on ice for 30 minutes.

281   The cells were scraped off the plates to dislodge lysate. Lysate was centrifuged at

282   13,000g for 5 minutes at 4ºC. The supernatant of lysate was stored at -20ºC.

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284   **Western blotting:**

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286   The nuclear and cytoplasm lysate samples were denatured at 95°C for 5minutes in 1x

287   Laemmli buffer prior to loading onto a 4-15% SDS-PAGE (4–15% Mini-PROTEAN®

288   TGX^{TM} Precast Protein Gels, Bio-Rad #4561085). 10uL Precision Plus Protein^{TM}

289   Kaleidoscope^{TM} Prestained Protein Standards (Bio-Rad, #1610375) or 10-15uL of

290   samples were loaded on to the gel. The gel was eletrophorised for 35-40mins at

291   200mV, in 1x running buffer (25mM Tris-Base, 190mM glycine, 0.1% SDS, pH 8.3).

292   Proteins were transferred onto a nitrocellulose membrane (Bio-Rad, Cat no. 162-

293   0115) at 400mA, 300V for 1 hour and 30 mins at 4°C, in 1x transfer buffer (25mM

294   Tris-Base, 190mM glycine, 20% methanol, pH 8.3). Primary antibodies (anti-FLAG

295   1:1000, Sigma Cat no. F1804-200UG, or anti-GAPDH1:1000, Millipore Cat no

296   MAB374) were diluted in 1% skim milk in PBS and incubated for 1 hour at RT with

297   shaking. After incubation, membranes were washed with 1xPBS+0.1%Tween for three

298   times 5 mins and once for 10 mins. After washing, membranes were incubated with

299   secondary antibody (1:5000 goat anti- mouse Ig-HRP, Sigma Cat no. A44161ML)

300   diluted in 1% skim milk in PBS, for 1 hour at RT with shaking, and then washed again

301   as above. Membrane was visualised under chemiluminescence with HRP substrate

302   (Millipore, Cat no. WBLUF0500) at various exposure times.

303   **Analysis and Statistics:**

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305   Using ImageJ 1.05i software, Western blot membranes were analyzed for the mean

306   band intensity for anti-FLAG (corresponding to the expression of NgAgo), as well as

307   for the loading control anti-GAPDH. The relative abundance of anti-FLAG was

308   determined as a ratio of the loading control to produce a FLAG:GAPDH ratio. These

309   ratios were then normalized to the negative control, containing no NgAgo. These

310   values were then analysed as listed below.

311   A Two-Way ANOVA using Tukey's multiple comparisons test was performed to

312   quantify changes in protein expression over time for each treatment. Paired T-tests

313   were performed to compare protein expression over time between NgAgo samples

314   with and without gDNA. A two-tailed Wilcoxon test was performed to examine

315   changes in degradation between samples with or without gDNA. All analyses were

316   conducted using the GraphPad software, Prism 7, with significance at $P \leq 0.05$.

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328   **Author contribution:** Designed the experiments: NCK, JL, LJ and GB. Performed the

329   experiments NCK, JL, LJ. Analyzed the data: NCK, JL, LJ and GB. Wrote the

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330   manuscript GB with the input from NCK, JL and LJ. All the authors read and

331   approved the manuscript.

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362   **Figure Legends:**

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364   **Figure 1**: No evidence for double strand break cleavage and editing from NgAgo. A)

365   DNA sequence indicating the locus targeted for the exon 26 of *Sptb*. The gDNA

366   sequence is indicated in red. B) Gel electrophoresis (1.5%) of *Sptb* blastocysts (n=6)

367   co-injected with nls-NgAgo-GK plasmid (2.5 ng/µl) and 2.5 ng/µl of gDNA. C57BL/6

368   DNA (B6) was also amplified as a control. The PCR product is 326 bp. C) T7

369   endonuclease 1 (T7E1) assay on the *Sptb* blastocysts indicating the absence of

370   heteroduplexes suggesting indels in 6 blastocysts co-injected with nls-NgAgo-GK and

371   gDNA. C57BL/6 (B6) non-edited control DNA was utilized as a negative control. A

372   positive control in mouse zygotes edited with CRISPR/Cas9 was used as a positive

373   control. The arrows indicate the presence of heteroduplexes suggesting a successful

374   editing of the DNA. D) Representative chromatogram of one *Sptb* blastocyst (#5)

375   suggesting no editing of the DNA under the DNA-guided NgAgo. E) Schematic

376   diagram representing the flag-nls-NgAgo-GK plasmid. The expression of NgAgo is

377   driven from a CMV promoter. A Flag tag was inserted in the 5' end of NgAgo

378   sequence. Two Sv40 nuclear localization signals were inserted in the 3' end of the

