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NmeCas9 is an intrinsically high-fidelity genome editing platform

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Running title: Intrinsically precise genome editing by NmeCas9

47   **ABSTRACT**

48

49   **Background:** The development of CRISPR genome editing has transformed biomedical research. Most

50   applications reported thus far rely upon the Cas9 protein from *Streptococcus pyogenes* SF370 (SpyCas9). With

51   many RNA guides, wild-type SpyCas9 can induce significant levels of unintended mutations at near-

52   cognate sites, necessitating substantial efforts toward the development of strategies to minimize off-target

53   activity. Although the genome-editing potential of thousands of other Cas9 orthologs remains largely

54   untapped, it is not known how many will require similarly extensive engineering to achieve single-site

55   accuracy within large (e.g. mammalian) genomes. In addition to its off-targeting propensity, SpyCas9 is

56   encoded by a relatively large (~4.2 kb) open reading frame, limiting its utility in applications that require

57   size-restricted delivery strategies such as adeno-associated virus vectors. In contrast, some genome-editing-

58   validated Cas9 orthologs (e.g. from *Staphylococcus aureus*, *Campylobacter jejuni*, *Geobacillus stearothermophilus* and

59   *Neisseria meningitidis*) are considerably smaller and therefore better suited for viral delivery.

60   **Results:** Here we show that wild-type NmeCas9, when programmed with guide sequences of natural

61   length (24 nucleotides), exhibits a nearly complete absence of unintended editing in human cells, even

62   when targeting sites that are prone to off-target activity with wildtype SpyCas9. We also validate at least

63   six variant protospacer adjacent motifs (PAMs), in addition to the preferred consensus PAM (5'-N_4GATT-

64   3'), for NmeCas9 genome editing in human cells.

65   **Conclusions:** Our results show that NmeCas9 is a naturally high-fidelity genome editing enzyme and

66   suggest that additional Cas9 orthologs may prove to exhibit similarly high accuracy, even without

67   extensive engineering.

68

69   **Keywords:** Cas9; CRISPR; sgRNA; Protospacer adjacent motif; off-target; *Neisseria meningitidis*

70

71 **BACKGROUND**

72

73   Over the past decade, clustered, regularly interspaced, short palindromic repeats (CRISPRs) have

74   been revealed as genomic sources of small RNAs (CRISPR RNAs, crRNAs) that specify genetic

75   interference in many bacteria and most archaea [1-3]. CRISPR sequences include "spacers," which often

76   match sequences of previously encountered invasive nucleic acids such as phage genomes and plasmids.

77   In conjunction with CRISPR-associated (Cas) proteins, crRNAs recognize target nucleic acids (DNA,

78   RNA, or both, depending on the system) by base pairing, leading to their destruction. The primary

79   natural function of CRISPR-Cas systems is to provide adaptive immunity against phages [4, 5] and other

80   mobile genetic elements [6]. CRISPR-Cas systems are divided into two main classes: Class 1, with large,

81   multi-subunit effector complexes, and Class 2, with single-protein-subunit effectors [7]. Both CRISPR-

82   Cas classes include multiple types based primarily on the identity of a signature effector protein. Within

83   Class 2, the Type II systems are the most abundant and the best characterized. The interference function

84   of Type II CRISPR-Cas systems requires the Cas9 protein, the crRNA, and a separate non-coding RNA

85   known as the trans-activating crRNA (tracrRNA) [8-10]. Successful interference also requires that the

86   DNA target (the "protospacer") be highly complementary to the spacer portion of the crRNA, and that

87   the PAM consensus be present at neighboring base pairs [11, 12].

88   Following the discovery that Type II interference occurs via double-strand breaks (DSBs) in the

89   DNA target [9], the Cas9 protein was shown to be the only Cas protein required for Type II interference,

90   to be manually reprogrammable via engineered CRISPR spacers, and to be functionally portable between

91   species that diverged billions of years ago [10]. Biochemical analyses with purified Cas9 confirmed its role

92   as a crRNA-guided, programmable nuclease that induces R-loop formation between the crRNA and one

93   dsDNA strand, and that cleaves the crRNA-complementary and noncomplementary strands with its

94   HNH and RuvC domains, respectively [13, 14]. *In vitro* cleavage reactions also showed that the tracrRNA

95   is essential for DNA cleavage activity, and that the naturally separate crRNA and tracrRNA could retain

96   function when fused into a single-guide RNA (sgRNA) [14]. Several independent reports then showed

3

97   that the established DSB-inducing activity of Cas9 could be elicited not only *in vitro* but also in living cells,

98   both bacterial [15] and eukaryotic [16-20]. As with earlier DSB-inducing systems [21], cellular repair of

99   Cas9-generated DSBs by either non-homologous end joining (NHEJ) or homology-directed repair (HDR)

100   enabled live-cell targeted mutagenesis, and the CRISPR-Cas9 system has now been widely adopted as a

101   facile genome-editing platform in a wide range of organisms [22-24]. In addition to genome editing,

102   catalytically inactivated Cas9 ("dead" Cas9, dCas9) retains its sgRNA-guided DNA binding function,

103   enabling fused or tethered functionalities to be delivered to precise genomic loci [25, 26]. Similar RNA-

104   guided tools for genome manipulations have since been developed from Type V CRISPR-Cas systems

105   that use the Cas12a (formerly Cpf1) enzyme [27].

106   Type II CRISPR-Cas systems are currently grouped into three subtypes (II-A, II-B and II-C) [7,

107   28]. The vast majority of Cas9 characterization has been done on a single Type II-A ortholog, SpyCas9,

108   in part due to its consistently high genome editing activity. SpyCas9's sgRNAs typically contain a 20-nt

109   guide sequence (the spacer-derived sequence that base pairs to the DNA target [8, 14]). The PAM

110   requirement for SpyCas9 is 5'-NGG-3' (or, less favorably, 5'-NAG-3'), after the 3' end of the

111   protospacer's crRNA-noncomplementary strand [8, 14]. Based on these and other parameters, many

112   sgRNAs directed against potentially targetable sites in a large eukaryotic genome also have near-cognate

113   sites available to it that lead to unintended, "off-target" editing. Indeed, off-target activity by SpyCas9 has

114   been well-documented with many sgRNA-target combinations [29, 30], prompting the development of

115   numerous approaches to limit editing activity at unwanted sites [31-36]. Although these strategies have

116   been shown to minimize off-targeting to various degrees, they do not always abolish it, and they can also

117   reduce on-target activity, at least with some sgRNAs. Furthermore, each of these approaches has required

118   extensive testing, validation, and optimization, and in some cases [33, 37, 38] depended heavily upon

119   prior high-resolution structural characterization [39-42].

120   Thousands of other Cas9 orthologs have been documented [7, 28, 43, 44], providing tremendous

121   untapped potential for additional genome editing capabilities beyond those offered by SpyCas9. Many

122   Cas9 orthologs will provide distinct PAM specificities, increasing the number of targetable sites in any

4

123   given genome. Many pair-wise Cas9 combinations also have orthogonal guides that load into one

124   ortholog but not the other, facilitating multiplexed applications [44-46]. Finally, some Cas9 orthologs

125   (especially those from subtype II-C) are hundreds of amino acids smaller than the 1,368 amino acid

126   SpyCas9 [7, 43, 44], and are therefore more amenable to combined Cas9/sgRNA delivery via a single

127   size-restricted vector such as adeno-associated virus (AAV) [47, 48]. Finally, there may be Cas9 orthologs

128   that exhibit additional advantages such as greater efficiency, natural hyper-accuracy, distinct activities,

129   reduced immunogenicity, or novel means of control over editing. Deeper exploration of the Cas9

130   population could therefore enable expanded or improved genome engineering capabilities.

131   We have used *N. meningitidis* (strain 8013) as a model system for the interference functions and

132   mechanisms of Type II-C CRISPR-Cas systems [49-52]. In addition, we and others previously reported

133   that the Type II-C Cas9 ortholog from *N. meningitidis* (NmeCas9) can be applied as a genome engineering

134   platform [46, 53, 54]. At 1,082 amino acids, NmeCas9 is 286 residues smaller than SpyCas9, making it

135   nearly as compact as SauCas9 (1,053 amino acids) and well within range of all-in-one AAV delivery. Its

136   spacer-derived guide sequences are longer (24 nts) than those of most other Cas9 orthologs [51], and like

137   SpyCas9, it cleaves both DNA strands between the third and fourth nucleotides of the protospacer

138   (counting from the PAM-proximal end). NmeCas9 also has a longer PAM consensus (5'-N_4GATT-3',

139   after the 3' end of the protospacer's crRNA-noncomplementary strand) [44, 46, 51-54], leading to a lower

140   density of targetable sites compared to SpyCas9. Considerable variation from this consensus is permitted

141   during bacterial interference [46, 52], and a smaller number of variant PAMs can also support targeting

142   in mammalian cells [53, 54]. Unlike SpyCas9, NmeCas9 has been found to cleave the DNA strand of

143   RNA-DNA hybrid duplexes in a PAM-independent fashion [52, 55], and can also catalyze PAM-

144   independent, spacer-directed cleavage of RNA [56]. Recently, natural Cas9 inhibitors (encoded by

145   bacterial mobile elements) have been identified and validated in *N. meningitidis* and other bacteria with

146   type II-C systems, providing for genetically encodable off-switches for NmeCas9 genome editing [57, 58].

