Adenine Base Editing *in vivo* with a Single Adeno-Associated Virus Vector

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Abstract

Base editors (BEs) have opened new avenues for the treatment of genetic diseases. However, advances in delivery approaches are needed to enable disease targeting of a broad range of tissues and cell types. Adeno-associated virus (AAV) vectors remain one of the most promising delivery vehicles for gene therapies. Currently, most BE/guide combinations and their promoters exceed the packaging limit (~5 kb) of AAVs. Dual-AAV delivery strategies often require high viral doses that impose safety concerns. In this study, we engineered an adenine base editor using a compact Cas9 from *Neisseria meningitidis* (Nme2Cas9). Compared to the well-characterized *Streptococcus pyogenes* Cas9-containing ABEs, Nme2-ABE possesses a distinct PAM (N_4CC) and editing window, exhibits fewer off-target effects, and can efficiently install therapeutically relevant mutations in both human and mouse genomes. Importantly, we showed that *in vivo* delivery of Nme2-ABE and its guide RNA by a single-AAV vector can revert the disease mutation and phenotype in an adult mouse model of tyrosinemia. We anticipate that Nme2-ABE, by virtue of its compact size and broad targeting range, will enable a range of therapeutic applications with improved safety and efficacy due in part to packaging in a single-vector system.

Key words: AAV, base editing, genome editing, CRISPR, sgRNA, deaminase

Introduction

Point mutations represent the largest class of known human pathogenic genetic variants [1,2]. Base editors (BEs), which comprise a single-guide RNA (sgRNA) loaded onto a Cas9 (nuclease-inactivated or nickase form) fused to a deaminase enzyme, enable precise installation of A•T to G•C (in the case of adenine base editors (ABEs) [3]) or C•G to T•A (in the case of cytidine base editors (CBEs) [4]) substitutions. In contrast to traditional nuclease-dependent genome editing approaches, base editors do not generate double-stranded DNA breaks (DSBs), do not require a DNA donor template, and are more efficient in editing non-dividing cells, making them attractive agents for *in vivo* therapeutic genome editing.

While robust editing has been achieved in many cultured mammalian cell systems, safe and effective *in vivo* delivery of base editors remains a major challenge. To date, both non-viral and viral delivery methods have shown great promise for delivering base editors for *in vivo* therapeutic purposes in rodents and primates [5,6]. For example, *in vivo* delivery via AAVs has achieved efficient editing in a wide range of tissue and cell types including liver [5–10], heart [11], muscle [12,13], retina [14,15], inner ear [16], and central nervous system (CNS) [17,18]. However, the large coding size (5.2 kb) of the best-characterized *Streptococcus pyogenes* Cas9 (SpyCas9)-containing BEs exceed the packaging limit of AAV (5 kb) [19,20]. Currently, *in vivo* delivery of base editors by AAV has been approached by splitting the SpyCas9 base editor between two AAVs and relying on the use of intein trans-splicing for the assembly of the full-length effector [13,16–18,21,22]. Although effective, this approach requires transduction of the target cell by both AAVs and successful *in trans* splicing of the two intein halves. The requirement to deliver two AAV vectors also increases the viral dosage needed for a treatment, which raises safety concerns and adds burdens to AAV manufacturing [23–25].

Compact Cas9 orthologs are ideal candidates for engineering base editors suitable for single-AAV delivery [26–28]. For example, single-AAV delivery of a domain-inlaid *Staphylococcus aureus* Cas9 (SauCas9) ABE has been reported in cultured HEK293 cells [29]. Previously, we developed a *Neisseria meningitidis* Cas9 (Nme2Cas9) as an *in vivo* genome editing platform [27,30]. Nme2Cas9 is a compact, naturally accurate genome editor with a distinct N_4CC PAM specificity. Recently, two other groups have successfully implemented Nme2-CBEs in cultured mammalian cells and in rabbits [31], as well as Nme2-ABEs in rice [32]. Here, we develop ABEs using Nme2Cas9 (Nme2-ABEs) and define their editing efficiencies, editing windows, and off-target activities in comparison to those of the widely applied Spy-ABEs in cultured mammalian cells. Next, we show that Nme2-ABE can edit multiple therapeutically significant loci, including one of the most common mutations occurring in Rett syndrome patients that cannot be targeted by other compact ABEs (e.g. ABEs derived from SauCas9 and SauCas9-KKH [33–36]) due to PAM restrictions. Lastly, by optimizing the promoter and the nuclear localization signals, we show that Nme2-ABE and its guide can be packaged into a single-AAV vector genome for *in vivo* delivery. One systematic administration of the single-AAV vector encoding both Nme2-ABE and sgRNA readily corrects the disease-causing mutation and phenotype in an adult mouse model of hereditary tyrosinemia type 1 (HT1).

Results

Development of Nme2-ABE and comparison of editing windows and off-target effects to those of Spy-ABE

First, to evaluate base editing efficiency in a streamlined manner, we developed an ABE reporter cell line in which a G-to-A mutation in the mCherry coding sequence generates a nonsense mutation. Adenine base editing can reverse the mutation and recover red fluorescence, and the editing efficiency can be readily measured by fluorescence-activated cell sorting (FACS) (**Figure 1a, Supplementary Figure 1**). Initially, we constructed Nme2-ABE7.10 by linking a TadA-TadA7.10 dimer from the Spy-ABE7.10 to the N-terminus of the Nme2Cas9 HNH nickase [3]. However, by plasmid transient transfection, Nme2-ABE7.10 showed poor activity in the ABE reporter cell line. Because the evolved TadA8e is highly active and compatible with a wide range of Cas9s [35], we next engineered Nme2-ABE8e by linking TadA8e monomer to the N-terminus of the Nme2Cas9 HNH nickase (**Figure 1b**). We found that Nme2-ABE8e supports robust editing activity in the ABE reporter cell line (**Figure 1c**). Next, to define the editing window and editing efficiency of Nme2-ABE8e, and to compare them to those parameters of Spy-ABE7.10 and Spy-ABE8e, we transfected HEK293T cells with plasmids expressing each ABE along with sgRNAs targeting 12 human genomic loci for Nme2-ABE8e [including eight dual-target sites (target sites followed by NGGNCC PAMs for both SpyCas9 and Nme2Cas9) [30] and four Nme2Cas9-specific target sites]. We found that this first-generation Nme2-ABE8e has a broad but shallow editing window that, due to modest activity, was difficult to define clearly (**Figure 1d**).

We next sought to understand the potential extent of off-target editing by Nme2-ABE8e. It has been shown that the major source of DNA off-target base editing is Cas9-dependent [37,38], caused by Cas9 binding and unwinding at near-cognate sequences. Because Nme2Cas9 is highly accurate during nuclease-driven editing in cells and *in vivo* [27,30,39], we hypothesized that Nme2-ABE8e would exhibit similar accuracy advantages relative to Spy-ABE8e. As an initial test, we systematically investigated the tolerance for nucleotide mismatches between the guide and the target sequence for the two effectors. We designed a panel of guides targeting the ABE reporter with single- and di-nucleotide mismatches with the target sequence for both Nme2-ABE8e and Spy-ABE8e and measured their activities by plasmid transfection and FACS (**Figure 1e**). Considering the differences in on-target efficiencies between the two effectors, we further normalized the activities of the mismatched guides to those of the perfectly complementary guides for each effector. We found that Nme2-ABE8e exhibited significantly lower off-target editing propensity than Spy-ABE8e: while single-nucleotide mismatches in the seed region (guide nucleotide positions 17-24 for Nme2Cas9, and 10-20 for SpCas9) and the majority of dinucleotide mismatches significantly compromised the editing efficiency of Nme2-ABE8e, these near-cognate sequences were mostly efficiently edited by Spy-ABE8e (**Figure 1e**).

Inhibition of base editing by anti-CRISPR proteins that limit DNA binding activity

The development of Nme2Cas9 base editing platforms raises the possibility that regulation strategies developed for nuclease-based editing could be similarly implemented in the case of base editing. One such strategy is the use of anti-CRISPR (Acr) proteins that limit Cas9 DNA-binding activity [40–43], which have been deployed to reduce both off-target [44] and off-tissue editing [45]. AcrIIC3 and AcrIIC4 have been reported to reduce Nme2Cas9 DNA binding activity [41,43,46] but have no effect on SpyCas9 [41,43]. To test whether such Acr proteins can

139 function as off-switches for Nme2-ABE8e base editing, we co-transfected the ABE reporter cell
140 line described above with plasmids expressing Nme2-ABE8e, sgRNA, and Acr proteins. Spy-
141 ABE7.10 and AcrIIA4 (an anti-CRISPR that prevents SpyCas9 DNA binding [40], nuclease
142 editing [40,44], and base editing [47]) was used as a positive control. Conversely, AcrE2, which
143 is a Type I-E Acr [43] that has no effect on SpyCas9 or Nme2Cas9 activity, was used as a
144 negative control. As expected, Spy-ABE7.10 base editing was reduced to background levels by
145 AcrIIA4, but AcrE2, AcrIIC3, and AcrIIC4 had no effect (**Supplementary Figure 3a**). By
146 contrast, Nme2-ABE8e editing was strongly inhibited by AcrIIC3 and AcrIIC4, but AcrE2 and
147 AcrIIA4 had no effect. These results confirm that these anti-CRISPRs can be effective off-
148 switches for Nme2-ABE8e editing.
149
150 Tissue-specific miRNAs, in combination with miRNA response elements [MREs] in the 3'UTR
151 of an Acr construct, have been used to restrict editing to cell types that express such miRNAs,
152 both in cultured cells [41,48] and *in vivo* [45]. To determine whether such a strategy could be
153 used to control Nme2-ABE8e, we inserted miR-122 MREs into the 3'UTRs of our AcrIIC3 and
154 AcrIIC4 constructs. Mir-122 is a hepatocyte-specific miRNA that is expressed in Huh7 cells but
155 not HEK293 cells, and has been used to validate this strategy for nuclease-based editing. Again,
156 AcrIIC3 and AcrIIC4 inhibited Nme2-ABE8e activity at an endogenous site in HEK293T cells,
157 and inhibition largely persisted even with the MREs (**Supplementary Figure 3b**), as expected
158 since miR-122 is not present to silence Acr expression. In Huh7 cells, which are transfected with
159 lower efficiencies, AcrIIC3 and AcrIIC4 again inhibited Nme2-ABE8e, but this inhibition of
160 editing activity was largely relieved by the insertion of the MRE122 sites. These results indicate
161 that miRNA-repressible anti-CRISPRs can be used to enforce the cell-type specificity of base
162 editing, as it can for nuclease editing.
163
164 **Installation of therapeutically relevant edits with Nme2-ABE8e**
165 We next tested the potential of Nme2-ABE8e to correct pathogenic mutations or to introduce
166 disease-suppressing mutations. One of the most common mutations that cause Rett syndrome is
167 a C•G to T•A base transition that produces a nonsense mutation in the human *MeCP2* gene
168 (c.502 C>T; p.R168X) [49–51]. Because the target adenine is within a cytidine-rich region, this
169 mutation is not readily accessible for ABEs based on other compact Cas9s such as SauCas9 and
170 SauCas9-KKH [33–36]. To test whether Nme2-ABE8e can correct this pathogenic mutation, we
171 electroporated Nme2-ABE8e mRNA with a synthetic sgRNA into a Rett syndrome patient-
172 derived fibroblast cell line that possesses this mutation. By amplicon deep sequencing, we found
173 that Nme2-ABE8e successfully edited the target adenine (A10) (**Figure 2a, 2b**). A bystander edit
174 at an upstream adenine (A16) causes a missense mutation (c.496 T>C; p.S166P), although this
175 occurs with only one-fourth the frequency of intended edit at A10. Because S166 has been shown
176 to be subject to phosphorylation in mice and is conserved from *X. laevis* to humans [52], further
177 investigation will be needed to determine whether bystander editing at A16 impairs functional
178 rescue of edited cells. However, as with other base editors [3,29,53–58], future protein
179 engineering efforts adjusting the editing window promise to provide greater control over Nme2-
180 ABE editing outcomes.
181
182 Next, we sought to generate a disease-suppressing mutation that has been shown to reverse
183 phenotypes of a validated Duchenne muscular dystrophy (DMD) mouse model (ΔEx51) [59].
184 The ΔEx51 mouse model was generated by deletion of the exon 51 in the *Dmd* gene, resulting in
185 a downstream premature stop codon in exon 52, causing the production of a nonfunctional