379   NgAgo sequence and in the 3' end of the flag tag. A Ploy A tail was appended to the

380   sequence in the 3' end a Neomycin cassette was added in the 3' end of the plasmid

381   sequence. F) DNA sequence indicating the targeting of the exon 3 from EMX1 human

382   sequence. The gDNA sequence is indicated in red. G) Gel electrophoresis (2%) of the

383   PCR for EMX1 in HEK293T cells at 8 and 12 hours post lipofection. The control

384   samples were: The DNA without transfection, the lipofection reagent (LTX) and

385   EMX1 DNA. The HEK293T cells were transfected with NgAgo alone, EMX1 gDNA

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386   alone or co-transfected with NgAgo and EMX1 gDNA. A control DNA was

387   successfully edited with CRISPR/Cas9 (+ Cas9 control) and was utilized as a negative

388   control (- Cas9 control). The top electrophoresis gel respresents the PCR only

389   whereas the bottom gel represents the T7E1 assay. The arrows indicate the formation

390   of heteroduplexes for CRISPR/Cas9 genome editing. H) Western blot of NgAgo

391   protein production with and without the co-transfection of the gDNA from 8 to 48

392   hours post lipofection. The staining was performed with a monoclonal Flag anti-

393   antibody and anti-GAPDH anti-antibody. The top band represents NgAgo at 103KDa.

394   GAPDH was utilized as a Housekeeper gene. The exposure time was 30 seconds. The

395   controls were the lipefection agent alone (LTX), lane 1 and the gDNA EMX1 alone

396   (lane 2). The smears under the NgAgo band show the degradation of the protein

397   stained with the Flag tag.

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409 **Table**

Gene	Microinjection mix	Zygotes injected	Blastocysts cultured	Targeted blastocysts (%)
Sptb	NgAgo 5 ng/µl + 2.5 ng/µl gDNA	8	8	0 (0%)
Sptb	NgAgo 5 ng/µl + 25 ng/µl gDNA	8	8	0 (0%)
Sptb	NgAgo 5 ng/µl + 50 ng/µl gDNA	6	6	0 (0%)
Sptb	NgAgo 10 ng/µl + 50 ng/µl gDNA	12	12	0 (0%)
Sptb	NgAgo 15 ng/µl + 50 ng/µl gDNA	15	15	0 (0%)
Tet-1	NgAgo 5 ng/µl + 25 ng/µl gDNA	10	10	0 (0%)
Tet-2	NgAgo 5 ng/µl + 2.5 ng/µl gDNA	7	7	0 (0%)
Tet-2	NgAgo 5 ng/µl + 25 ng/µl gDNA	7	7	0 (0%)
Tet-3	NgAgo 5 ng/µl + 2.5 ng/µl gDNA	13	13	0 (0%)
Sptb	50 ng/µl Cas9 + 0.6 pMol CrRNA & TracRNA	6	6	3(50%)

410

411 **Table 1**: Generation of edited mouse blastocyst using NgAgo and CRISPR/Cas9

412 genome editing technologies. NgAgo circular DNA was co-injected into the mouse

413 zygotes with various concentrations of gDNA. Cas9 Ribonucleoprotein (RNP) was

414 injected as controls for *Sptb* targeting the same genomic sequence as NgAgo.

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Gene	Microinjection mix	Zygotes injected	Zygotes Trasferred	Newborn pups	Targeted pups (%)
Sptb	NgAgo 5 ng/µl + 50 ng/µl gDNA	68	43	9	0 (0%)
Sptb	NgAgo 5 ng/µl + 25 ng/µl gDNA	27	22	12	0 (0%)
Sptb	5 ng/µl Plasmid DNA px330	49	41	10	6(60%)
Tet2	NgAgo 5 ng/µl + 25 ng/µl gDNA	66	50	4	0 (0%)

417

418 **Table 2**: Generation of knockout mice using NgAgo and CRISPR/Cas9 genome

419 editing technologies. Various concentrations of gDNA were co-injected with NgAgo

