No evidence for genome editing in mouse zygotes and HEK293T human cell line using the DNA-guided *Natronobacterium gregoryi* Argonaute (NgAgo).

Short title: No evidence for genome editing using NgAgo

Authors: Nay Chi Khin, Jenna L. Lowe, Lora M. Jensen and Gaetan Burgio

Department of Immunology and Infectious diseases, the John Curtin School of Medical Research, The Australian National University, Canberra, Australia.

Corresponding author:

Gaetan Burgio:

The John Curtin School of Medical Research

The Australian National University

131 Garran Road, Acton, ACT 2601, Australia

Ph: +61 2 6125 9428

email: Gaetan.burgio@anu.edu.au

Twitter handle @GaetanBurgio

Abstract: A recently published research article reported that the extreme halophile archaebacterium *Natronobacterium gregoryi* Argonaute enzyme (NgAgo) could cleave the cellular DNA under physiological temperature conditions in cell line and be implemented as an alternative to CRISPR/Cas9 genome editing technology. We assessed this claim in mouse zygotes for four loci (*Sptb*, *Tet-1*, *Tet-2* and *Tet-3*) and in the human HEK293T cell line for the EMX1 locus. Over 100 zygotes were microinjected with nls-NgAgo-GK plasmid provided from Addgene and various concentrations of 5'-phosphorylated guide DNA (gDNA) from 2.5 ng/µl to 50 ng/µl and cultured to blastocyst stage of development. The presence of indels was verified using T7 endonuclease 1 assay (T7E1) and Sanger sequencing. We reported no evidence of successful editing of the mouse genome. We then assessed the lack of editing efficiency in HEK293T cell line for the EMX1 endogenous locus by monitoring the NgAgo protein expression level and the editing efficiency by T7E1 assay and Sanger sequencing. We reported that the NgAgo protein was expressed from 8 hours to a maximum expression at 48 hours post-transfection, confirming the efficient delivery of the plasmid and the gDNA but no evidence of successful editing of EMX1 target in all transfected samples. Together our findings indicate that we failed to edit using NgAgo.

Introduction

Type II CRISPR/Cas9 genome editing system offers the ability to efficiently and precisely edit DNA using a combination of the Cas9 endonuclease enzyme and a single guide RNA gRNA [1]. However the requirement of a specific protospacer adjacent motif (PAM) sequence limits the ability of the Cas9 enzyme to edit any nucleotide of a genome. Recently a report described a novel genome editing technology based on the archaebacterium *Natrnobacterium gregoryi* Argonaute (NgAgo) enzyme. Gao et al. described an endonuclease activity for NgAgo, which has the ability to create a site-specific double strand break in the DNA under the guidance of the 24 nucleotide- 5'-phosphorylated single stranded DNA (gDNA) which binds to the endogenous DNA [2]. Gao et al. demonstrated the high efficiency of NgAgo to edit the genome under physiological temperature condition without the requirement of a PAM [2]. Interestingly, Gao et al. demonstrated the intolerance of NgAgo to guide-target mismatches leading to negligible off-target effects. With such ability to target any nucleotide in the genome and with an equivalent efficiency to CRISPR/Cas9 genome editing technology, NgAgo has an undeniably important therapeutic potential [3]. We sought to assess the efficiency of NgAgo in two different systems: mouse zygotes and the HEK293T human cell line to determine the suitability of NgAgo as an alternative to the CRISPR/Cas9 genome editing tool. Here we report our attempts to edit the mouse and human genomes using NgAgo. We synthetized the gDNA and co-microinjected in mouse zygotes with the nls-NgAgo-GK plasmid vector provided by Gao et al. We also co-transfected the gDNA with a modified Flag-nls-NgAgo-GK plasmid into HEK293T cells and assessed the DNA editing using T7 endonuclease assay and Sanger sequencing. We monitored the

expression of the protein for the first 48 hours post-transfection in HEK293T cells. We found no evidence for a double strand break and editing of the DNA under various conditions and optimizations. We concluded that we failed to edit the genome using NgAgo.