147   These "anti-CRISPR" (Acr) proteins [59] enable temporal, spatial, or conditional control over the

148   NmeCas9 system. Natural inhibitors of Type II-A systems have also been discovered in *Listeria*

5

149   *monocytogenes* [60] and *Streptococcus thermophilus* [61], some of which are effective at inhibiting SpyCas9.

150   The longer PAM consensus, longer guide sequence, or enzymological properties of NmeCas9

151   could result in a reduced propensity for off-targeting, and targeted deep sequencing at bioinformatically

152   predicted near-cognate sites is consistent with this possibility [54]. A high degree of genome-wide

153   specificity has also been noted for the dNmeCas9 platform [62]. However, the true, unbiased accuracy of

154   NmeCas9 is not known, since empirical assessments of genome-wide off-target editing activity

155   (independent of bioinformatics prediction) have not been reported for this ortholog. Here we define and

156   confirm many of the parameters of NmeCas9 editing activity in mammalian cells including PAM

157   sequence preferences, guide length limitations, and off-target profiles. Most notably, we use two empirical

158   approaches (GUIDE-seq [63] and SITE-Seq [64] to define NmeCas9 off-target profiles and find that

159   wild-type NmeCas9 is a high-fidelity genome editing platform in mammalian cells, with far lower levels of

160   off-targeting than wild-type SpyCas9. These results further validate NmeCas9 as a genome engineering

161   platform, and suggest that continued exploration of Cas9 orthologs could identify additional RNA-guided

162   nucleases that exhibit favorable properties, even without the extensive engineering efforts that have been

163   applied to SpyCas9 [31, 34, 35].

164

165   **RESULTS**

166

167   **Co-expressed sgRNA increases NmeCas9 accumulation in mammalian cells**

168   Previously we demonstrated that NmeCas9 (derived from *N. meningitidis* strain 8013 [51]) can

169   efficiently edit chromosomal loci in human stem cells using either dual RNAs (crRNA + tracrRNA) or a

170   sgRNA [53]. To further define the efficacy and requirements of NmeCas9 in mammalian cells, we first

171   constructed an all-in-one plasmid (pEJS15) that delivers both NmeCas9 protein and a sgRNA in a single

172   transfection vector, similar to our previous all-in-one dual-RNA plasmid (pSimple-Cas9-Tracr-crRNA;

173   Addgene #47868) [53]. The pEJS15 plasmid expresses NmeCas9 fused to a C-terminal single-HA epitope

174   tag and nuclear localization signal (NLS) sequences at both N- and C-termini under the control of the

6

175   elongation factor-1α (EF1α) promoter. The sgRNA cassette (driven by the U6 promoter) includes two

176   *BsmB*I restriction sites that are used to clone a spacer of interest from short, synthetic oligonucleotide

177   duplexes. First, we cloned three different bacterial spacers (spacers 9, 24 and 25) from the endogenous *N.*

178   *meningitidis* CRISPR locus (strain 8013) [51, 52] to express sgRNAs that target protospacer (ps) 9, ps24 or

179   ps25, respectively (Supplemental Fig. 1A). None of these protospacers have cognate targets in the human

180   genome. We also cloned a spacer sequence to target an endogenous genomic N̲meCas9 t̲arget s̲ite (NTS)

181   from chromosome 10 that we called N-TS3 (Table 1). Two of the resulting all-in-one plasmids

Site	Gene or locus	Spacer Sequence of sgRNA	Target site, with PAM
NTS1B	*SLC9A9*	GGGCAUCAUGAUUUUGAACUCCCU	CCTTGGCATCATGATTTTGAACTCCCTATGTGATTCTA
NTS1C	*SLC9A9*	GUGGUCUGGGGUACAGCCUUGGCA	TACTUGGTCTGGGGTACAGCCTTGGCATCATGATTTTG
NTS1C–OT1	*PHKG2*	GCGGUGUGAGGUACAGCCUUGGCA	TAATCGGTGTGAGGTACAGCCTTGGCATCAGGATTTCT
NTS3	*AL158836*	GAUGCUCAGAAAGAGGAAGCUGGU	GGGGATGCTCAGAAAGAGGAAGCTGGTTTATGATTGGA
NTS4B	*FLJ00328*	GGACAGGAGUCGCCAGAGGCCGGU	GCAGGACAGGAGTCGCCAGAGGCCGGTGGTGGATTTCC
NTS4C	*FLJ00328*	GGGGCUGGCUCCACGUCGCGCCGC	TGCGGGGCTGGCTCCACGTCGCGCCGCGGCGGTTTGGG
NTS5	*AF064860*	GAAACAGACUCGCAAGACUUCAGA	GACAAAACAGACTCGCAAGACTTCAGATACAGATTCCA
NTS7	*LOC100505797*	GAGGGAGAGAGGUGAGCGGAUGAA	GCAAAGGGAGAGAGGTGAGCGGATGAAGGGAGATTGGT
NTS8	*ESPN*	GGACGCAAUUCCAGAGGUGAUGGG	CGGCGACGCAATTCCAGAGGTGATGGGGAGTGATTGTC
NTS9	*ZNHIT2*	GGCGCUGUGUUUUCGCAAAGCUUC	CGGCGCGCTGTGTTTTCGCAAAGCTTCCGAGGATTCTC
NTS10	*HHLA1*	GCAGCCAAGUUUGAGAACUGCUGU	TGTGCAGCCAAGTTTGAGAACTGCTGTTACAGATTTCC
NTS11	*SMARCB1*	GUUCCAGUUGGGAAGGGCCAGUGC	TAGATTCCAGTTGGGAAGGGCCAGTGCCTCCGATTCCA
NTS21	*TNNC1*	GCCAGAGCUGCCGCCAGACAGUGA	CAGTCCAGAGCTGCCGCCAGACAGTGATGCTGTCTTGG
NTS25	*AC193513*	GGUUUCUCAUCCUGUCUUCUGCCU	CCGCGTTTCTCATCCTGTCTTCTGCCTAGTGGATATGT
NTS26	*LOC105378512*	GUUCAAAAGUAGCGGGCGCUAGGC	GTACTTCAAAAGTAGCGGGCGCTAGGCGGGTGTTTCTG
NTS27	*TIE1*	GUUCUCCAAGCCCUCGGACCUCGU	CGGCTTCTCCAAGCCCTCGGACCTCGTGGGCGTCTTCT
NTS30	*NEK8*	GGGGCUCCGGAGCCCACCCCAGGA	CGCGGGGCTCCGGAGCCCACCCCAGGACCAGGACTTAG
NTS31	*POC1A*	GUGGGAAGUGUAGCUCCACCUUCC	ATGTTGGGAAGTGTAGCTCCACCTTCCTCCAGACATAG
NTS32	*VEGFA*	GCCCCGGCUCUGGCUAAAGAGGGA	CACACCCCGGCTCTGGCTAAAGAGGGAATGGGCTTTGG
NTS33	*VEGFA*	GCGGGGAGAAGGCCAGGGGUCACU	GGAGCGGGGAGAAGGCCAGGGGTCACTCCAGGATTCCA
NTS55	*CYBB*	GCUGGAUUACUGUGUGGUAGAGGG	CTAGCTGGATTACTGTGTGGTAGAGGGAGGTGATTAGC
NTS58	*AAVS1*	GUUUGCCUGGACACCCCGUUCUCC	TTTCTTTGCCTGGACACCCCGTTCTCCTGTGGATTCGG
NTS59	*AAVS1*	GACCCCACAGUGGGGCCACUAGGG	CTCCACCCCACAGTGGGGCCACTAGGGACAGGATTGGT
STS60	*AAVS1*	GUUAAUGUGGCUCUGGUUCU	CCGGTTAATGTGGCTCTGGTTCTGGGTAC
STS61	*AAVS1*	GUCCCCUCCACCCCACAGUG	TCTGTCCCCTCCACCCCACAGTGGGGCCA
STS62	*AAVS1*	GGGGCCACUAGGGACAGGAU	AGTGGGGCCACTAGGGACAGGATTGGTGA
NTS63	*AAVS1*	GAGUUAGAACUCAGGACCAACUUA	CCAAAGTTAGAACTCAGGACCAACTTATTCTGATTTTG
NTS64	*Rosa26*	GGCAGAUCACGAGGGAAGAGGGGG	AGTTGCAGATCACGAGGGAAGAGGGGGAAGGGATTCTC

182   **Table 1.** NmeCas9 or SpyCas9 guide and target sequences used in this study. NTS, NmeCas9 target site; STS,
183   SpyCas9 target site. The sgRNA spacer sequences (5'→3') are shown with their canonical lengths, and with a 5'-
184   terminal G residue; non-canonical lengths are described in the text and figures. Target site sequences are also 5'→3'
185   and correspond to the DNA strand that is non-complementary to the sgRNA, with PAM sequences underlined.
186
187   (spacer9/sgRNA and N-TS3/sgRNA), as well as a plasmid lacking the sgRNA cassette, were transiently

188   transfected into HEK293T cells for 48 hours, and NmeCas9 expression was assessed by anti-HA western

189   blot (Fig. 1A). As a positive control we also included a sample transfected with a SpyCas9-expressing

190   plasmid (triple-HA epitope-tagged, and driven by the cytomegalovirus (CMV) promoter) [65] (Addgene

191   #69220). Full-length NmeCas9 was efficiently expressed in the presence of both sgRNAs (lanes 3 and 4).

192   However, the abundance of the protein was much lower in the absence of sgRNA (lane 2). A different

193   Type II-C Cas9 (CdiCas9) was shown to be dramatically stabilized by its cognate sgRNA when subjected

194   to proteolysis *in vitro* [55]; if similar resistance to proteolysis occurs with NmeCas9 upon sgRNA binding, it

195   could explain some or all of the sgRNA-dependent increase in cellular accumulation.