186 truncated dystrophin protein. Previously, it has been shown that the *Dmd* reading frame can be
187 restored by skipping exon 50 by adenine base editing (**Figure 2c**) [13]. However, *in vivo* base
188 editing using ABE_{max}-SpCas9-NG delivered by dual-AAV vectors was limited to local muscle
189 injection due to the high viral dosage required to achieve therapeutic benefit. We identified a
190 guide design for Nme2-ABE8e to target the adenine (A7) within the splice donor site downstream
191 of exon 50 (**Figure 2c**). By plasmid transfection in the mouse N2a cell line and amplicon deep
192 sequencing, we found that Nme2-ABE8e can generate 17.67 ± 4.57% editing at A7 (**Figure 2d**).
193 The efficient editing at multiple bystander adenines is not a concern in this case as those adenines
194 are within the skipped exon 50 or the intron.
195
196 **Optimization of an Nme2-ABE8e construct for single AAV delivery**
197 Previously, we showed that Nme2Cas9 with one or two sgRNAs can be packaged into a single
198 AAV vector and support efficient editing *in vivo* [27,30]. We reasoned, based upon the compact
199 sizes of Nme2Cas9 and TadA8e, that Nme2-ABE8e with a sgRNA could be packaged into a
200 single AAV for *in vivo* delivery. To achieve this, we first replaced NmeCas9 with Nme2-ABE8e in
201 the minimized all-in-one AAV vector reported previously [27]. We attached one cMyc NLS
202 sequence on each terminus of Nme2-ABE8e while retaining the original promoters for effector
203 and sgRNA expression (**Figure 3a,** 2x cMyc). By transient transfection of vector backbone
204 plasmids, the single-AAV construct successfully edited the ABE reporter cell line. To further
205 improve Nme2-ABE editing efficiency, we then tested three different NLS configurations: 1) one
206 cMyc NLS on the N-terminus and two cMyc NLSs on the C-terminus (3x cMyc); 2) one Ty1
207 NLS, which derived from the yeast Ty1 retrotransposon that supports robust nuclear localization
208 in dPspCas13b fusion proteins, on the N-terminus (Ty1) [60]; and 3) one bipartite SV40 NLS
209 (BP_SV40) on each terminus (2x BP_SV40) (**Figure 3a**) [61]. When transfecting the vector
210 plasmid into the ABE reporter cell line, the 2x BP_SV40 construct showed the highest editing
211 efficiency (**Figure 3b**).
212
213 The total length of the vector constructed with the 2xBP_SV40 NLS, hereafter Nme2-ABE8e-
214 U6, is 4998 bp, very close to the packaging limit of AAV. To test whether we could further
215 reduce the vector size without significantly compromising editing efficiency, we turned to a
216 recently reported "miniU6" promoter that has been shown to support sgRNA expression and
217 achieve comparable editing efficiencies as with the complete U6 promoter [62]. Upon
218 replacement of the U6 promoter with miniU6 promoter, the vector, hereafter Nme2-ABE8e-
219 miniU6, was shortened to 4860 bp, within the packaging limit of AAV (**Figure 3c**). Both
220 constructs induced robust editing in the ABE reporter cell line via transient transfection of the
221 vector plasmids (**Figure 3c**).
222
223 To avoid potential ABE reporter-specific effects, we further tested both single-AAV vector
224 backbone plasmids at two endogenous target sites: 1) one of the human dual-target sites, DS12,
225 and 2) a previously reported Nme2Cas9 target site in the mouse *Rosa26* gene [45]. By plasmid
226 transfection in human HEK293T or mouse N2a cells, we observed significant editing at these
227 loci by both vectors, although the Nme2-ABE8e-miniU6 vector was somewhat less efficient
228 (**Figure 3c**). We thus chose both vector designs for the subsequent *in vivo* study.
229
230 **Hydrodynamic injection of single-AAV vector plasmids corrects the disease**
231 **mutation and phenotype in an adult mouse model of HT1**

232 To test the *in vivo* editing efficiency and therapeutic potential of the single-AAV constructs, we
233 chose to target a pathogenic mutation associated with the liver disease HT1. HT1 is caused by
234 mutations in fumarylacetoacetate hydrolase (FAH), which catalyzes one step of the tyrosine
235 catabolic pathway. FAH deficiency leads to accumulations of toxic fumarylacetoacetate and
236 succinyl acetoacetate, causing liver, kidney, and CNS damage [63]. The *Fah*$^{PM/PM}$ mouse model
237 possesses a G•C to A•T point mutation in the last nucleotide of exon 8, which causes skipping of
238 exon 8 and FAH deficiency (**Figure 4a**). Without treatment, FAH deficient mice will rapidly
239 lose weight and eventually die. The *Fah*$^{PM/PM}$ mouse can be treated with 2-(2-nitro-4-
240 trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC), an inhibitor of an enzyme upstream
241 within the tyrosine degradation pathway, which prevents toxin accumulation [64]. Previously, we
242 and others have tested various *in vivo* gene-editing tools to treat the *Fah*$^{PM/PM}$ mouse model,
243 including Cas9-directed HDR [27,65,66], base editing [67,68], microhomology-directed end
244 joining [69], and prime editing [70]. Among these, multiple approaches including AAV, lipid
245 nanoparticle (LNP), and plasmid hydrodynamic tail-vein injection have been used to deliver the
246 gene-editing agents into this mouse model. However, caveats should be considered when
247 comparing the efficiencies of different gene-editing strategies: only the initial editing efficiency
248 (measured before NTBC withdrawal) reflects the activity of the gene-editing agents, because after
249 NTBC withdraw, the hepatocytes in which *Fah* has been repaired will show clonal expansion
250 over time due to their survival advantage.
251
252 We first validated a guide targeting the point mutation by electroporation of the single-AAV
253 vector plasmids, either Nme2-ABE8e-U6 or Nme2-ABE8e-miniU6, into mouse embryonic
254 fibroblasts (MEFs) isolated from a *Fah*$^{PM/PM}$ mouse (**Figure 4b**). We detected modest but
255 significant editing by both vectors at the target adenine at position 13 (A13), despite low (~12%)
256 plasmid electroporation efficiencies. We also observed higher levels of bystander editing at A16
257 with both vectors, and a lower level of bystander editing at A10 for Nme2-ABE8e-U6. Bystander
258 editing at A10 (which changes an active-site-proximal serine into an alanine) has been observed
259 previously with Spy-ABE as well [67]. The effect (if any) of the intronic A16 edit on intron 8
260 splice donor activity has not been defined (**Figure 4c**).
261
262 To test our single-AAV vectors of Nme2-ABE8e *in vivo*, we first performed hydrodynamic tail-
263 vein injections of the AAV-vector plasmids into 10-week-old HT1 mice, or PBS injection as a
264 negative control group [71]. We also injected plasmids expressing Spy-RA6.3, which is a codon-
265 optimized Spy-ABE, as a positive control [67]. Seven days post-injection, we sacrificed 2 mice
266 from each experimental group, and 1 mouse from each control group, to measure the editing
267 efficiency before hepatocyte expansion. We then withdrew NTBC for the rest of the mice for
268 long-term phenotypic study**.** Before NTBC withdrawal, anti-FAH immunohistochemistry (IHC)
269 staining showed 4.58 ± 1.1% FAH$^+$ hepatocytes from the group that was injected with the
270 Nme2-ABE8e-U6 plasmid, and 1.71 ± 0.49% from the group injected with the Nme2-ABE8e-
271 miniU6 plasmid (**Figure 4d**). The mouse injected with Spy-RA6.3 plasmid showed 4.5% FAH$^+$
272 hepatocytes, consistent with previously reported data [67] (**Figure 4d**). After NTBC withdrawal,
273 we monitored body weight changes. The PBS injected mice rapidly lost body weight after NTBC
274 withdrawal and were euthanized. By contrast, mice injected with either the Nme2-ABE8e-U6 or
275 Nme2-ABE8e-miniU6 plasmid gradually gained body weight, suggesting rescue of the
276 pathological phenotype (**Figure 4e)**. Forty days after NTBC withdrawal, we sacrificed mice
277 from all surviving groups. To determine whether Nme2-ABE8e successfully corrects the *Fah*
278 splicing defect, we extracted total RNA from the livers and performed reverse transcription PCR

279 (RT-PCR) using primers that spanned exons 5 and 9. By contrast to the PBS-injected mice,
280 which only showed a 305 bp PCR product corresponding to the truncated mRNA lacking exon
281 8, we observed that the 405 bp PCR product (containing exon 8) predominated in the Nme2-
282 ABE treated mice (**Figure 4f**). Sanger sequencing of the 405 bp bands further confirmed the
283 presence of the corrected G residue at the end of exon 8 (**Figure 4g**). When performing anti-
284 FAH IHC staining, we observed expansion of FAH^+ hepatocytes in the groups that were injected
285 with either of the single-AAV vector plasmids (**Figure 4h**). Amplicon deep sequencing of
286 genomic DNA from the livers of treated mice again provided evidence for Nme2-ABE activity
287 (**Figure 4i**). By contrast to the efficiencies achieved in the MEF cells, we observed a lower
288 editing at A16 and no significant editing at A10, likely due to partial (A16) or complete (A10)
289 selection against mice harboring bystander edits at those positions. These data indicate that our
290 Nme2-ABE8e single-AAV vector plasmids can correct the disease genotype and phenotype of the
291 *Fah*$^{PM/PM}$ mice *in vivo*.
292
293 ***In vivo* base editing by single AAV delivered Nme2-ABE8e in the *Fah*$^{PM/PM}$ mice**
294 Encouraged by the initial results, we packaged AAV9 with the Nme2-ABE8e-U6 construct, as
295 well as the Nme2-ABE8e-miniU6 construct, considering that the relatively smaller size of the
296 latter may potentially benefit packaging efficiency (**Figure 5a**). However, both constructs
297 yielded similar vector titres. Next, to confirm the AAV genome integrity, we performed AAV
298 genomic DNA extraction and alkaline gel electrophoresis. We did not observe any sign of
299 genome truncation (**Supplemental Figure 4a**). We then tail-vein injected 8-week-old
300 *Fah*$^{PM/PM}$ mice at a dosage of 4×10^{11} vg per mouse. We kept the mice on NTBC for one month
301 before analyzing the editing efficiency . One month after AAV injection and before NTBC
302 withdrawal, we sacrificed the mice and performed IHC staining using an anti-FAH antibody.
303 The negative control groups injected with AAV9 expressing Nme2-ABE and a sgRNA targeting
304 the *Rosa26* gene did not show any FAH^+ hepatocytes. In contrast, we observed 6.49 ± 2.08%
305 FAH^+ hepatocytes in the AAV9-Nme2-ABE8e-U6-*Fah* treated group, and 1.62 ± 0.49% FAH^+
306 hepatocytes in AAV9-Nme2-ABE8e-miniU6-*Fah* treated group (**Figure 5b**). Because repair of
307 1/100,000 hepatocytes was reported to rescue the phenotype, both AAV constructs achieved
308 efficiencies that were well above the therapeutic threshold [72]. Moreover, the percentage of
309 edited hepatocytes by AAV9-Nme2-ABE8e-U6-*Fah* was higher than what has been reported
310 previously by other genome editing strategies [65–67,70]. By targeted deep sequencing, the
311 editing efficiency at the target adenine (A13) in the AAV9-Nme2-ABE8e-U6 treated group is
312 0.34 ± 0.14%, while no significant editing was observed in the AAV9-Nme2-ABE8e-miniU6
313 treated group, possibly due to low efficiency that was below the detection limit of amplicon deep
314 sequencing (**Figure 5c**). The reason for the higher frequency of FAH^+ hepatocytes than the
315 frequency of editing at the DNA level is likely due to hepatocyte polyploidy [73], as well as the
316 presence of genomic DNA from nonparenchymal cells. Similar distinctions in FAH^+ frequencies
317 and genomic readouts were also observed in previous studies using this mouse model [67,70]. We
318 also measured the efficiency of insertions and deletions (Indels) by amplicon deep sequencing. We
319 did not detect any above-background level of indel, indicating that single-AAV delivered Nme2-
320 ABE8e can install precise editing *in vivo* without generating unwanted indels (**Figure 5c**).
321
322 Nme2Cas9 is known to be highly accurate in nuclease based editing in cells [27,30,39], and our
323 mismatch scanning experiments (**Figure 1e**) strongly suggest that the same will be true *in vivo*.
324 To evaluate potential Cas9-dependent off-target effects in the AAV9-injected mice, we searched
325 for genome-wide off-target sites for Nme2Cas9 using Cas-OFFinder [74], allowing for up to 6

326 mismatches. We then performed amplicon deep sequencing in the AAV9 treated livers at the two
327 top-ranking potential off-target sites, each including 5 mismatches. We did not detect any above-
328 background A•T-to-G•C editing at these sites (**Supplementary Figure 5**).
329

Discussion

Rapidly evolving and precise genome editing tools such as base editors and prime editors possess great potential to address the root causes of human genetic diseases [2,75,76]. However, the safe and effective delivery of genome editors remains a major challenge. Viral delivery using AAV, which is the only FDA-approved *in vivo* gene therapy vector to date, has a limited packaging capacity. Previous studies using AAV to deliver base editors or prime editors have been reported to be effective in rodents [7,8,10–13,15–18,21], but all of them required multiple vectors and most of them required high viral dosage [7,10–13,17,18]. To date, AAV administered at high dose has been reported to relate to severe toxicity or even death in nonhuman primates, piglets, and humans [23,24,77–79]. Engineering single-AAV deliverable genome editing tools has the potential to achieve therapeutic benefits at lower dosage, which would not only ease manufacturing burdens but also reduce likelihoods of serious adverse events [80,81].