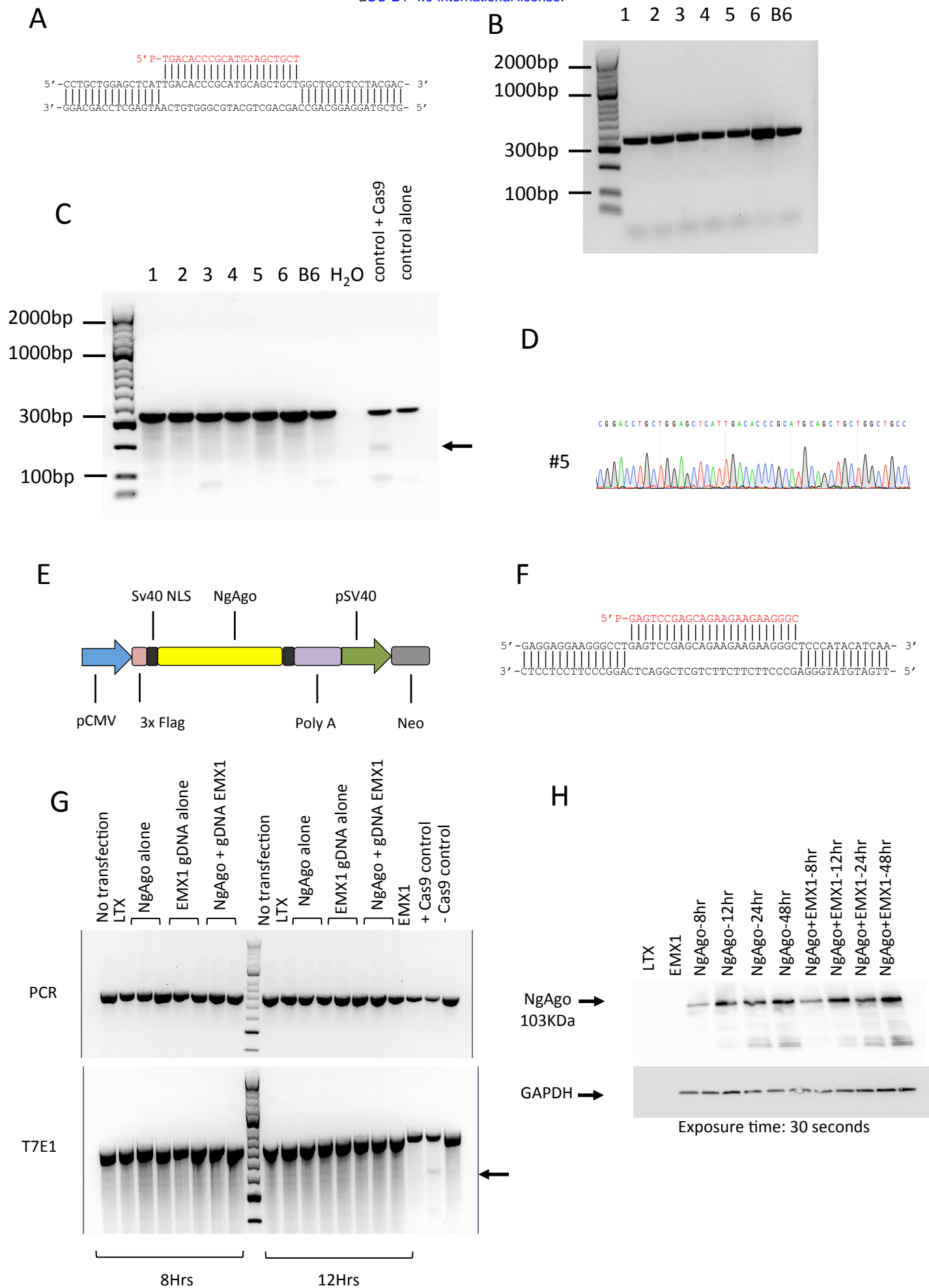420 plasmid or px330-U6-Chimeric_BB-CBh-hSpCas9 circular DNA as control.

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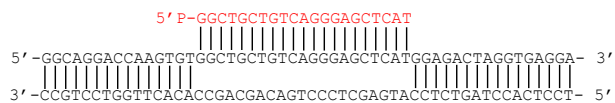| Primer Name	Sequence
Sptb-F	5'-GCTACGTGACAAGTTCCGAGA-3'
Sptb-R	5'- GGTGGAAGGACTCAGCAGTG -3'
5'P-Sptb	5'-P-TGACACCCGCATGCAGCTGCT-3'
Tet1-F	5'-GTGTCAGGTTCAAGGCCATC-3'
Tet1-R	5'-ACGGGCGAGTTAGGGTTAAA-3'
5'P-Tet1	5'-P-GGCTGCTGTCAGGGAGCTCAT-3'
Tet2-F	5'-TGTCCAGCAGGATAAAGCAA-3'
Tet2-R	5'-ACCTGGATTGCATCCTTCAC-3'
5'P-Tet2	5'-P-GAAAGTGCCAACAGATATCCA-3'
Tet3-F	5'-GTGGAACAGGAGCAGAGGAG-3'
Tet3-R	5'-CCCGTGATGGTGAATGTCTA-3'
5'P-Tet3	5'-P-CAAGGAGGGGAAGAGTTCTCG-3'
EMX1-F	5'-GGGGCCCTAACCCTATGTA-3'
EMX1-R	5'-AGGGAGATTGGAGACACGGA-3'
5'P-EMX1	5'P-GAGTCCGAGCAGAAGAAGAAGGGC-3'