196

Efficient editing in mammalian cells by NmeCas9

198   To establish an efficient test system for NmeCas9 activity in mammalian cells, we used a co-

199   transfected fluorescent reporter carrying two truncated, partially overlapping GFP fragments that are

200   separated by a cloning site [66] into which we can insert target protospacers for NmeCas9. Cleavage

201   promotes a single-strand-annealing-based repair pathway that generates an intact GFP open reading

202   frame (ORF), leading to fluorescence [66] that can be scored after 48 hours by flow cytometry. We

203   generated reporters carrying three validated bacterial protospacers (ps9, ps24 and ps25, as described

204   above) [51, 52] for transient cotransfection into HEK293T cells along with the corresponding

205   NmeCas9/sgRNA constructs. Figure 1B shows that all three natural protospacers of NmeCas9 can be

206   edited in human cells and the efficiency of GFP induction was comparable to that observed with SpyCas9

207   (Fig. 1B).

208   Next, we reprogrammed NmeCas9 by replacing the bacterially-derived spacers with a series of

209   spacers designed to target eleven human chromosomal sites with an N_4GATT PAM (Table 1). These

210   sgRNAs induced insertion/deletion (indel) mutations at all sites tested, except NTS10 (Fig. 1C, lanes 23-

211   25), as determined by T7 Endonuclease 1 (T7E1) digestion (Fig. 1C). The editing efficiencies ranged from

212   5% for NTS1B site to 47% in the case of NTS33 (Fig. 1D), though T7E1 tends to underestimate the true

213   frequencies of indel formation [67]. These data confirm that NmeCas9 can induce, with variable

214   efficiency, edits at many genomic target sites in human cells. Furthermore, we demonstrated NmeCas9

8

215   genome editing in multiple cell lines and via distinct delivery modes. Nucleofection of NmeCas9

216   ribonucleoprotein (RNP) (loaded with an *in vitro*-transcribed sgRNA) led to indel formation at three sites in

217   K562 chronic myelogenous leukemia cells and in hTERT-immortalized human foreskin fibroblasts (gift

218   from Dr. Job Dekker) (Fig. 1E). In addition, mouse embryonic stem cells (mESCs) and HEK293T cells

219   were transduced with a lentivirus construct expressing NmeCas9. In these cells, transient transfection of

220   plasmids expressing a sgRNA led to genome editing (Fig. 1E). Collectively, our results show that

221   NmeCas9 can be used for genome editing in a range of human or mouse cell lines via plasmid

222   transfection, RNP delivery, or lentiviral transduction.

223

224   **Functionality of truncated sgRNAs with NmeCas9**

225   SpyCas9 can accommodate limited variation in the length of the guide region (normally 20

226   nucleotides) of its sgRNAs [68-71], and sgRNAs with modestly lengthened (22-nt) or shortened (17−18-nt)

227   guide regions can even enhance editing specificity by reducing editing at off-target sites by a greater

228   degree than they affect editing at the on-target site [68, 69]. To test the length dependence of the

229   NmeCas9 guide sequence (normally 24 nucleotides; [51]) during mammalian editing, we constructed a

230   series of sgRNAs containing 18, 19, 20, 21, 22, 23, and 24 nucleotides of complementarity to ps9 cloned

231   into the split-GFP reporter plasmid (Supplemental Fig. 1B). All designed guides started with two guanine

232   nucleotides (resulting in 1-2 positions of target non-complementarity at the very 5' end of the guide) to

233   facilitate transcription and to test the effects of extra 5'-terminal G residues, analogous to the SpyCas9

234   "GGN$_{20}$" sgRNAs [68]. We then measured the abilities of these sgRNAs to direct NmeCas9 cleavage of

235   the reporter in human cells. sgRNAs that have 20−23 nucleotides of target complementarity showed

236   activities comparable to the sgRNA with the natural 24 nucleotides of complementarity, whereas sgRNAs

237   containing 18 or 19 nucleotides of complementarity show lower activity (Fig. 2A).

238   We next used a native chromosomal target site (NTS33 in *VEGFA*, as in Figs. 1C and 1D) to test

239   the editing efficiency of NmeCas9 spacers of varying lengths (Supplemental Fig. 1C). sgRNA constructs

240   included one or two 5'-terminal guanine residues to enable transcription by the U6 promoter, sometimes

9

241   resulting in 1–2 nucleotides of target non-complementarity at the 5' end of the guide sequence. sgRNAs

242   with 20, 21, or 22 nucleotides of target complementarity (GGN_{18}, GGN_{19}, and GGN_{20}, respectively)

243   performed comparably to the natural guide length (24 nucleotides of complementarity, GN_{23}) at this site

244   (Fig. 2B-C), and within this range, the addition of 1–2 unpaired G residues at the 5' end had no adverse

245   effect. These results are consistent with the results obtained with the GFP reporter (Fig. 2A). sgRNAs with

246   guide lengths of 19 nucleotides or shorter, along with a single mismatch in the first or second position

247   (GGN_{17}, GGN_{16}, and GGN_{15}), did not direct detectable editing, nor did a sgRNA with perfectly matched

248   guide sequences of 17 or 14 nucleotides (GN_{16} and GN_{13}, respectively) (Fig. 2B-C). However, a 19-nt

249   guide with no mismatches (GN_{18}) successfully directed editing, albeit with slightly reduced efficiency.

250   These results indicate that 19–26-nt guides can be tolerated by NmeCas9, but that activity can be

251   compromised by guide truncations from the natural length of 24 nucleotides down to 17–18 nucleotides

252   and smaller, and that single mismatches (even at or near the 5'-terminus of the guide) can be

253   discriminated against with a 19-nt guide.

254   The target sites tested in Figs. 2A and 2B-C are both associated with a canonical N_4GATT PAM,

255   but efficient NmeCas9 editing at mammalian chromosomal sites associated with N_4GCTT [53] and other

256   variant PAMs [[54; also see below] has also been reported. To examine length dependence at a site with

257   a variant PAM, we varied guide sequence length at the N_4GCTT-associated NTS32 site (also in *VEGFA*).

258   In this experiment, each of the guides had two 5'-terminal G residues, accompanied by 1–2 terminal

259   mismatches with the target sequence (Supplemental Fig. 1D). At the NTS32 site, sgRNAs with 21–24

260   nucleotides of complementarity (GGN_{24}, GGN_{23}, GGN_{22}, and GGN_{21}) supported editing, but shorter

261   guides (GGN_{20}, GGN_{19}, and GGN_{18}) did not (Fig. 2D-E). We conclude that sgRNAs with 20 nucleotides

262   of complementarity can direct editing at some sites (Fig. 2B-C) but not all (Fig. 2D-E). It is possible that

263   this minor variation in length dependence can be affected by the presence of mismatched 5'-terminal G

264   residues in the sgRNA, the adherence of the target to the canonical N_4GATT PAM consensus, or both,

265   but the consistency of any such relationship will require functional tests at much larger numbers of sites.

266   Nonetheless, NmeCas9 guide truncations of 1–3 nucleotides appear to be functional in most cases, in

267   agreement with the results of others [54].

268

PAM specificity of NmeCas9 in human cells

270   During native CRISPR interference in bacterial cells, considerable variation in the N_4GATT

271   PAM consensus is tolerated: although the G1 residue ($N_4$$\underline{G}$ATT) is strictly required, virtually all other

272   single mutations at A2 (N_4G$\underline{A}$TT), T3 (N_4GA$\underline{T}$T), and T4 (N_4GAT$\underline{T}$) retain at least partial function in

273   licensing bacterial interference [46, 52]. In contrast, fewer NmeCas9 PAM variants have been validated

274   during genome editing in mammalian cells [53, 54]. To gain more insight into NmeCas9 PAM flexibility

275   and specificity in mammalian cells, and in the context of an otherwise identical target site and an invariant

276   sgRNA, we employed the split-GFP readout of cleavage activity described above. We introduced single-

277   nucleotide mutations at every position of the PAM sequence of ps9, as well as all double mutant

278   combinations of the four most permissive single mutants, and then measured the ability of NmeCas9 to

279   induce GFP fluorescence in transfected HEK293T cells. The results are shown in Fig. 3A. As expected,

280   mutation of the G1 residue to any other base reduced editing to background levels, as defined by the

281   control reporter that lacks a protospacer [(no ps), see Fig. 3A]. As for mutations at the A2, T3 and T4

282   positions, four single mutants (N_4GCTT, N_4GTTT, N_4GACT, and N_4GATA) and two double mutants

283   (N_4GTCT and N_4GACA) were edited with efficiencies approaching that observed with the N_4GATT

284   PAM. Two other single mutants (N_4GAGT and N_4GATG), and three double mutants (N_4GCCT,

285   N_4GCTA, and N_4GTTA) gave intermediate or low efficiencies, and the remaining mutants tested were at

286   or near background levels. We note that some of the minimally functional or non-functional PAMs (e.g.

287   N_4GAAT and N_4GATC) in this mammalian assay fit the functional consensus sequences defined

288   previously in *E. coli* [46].