Engineering new genome editors based on compact Cas9s is an alternative approach to address this issue. Previously, single-AAV delivery of Cas9 nucleases has been reported to generate NHEJ-based editing *in vivo* [26,30,82]. Precision editing via HDR has also been achieved with single-AAV systems, albeit with low efficiencies [27,83]. The only single-AAV BE system reported previously (which was not tested *in vivo*) was based on SauCas9, which has limited targeting range due to its PAM constraints. In this study, we constructed and characterized a compact, accurate adenine base editor, Nme2-ABE8e, which can target many sites that are inaccessible to SauCas9 base editors due to their distinct PAMs. We showed that Nme2-ABE8e with a sgRNA can be packaged into a single AAV vector, and a single intravenous injection in an adult disease mouse model of tyrosinemia reversed both the disease mutation and phenotypes. Furthermore, the dosage used in this study (4×10^{11} per mouse or 2×10^{13} vg/kg) is well below the 1×10^{14} vg/kg systemic doses that have been tolerated in clinical trials [77,78].

Although the editing efficiency was modest at the *Fah* disease locus, we reached therapeutic thresholds for HT1. Furthermore, the initial editing efficiency of the AAV9 delivered Nme2-ABE8e-U6 construct exceeded that reported previously using other precision genome editing tools and delivery approaches [65–67,70]. Future optimization of this system promises to improve efficiency. There are several potential explanations for the low *in vivo* editing efficiency we achieved with our first-generation Nme2Cas9 ABE that suggest directions for future improvement. First, structural analyses of Nme2Cas9 [84] indicate that the position of the N-terminally fused TadA8e domain relative to the predicted path of the displaced strand is not optimal. With other effectors, domain-inlaid deaminase fusions have proven to be advantageous in some contexts [29,54], and the same is likely to be true with Nme2-ABEs. Second, as with other BE effectors, editing efficiencies are target-site specific. While we observed only around 0.3% editing at the *Fah* locus *in vivo* by AAV9-Nme-ABE8e-U6 delivery, editing at the *Rosa26* locus was ~5% (**Supplementary Figure 4b**), indicating that more efficiently edited sites can be identified. Third, the current Nme2-ABE8e has a wide editing window that could result in increased bystander editing. For example, we observed higher editing at a bystander adenine (A16) at the *Fah* locus compared to the on-target adenine at A13. Optimizations of linker composition and length [3,53], as well as domain-inlaid systems [29,54–58], may confer greater control over the editing window. Finally, future optimization of the single-AAV vector construct, such as changing the transgene cassette orientations, may potentially increase expression levels of the BE effector, the sgRNA, or both [18,85].

377
378 Our studies also suggest factors that must be considered for optimal guide expression. In contrast
379 to a previous study that showed no significant difference between the U6 and miniU6 promoters
380 in supporting Cas9 editing efficiency [62], we found that the construct with the miniU6 promoter
381 was consistently less efficient than that with the U6 promoter, including via AAV delivery.
382 Because the previous study compared these promoters in T cells by lentivirus transduction, our
383 observations may only apply to certain delivery strategies and cellular contexts.
384
385 In summary, we have engineered and characterized Nme2-ABE8e editing in mammalian cell
386 culture, and achieved *in vivo* delivery by a single AAV vector in an adult mouse model of
387 tyrosinemia. To our knowledge, this is the first single-AAV-delivered *in vivo* base editing reported
388 to date [29]. We anticipate that Nme2-ABE8e, with its distinct PAM specificity, editing window,
389 and high accuracy, will provide additional targetability, safety, and therapeutic potential for
390 genome engineering applications.
391

392 **Methods**
393
394 **Cell culture.** HEK293T cells (ATCC CRL-3216), ABE reporter cells, MEF cells, and mouse
395 N2a cells (ATCC CCL-131) were cultured in in Dulbecco's Modified Eagle Media (DMEM,
396 Genesee Scientific Cat. #: 25-500) supplemented with 10% Fetal Bovine Serum (Gibco Cat. #:
397 26140079). Rett syndrome human patient-derived fibroblasts (hPDFs) were obtained from the
398 Rett Syndrome Research Trust and cultured with DMEM supplemented with 15% FBS and 1x
399 non-essential amino acids (Gibco Cat. #: 11140050). All cells were incubated in a 37°C
400 incubator with 5% CO_2.
401
402 **Molecular cloning.** To generate the CMV-Nme2-ABE8e and the CMV-Nme2-ABE7.10
403 plasmids used in **Figure 1**, the Nme2-ABE8e, Nme2-ABE7.10, Spy-ABE8e, and Spy-ABE7.10
404 constructs were cloned into the pCMV-PE2 vector backbone (Addgene #132775) by Gibson
405 assembly. Briefly, the pCMV-PE2 plasmid was digested with NotI and PmeI restriction enzymes,
406 and the plasmid backbone was then Gibson-assembled with five fragments: N-terminal NLS,
407 TadA8e (for Nme2-ABE8e or Spy-ABE8e), or TadA-TadA*7.10 (for Nme2-ABE7.10 or Spy-
408 ABE7.10), the linker, Nme2Cas9-D16A or SpCas9-D10A nickase, and the C-terminal NLS. The
409 ABE reporter construct was cloned by site-directed mutagenesis to change the 47th amino acid
410 (Glutamine, CAG) of the mCherry coding sequence to a stop codon (TAG). The ABE reporter
411 was further cloned into a lentiviral transfer vector backbone (Addgene #99373) by Gibson
412 assembly. The sgRNA expression plasmids used in **Figure 1** were cloned from pBluescriptSKII
413 (Addene #74705) in two steps. First, pBluescriptSKII was digested by NotI and XbaI, and a gene
414 fragment that contains a U6 promoter, a type-IIS restriction cloning site (BfuAI), and a
415 tracrRNA was assembled into the backbone by Gibson assembly. The plasmids were further
416 digested with BfuAI and ligated to the annealed oligos to insert the guide sequences. The single-
417 AAV vector plasmids in **Figure 3a** were cloned from the Addgene #119924 plasmid by
418 replacing the Nme2Cas9 sequence with the Nme2-ABE8e sequence, and then subsequently re-
419 cloned to encode different NLS configurations and the miniU6 promoter by restriction enzyme
420 digestion and Gibson assembly. The NLS configuration of Nme2-ABE8e mRNA used in **Figure**
421 **2b** was 2x_BPSV40, and the plasmid used in **Figure 2d** was the single-AAV Nme2-ABE8e-U6
422 plasmid shown in **Figure 3a** (2xBP_SV40). Most of the plasmids used in **Supplementary**
423 **Figure 3a** were previously deposited in Addgene (AcrIIC3, Addgene #85713; AcrIIC4,
424 Addgene #113434; Acr-E2, Addgene #85677). To clone the plasmid expressing AcrIIA4,
425 Addgene #85713 plasmid was digested with XhoI and BamHI and Gibson-assembled with a
426 gene fragment containing AcrIIA4 coding sequence [46]. In **Supplementary Figure 3b**, the
427 AcrIIC3-MRE122 plasmid was cloned from Addgene plasmid #129531 by replacing the *Rosa26*-
428 targeting guide with the DS12-targeting guide through restriction enzyme cloning. Subsequent
429 replacement of the coding sequence of AcrIIC3 with AcrIIC4 generated the AcrIIC4-MRE122
430 plasmid. Sequences of plasmids first described in this paper can be found in the
431 **Supplementary note** and will be made available from Addgene.
432
433 **ABE reporter HEK293T cell line.** Lentivirus was produced following instructions from
434 Addgene (https://www.addgene.org/protocols/lentivirus-production/). Briefly, HEK293T cells
435 were transfected with the transfer vector and the packaging plasmids psPAX2 (Addgene #12260)
436 and pMD2.G (Addgene #12259), using Lipofectamine 3000 (ThermoFisher Cat. #: L3000015).
437 Two days later, the medium was collected and filtered through a 0.45 μm filter (Cytiva Cat. #:
438 6780-25040) to remove cell debris. The viral titer was determined using Lenti-X™GoStix™

439 (Takara Bio Cat. #: 631280). HEK293T cells were transduced with lentivirus encoding the ABE
440 reporter at varying dilutions (1:10, 1:100, 1:500, and 1:1000) in the presence of 8 µg/ml
441 polybrene (Millipore Sigma Cat. #: TR-1003-G). Three days after transduction, the media was
442 removed, and fresh media was supplemented with 2 µg/ml Puromycin to select cells expressing
443 the full-length reporter construct. Seven days after selection, the puromycin-resistant cells were
444 collected. Single-cell clones were established by serial dilution in 96-well plates.
445
446 **Fluorescent reporter assay.** Forty-eight hours after transfection, cells were trypsinized and
447 harvested into microcentrifuge tubes. After centrifuging at 300 x g for 3 mins, cells were
448 resuspended into 150ul 1xPBS for flow cytometry analysis (MACSQuant VYB). For each
449 sample, 10,000 events were counted for FACS analysis. Data was analyzed using Flowjo v10.
450 Representative gating strategy can be found in **Supplementary Figure 2**.
451
452 ***In vitro* transcription of Nme2-ABE8e mRNA.** Nme2-ABE8e mRNA was *in vitro*
453 transcribed using the NEB HiScribe T7 RNA synthesis kit, from 500ng of a linearized template.
454 Uridine was fully substituted with 1-methylpseudouridine, and mRNA was capped co-
455 transcriptionally using CleanCap AG analog (TriLink Biotechnologies Cat. #N-1081 and N-
456 7113, respectively). All enzymes were purchased from New England Biolabs. Transcription was
457 conducted according to the manufacturer's protocol with the following amendments:
458 transcriptions were completed in 1× NEB HiScribe transcription buffer, and 4 mM CleanCap
459 AG was used during transcription. Transcription reactions were incubated at 37 °C for 2 hours
460 then treated with 0.4 U/µl DNase I (final concentration) for 15 min at 37 °C. mRNAs were
461 purified with NEB Monarch RNA purification columns and treated with pyrophosphatase for 1
462 hour with 0.25 U/µg Antarctic phosphatase (final concentration) in 1X Antarctic phosphatase
463 buffer. The final product was further purified with a NEB Monarch RNA column and eluted in
464 water.
465
466 **Transfection and electroporation**. For plasmid transfection, cells were seeded in 24-well
467 plates at 80,000 cells per well in culture media and incubated overnight. Briefly, plasmids were
468 transfected at 400ng per well when targeting the ABE reporter, and 1ug per well for the
469 endogenous target sites. Specifically, in **Figure 1c**, **1e**, **2d**, **3b**, **3c**, and **Supplementary**
470 **Figure 3**, plasmids were transfected using Lipofectamine3000 (ThermoFisher Cat #L3000001),
471 while in **Figure 1d** and **Supplementary Figure 2**, plasmids were transfected using
472 Lipofectamine2000 (ThermoFisher Cat #11668030), following the manufacturer's protocols.
473 Electroporation was performed using Neon TransfectionSystem 10 ul kit (ThermoFisher Cat #:
474 MPK 1096), with the following electroporation parameters: Pulse voltage (1650 v), Pulse width
475 (20 ms), Pulse number (1). Specifically, in **Figure 2a**, 264 ng Nme2-ABE8e mRNA and 100
476 pmole of sgRNA were electroporated into 50,000 Rett Syndrome hPDFs, while in **Figure 4c**, 1
477 ug plasmid DNA was electroporated into 100,000 MEF cells.
478
479 **AAV production**. AAV vector packaging was done at the Viral Vector Core of the Horae Gene
480 Therapy Center at the UMass Chan Medical School as previously described [86]. Constructs
481 were packaged in AAV9 capsids and viral titers were determined by digital droplet PCR and gel
482 electrophoresis followed by silver staining.
483
484 **AAV genomic DNA extraction and alkaline agarose gel electrophoresis.** Genomic
485 DNAs were extracted from 10^{11} vg AAV by incubating with 20 units of DNase I (ThermoFisher