423

424

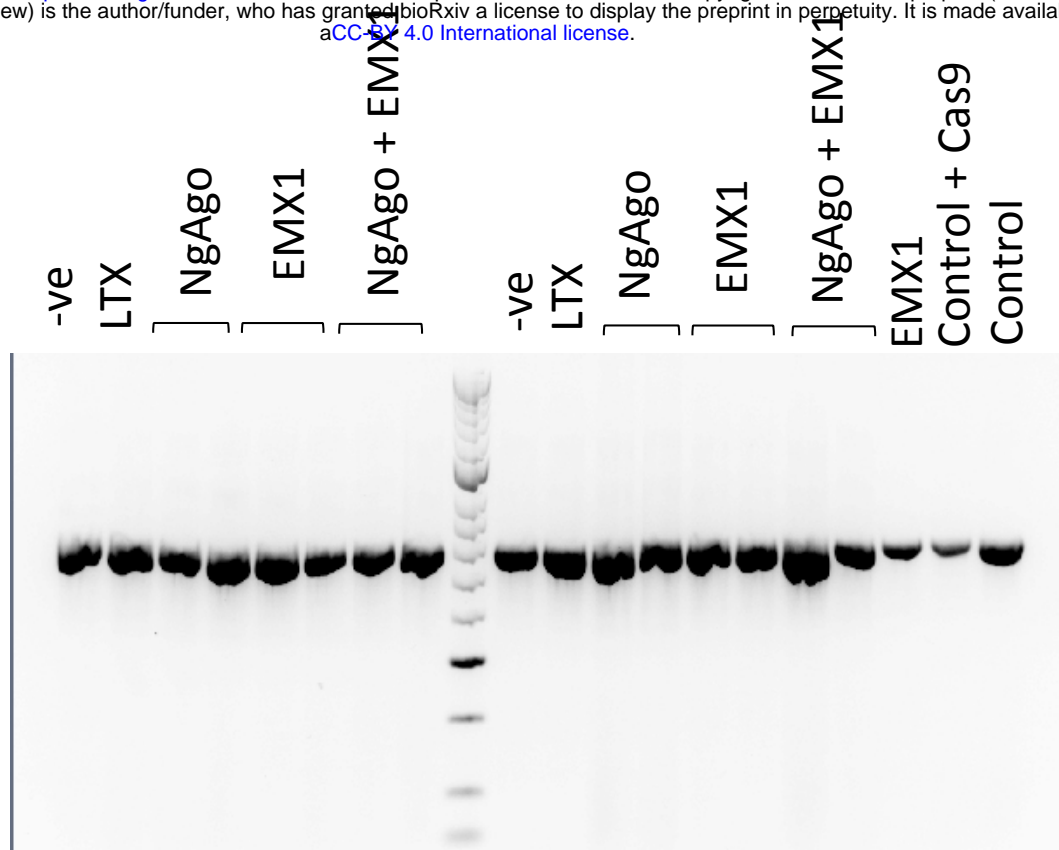425   **Table 3**: List of oligonucleotides used in this study.

426

A

```
               5' P-GGCTGCTGTCAGGGAGCTCAT
               |||||||||||||||||||||
5'-GGCAGGACCAAGTGTGGCTGCTGTCAGGGAGCTCATGGAGACTAGGTGAGGA- 3'
  |||||||||||||||                      ||||||||||||||||
3'-CCGTCCTGGTTCACACCGACGACAGTCCCTCGAGTACCTCTGATCCACTCCT- 5'
```

B

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                    5' P-GAAAGTGCCAACAGATATCCA
                    |||||||||||||||||||||
5'-GTCACAGAAACACGTGAAAGTGCCAACAGATATCCAGGCTGCAGAATCGGAG- 3'
  |||||||||||||                      ||||||||||||||
3'-CAGTGTCTTTGTGCACTTTCACGGTTGTCTATAGGTCCGACGTCTTAGCCTC- 5'
```

C

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              5' P-CAAGGAGGGGAAGATTCTGGA
              |||||||||||||||||||||
5'-GGTCATCTACACGGGCAAGGAGGGGAAGATTCTGGAGGCTGTCCCATCGCCA- 3'
  ||||||||||||||                   ||||||||||||||
3'-CCAGTAGATGTGCCCGTTCCTCCCCTTCTAAGACCTCCGACAGGGTAGCGGT- 5'
```

Khin et al. Supplementary Figure 1

A

24Hrs   48Hrs

B

24Hrs   48Hrs

Khin et al. Supplementary Figure 2

Khin et al. Supplementary Figure 3