289   We then used T7E1 analysis to validate genome editing at eight native chromosomal sites

290   associated with the most active PAM variants (N_4GCTT, N_4GTTT, N_4GACT, N_4GATA, N_4GTCT, and

291   N_4GACA). Our results with this set of targets indicate that all of these PAM variants tested except

292   N_4GACA support chromosomal editing (Fig. 3B and C).

11

293

Apo NmeCas9 is not genotoxic to mammalian cells

295   NmeCas9 and several other type II-C Cas9 orthologs have been shown to possess an RNA-

296   dependent ssDNA cleavage (DNase H) activity *in vitro* [52, 55]. R-loops (regions where an RNA strand

297   invades a DNA duplex to form a DNA:RNA hybrid, with the other DNA strand displaced) occur

298   naturally during transcription and other cellular processes [72]. Since DNase H activity is independent of

299   the tracrRNA or the PAM sequence, it is theoretically possible that it could degrade naturally-occurring

300   R-loops in living cells. Global degradation of R-loops in cells could result in an increase in DNA damage

301   detectable by increased γH2AX staining [73]. To test whether the DNase H activity of NmeCas9 could

302   lead to an increase in γH2AX, we transduced mouse embryonic stem cells E14 (mESCs) with lentiviral

303   plasmids expressing NmeCas9 and dNmeCas9 (which lacks DNase H activity; [52]). mESCs are ideal for

304   this purpose as R-loops have been extensively studied in these cells and have been shown to be important

305   for differentiation [74]. We performed γH2AX staining of these two cell lines and compared them to

306   wildtype E14 cells. As a positive control for γH2AX induction, we exposed wildtype E14 cells to UV, a

307   known stimulator of the global DNA damage response. Immunofluorescence microscopy of cells

308   expressing NmeCas9 or dNmeCas9 exhibited no increase in γH2AX foci compared to wildtype E14,

309   suggesting that sustained NmeCas9 expression is not genotoxic (Supplemental Fig. 2A). In contrast, cells

310   exposed to UV light showed a significant increase in γH2AX levels. Flow cytometric measurements of

311   γH2AX immunostaining confirmed these results (Supplemental Fig. 2B). These data suggest that

312   NmeCas9 expression does not lead to a global DNA damage response in mESCs.

313

Comparative analysis of NmeCas9 and SpyCas9

315   SpyCas9 is by far the best-characterized Cas9 orthologue, and is therefore the most informative

316   benchmark when defining the efficiency and accuracy of other Cas9s. To facilitate comparative

317   experiments between NmeCas9 and SpyCas9, we developed a matched Cas9 + sgRNA expression system

318   for the two orthologs. This serves to minimize the expression differences between the two Cas9s in our

319   comparative experiments, beyond those differences dictated by the sequence variations between the

320   orthologues themselves. To this end, we employed the separate pCSDest2-SpyCas9-NLS-3XHA-NLS

321   (Addgene #69220) and pLKO.1-puro-U6sgRNA-BfuA1 (Addgene #52628) plasmids reported previously

322   for the expression of SpyCas9 (driven by the CMV promoter) and its sgRNA (driven by the U6 promoter),

323   respectively [58, 65]. We then replaced the bacterially-derived SpyCas9 sequence (i.e., not including the

324   terminal fusions) with that of NmeCas9 in the CMV-driven expression plasmid. This yielded an

325   NmeCas9 expression vector (pEJS424) that is identical to that of the SpyCas9 expression vector in every

326   way (backbone, promoters, UTRs, poly(A) signals, terminal fusions, etc.) except for the Cas9 sequence

327   itself. Similarly, we replaced the SpyCas9 sgRNA cassette in pLKO.1-puro-U6sgRNA-BfuA1 with that of

328   the NmeCas9 sgRNA [46, 53], yielding the NmeCas9 sgRNA expression plasmid pEJS333. This matched

329   system facilitates direct comparisons of the two enzymes' accumulation and activity during editing

330   experiments. To assess relative expression levels of the identically-tagged Cas9 orthologs, the two plasmids

331   were transiently transfected into HEK293T cells for 48 hours, and the expression of the two proteins was

332   monitored by anti-HA western blot (Fig. 4A). Consistent with our previous data (Fig. 1A), analyses of

333   samples from identically transfected cells show that NmeCas9 accumulation is stronger when co-expressed

334   with its cognate sgRNA (Fig. 4A, compare lane 6 to 4 and 5), whereas SpyCas9 is not affected by the

335   presence of its sgRNA (lanes 1-3).

336   For an initial comparison of the cleavage efficiencies of the two Cas9s, we chose three previously

337   validated SpyCas9 guides targeting the *AAVS1* "safe harbor" locus [20, 75] and used the CRISPRseek

338   package [76] to design three NmeCas9 guides targeting the same locus within a region of ~700 base pairs

339   (Supplemental Fig. 3A). The matched Cas9/sgRNA expression systems described above were used for

340   transient transfection of HEK293T cells. T7E1 analysis showed that the editing efficiencies were

341   comparable, with the highest efficiency being observed when targeting the NTS59 site with NmeCas9

342   (Fig. 4B and Supplemental Fig. 3B).

343   To provide a direct comparison of editing efficiency between the SpyCas9 and NmeCas9 systems,

344   we took advantage of the non-overlapping PAMs of SpyCas9 and NmeCas9 (NGG and N_4GATT,

13

345   respectively). Because the optimal SpyCas9 and NmeCas9 PAMs are non-overlapping, it is simple to

346   identify chromosomal target sites that are compatible with both orthologues, i.e. that are dual target sites

347   (DTSs) with a composite PAM sequence of NGGNGATT that is preferred by both nucleases. In this

348   sequence context, both Cas9s will cleave the exact same internucleotide bond (N**N/N**NN<u>NGGNGATT</u>;

349   cleaved junction in bold, and PAM region underlined), and both Cas9s will have to contend with the

350   exact same sequence and chromatin structural context. Furthermore, if the target site contains a G residue

351   at position -24 of the sgRNA-noncomplementary strand (relative to the PAM) and another at position -20,

352   then the U6 promoter can be used to express perfectly-matched sgRNAs for both Cas9 orthologues. Four

353   DTSs with these characteristics were used in this comparison (Supplemental Fig. 4A). We had previously

354   used NmeCas9 to target a site (NTS7) that happened also to match the SpyCas9 PAM consensus, so we

355   included it in our comparative analysis as a fifth site, even though it has a predicted rG-dT wobble pair at

356   position -24 for the NmeCas9 sgRNA (Supplemental Fig. 4A).

357   We set out next to compare the editing activities of both Cas9 orthologs programmed to target the

358   five chromosomal sites depicted in Supplemental Fig. 4A, initially via T7E1 digestion. SpyCas9 was more

359   efficient than NmeCas9 at generating lesions at the DTS1 and DTS8 sites (Fig. 4C, lanes 1-2 and 13-14).

360   In contrast, NmeCas9 was more efficient than SpyCas9 at the DTS3 and NTS7 sites (Fig. 4C, lanes 5-6

361   and 17-18). Editing at DTS7 was approximately equal with both orthologs (Fig. 4C, lanes 9-10). Data

362   from three biological replicates of all five target sites are plotted in Fig. 4D. The remainder of our

363   comparative studies focused on DTS3, DTS7, and DTS8, as they provided examples of target sites with

364   NmeCas9 editing efficiencies that are greater than, equal to, or lower than those of SpyCas9, respectively.

365   At all three of these sites, the addition of an extra 5'-terminal G residue had little to no effect on editing by

366   either SpyCas9 or NmeCas9 (Supplemental Fig. 4B). Truncation of the three NmeCas9 guides down to

367   20 nucleotides (all perfectly matched) again had differential effects on editing efficiency from one site to

368   the next, with no reduction in DTS7 editing, partial reduction in DTS3 editing, and complete loss of

369   DTS8 editing (Supplemental Fig. 4B).

370

Assessing the genome-wide precision of NmeCas9 editing

371

372   All Cas9 orthologs described to date have some propensity to edit off-target sites lacking perfect

373 complementarity to the programmed guide RNA, and considerable effort has been devoted to developing

374 strategies (mostly with SpyCas9) to increase editing specificity (reviewed in [31, 34, 35]). In comparison

375 with SpyCas9, orthologs such as NmeCas9 that employ longer guide sequences and that require longer

376 PAMs have the potential for greater on-target specificity, possibly due in part to the lower density of near-

377 cognate sequences. As an initial step in exploring this possibility, we used CRISPRseek [76] to perform a

378 global analysis of potential NmeCas9 and SpyCas9 off-target sites with six or fewer mismatches in the

379 human genome, using sgRNAs specific for DTS3, DTS7 and DTS8 (Fig. 5A) as representative queries.

380 When allowing for permissive and semi-permissive PAMs (NGG, NGA, and NAG for SpyCas9;

381 N_4GHTT, N_4GACT, N_4GAYA, and N_4GTCT for NmeCas9), potential off-target sites for NmeCas9

382 were predicted with two to three orders of magnitude lower frequency than for SpyCas9 (Table 2).

383 Furthermore, NmeCas9 off-target sites with fewer than five mismatches were rare (two sites with four

Number of mismatches	SpyCas9 sites (NGG, NGA, NAG PAMs)			NmeCas9 sites (N_4GATT, N_4GCTT, N_4GTTT, N_4GACT, N_4GATA, N_4GTCT, N_4GACA PAMs)		
	DTS3	DTS7	DTS8	DTS3	DTS7	DTS8
1	0	0	0	0	0	0
2	4	2	2	0	0	0
3	45	52	60	0	0	0
4	680	500	772	0	2	0
5	6,691	4,116	7,325	4	5	25
6	45,897	26,474	52,547	17	61	129
Total	**53,317**	**31,144**	**60,706**	**21**	**68**	**154**

384 **Table 2.** Number of predicted near-cognate sites in the human genome for the three dual target sites (DTS3, DTS7
385 and DTS8) analyzed in this study. These potential off-target sites differ from the on-target site by six or fewer
386 mismatches, as listed on the left, and include the functional or semi-functional PAMs shown at the top.