486 Cat. #: EN0521) at 37 °C for 30 minutes and then with an equal volume of 2 x Pronase solution
487 (Sigma-Aldrich, Cat. # 10165921001) for 4 hours. The genomic DNA was subsequently purified
488 by phenol-chloroform (ThermoFisher Cat. #:15593-049) extraction and ethanol precipitation.
489 DNA pellets were resuspended in water and analyzed by alkaline agarose gel electrophoresis and
490 SYBR Gold staining.
491
492 **Mouse experiments.** All animal study protocols were approved by the Institutional Animal
493 Care and Use Committee (IACUC) at UMass Chan Medical School. The *Fah*$^{PM/PM}$ mice were
494 kept on water supplemented with 10 mg/L 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-
495 cyclohexanedione (NTBC; Sigma-Aldrich, Cat. #: PHR1731-1G). Mice with more than 20%
496 weight loss were humanely euthanized according to IACUC guidelines. For hydrodynamic tail
497 vein injections, plasmids were prepared by EndoFree Plasmid Maxi Kit (Qiagen Cat. #12362).
498 Briefly, 30 ug SpCas8-RA6.3 and 30 ug sgRNA plasmids, or 60 ug Nme2-ABE8e single AAV
499 plasmids were suspended in 2 ml saline and injected via tail vein within 5-7 seconds into 10-
500 week-old *Fah*$^{PM/PM}$ mice. Mice were euthanized 7 days after injection and livers were collected
501 for analysis. For AAV injection, a dosage of 4 x 10^{11} vg per mouse (in 200 ul saline) was tail-vein
502 injected into 8-week-old *Fah*$^{PM/PM}$ mice.
503
504 **Genomic DNA extraction from cultured cells and mouse liver.** For cultured cells,
505 genomic DNAs were extracted 72 hours after plasmid transfection, or 48 hours after mRNA and
506 sgRNA electroporation. Briefly, cell culture media were aspirated, and cells were washed with 1x
507 PBS. Genomic DNAs were prepared using QuickExtract DNA Extraction Solution (Lucigen)
508 following the manufacturer's protocols. To extract genomic DNA from mouse livers, all 5 lobes
509 of mouse liver were combined and pulverized in liquid nitrogen, and 15 mg of the tissue from
510 each mouse liver was used for genomic DNA extraction using GenElute Mammalian Genomic
511 DNA Miniprep Kit (Millipore Sigma Cat. #: G1N350).
512
513 **Amplicon deep sequencing and data analysis**. Genomic DNA was amplified by PCR
514 using Q5 High-Fidelity 2X Master Mix (NEB Cat. #: M0492) for 20 cycles. One microliter of
515 the unpurified PCR product was used as a template for 20 cycles of barcoding PCR. The
516 barcoding PCR reactions were further pooled and gel-extracted using Zymo gel extraction kit
517 and DNA clean & concentrator (Zymo research Cat. #: 11-301 and 11-303) and quantified by
518 Qubit 1x dsDNA HS assay kits (Thermo Fisher Scientific, Cat #: Q32851). Sequencing of the
519 pooled amplicons was performed using an Illumina MiniSeq system (300-cycles, FC-420-1004)
520 following the manufacturer's protocol. The raw MiniSeq output was de-multiplexed using
521 bcl2fastq2 (Illumina, version 2.20.0) with the flag --barcode-mismatches 0. To align the
522 generated fastq files and to quantify editing efficiency, CRISPResso2 [87] (version 2.0.40) was
523 used in batch mode with base editor output and the following flags: -w 15, -q 30. Indel frequency
524 = (insertions reads + deletions reads)/all aligned reads x 100%.
525
526 **Immunohistochemistry (IHC).** Mice were euthanized by CO_2 asphyxiation and livers were
527 fixed with 10% neutral buffered formalin (Epredia Cat. #: 5735), sectioned at 10um, and stained
528 with hematoxylin and eosin for pathology analysis. For IHC, liver sections were dewaxed,
529 rehydrated, and stained using an anti-FAH antibody (Abcam Cat. #: ab83770) at 1:400 dilution
530 as described previously [67].
531

532 **Reverse transcription PCR**. All 5 lobes of each mouse liver were combined and pulverized in
533 liquid nitrogen. Total RNA was extracted from 50 mg of liver tissue using TRIzol reagent
534 (ThermoFisher Cat. #: 15596026) and reverse-transcribed using SuperScript III First-Strand
535 Synthesis System (ThermoFisher Cat. #:18080051). PCR was performed using primers
536 previously described [67].
537
538 **Author contributions**
539
540 H.Z., X.D.G., and E.J.S conceived the study. H.Z. designed, performed, and analyzed the *in vivo*
541 experiments. H.Z., N.B., O.O., P.L., and X.D.G., designed, performed, and analyzed the *in vitro*
542 experiments. T.R. and H.C. analyzed the deep sequencing data. E.J.S., W.X., and S.A.W.
543 supervised research. H.Z. and E.J.S. wrote the manuscript with contributions from N.B., P.L.,
544 and H.C. All authors edited the manuscript.
545
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565
566 **Declaration of Competing Interests**
567
568 H.Z., N.B., P.L., X.D.G., S.A.W., W.X., and E.J.S. are co-inventors on patent filings related to
569 this work. E.J.S. is a co-founder and Scientific Advisor Board member of Intellia Therapeutics.
570

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Figures

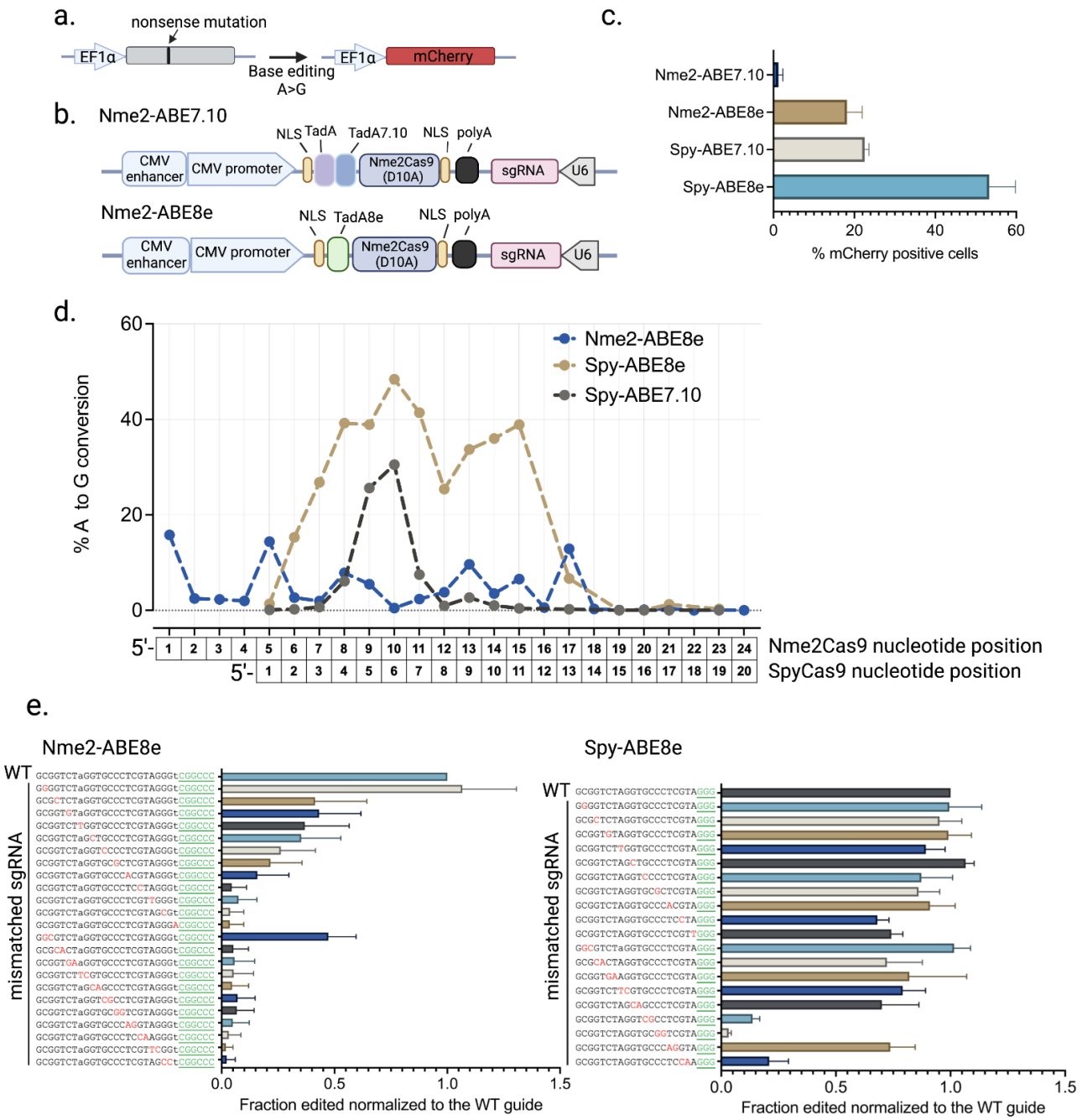


Figure 1

a). Schematic representation of the ABE reporter HEK293T cell line. **b).** Schematic representation of the Nme2-ABE constructs. **c).** Comparison of editing efficiencies of Nme2-ABEs to those of Spy-ABEs in the ABE reporter cell line by plasmid transient transfection (n = 3 biological replicates). **d).** Summary of editing windows and comparison of editing efficiencies for the ABEs at endogenous genomic loci. Each data point represents the mean A-to-G editing efficiency at the indicated position of the spacer across 12 Nme2Cas9 target sites and 8 SpyCas9 target sites, respectively (n = 3 biological replicates). Summary of the individual A-to-G conversion and indel efficiencies at each target site can be found in **Supplementary Figure 2**. **e).** Comparison of Nme2-ABE8e mismatch tolerance to that of Spy-ABE8e in the ABE reporter cell line. The activities of the effectors with the mismatched guides are normalized to that of the perfectly complementary (WT) guide. Red, mismatched nucleotides; green, PAM sequence (n = 3 biological replicates).