Results and discussion

To assess NgAgo efficiency to create a double strand break under the guidance of a single gDNA as described in Gao et al. [2] we used mouse zygotes as a system model. We firstly co-injected mouse zygotes with the nls-NgAgo-GK plasmid and the gDNA targeting four different genes (*Sptb*, *Tet1*, *Tet2* and *Tet3*). The gDNA selected were previously shown to edit efficiently (over 60% efficiency) as an sgRNA with CRISPR/Cas9 mediated genome editing [4]. Initially, we targeted exon 26 of *Sptb* in mouse zygotes (Figure 1A). We titrated the gDNA at various concentrations (2.5, 25 or 50 ng/µl) and co-injected with 5, 10 or 15 ng/µl of nls-NgAgo-GK plasmid, provided by Gao et al. and available at Addgene, into the pronucleus of the fertilized zygotes. The zygotes were cultured for 4 days to the blastocyst stage of development. No abnormality in the development was found in these embryos. From the 49 bastocysts that were genotyped (Table 1), we found the amplification of a band corresponding to the expected amplicon length for *Sptb* (Figure 1B presented on NgAgo 5 ng/µl and 50 ng/µl of gDNA). We performed a T7 endonuclease assay (T7E1) and Sanger sequencing to identify indels. We could not identify any indels from the T7E1 assay (Figure 1C) and the Sanger sequencing (Figure 1D) on all blastocysts. We then hypothesized the lack of editing could be gene specific, hence we decided to assess three other genes; exon 5 of

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

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.
154

155 ## Material and Methods:

156

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

158

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 :**

172

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.

190

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.
207
208 **Genotyping:**
209
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.
229
230 **T7 endonuclease assay.**
231
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:**
238

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:**
253
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

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.
273
274 **Genomic DNA extraction and protein extraction from HEK293T cells:**
275
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.
283
284 **Western blotting:**

285

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™ Precast Protein Gels, Bio-Rad #4561085). 10uL Precision Plus Protein™
289 Kaleidoscope™ 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:**

304

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 $P \leq 0.05$.
317

318 **Acknowledgments:** This work was supported from the National Collaborative
319 Research Infrastructure (NCRIS) through the Australian Phenomics Network in
320 Australia. The funders have no role in study design, data collection and analysis,
321 decision to publish or preparation of the manuscript. We would like to thank all the
322 comments, anonymous or not on the initial blogpost, on Twitter and/or on a Google
323 discussion group dedicated to genome editing technology that have led to stimulating,
324 interesting and fruitful discussions on this technique. Finally we would like to thanks 3
325 anonymous reviewers for their helpful comments that has improved the quality of the
326 manuscript.
327

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

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

References:

1. Komor AC, Badran AH, Liu DR (2016) CRISPR-Based Technologies for the Manipulation of Eukaryotic Genomes. Cell.
2. Gao F, Shen XZ, Jiang F, Wu Y, Han C (2016) DNA-guided genome editing using the Natronobacterium gregoryi Argonaute. Nat Biotechnol 34: 768-773.
3. Martinez-Galvez G, Ata H, Campbell JM, Ekker SC (2016) ssDNA and the Argonautes: The Quest for the Next Golden Editor. Hum Gene Ther 27: 419-422.
4. Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, et al. (2013) One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell 153: 910-918.
5. Burgess S, Cheng L, Gu F, Huang J, Huang Z, et al. (2016) Questions about NgAgo. Protein Cell.
6. Qi J, Dong Z, Shi Y, Wang X, Qin Y, et al. (2016) NgAgo-based fabp11a gene knockdown causes eye developmental defects in zebrafish. Cell Res.
7. Lee SH, Turchiano G, Ata A, Nowsheen S, Romito M, et al. (2016) Failure to detect DNA-guided genome editing using Natronobacterium gregoryi Argonaute. Nat Biotechnol.
8. Richardson L, Stevenson P, Venkataraman S, Yang Y, Burton N, et al. (2014) EMAGE: Electronic Mouse Atlas of Gene Expression. Methods Mol Biol 1092: 61-79.
9. Cong L, Ran FA, Cox D, Lin S, Barretto R, et al. (2013) Multiplex genome engineering using CRISPR/Cas systems. Science 339: 819-823.

Figure Legends:

363

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

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.
398
399
400
401
402
403
404
405
406
407
408

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.

415

416

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.