387 mismatches) for DTS7, and non-existent for DTS3 and DTS8 (Table 2). Even when we relaxed the

388 NmeCas9 PAM requirement to N_4GN_3, which includes some PAMs that enable only background levels of

389 targeting (e.g. N_4GATC (Fig. 3A)), the vast majority of predicted off-target sites (>96%) for these three

390 guides had five or more mismatches, and none had fewer than four mismatches (Fig. 5A). In contrast, the

391 SpyCas9 guides targeting DTS3, DTS7, and DTS8 had 49, 54, and 62 predicted off-target sites with

15

392   three or fewer mismatches, respectively (Table 2). As speculated previously [53, 54], these bioinformatic

393   predictions suggest the intriguing possibility that the NmeCas9 genome editing system may induce very

394   few undesired mutations, or perhaps none, even when targeting sites that induce substantial off-targeting

395   with SpyCas9.

396   Although bioinformatic predictions of off-targeting can be useful, it is well established that off-

397   target profiles must be defined experimentally in a prediction-independent fashion due to our limited

398   understanding of target specificity determinants, and the corresponding inability of algorithms to predict

399   all possible sites successfully [31, 34, 35]. The need for empirical off-target profiling is especially acute

400   with Cas9 orthologs that are far less thoroughly characterized than SpyCas9. A previous report used PCR

401   amplification and high-throughput sequencing to detect the frequencies of lesions at 15-20 predicted

402   NmeCas9 off-target sites for each of three guides in human cells, and found only background levels of

403   indels in all cases, suggesting a very high degree of precision for NmeCas9 [54]. However, this report

404   restricted its analysis to candidate sites with N_4GNTT PAMs and three or fewer mismatches (or two

405   mismatches combined with a 1-nt bulge) in the PAM-proximal 19 nucleotides, leaving open the possibility

406   that legitimate off-target sites that did not fit these specific criteria remained unexamined. Accordingly,

407   empirical and minimally-biased off-target profiles have never been generated for any NmeCas9/sgRNA

408   combination, and the true off-target propensity of NmeCas9 therefore remains unknown. At the time we

409   began this work, multiple methods for prediction-independent detection of off-target sites had been

410   reported including GUIDE-seq, BLESS, Digenome-Seq, HTGTS, and IDLV capture, each with their

411   own advantages and disadvantages (reviewed in [31, 34, 35]); additional methods (SITE-Seq [64],

412   CIRCLE-seq [77], and BLISS [78]) have been reported more recently. Initially we chose to apply

413   GUIDE-seq [63], which takes advantage of oligonucleotide incorporation into double-strand break sites,

414   for defining the off-target profiles of both SpyCas9 and NmeCas9 when each is programmed to edit the

415   DTS3, DTS7 and DTS8 sites (Fig. 4C-D) in the human genome.

416   After confirming that the co-transfected double-stranded oligodeoxynucleotide (dsODN) was

417   incorporated efficiently at the DTS3, DTS7 and DTS8 sites during both NmeCas9 and SpyCas9 editing

16

418   (Supplemental Fig. 4C), we then prepared GUIDE-seq libraries for each of the six editing conditions, as

419   well as for the negative control conditions (i.e., in the absence of any sgRNA) for both Cas9 orthologs.

420   The GUIDE-seq libraries were then subjected to high-throughput sequencing, mapped, and analyzed as

421   described [79] (Fig. 5B-C). On-target editing with these guides was readily detected by this method, with

422   the number of independent reads ranging from a low of 167 (NmeCas9, DTS8) to a high of 1,834

423   (NmeCas9, DTS3) (Fig. 5C and Supplemental Table 2).

424   For our initial analyses, we scored candidate sites as true off-targets if they yielded two or more

425   independent reads and had six or fewer mismatches with the guide, with no constraints placed on the

426   PAM match at that site. For SpyCas9, two of the sgRNAs (targeting DTS3 and DTS7) induced

427   substantial numbers of off-target editing events (271 and 54 off-target sites, respectively (Fig. 5B)) under

428   these criteria. The majority of these SpyCas9 off-target sites (88% and 77% for DTS3 and DTS7,

429   respectively) were associated with a canonical NGG PAM. Reads were very abundant at many of these

430   loci, and at five off-target sites (all with the DTS3 sgRNA) even exceeded the number of on-target reads

431   (Fig. 5C). SpyCas9 was much more precise with the DTS8 sgRNA: we detected a single off-target site

432   with five mismatches and an NGG PAM, and it was associated with only three independent reads, far

433   lower than the 415 reads that we detected at the on-target site (Fig. 5C and Supplemental Table 2).

434   Overall, the range of editing accuracies that we measured empirically for SpyCas9 – very high (e.g.

435   DTS8), intermediate (e.g. DTS7), and poor (e.g. DTS3) – are consistent with the observations of other

436   reports using distinct guides (reviewed in [31, 34, 35]).

437   In striking contrast, GUIDE-seq analyses with NmeCas9, programmed with sgRNAs targeting

438   the exact same three sites, yielded off-target profiles that were exceptionally specific in all cases (Fig. 5B-

439   C). For DTS3 and DTS8 we found no reads at any site with six or fewer guide mismatches; for DTS7 we

440   found one off-target site with four mismatches (three of which were at the PAM-distal end; see

441   Supplemental Table 2), and even at this site there were only 12 independent reads, ~100x fewer than the

442   1,222 reads detected at DTS7 itself. This off-target site was also associated with a PAM (N_4GGCT) that

443   would be expected to be poorly functional, though it could also be considered a "slipped" PAM with a

17

444   more optimal consensus but variant spacing (N_5GCTT). Purified, recombinant NmeCas9 has been

445   observed to catalyze DNA cleavage *in vitro* at a site with a similarly slipped PAM [52]. To explore the off-

446   targeting potential of NmeCas9 further, we decreased the stringency of our mapping to allow detection of

447   off-target sites with up to 10 mismatches. Even in these conditions, only four (DTS7), 15 (DTS8), and 16

448   (DTS3) candidate sites were identified, most of which had only four or fewer reads (Fig. 5C) and were

449   associated with poorly functional PAMs (Supplemental Table 2). We consider it likely that most if not all

450   of these low-probability candidate off-target sites represent background noise caused by spurious priming

451   and other sources of experimental error.

452   As an additional test of off-targeting potential, we repeated the DTS7 GUIDE-seq experiments

453   with both SpyCas9 and NmeCas9, but this time using a different transfection reagent (Lipofectamine3000

454   rather than Polyfect). These repeat experiments revealed that >96% (29 out of 30) of off-target sites with

455   up to five mismatches were detected under both transfection conditions for SpyCas9 (Supplemental Table

456   1). However, the NmeCas9 GUIDE-seq data showed no overlap between the potential sites identified

457   under the two conditions, again suggesting that the few off-target reads that we did observe are unlikely to

458   represent legitimate off-target editing sites.

459   To confirm the validity of the off-target sites defined by GUIDE-seq, we designed primers

460   flanking candidate off-target sites identified by GUIDE-seq, PCR-amplified those loci following standard

461   genome editing (i.e., in the absence of co-transfected GUIDE-seq dsODN) (3 biological replicates), and

462   then subjected the PCR products to high-throughput sequencing to detect the frequencies of Cas9-

463   induced indels. For this analysis we chose the top candidate off-target sites (as defined by GUIDE-seq read

464   count) for each of the six cases (DTS3, DTS7 and DTS8, each edited by either SpyCas9 or NmeCas9). In

465   addition, due to the low numbers of off-target sites and the low off-target read counts observed during the

466   NmeCas9 GUIDE-seq experiments, we analyzed the top two predicted off-target sites for the three

467   NmeCas9 sgRNAs, as identified by CRISPRseek (Fig. 5A and Table 2) [76]. On-target indel formation

468   was detected in all cases, with editing efficiencies ranging from 7% (DTS8, with both SpyCas9 and

469   NmeCas9) to 39% (DTS3 with NmeCas9) (Fig. 5D). At the off-target sites, our targeted deep-sequencing

470   analyses largely confirmed our GUIDE-seq results: SpyCas9 readily induced indels at most of the tested

471   off-target sites when paired with the DTS3 and DTS7 sgRNAs, and in some cases the off-target editing

472   efficiencies approached those observed at the on-target sites (Fig. 5D). Although some SpyCas9 off-

473   targeting could also be detected with the DTS8 sgRNA, the frequencies were much lower (<0.1% in all

474   cases). Off-target lesions induced by NmeCas9 were far less frequent in all cases, even with the DTS3

475   sgRNA that was so efficient at on-target mutagenesis: many off-target sites exhibited lesion efficiencies

476   that were indistinguishable from background, and never rose above ~0.02% (Fig. 5D). These results, in

477   combination with the GUIDE-seq analyses described above, reveal wild-type NmeCas9 to be an

478   exceptionally precise genome editing enzyme.

479   To explore NmeCas9 editing accuracy more deeply, we chose 16 additional NmeCas9 target sites

480   across the genome, 10 with canonical N_4GATT PAMs and six with variant functional PAMs

481   (Supplemental Table 9). We then performed GUIDE-seq and analyses of NmeCas9 editing at these sites.