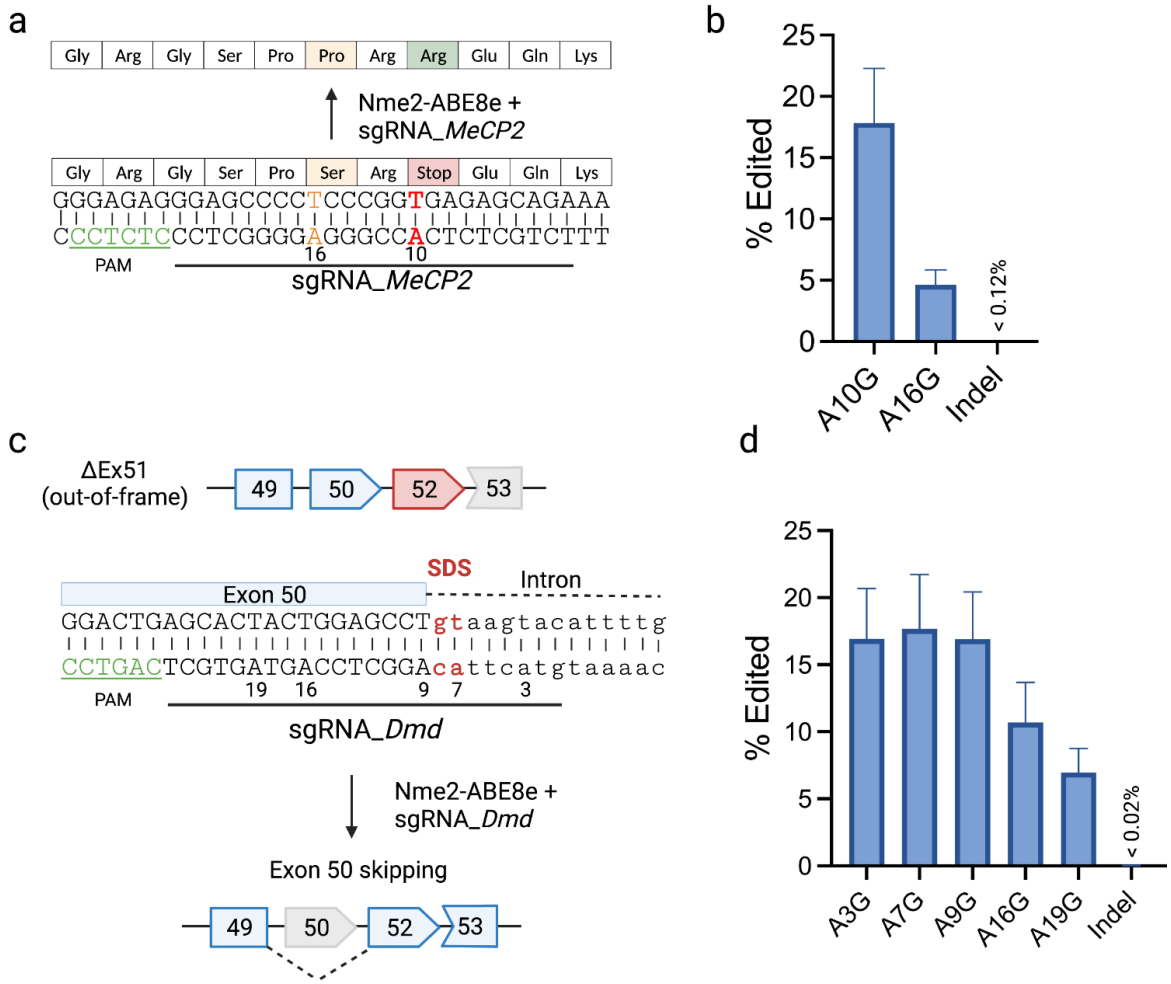


Figure 2

a). Schematic representation of a nonsense mutation in the human *MeCP2* gene (c.502 C>T; p.R168X) that causes Rett Syndrome. The black underline denotes the target sequence of an Nme2-ABE8e guide for reverting the mutant A to G (wildtype) at position 10 (red, bold). The PAM region is underlined in green. A bystander edit at position 16 (orange) can generate a missense mutation (c.496 T>C; p.S166P). **b).** Amplicon deep sequencing quantifying the editing efficiency in Rett patient-derived fibroblasts transfected with the Nme2-ABE8e mRNA and the synthetic sgRNA_*MeCP2* noted in **a** (n = 3 biological replicates). **c).** Schematic representation of the exon skipping strategy that restores the reading frame of the mouse *Dmd* transcript. Deletion of exon 51 (ΔEx51) can alter the reading frame and generate a premature stop codon in exon 52 (red). Adenine base editing at the splice site of exon 50 (red) by Nme2-ABE8e can cause exon 50 skipping (gray) and restore the *Dmd* reading frame. The PAM region is underlined in green. **d).** Amplicon deep sequencing quantifies the editing efficiency at the target site in mouse N2a cells transfected with the Nme2-ABE8e and sgRNA_*Dmd* expression plasmid (n = 3 biological replicates).

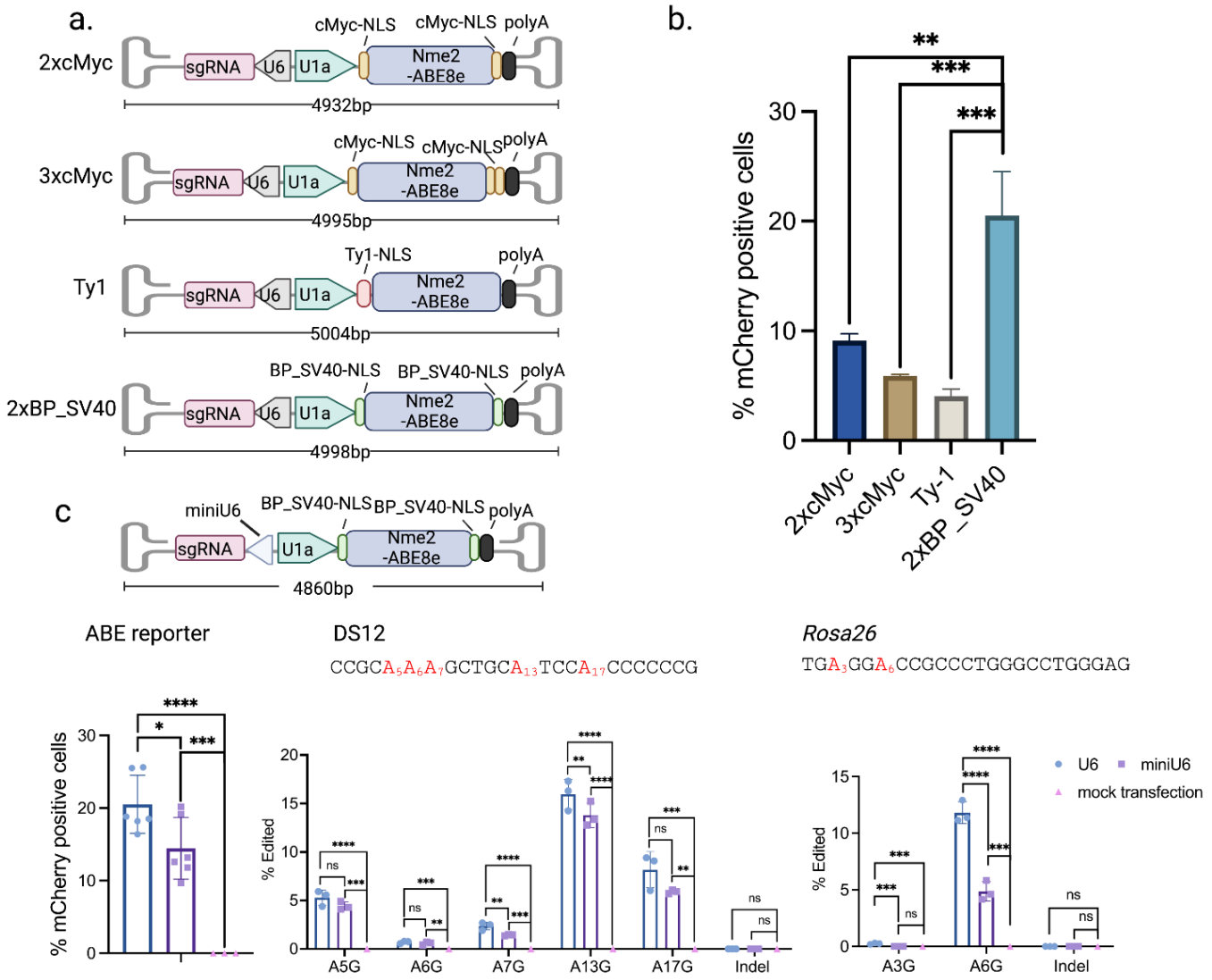
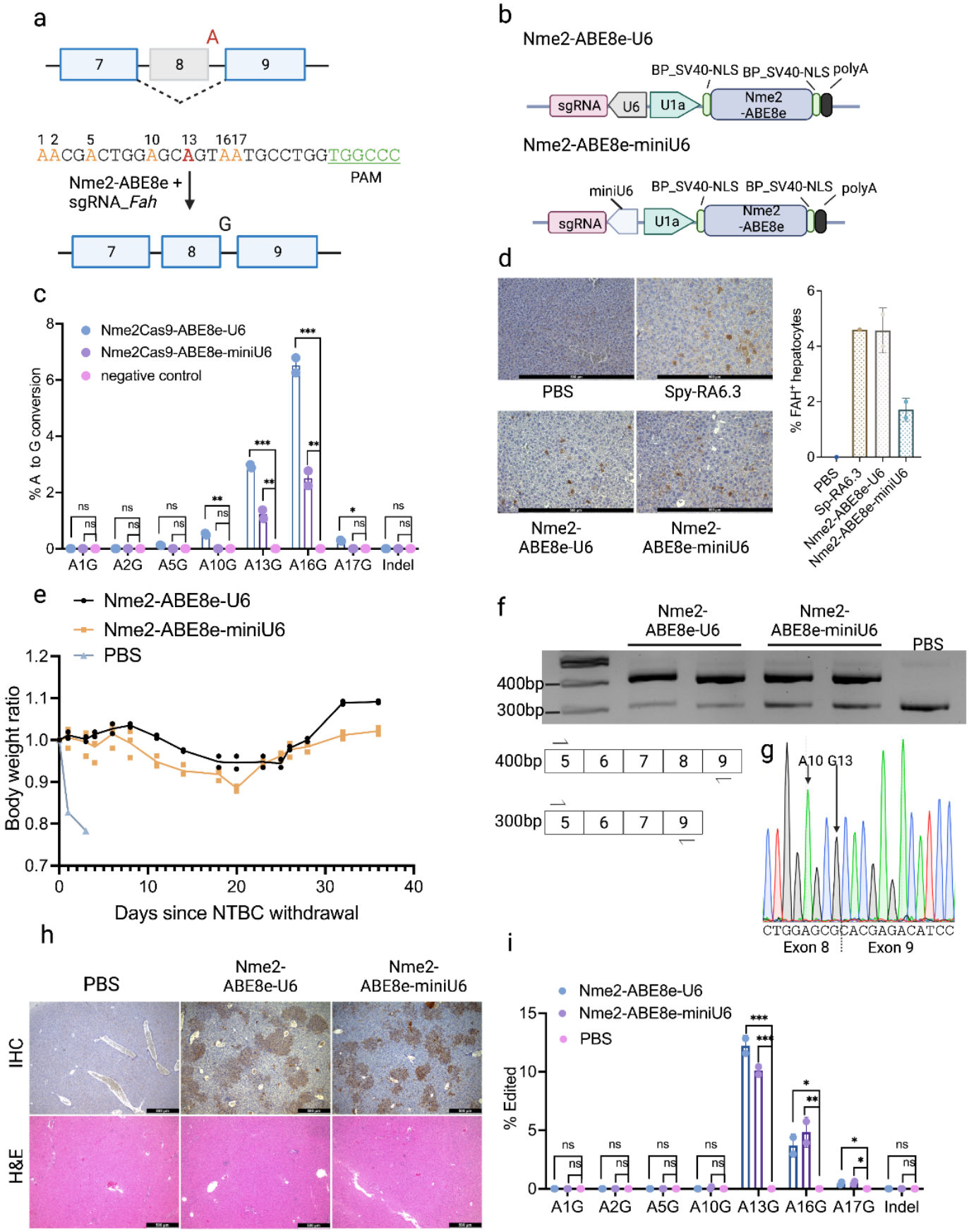