421

422

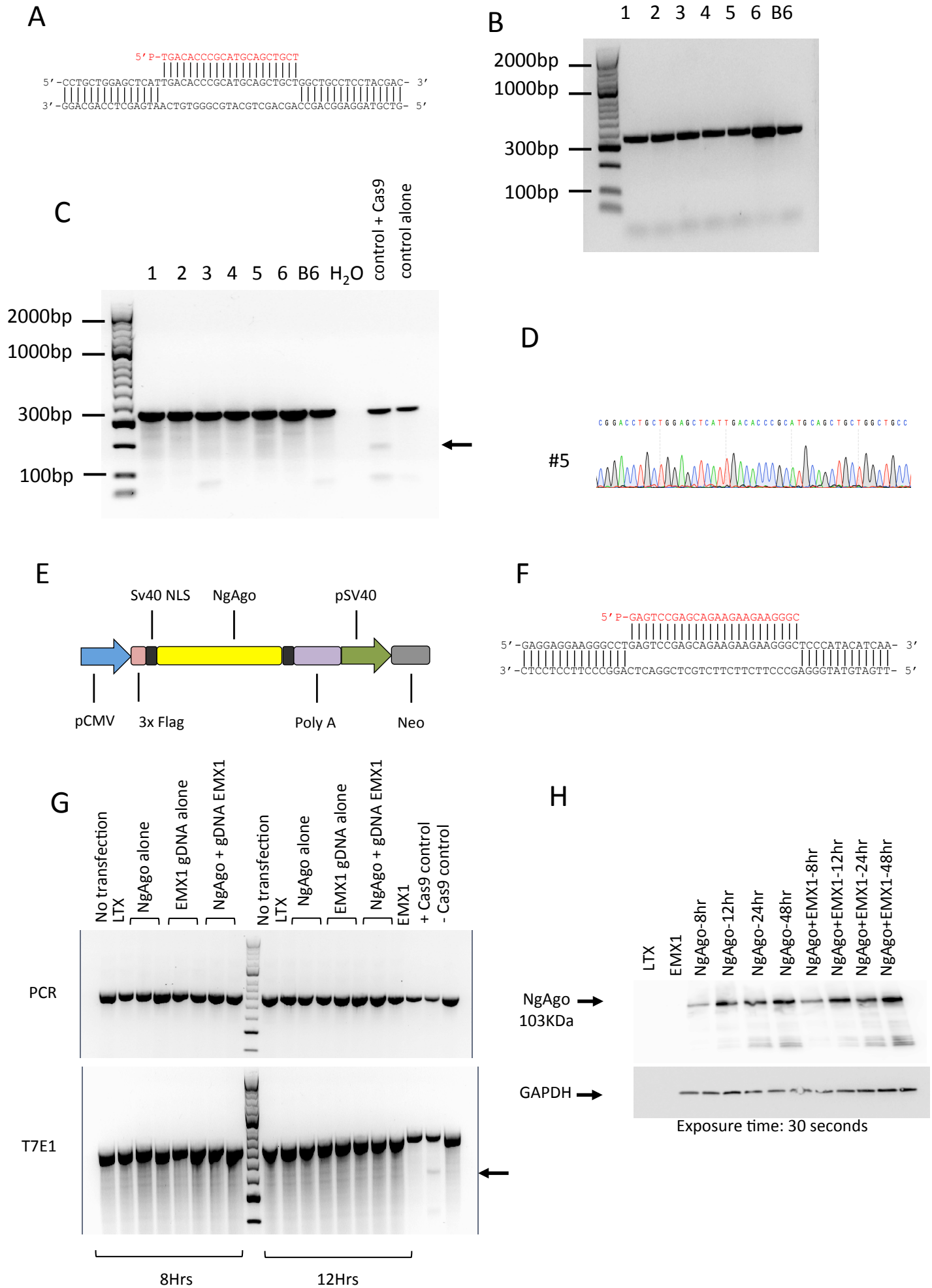
Primer Name	Sequence
Sptb-F	5’-GCTACGTGACAAGTTCCGAGA-3’
Sptb-R	5’- GGTGGAAGGACTCAGCAGTG -3’
5’P-Sptb	5’-P-TGACACCCGCATGCAGCTGCT-3’
Tet1-F	5’-GTGTCAGGTTCAAGGCCATC-3’
Tet1-R	5’-ACGGGCGAGTTAGGGTTAAA-3’
5’P-Tet1	5’-P-GGCTGCTGTCAGGGAGCTCAT-3’
Tet2-F	5’-TGTCCAGCAGGATAAAGCAA-3’
Tet2-R	5’-ACCTGGATTGCATCCTTCAC-3’
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-TGACACCCGCATGCAGCTGCT
5'-CCTGCTGGAGCTCATTGACACCCGCATGCAGCTGCTGGCTGCCTCCTACGAC- 3'
3'-GGACGACCTCGAGTAACTGTGGGCGTACGTCGACGACCGACGGAGGATGCTG- 5'
B
1 2 3 4 5 6 B6
2000bp
1000bp
300bp
100bp
C
1 2 3 4 5 6 B6 H2O
control + Cas9
control alone
2000bp
1000bp
300bp
100bp
D
#5
E
Sv40 NLS
NgAgo
pSV40
pCMV
3x Flag
Poly A
Neo
F
5'P-GAGTCCGAGCAGAAGAAGAAGGGC
5'-GAGGAGGAAGGGCCTGAGTCCGAGCAGAAGAAGAAGGGCTCCCATACATCAA- 3'
3'-CTCCTCCTTCCCGGACTCAGGCTCGTCTTCTTCTTCCCGAGGGTATGTAGTT- 5'
G
No transfection
LTX
NgAgo alone
EMX1 gDNA alone
NgAgo + gDNA EMX1
No transfection
LTX
NgAgo alone
EMX1 gDNA alone
NgAgo + gDNA EMX1
EMX1
+ Cas9 control
- Cas9 control
PCR
T7E1
8Hrs
12Hrs
H
LTX
EMX1
NgAgo-8hr
NgAgo-12hr
NgAgo-24hr
NgAgo-48hr
NgAgo+EMX1-8hr
NgAgo+EMX1-12hr
NgAgo+EMX1-24hr
NgAgo+EMX1-48hr
NgAgo
103KDa
GAPDH
Exposure time: 30 seconds