482   GUIDE-seq analysis readily revealed editing at each of these sites, with on-target read counts ranging

483   from ~100 to ~5,000 reads (Fig. 6A). More notably, off-target reads were undetectable by GUIDE-seq

484   with 14 out of the 16 sgRNAs (Fig. 6B). Targeted deep sequencing of PCR amplicons, which is a more

485   quantitative readout of editing efficiency than either GUIDE-seq or T7E1 analysis, confirmed on-target

486   editing in all cases, with indel efficiencies ranging from ~5-85% (Fig. 6C).

487   The two guides with off-target activity (NTS1C and NTS25) had only two and one off-target sites,

488   respectively (Fig. 6B and Supplemental Fig. 5). Off-target editing was confirmed by high-throughput

489   sequencing and analysis of indels (Fig. 6D). Compared with the on-target site (perfectly matched at all

490   positions other than the 5'-terminal guide nucleotide, and with an optimal N_4GATT PAM), the efficiently

491   targeted NTS1C-OT1 had two wobble pairs and one mismatch (all in the nine PAM-distal nucleotides),

492   as well as a canonical N_4GATT PAM (Fig. 6E and Supplemental Table 2). The weakly edited NTS1C-

493   OT2 site had only a single mismatch (at the 11th nucleotide, counting in the PAM-distal direction), but

494   was associated with a non-canonical N_4GGTT (or a "slipped" N_5GTTT) PAM (Fig. 6E and

495   Supplemental Table 2). NTS25 with an N_4GATA PAM was the other guide with a single off-target site

19

496   (NTS25-OT1), where NmeCas9 cleaved and edited up to ~1,000x less efficiently than at the on-target site

497   (Fig. 6D). This minimal amount of off-target editing arose despite the association of NTS25-OT1 with an

498   optimal N_4GATT PAM, unlike the variant N_4GATA PAM that flanks the on-target site. Overall, our

499   GUIDE-seq and sequencing-based analyses demonstrate that NmeCas9 genome editing is exceptionally

500   accurate: we detected and confirmed cellular off-target editing with only two of the 19 guides tested, and

501   even in those two cases, only one or two off-target sites could be found for each. Furthermore, of the three

502   bona fide off-target sites that we identified, only one generated indels at substantial frequency (11.6%);

503   indel frequencies were very modest (0.3% or lower) at the other two off-target sites.

504   We next sought to corroborate and expand on our GUIDE-seq results with a second prediction-

505   independent method. We applied the SITE-Seq™ (Caribou Biosciences, Inc., Berkeley, CA) assay, a

506   biochemical-based method that does not rely on cellular events such as DNA repair, thus potentially

507   enabling a more thorough profiling of genome-wide specificity [63]. SITE-Seq libraries were prepared for

508   the three dual target sites with both Cas9 orthologues as well as for twelve of the NmeCas9-only target

509   sites. SITE-Seq was performed on HEK293T genomic DNA (gDNA) treated with a range of RNP

510   concentrations (4 nM – 256 nM) previously shown to discriminate high and low probability cellular off-

511   targets [63]. Finally, the resulting libraries were sequenced, aligned, and then analyzed as previously

512   described [63].

513   Negative controls without RNP recovered zero sites across any concentrations, whereas SpyCas9

514   assembled with sgRNAs targeting DTS3, DTS7, or DTS8 recovered hundreds (at 4 nM RNP) to

515   thousands (at 256 nM RNP) of biochemical off-target sites (Fig. 6F). In contrast, NmeCas9 assembled

516   with sgRNAs targeting the same three sites recovered only their on-target sites at 4 nM RNP and at most

517   29 off-target sites at 256 nM RNP (Fig. 6F). Moreover, the 12 additional NmeCas9 target sites showed

518   similarly high specificity: eight samples recovered only the on-target sites at 4 nM RNP and six of those

519   recovered no more than nine off-targets at 256 nM RNP (Supplemental Fig. 6A). Across NmeCas9

520   RNPs, off-target sequence mismatches appeared enriched in the 5' end of the sgRNA target sequence

521   (Supplemental Table 4). Finally, three of the NmeCas9 RNPs (NTS30, NTS4C, and NTS59) required

522   elevated concentrations to retrieve their on-targets, potentially due to poor sgRNA transcription and/or

523   RNP assembly. These RNPs were therefore excluded from further analysis.

524   We next performed cell-based validation experiments to investigate whether any of the

525   biochemical off-targets were edited in cells. Since NmeCas9 recovered only ~100 biochemical off-targets

526   across all RNPs and concentrations, we could examine each site for editing in cells. SpyCas9 generated

527   >10,000 biochemical off-targets across all DTS samples, preventing comprehensive cellular profiling.

528   Therefore, for each RNP we selected 96 of the high cleavage sensitivity SITE-Seq sites (i.e., recovered at

529   all concentrations tested in SITE-Seq) for examination, as we predicted those were more likely to

530   accumulate edits in cells [63] (Supplemental Table 5). Sites were randomly selected within this cohort

531   and only included a subset of the GUIDE-seq validation test set sites (1/8 and 5/8 overlapping sites for

532   DTS3 and DTS7, respectively). Additionally, SITE-Seq and GUIDE-seq validations were performed on

533   the same gDNA samples to facilitate comparisons between data sets.

534   Across all NmeCas9 RNPs, only three cellular off-targets were observed. These three all

535   belonged to the NTS1C RNP, and two of them had also been detected with GUIDE-seq. Of note, all

536   high cleavage sensitivity SITE-Seq sites (i.e., all on-targets and the single prominent NTS1C off-target,

537   NTS1C-OT1) showed editing in cells. Conversely, SITE-Seq sites with low cleavage sensitivity, defined

538   as being recovered at only 64 nM and/or 256 nM RNP, were rarely found as edited (2/93 sites).

539   Importantly, this suggests that we identified all or the clear majority of NmeCas9 cellular off-targets, albeit

540   at our limit of detection. Across all SpyCas9 RNPs, 14 cellular off-targets were observed (8/70 sites for

541   DTS3, 6/83 sites for DTS7, and 0/79 sites for DTS8) (Supplemental Table 5). Since our data set was

542   only a subset of the total number of high cleavage sensitivity SITE-Seq sites, and excluded many of the

543   GUIDE-seq validated sites, we expect that sequencing all SITE-Seq sites would uncover additional

544   cellular off-targets. Taken together, these data corroborate our GUIDE-seq results, suggesting that

545   NmeCas9 can serve as a highly specific genome editing platform.

546

547   **Indel spectrum at NmeCas9-edited sites**

21

548   Our targeted deep sequencing data at the three dual target sites (Fig. 5D, Supplemental Fig. 4A

549   and Supplemental Table 5) enabled us to analyze the spectrum of insertions and deletions generated by

550   NmeCas9, in comparison with those of SpyCas9 when editing the exact same sites (Supplemental Figs. 6B

551   and 7-9). Although small deletions predominated at all three sites with both Cas9 orthologs, the frequency

552   of insertions was lower for NmeCas9 than it was with SpyCas9 (Supplemental Figs. 6B and 7-9). For both

553   SpyCas9 and NmeCas9, the vast majority of insertions were only a single nucleotide (Supplemental Fig.

554   8). The sizes of the deletions varied from one target site to the other for both Cas9 orthologs. Our data

555   suggest that at Cas9 edits, deletions predominated over insertions and the indel size varies considerably

556   site to site (Supplemental Figs. 6B, 10 and 11).

557

558   Truncated sgRNAs reduce off-target cleavage by NmeCas9

559   Although NmeCas9 exhibits very little propensity to edit off-target sites, for therapeutic

560   applications it may be desirable to suppress even the small amount of off-targeting that occurs (Fig. 6).

561   Several strategies have been developed to suppress off-targeting by SpyCas9 [31, 34, 35], some of which

562   could be readily applied to other orthologs. For example, truncated sgRNAs (tru-sgRNAs) sometimes

563   suppress off-target SpyCas9 editing more than they suppress on-target editing [69]. Because 5'-terminal

564   truncations are compatible with NmeCas9 function (Fig. 2), we tested whether NmeCas9 tru-sgRNAs can

565   have similar suppressive effects on off-target editing without sacrificing on-target editing efficiency.

566   First, we tested whether guide truncation can lead to NmeCas9 editing at novel off-target sites (i.e.

567   at off-target sites not edited by full-length guides), as reported previously for SpyCas9 [69]. Our earlier

568   tests of NmeCas9 on-target editing with tru-sgRNAs used guides targeting the NTS33 (Fig. 2B-C) and

569   NTS32 (Fig. 2D-E) sites. GUIDE-seq did not detect any NmeCas9 off-target sites during editing with full-

570   length NTS32 and NTS33 sgRNAs (Fig. 6). We again used GUIDE-seq with a subset of the validated

571   NTS32 and NTS33 tru-sgRNAs to determine whether NmeCas9 guide truncation leads to off-target

572   editing at new sites, and found none (Supplemental Fig. 12). Although we cannot rule out the possibility

573   that other NmeCas9 guides could be identified that yield novel off-target events upon truncation, our

574   results suggest that *de novo* off-targeting by NmeCas9 tru-sgRNAs is unlikely to be a pervasive problem.

575   The most efficiently edited off-target site from our previous analyses was NTS1C-OT1, providing

576   us with our most stringent test of off-target suppression. When targeted by the NTS1C sgRNA, NTS1C-

577   OT1 has one rG-dT wobble pair at position -16 (i.e., at the 16th base pair from the PAM-proximal end of

578   the R-loop), one rC-dC mismatch at position -19, and one rU-dG wobble pair at position -23 (Fig. 6E).