Figure 3

a). Schematic representation of single AAV constructs with different NLS configurations. **b).** Comparison of different NLS configurations by plasmid transfection in the ABE reporter cell line. **c).** Comparison of Nme2-ABE activity when sgRNA expression is driven by the U6 or miniU6 promoters in the 2xBP_SV40 NLS construct targeting the ABE reporter site (left) or endogenous human (middle) and mouse (right) genomic sites by plasmid transfection in cultured cells followed by amplicon deep sequencing (n = 3 biological replicates). Data represent mean ± SD; ns, P > 0.05; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 (one-way ANOVA).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AACGACTGGAGCAGTAATGCCTGGTGGCCC
PAM
Nme2-ABE8e +
sgRNA_Fah
G
b
Nme2-ABE8e-U6
BP_SV40-NLS BP_SV40-NLS polyA
sgRNA U6 U1a Nme2 -ABE8e
Nme2-ABE8e-miniU6
miniU6 BP_SV40-NLS BP_SV40-NLS polyA
c
Nme2Cas9-ABE8e-U6
Nme2Cas9-ABE8e-miniU6
negative control
% A to G conversion
A1G A2G A5G A10G A13G A16G A17G Indel
d
PBS
Spy-RA6.3
Nme2-ABE8e-U6
Nme2-ABE8e-miniU6
% FAH+ hepatocytes
e
Nme2-ABE8e-U6
Nme2-ABE8e-miniU6
PBS
Body weight ratio
Days since NTBC withdrawal
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Nme2-ABE8e-U6
Nme2-ABE8e-miniU6
PBS
400bp
300bp
g
A10 G13
CTGGAGCGCACGAGACATCC
Exon 8
Exon 9
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PBS
Nme2-ABE8e-U6
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A1G A2G A5G A10G A13G A16G A17G Indel

Figure 4

a). Illustration of the pathogenic point mutation in the *Fah*$^{PM/PM}$ mouse model that causes exon 8 skipping of the *Fah* gene, and the guide design for Nme2-ABE8e to correct the point mutation. Red and bold, target adenine; orange, other bystander adenines; green and underlined, PAM. **b).** Illustration of constructs of the single-AAV vector plasmids used in *in vivo* studies. **c).** Editing efficiencies at the *Fah* mutant site by AAV plasmid electroporation in MEF cells derived from the *Fah*$^{PM/PM}$ mouse. Data are from amplicon deep sequencing (n = 2 biological replicates). **d).** Anti-FAH immunohistochemistry (IHC) staining showing FAH^{+} hepatocytes, before NTBC withdrawal, in the *Fah*$^{PM/PM}$ mouse hydrodynamically injected with the indicated plasmid. The bar graph quantifies the percentage of FAH^{+} hepatocytes detected by IHC (n = 2 mice per group). Scale bars, 500 μm. **e).** Body weight plot of mice injected with the single-AAV vector plasmid showing gradual weight gain over a month after NTBC withdrawal. **f).** RT–PCR analysis of the plasmid- or PBS-injected mouse livers using primers that span exons 5 and 9. The wild-type amplicon is 405 bp and exon 8 skipped amplicon is 305 bp. **g).** Representative Sanger sequencing trace of the 405 bp RT-PCR band. **h).** Anti-FAH IHC staining showing expansion of FAH^{+} hepatocytes 40 days post NTBC withdrawal. Scale bars, 500 μm. **i).** Quantification of the editing efficiency by amplicon deep sequencing of genomic DNA of the treated mouse livers harvested 40 days post NTBC withdrawal. **d-i**, n = 2 mice per group). Data represent mean ± SD; ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (one-way ANOVA).

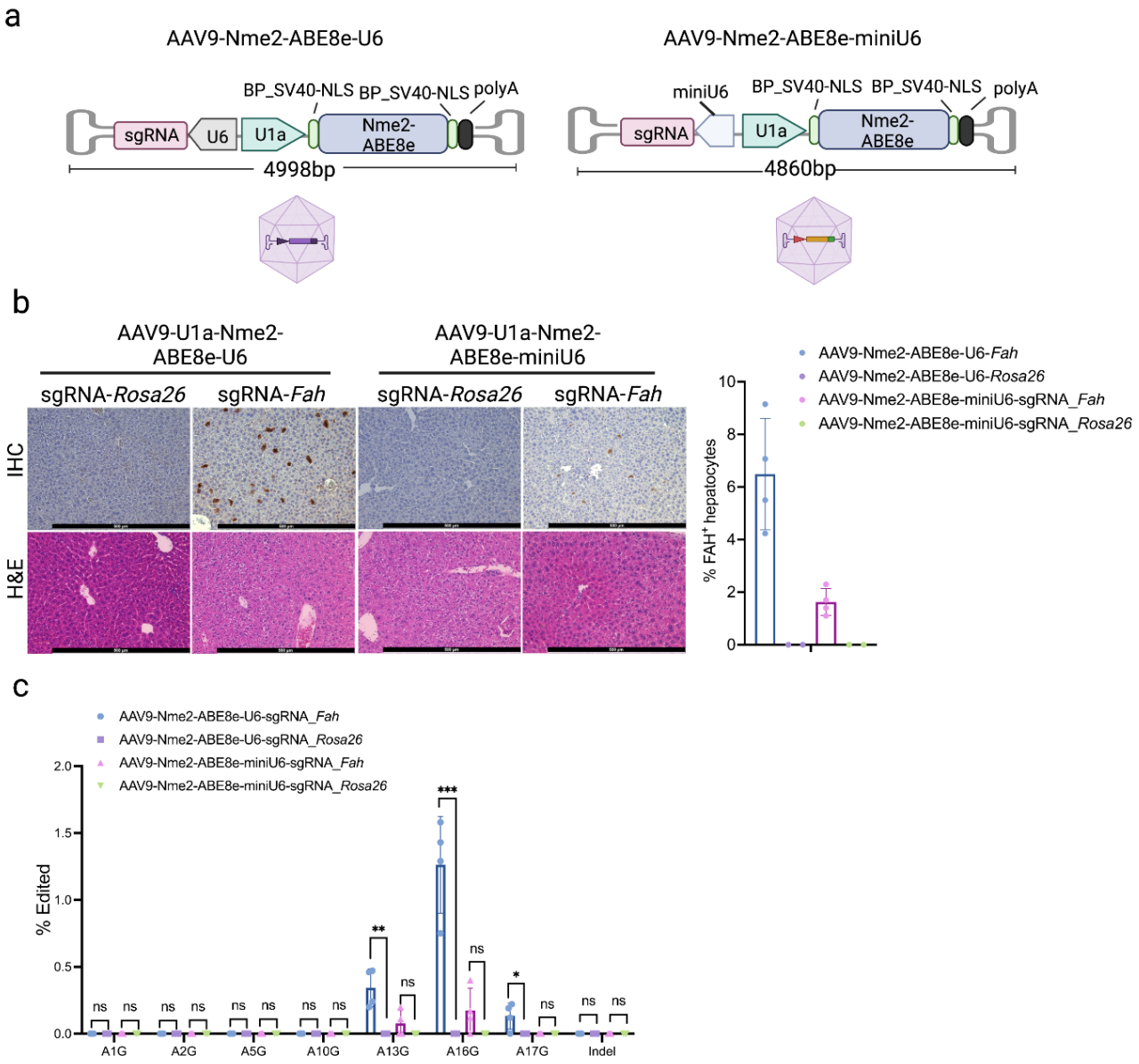
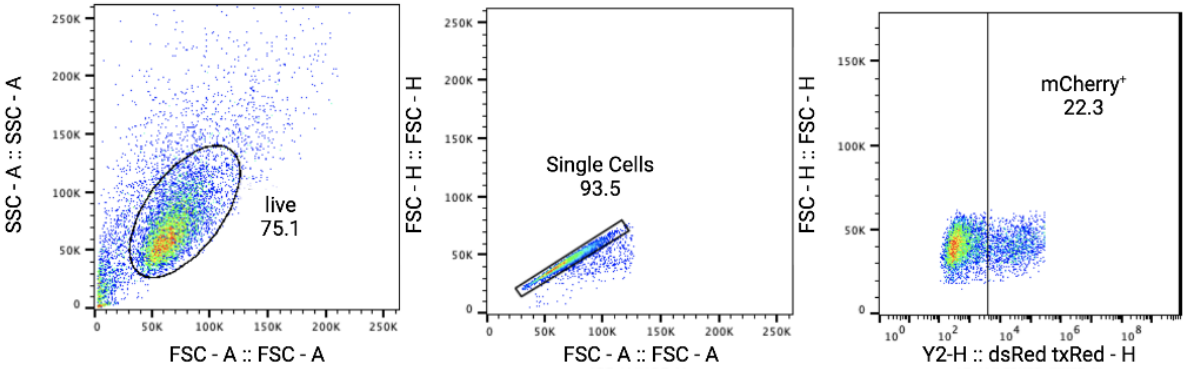
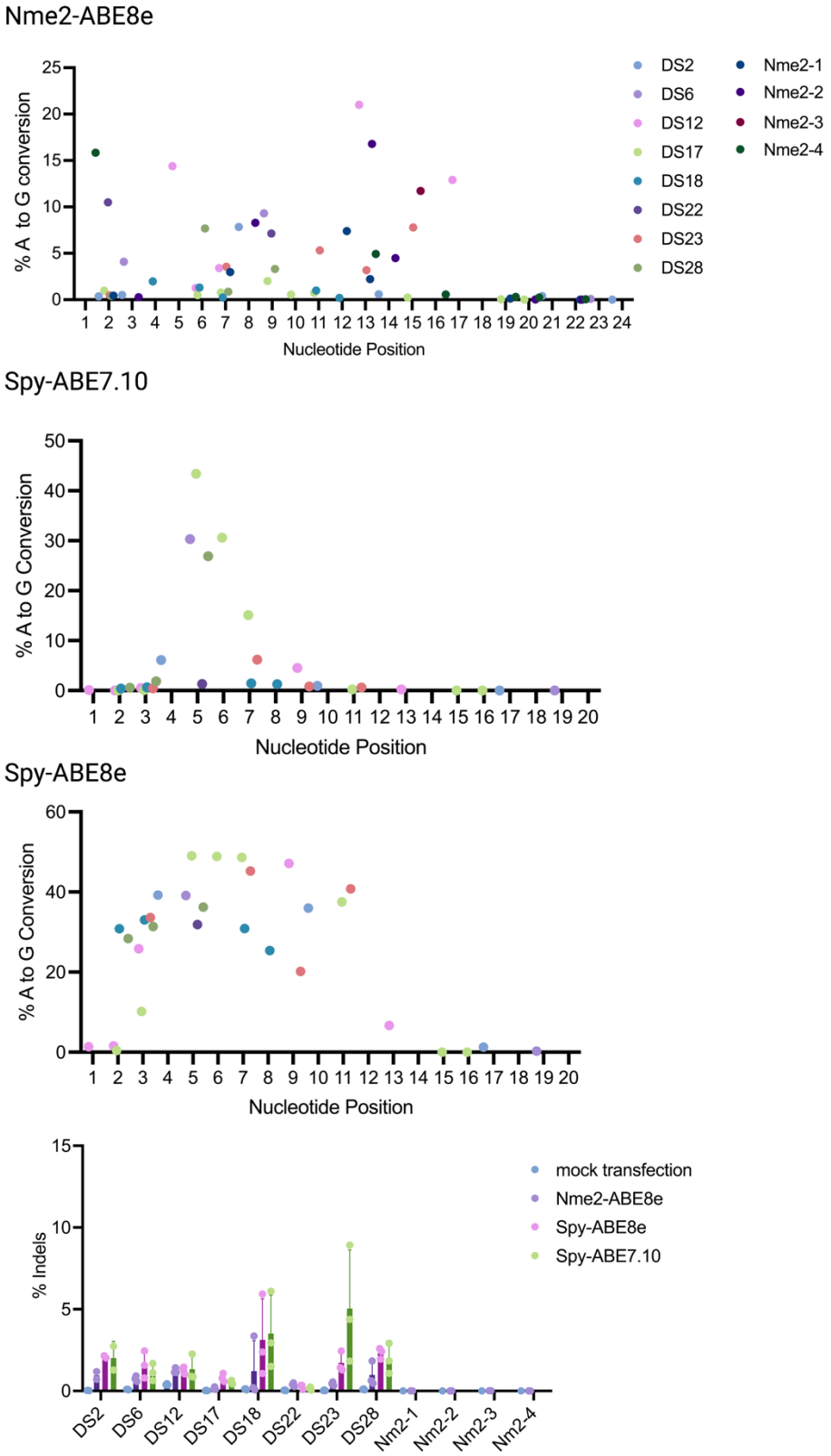


Figure 5

a). Schematic representation of the AAV constructs. **b).** Anti-FAH immunohistochemistry (IHC) staining showing FAH$^+$ hepatocytes, before NTBC withdrawal, in the *Fah*$^{PM/PM}$ mouse injected with AAV9 expressing Nme2-ABE8e with a sgRNA targeting either the *Fah* gene, or the *Rosa26* gene that serves as a negative control. Scale bar, 500 µm. The bar graph quantifies the percentage of FAH$^+$ hepatocytes detected by IHC (n = 4 mice per group). **c).** Quantification of the editing efficiency by amplicon deep sequencing of genomic DNA from AAV9-injected mouse livers harvested before NTBC withdrawal (n = 4 mice per group). Data represent mean ± SD; ns, P > 0.05; *, P < 0.05; **, P < 0.01; ***, P < 0.001 (one-way ANOVA).