A

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

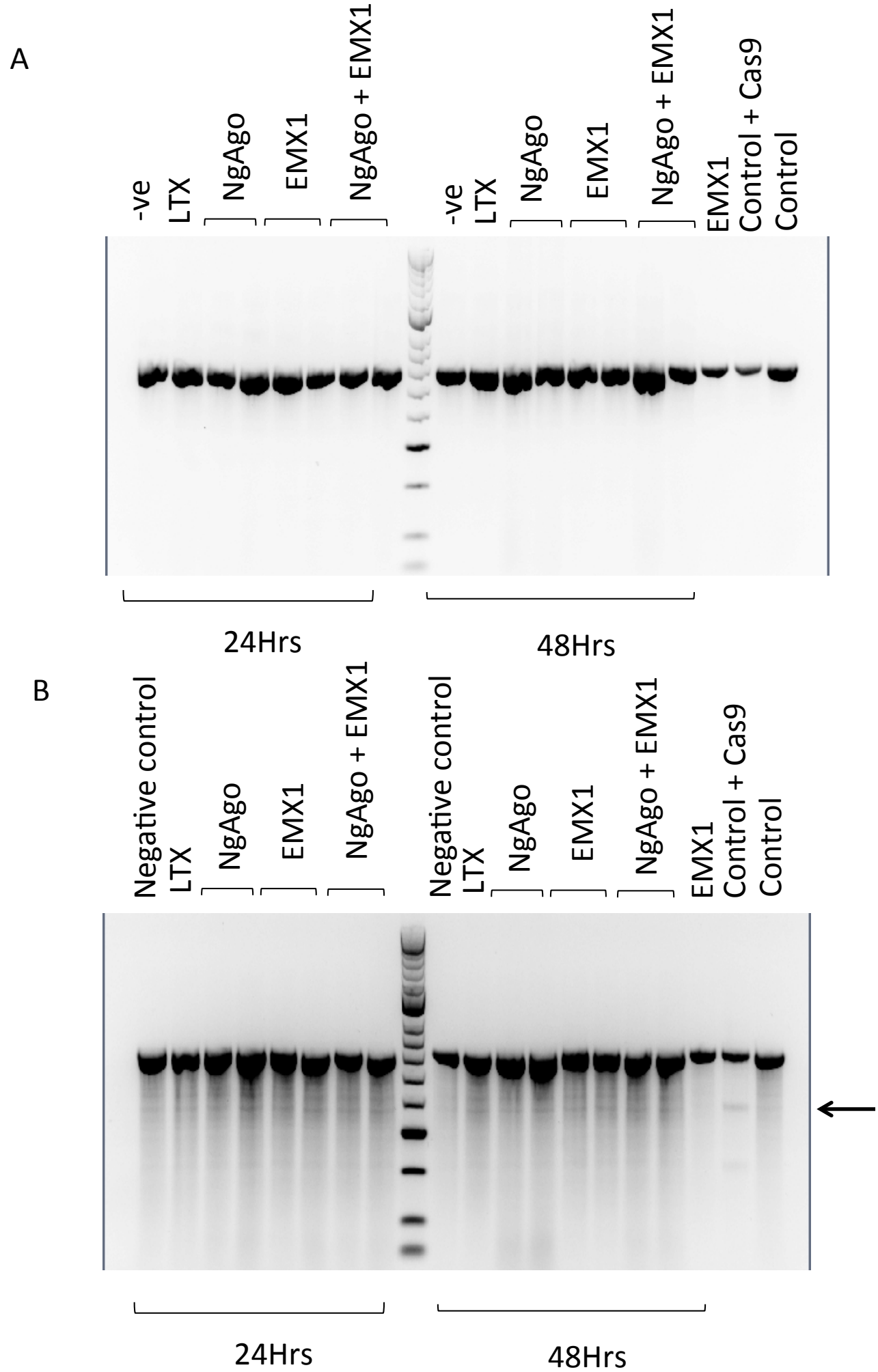
B

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

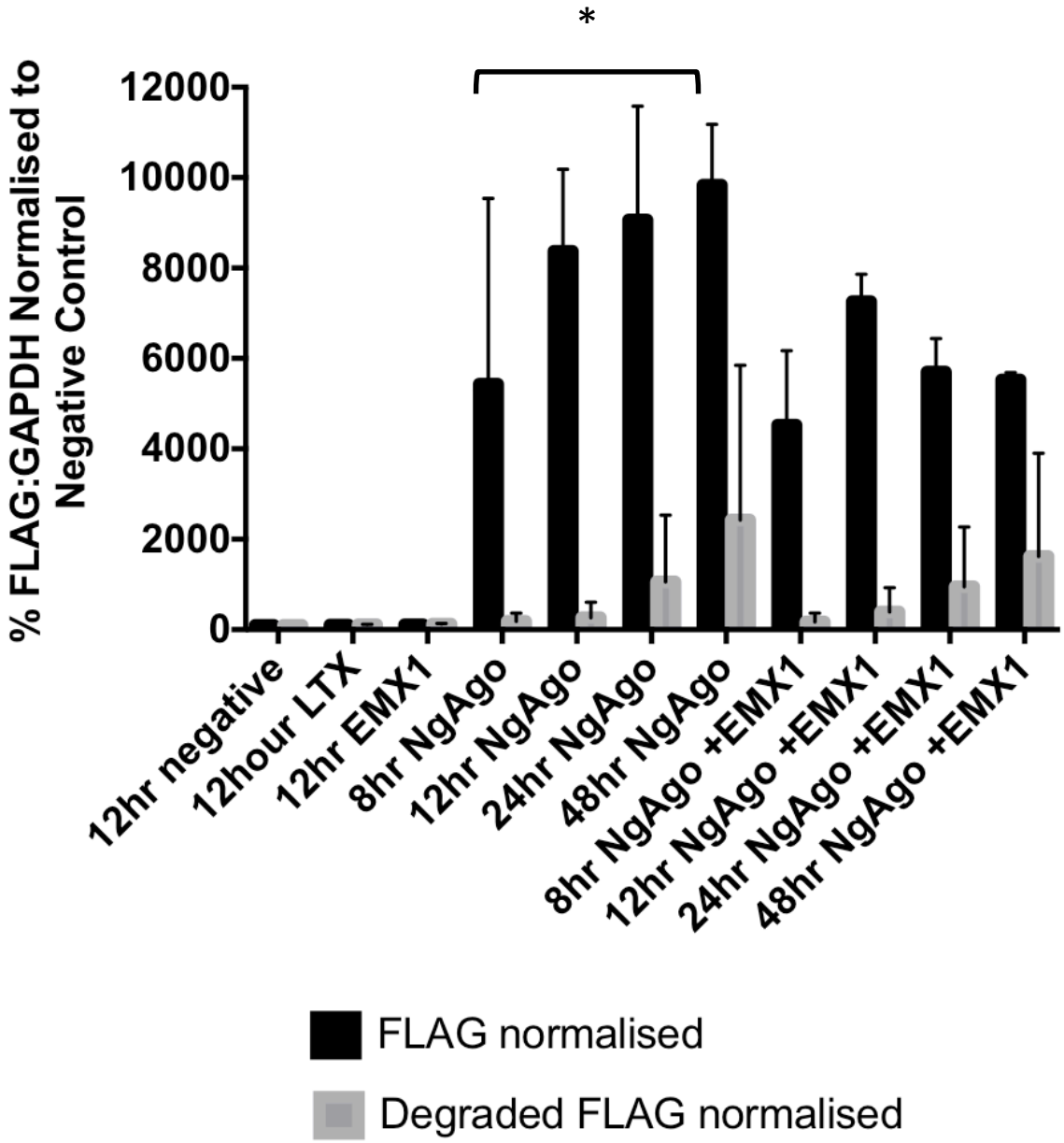
C

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

Khin et al. Supplementary Figure 1



Khin et al. Supplementary Figure 2



Khin et al. Supplementary Figure 3