579   We generated a series of NTS1C-targeting sgRNAs with a single 5'-terminal G (for U6 promoter

580   transcription) and spacer complementarities ranging from 24 to 15 nucleotides (GN_{24} to GN_{15},

581   Supplemental Fig. 13A, top panel). Conversely, we designed a similar series of sgRNAs with perfect

582   complementarity to NTS1C-OT1 (Supplemental Fig. 13B, top panel). Consistent with our earlier results

583   with other target sites (Fig. 2), T7E1 analyses revealed that both sets of guides enabled editing of the

584   perfectly-matched on-target site with truncations down to 19 nucleotides (GN_{18}), but that shorter guides

585   were inactive. On-target editing efficiencies at both sites were comparable across the seven active guide

586   lengths (GN_{24} through GN_{18}), with the exception of slightly lower efficiencies with the GN_{19} guides

587   (Supplemental Fig. 13A & B, middle and bottom panels).

588   We then used targeted deep sequencing to test whether off-target editing is reduced with the

589   truncated sgRNAs. With both sets of sgRNAs (perfectly complementary to either NTS1C or NTS1C-

590   OT1), we found that off-targeting at the corresponding near-cognate site persisted with the four longest

591   guides (GN_{24}, GN_{23}, GN_{22}, GN_{21}; Fig. 7). However, off-targeting was abolished with the GN_{20} guide,

592   without any significant reduction in on-target editing efficiencies (Fig. 7). Off-targeting was also absent

593   with the GN_{19} guide, though on-target editing efficiency was compromised. These results, albeit from a

594   limited data set, indicate that truncated sgRNAs (especially those with 20 or 19 base pairs of guide/target

595   complementarity, 4-5 base pairs fewer than the natural length) can suppress even the limited degree of off-

596   targeting that occurs with NmeCas9.

597   Unexpectedly, even though off-targeting at NTS1C-OT1 was abolished with the GN_{20} and GN_{19}

598   truncated NTS1C sgRNAs, truncating by an additional nucleotide (to generate the GN_{18} sgRNA) once

23

599   again yielded NTS1C-OT1 edits (Fig. 7A). This could be explained by the extra G residue at the 5'-

600   terminus of each sgRNA in the truncation series (Supplemental Fig. 13). With the NTS1C GN_{19} sgRNA,

601   both the 5'-terminal G residue and the adjacent C residue are mismatched with the NTS1C-OT1 site. In

602   contrast, with the GN_{18} sgRNA, the 5'-terminal G is complementary to the off-target site. In other words,

603   with the NTS1C GN_{19} and GN_{18} sgRNAs, the NTS1C-OT1 off-target interactions (which are identical in

604   the PAM-proximal 17 nucleotides) include two additional nucleotides of non-complementarity or one

605   additional nucleotide of complementarity, respectively. Thus, the more extensively truncated GN_{18}

606   sgRNA has *greater* complementarity with the NTS1C-OT1 site than the GN_{19} sgRNA, explaining the re-

607   emergence of off-target editing with the former. This observation highlights the fact that the inclusion of a

608   5'-terminal G residue that is mismatched with the on-target site, but that is complementary to a C residue

609   at an off-target site, can limit the effectiveness of a truncated guide at suppressing off-target editing,

610   necessitating care in truncated sgRNA design when the sgRNA is generated by cellular transcription. This

611   issue is not a concern with sgRNAs that are generated by other means (e.g. chemical synthesis) that do not

612   require a 5'-terminal G. Overall, our results demonstrate that NmeCas9 genome editing is exceptionally

613   precise, and even when rare off-target editing events occur, tru-sgRNAs can provide a simple and effective

614   way to suppress them.

615

616   **DISCUSSION**

617   The ability to use Type II and Type V CRISPR-Cas systems as RNA-programmable DNA-

618   cleaving systems [13, 14, 27] is revolutionizing many aspects of the life sciences, and holds similar promise

619   for biotechnological, agricultural, and clinical applications. Most applications reported thus far have used

620   a single Cas9 ortholog (SpyCas9). Thousands of additional Cas9 orthologs have also been identified [28],

621   but only a few have been characterized, validated for genome engineering applications, or both. Adding

622   additional orthologs promises to increase the number of targetable sites (through new PAM specificities),

623   extend multiplexing possibilities (for pairwise combinations of Cas9 orthologs with orthogonal guides), and

624   improve deliverability (for the more compact Cas9 orthologs). In addition, some Cas9s may show

24

625   mechanistic distinctions (such as staggered vs. blunt dsDNA breaks) [80], greater protein stability *in vivo*,

626   improved control mechanisms (e.g. via multiple anti-CRISPRs that act at various stages of the DNA

627   cleavage pathway) [57, 58, 60, 61, 81-83], and other enhancements. Finally, some may exhibit a greater

628   natural propensity to distinguish between on- vs. off-target sites during genome editing applications,

629   obviating the need for extensive engineering (as was necessary with SpyCas9) to attain the accuracy

630   needed for many applications, especially therapeutic development.

631   Here we have further defined the properties of NmeCas9 during editing in human cells, including

632   validation and extension of previous analyses of guide length and PAM requirements [46, 53, 54].

633   Intriguingly, the tolerance to deviations from the $N_4G(A/C)TT$ natural PAM consensus [51] observed *in*

634   *vitro* and in bacterial cells [46, 52] is considerably reduced in the mammalian context, i.e. fewer PAM

635   variations are permitted during mammalian editing. The basis for this context-dependent difference is not

636   clear but may be due in part to the ability to access targets within eukaryotic chromatin, or to decreased

637   expression levels relative to potential DNA substrates, since lower SpyCas9/sgRNA concentrations have

638   been shown to improve accuracy [30, 84, 85]. We have also found that steady-state NmeCas9 levels in

639   human cells are markedly increased in the presence of its cognate sgRNA, suggesting that sgRNA-loaded

640   NmeCas9 is more stable than *apo* NmeCas9. An increased proteolytic sensitivity of *apo* Cas9 relative to the

641   sgRNA-bound form has been noted previously for a different Type II-C ortholog (*Corynebacterium diphtheria*

642   Cas9, CdiCas9 [55]).

643   A previous report indicated that NmeCas9 has high intrinsic accuracy, based on analyses of

644   candidate off-target sites that were predicted bioinformatically [54]. However, the true genome-wide

645   accuracy of NmeCas9 was not assessed empirically, as is necessary given well-established imperfections in

646   bioinformatic predictions of off-targeting [31, 34, 35]. We have used GUIDE-seq [63] and SITE-Seq [64]

647   to define the genome-wide accuracy of wild-type NmeCas9, including side-by-side comparisons with

648   wildtype SpyCas9 during editing of identical on-target sites. We find that NmeCas9 is a consistently high-

649   accuracy genome editor, with off-target editing undetectable above background with 17 out of 19

650   analyzed sgRNAs, and only one or three verified off-target edits with the remaining two guides. We

25

651   observed this exquisite specificity by NmeCas9 even with sgRNAs that target sites (DTS3 and DTS7 (see

652   Fig. 5)) that are highly prone to off-target editing when targeted with SpyCas9. Of the four off-target sites

653   that we validated, three accumulated <1% indels. Even with the one sgRNA that yielded a significant

654   frequency of off-target editing (NTS1C, which induced indels at NTS1C-OT1 with approximately half

655   the efficiency of on-target editing), the off-targeting with wild-type NmeCas9 could be easily suppressed

656   with truncated sgRNAs. Our ability to detect NTS25-OT1 editing with GUIDE-seq, despite its very low

657   (0.06%) editing efficiency based on high-throughput sequencing, indicates that our GUIDE-seq

658   experiments can identify even very low-efficiency off-target editing sites. Similar considerations apply to

659   our SITE-Seq analyses. We observed high accuracy even when NmeCas9 is delivered by plasmid

660   transfection, a delivery method that is associated with higher off-target editing than more transient

661   delivery modes such as RNP delivery [86, 87].

662   The two Type II-C Cas9 orthologs (NmeCas9 and CjeCas9) that have been validated for

663   mammalian genome editing and assessed for genome-wide specificity [47, 54] (this work) have both

664   proven to be naturally hyper-accurate. Both use longer guide sequences than the 20-nucleotide guides

665   employed by SpyCas9, and both also have longer and more restrictive PAM requirements. For both Type

666   II-C orthologs, it is not yet known whether the longer PAMs, longer guides, or both account for the

667   limited off-target editing. Type II-C Cas9 orthologs generally cleave dsDNA more slowly than SpyCas9

668   [49, 55], and it has been noted that lowering k_{cat} can, in some circumstances, enhance specificity [88].

669   Whatever the mechanistic basis for the high intrinsic accuracy, it is noteworthy that it is a property of the

670   native proteins, without a requirement for extensive engineering. This adds to the motivation to identify

671   more Cas9 orthologs with human genome editing activity, as it suggests that it may be unnecessary in

672   many cases (perhaps especially among Type II-C enzymes) to invest heavily in structural and mechanistic

673   analyses and engineering efforts to attain sufficient accuracy for many applications and with many desired

674   guides, as was done with (for example) SpyCas9 [32, 33, 37, 38, 65]. Although Cas9 orthologs with more

675   restrictive PAM requirements (such as NmeCas9, CjeCas9, and GeoCas9) by definition will afford lower

676   densities of potential target sites than SpyCas9 (which also usually affords the highest on-target editing

677   efficiencies among established Cas9 orthologs), the combined targeting possibilities for multiple such

678   Cas9s will increase the targeting options available within a desired sequence window, with little propensity

679   for off-targeting. The continued exploration of natural Cas9 variation, especially for those orthologs with

680   other advantages such as small size and anti-CRISPR off-switch control, therefore has great potential to

681   advance the CRISPR genome editing revolution.