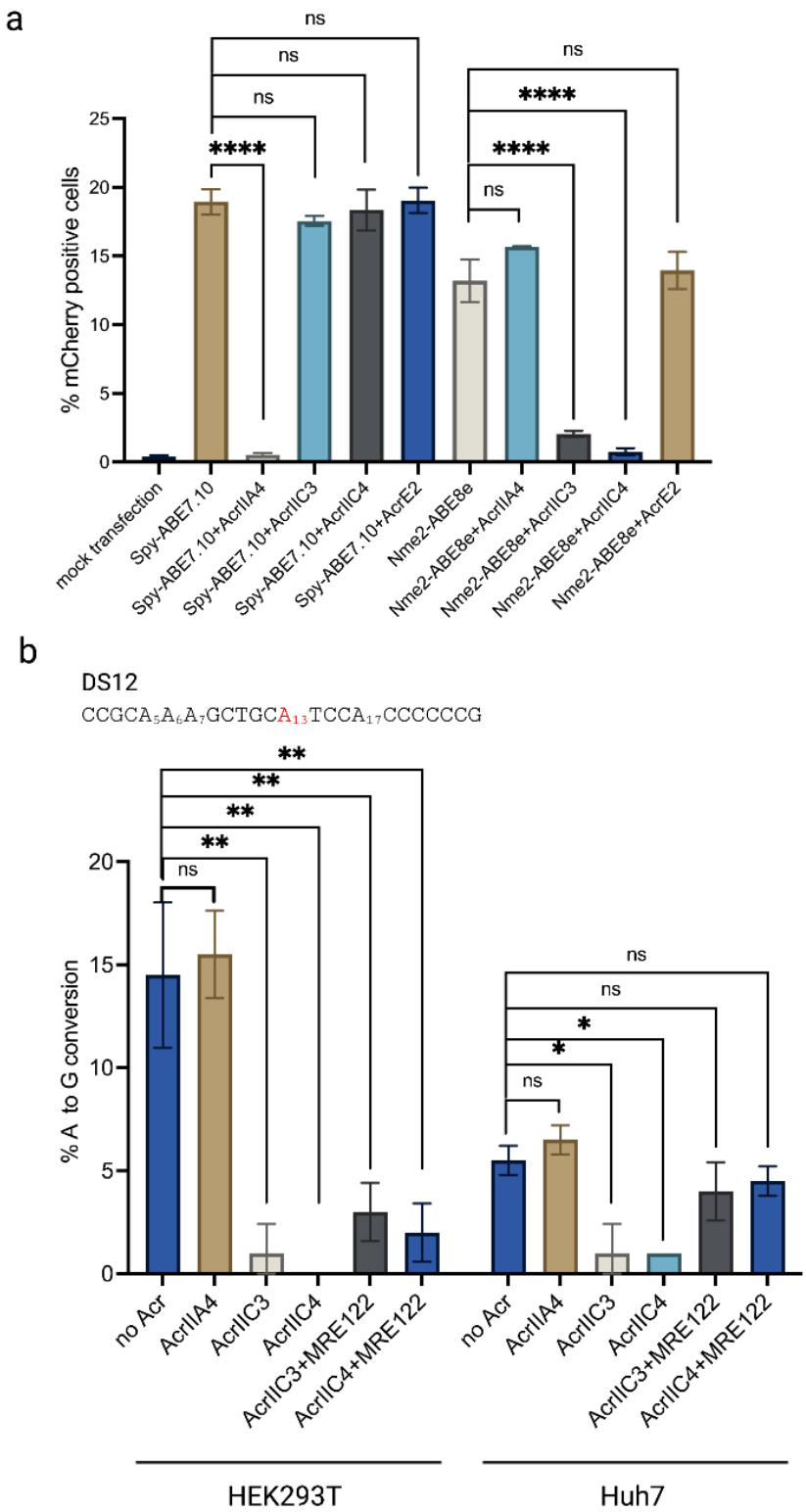


Supplementary Figure 1
Representative flow cytometry gating strategy for the ABE reporter cell line.



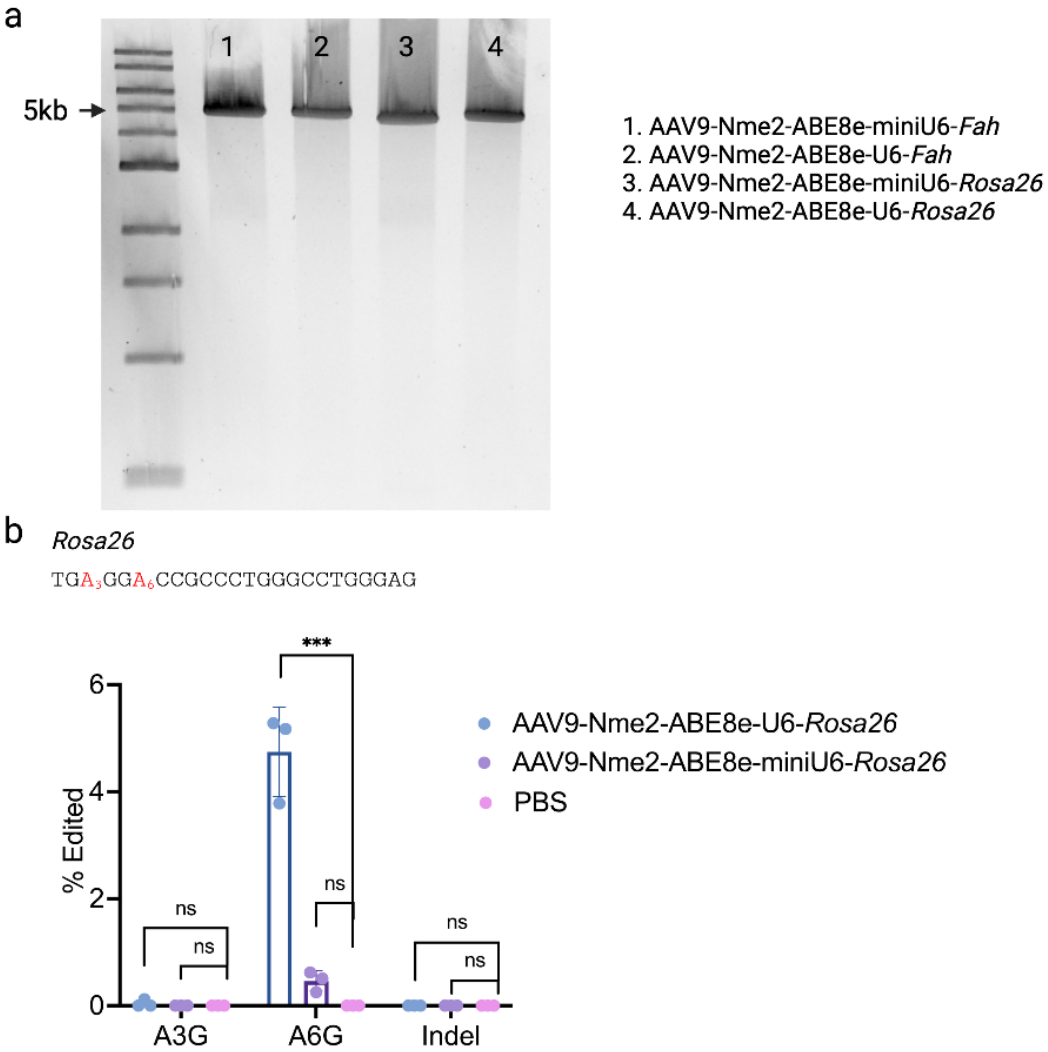
Supplementary Figure 2

Summary of the individual A-to-G conversion efficiency and the indel efficiency (bottom) at 12 target sites for Nme2-ABE8e, which includes 8 dual-target sites (DS 2 - 28) and 4 Nme2Cas9-specific target sites (Nme2 1 - 4), and 8 dual-target sites for Spy-ABE7.10 and Spy-ABE8e. Each data point represents the A-to-G conversion efficiency at the indicated nucleotide position measured by amplicon deep sequencing (n = 3 biological replicates).



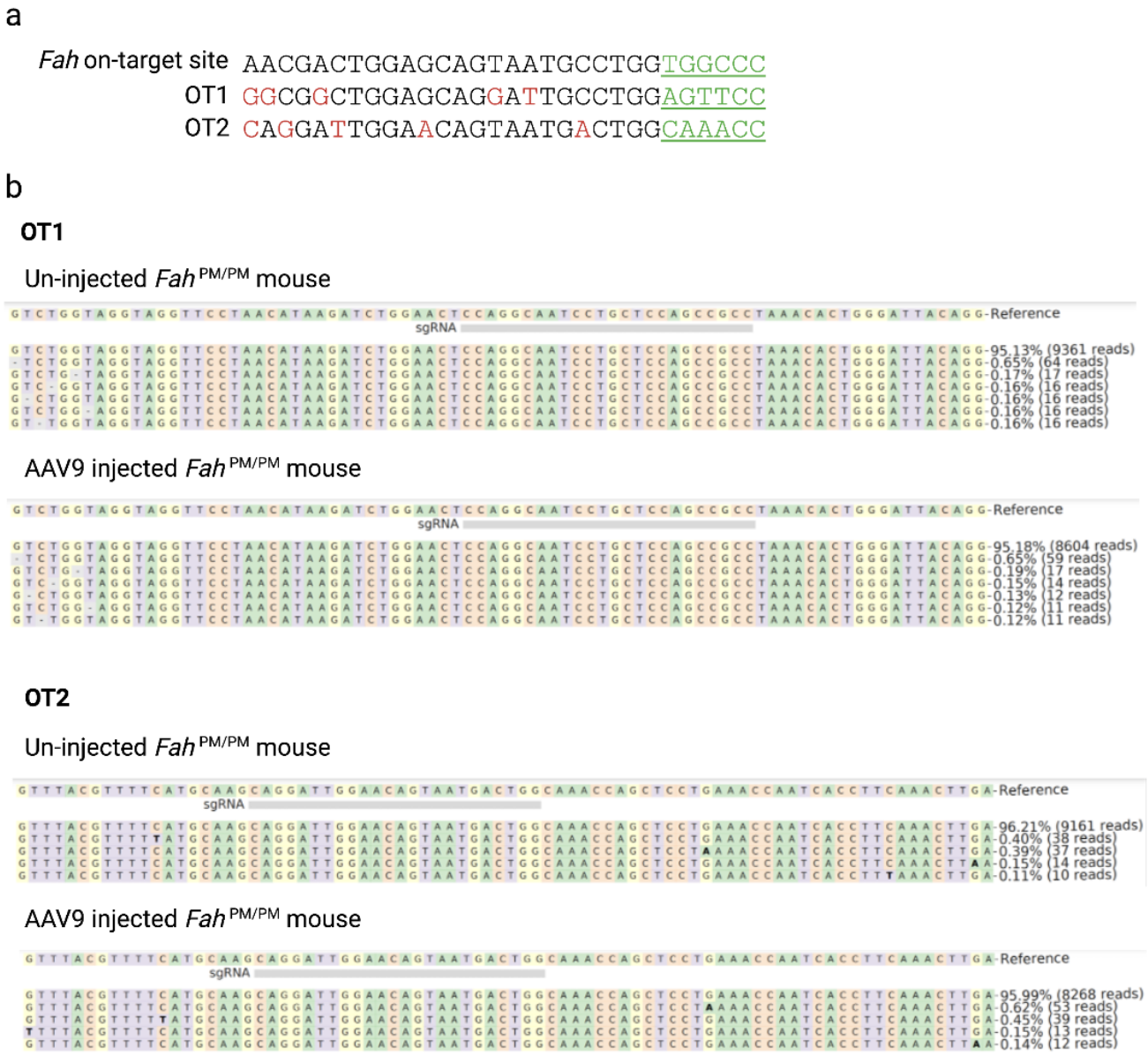
Supplementary Figure 3

a). Anti-CRISPR proteins inhibit the activity of either Spy-ABE7.10 or Nme2-ABE8e in the ABE reporter cell line as a function of their previously defined nuclease specificity (n = 2 biological replicates). **b).** Editing efficiency at the A12 (the highest edited adenine within the target) of the DS12 site by Nme2-ABE8e controlled by microRNA-repressible anti-CRIPSR proteins. Data generated by Sanger sequencing and EditR analysis (n = 2 biological replicates). Data represent mean ± SD; ns, P > 0.05; *, P < 0.05; **, P < 0.01; ****, P < 0.0001 (one-way ANOVA).



Supplementary Figure 4

a). Alkaline gel electrophoresis of AAV9 genomic DNA. **b).** Quantification of the editing efficiency at the *Rosa26* locus by amplicon deep sequencing using mouse liver injected with indicated AAV (n = 3 mice per group). Data represent mean ± SD; ns, P > 0.05 ***; P < 0.001 (one-way ANOVA).



Supplementary Figure 5

a). Sequence of the *Fah* on-target site and two top-rated Cas-OFFinder predicted off-target sites for Nme2-ABE8e. Bases that are different from the on-target site are labeled in red. PAM, green, underlined. **b).** Representative amplicon deep sequencing reads at the predicted off-target sites in mice injected with AAV9 expressing Nme2-ABE8e and sgRNA-*Fah*.

Supplementary note

Nucleotide sequences of plasmids

Nme2-ABE7.10 construct in **Figure 1**

Legend: CMV enhancer, CMV promoter, NLS, **TadA**, linker, TadA7.10, Nme2Cas9-D16A, 3xHA, poly-A signal

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Nme2-ABE8e construct in **Figure 1**

Legend: CMV enhancer, CMV promoter, NLS, TadA8e, linker, Nme2Cas9-D16A, 3xHA, poly-A signal

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Spy-ABE7.10 construct in **Figure 1**

Legend: CMV enhancer, CMV promoter, NLS, **TadA**, linker, TadA7.10, SpyCas9-D10A, 3xHA, poly-A signal

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TGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGG
CATGCTGGGGATGCGGTGGGCTCTATGG

Spy-ABE8e construct in **Figure 1**

Legend: CMV enhancer, CMV promoter, NLS, TadA8e, linker, SpyCas9-D10A, 3xHA, poly-A signal

GACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTA
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GTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCAT
GCTGGGGATGCGGTGGGCTCTATGG

U6-sgRNA construct for Nme2-ABE in **Figure 1**
Legend: U6 promoter, **guide sequence**, guide RNA scaffold, U6 termination signal

GAGGGCCTATTTCCCATGATTCCTTCATATTTGCATATACGATACAAGGCTGTTAGAGAGATAATTGGAATTAATTTGACT
GTAAACACAAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGT
TTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGAC**NN**
NNNNNNNNNNNNNNNNNNNNNNGTTGTAGCTCCCTTTCTCATTTCGGAAACGAAATGAGAACCGTTGCTACAATAAG
GCCGTCTGAAAAGATGTGCCGCAACGCTCTGCCCCTTAAAGCTTCTGCTTTAAGGGGCATCGTTTATTTTTTT

U6-sgRNA construct for Spy-ABE in **Figure 1**
Legend: U6 promoter**, guide sequence**, guide RNA scaffold, U6 termination signal
GAGGGCCTATTTCCCATGATTCCTTCATATTTGCATATACGATACAAGGCTGTTAGAGAGATAATTGGAATTAATTTGACT
GTAAACACAAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGT
TTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGAC**NN**
NNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAG
TGGCACCGAGTCGGTGCTTTTTTT

Single-AAV vector with 2xcMyc NLS in **Figure 3a**
Legend: ITR, U1a promoter, cMyc NLS, TadA8e, linker, Nme2Cas9-D16A, short poly-A signal, U6 promoter, **guide sequence**, guide RNA scaffold, U6 termination signal
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GTTTCGGGAGCTACAAC**NNNNNNNNNNNNNNNNNNNNNNNNNN**GGTGTTTCGTCCTTTCCACAAGATATATAAAGCCA
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gg

Single-AAV vector with 3xcMyc NLS in **Figure 3a**

Legend: ITR, U1a promoter, cMyc NLS, TadA8e, linker, Nme2Cas9-D16A, short poly-A signal, U6 promoter, **guide sequence**, guide RNA scaffold, U6 termination signal

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TAAACGATGCCCCTTAAAGCAGAAGCTTTAAGGGGCAGAGCGTTGCGGCACATCTTTTCAGACGGCCTTATTGTAGCAAC
GTTTCGGGAGCTACAAC**NNNNNNNNNNNNNNNNNNNNNNNNN**GGTGTTTCGTCCTTTCCACAAGATATATAAAGCCA
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Single-AAV vector with Ty1 NLS in **Figure 3a**

Legend: ITR, U1a promoter, Ty1 NLS, TadA8e, linker, Nme2Cas9-D16A, short poly-A signal, U6 promoter, **guide sequence**, guide RNA scaffold, U6 termination signal

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TAAACGATGCCCCTTAAAGCAGAAGCTTTAAGGGGCAGAGCGTTGCGGCACATCTTTTCAGACGGCCTTATTGTAGCAAC
GTTTCGGGAGCTACAAC**NNNNNNNNNNNNNNNNNNNNNNNNN**GGTGTTTCGTCCTTTCCACAAGATATATAAAGCCA
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Single-AAV vector with 2xBP_SV40 NLS in **Figure 2d, 3a, and Supplementary Figure 3**
Legend: ITR, U1a promoter, BP_SV40 NLS, TadA8e, linker, Nme2Cas9-D16A, short poly-A signal, U6 promoter, **guide sequence**, guide RNA scaffold, U6 termination signal

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GTTTCGGGAGCTACAAC**NNNNNNNNNNNNNNNNNNNNNNNNNN**GGTGTTTCGTCCTTTCCACAAGATATATAAAGCCA
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Single-AAV vector with miniU6 promoter in **Figure 3c**
Legend: ITR, U1a promoter, BP_SV40 NLS, TadA8e, linker, Nme2Cas9-D16A, short poly-A signal, miniU6 promoter, **guide sequence**, guide RNA scaffold, U6 termination signal

Cctgcaggcagctgcgcgctcgctcgctcactgaggccgcccgggcaaagcccgggcgtcgggcgacctttggtcgcccggcctcagtgagcgagcgagcgc
gcagagagggagtggccaactccatcactaggggttcctgcggcctctagaGTTTAAACAAAAAAA
TAAACGATGCCCCTTAAAGCAGAAGCTTTAAGGGGCAGAGCGTTGCGGCACATCTTTTCAGACGGCCTTATTGTAGCAAC
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TATTTTCATTAGATCTGTGTGTTGGTTTTTTGTGTAAGCgcggccgcaggaacccctagtgatggagttggccactccctctctgcgcgctcgct
cgctcactgaggccgggcgaccaaaggtcgcccgacgcccgggctttgcccgggcggcctcagtgagcgagcgagcgcgcagctgcctgcagg

AcrIIA4 plasmid in **Supplementary Figure 3**
Legend: CMV IE94 promoter, AcrIIA4, SV40 polyA signal,
GACCATAGCCAATTCAATATGGCGTATATGGACTCATGCCAATTCAATATGGTGGATCTGGACCTGTGCCAATTCAATATG
GCGTATATGGACTCGTGCCAATTCAATATGGTGGATCTGGACCCCAGCCAATTCAATATGGCGGACTTGGCACCATGCCA
ATTCAATATGGCGGACTTGGCACTGTGCCAACTGGGGAGGGGTCTACTTGGCACGGTGCCAAGTTTGAGGAGGGGTCTT
GGCCCTGTGCCAAGTCCGCCATATTGAATTGGCATGGTGCCAATAATGGCGGCCATATTGGCTATATGCCAGGATCAATA
TATAGGCAATATCCAATATGGCCCTATGCCAATATGGCTATTGGCCAGGTTCAATACTATGTATTGGCCCTATGCCATATA
GTATTCCATATATGGGTTTTCCTATTGACGTAGATAGCCCCTCCCAATGGGCGGTCCCATATACCATATATGGGGCTTCCT
AATACCGCCCATAGCCACTCCCCCATTGACGTCAATGGTCTCTATATATGGTCTTTCCTATTGACGTCATATGGGCGGTCCT
ATTGACGTATATGGCGCCTCCCCCATTGACGTCAATTACGGTAAATGGCCCGCCTGGCTCAATGCCCATTGACGTCAATAG
GACCACCCACCATTGACGTCAATGGGATGGCTCATTGCCCATTCATATCCGTTCTCACGCCCCCTATTGACGTCAATGACG
GTAAATGGCCCACTTGGCAGTACATCAATATCTATTAATAGTAACTTGGCAAGTACATTACTATTGGAAGGACGCCAGGG
TACATTGGCAGTACTCCCATTGACGTCAATGGCGGTAAATGGCCCGCGATGGCTGCCAAGTACATCCCCATTGACGTCAA
TGGGGAGGGGCAATGACGCAAATGGGCGTTCCATTGACGTAAATGGGCGGTAGGCGTGCCTAATGGGAGGTCTATATA
AGCAATGCTCGTTTAGGGAACATGAACATTAACGACCTCATACGAGAGATTAAGAACAAAGATTACACCGTCAAACTGTC
AGGAACTGATAGTAACTCAATCACCCAGCTTATTATCAGGGTAAACAATGATGGGAATGAATATGTGATATCTGAGAGCG
AAAACGAGTCTATCGTCGAGAAATTCATTTCCGCTTTTAAGAACGGGTGGAATCAGGAATATGAGGATGAAGAAGAATTT
TACAATGACATGCAGACGATCACGTTGAAAAGTGAACTGAACTAACTCGAGCCTCTAGAACTATAGTGAGTCGTATTACG
TAGATCCAGACATGATAAGATACATTGATGAGTTTGGACAAACCACAACTAGAATGCAGTGAAAAAAATGCTTTATTTGT
GAAATTTGTGATGCTATTGCTTTATTTGTAACCATTATAAGCTGCAATAAACAAGTT

AcrIIC4-MRE122 plasmid in **Supplementary Figure 3b**
Legend: ITR, CMV enhancer, CB promoter, SV40 interon, AcrIIC4, FLAG, SV40 NLS, 3 x MRE122, BGh polyA, U6 promoter, **guide sequence**, guide RNA scaffold, U6 termination signal
ctgcgcgctcgctcgctcactgaggccgcccgggcaaagcccgggcgtcgggcgacctttggtcgcccggcctcagtgagcgagcgagcgcgcagagaggga
gtgtagccatgctctaggaagatcaattcggtacaattcagctagcGTTTAAACAAAAAAATAAACGATGCCCCTTAAAGCAGAAGCTTTA
AGGGGCAGAGCGTTGCGGCACATCTTTTCAGACGGCCTTATTGTAGCAACGTTTCGGGAGCTACAAC**NNNNNNNNNNNN**
NNNNNNNNNNggtgtttcgtcctttccacaagatatataaagccaagaaatcgaaatactttcaagttacggtaagcatatgatagtccattttaaaaca
taattttaaaactgcaaactacccaagaaattattactttctacgtcacgtattttgtactaatatctttgtgtttacagtcaaattaattctaattatctctctaaca
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GCGCGGTTGAACAGTTTAGCAGCGCGCGTGACTTCTTTAAGAACGAGAACTACAGCAAAGAGCTGGCGGAAAAGTTCAA
CAAAGAAGCGGTGAACGAGGCGGTTGAAAAGCTGCAAAAAGCGATCGATCTGGCGGAAAAACAGGGCATTCAATTTggc
ggggattataaggatgatgatgataaggctccCCTAAGAAGAAGAGAAAGGTGTGATAAAGCGGCCATCAAGCTtGACAAACACC

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gcatcgcattgtctgagtaggtgtcattctattctggggggtggggtggggcaggacagcaagggggaggattgggaagacaattaggtagataagtagcatg
gcgggttaatcattaactacaaggaacccctagtgatggagttggccactccctctctgcgcgctcgctcgctcactgaggccgggcgaccaaaggtcgcccga
cgcccgggctttgcccgggcggcctcagtgagcgagcgagcgcgcag