682

683   **CONCLUSIONS**

684   NmeCas9 is an intrinsically high-accuracy genome editing enzyme in mammalian cells, and the limited

685   off-target editing that occurs can (at least in some cases) be suppressed by guide truncation. Continued

686   exploration of Cas9 orthologs could therefore yield additional enzymes that do not require extensive

687   characterization and engineering to prevent off-target editing.

688

689   **METHODS**

690   **Plasmids**

691   Two plasmids for the expression of NmeCas9 were used in this study. The first construct (used in Figs. 1

692   and 2) was derived from the plasmid pSimpleII where NmeCas9 was cloned under the control of the

693   elongation factor-1α promoter, as described previously [53]. The *Cas9* gene in this construct expresses a

694   protein with two NLSs and an HA tag. To make an all-in-one expression plasmid, a fragment containing

695   a *BsmB*I-crRNA cassette linked to the tracrRNA by six nucleotides, under the control of U6 RNA

696   polymerase III promoter, was synthesized as a gene block (Integrated DNA Technologies) and inserted

697   into pSimpleII, generating the pSimpleII-Cas9-sgRNA-*BsmB*I plasmid that includes all elements needed

698   for editing. To insert specific spacer sequence into the crRNA cassette, synthetic oligonucleotides were

699   annealed to generate a duplex with overhangs compatible with those generated by *BsmB*I digestion of the

700   pSimpleII-Cas9-sgRNA-*BsmB*I plasmid. The insert was then ligated into the *BsmB*I-digested plasmid. For

701   Figs. 3-7, NmeCas9 and SpyCas9 constructs were expressed from the pCS2-Dest Gateway plasmid under

702   the control of the CMV IE94 promoter [89]. All sgRNAs used with pCS2-Dest-Cas9 were driven by the

703   U6 promoter in pLKO.1-puro [90]. The M427 GFP reporter plasmid [66] was used as described [65].

704

Cell culture, transfection, and transduction

706   HEK293T were cultured in DMEM with 10% FBS and 1% Penicillin/Streptomycin (Gibco) in a 37°C

707   incubator with 5% CO_2. K562 cells were grown in the same conditions but using IMDM. HFF cells were

708   grown in the same conditions but using DMEM with Glutamax and 20% FBS without antibiotics. mESCs

709   were grown in DMEM supplemented with 10% FBS, glutamine beta-ME and LIF. For transient

710   transfection, we used early to mid-passage cells (passage number 4-18). Approximately 1.5×10^5 cells were

711   transfected with 150 ng Cas9-expressing plasmid, 150 ng sgRNA-expressing plasmid and 10 ng mCherry

712   plasmid using Polyfect transfection reagent (Qiagen) in a 24-well plate according to the manufacturer's

713   protocol. For the GFP reporter assay, 100 ng M427 plasmid was included in the co-transfection mix.

714   Transduction was done as described previously [91].

715

Western blotting

717   48 h after transfection, cells were harvested and lysed with 50 µl of RIPA buffer. Protein concentration

718   was determined with the BCA kit (Thermo Scientific) and 12 µg of proteins were used for electrophoresis

719   and blotting. The blots were probed with anti-HA (Sigma, H3663) and anti-GAPDH (Abcam, ab9485) as

720   primary antibodies, and then with horseradish peroxidase–conjugated anti-mouse IgG (Thermoscientific,

721   62-6520) or anti-rabbit IgG (Biorad, 1706515) secondary antibodies, respectively. Blots were visualized

722   using the Clarity Western ECL substrate (Biorad, 170-5060).

Flow cytometry

724   The GFP reporter was used as described previously [65]. Briefly, cells were harvested 48 hours after

725   transfection and used for FACS analysis (BD Accuri 6C). To minimize the effects of differences in the

726   efficiency of transfection among samples, cells were initially gated for mCherry-expression, and the

727   percentage of GFP-expressing cells were quantified within mCherry positive cells. All experiments were

728   performed in triplicate with data reported as mean values with error bars indicating the standard error of

729   the mean (s.e.m.).

Genome editing

731   72 hours after transfection, genomic DNA was extracted via the DNeasy Blood and Tissue kit (Qiagen),

732   according to the manufacturer's protocol. 50 ng DNA was used for PCR-amplification using primers

733   specific for each genomic site (Supplemental Table 9) with High Fidelity 2X PCR Master Mix (New

734   England Biolabs). For T7E1 analysis, 10 μl of PCR product was hybridized and treated with 0.5 μl T7

735   Endonuclease I (10 U/μl, New England Biolabs) in 1X NEB Buffer 2 for 1 hour. Samples were run on a

736   2.5% agarose gel, stained with SYBR-safe (ThermoFisher Scientific), and quantified using the

737   ImageMaster-TotalLab program. Indel percentages are calculated as previously described [92, 93].

738   Experiments for T7E1 analysis are performed in triplicate with data reported as mean ± s.e.m. For indel

739   analysis by TIDE, 20 ng of PCR product is purified and then sequenced by Sanger sequencing. The trace

740   files were subjected to analysis using the TIDE web tool (https://tide.deskgen.com).

741

Expression and purification of NmeCas9

743   NmeCas9 was cloned into the pMCSG7 vector containing a T7 promoter followed by a 6xHis tag and a

744   tobacco etch virus (TEV) protease cleavage site. Two NLSs on the C-terminus of NmeCas9 and another

745   NLS on the N-terminus were also incorporated. This construct was transformed into the Rosetta 2 DE3

746   strain of *E. coli*. Expression of NmeCas9 was performed as previously described for SpyCas9 [14]. Briefly,

747   a bacterial culture was grown at 37°C until an OD600 of 0.6 was reached. At this point the temperature

748   was lowered to 18°C followed by addition of 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) to

749   induce protein expression. Cells were grown overnight, and then harvested for purification. Purification of

750   NmeCas9 was performed in three steps: Nickel affinity chromatography, cation exchange

751   chromatography, and size exclusion chromatography. The detailed protocols for these can be found in

752   [14].

753

RNP delivery of NmeCas9

RNP delivery of NmeCas9 was performed using the Neon transfection system (ThermoFisher).

Approximately 40 picomoles of NmeCas9 and 50 picomoles of sgRNA were mixed in buffer R and

incubated at room temperature for 30 minutes. This preassembled complex was then mixed with 50,000 –

150,000 cells, and electroporated using 10 µL Neon tips. After electroporation, cells were plated in pre-

warmed 24-well plates containing the appropriate culture media without antibiotics. The number of cells

used and pulse parameters of electroporation were different for different cell types tested. The number of

cells used were 50,000, 100,000, and 150,000 for PLB985 cells, HEK293T cells, and K562/HFF cells

respectively. Electroporation parameters (voltage, width, number of pulses) were 1150 v, 20 ms, 2 pulses

for HEK293T cells; 1000 v, 50 ms, 1 pulse for K562 cells; 1350 v, 35 ms, 1 pulse for PLB985 cells; and

1700 v, 20 ms, 1pulse for HFF cells.

765

γH2AX immunofluorescence staining and flow cytometry

For immunofluorescence, mouse embryonic stem cells (mESCs) were crosslinked with 4%

paraformaldehyde and stained with anti-γH2AX (LP BIO, AR-0149-200) as primary antibody and Alexa

Fluor® 488 goat anti-rabbit IgG (Invitrogen, A11034) as secondary antibody. DNA was stained with

DAPI. For a positive control, E14 cells were irradiated with 254 nm UV light (3 mJ/cm2). Images were

taken by a Nikon Eclipse E400 and representative examples were chosen.

For flow cytometry, cells were fixed with 70% ethanol, primary and secondary antibody were as

described above for immunofluorescence, and DNA was stained with propidium iodide. Cells were

analyzed by BD FACSCalibur. The box plot was presented with the bottom line of the box representing

the first quartile, the band inside box indicating the median, the top line being the third quartile, the

bottom end of whisker denoting data of first quartile minus 1.5 times of interquartile range (no less than

0), and the top end of the whisker indicating data of third quartile plus 1.5 times of interquartile. Outliers

are not shown. All experiments were performed in duplicate.

779

CRISPRseek analysis of potential off-target sites

Global off-target analyses for DTS3, DTS7, and DTS8 with NmeCas9 sgRNAs were performed using the

Bioconductor package CRISPRseek 1.9.1 [76] with parameter settings tailored for NmeCas9. Specifically,

all parameters are set as default except the following: gRNA.size = 24, PAM = "NNNNGATT",

PAM.size = 8, RNA.PAM.pattern = "NNNNGNNN$", weights = c(0, 0, 0, 0, 0, 0, 0.014, 0, 0, 0.395,

0.317, 0, 0.389, 0.079, 0.445, 0.508, 0.613, 0.851, 0.732, 0.828, 0.615, 0.804, 0.685, 0.583),

max.mismatch = 6, allowed.mismatch.PAM = 7, topN = 10000, min.score = 0.   This setting means that

all seven permissive PAM sequences (N_4GATT, N_4GCTT, N_4GTTT, N_4GACA, N_4GACT, N_4GATA,

N_4GTCT) were allowed and all off-targets with up to 6 mismatches were collected [the sgRNA length was

changed from 20 to 24; four additional zeros were added to the beginning of the weights series to be

consistent with the gRNA length of 24; and topN (the number of off-target sites displayed) and min.score

(the minimum score of an off-target to be included in the output) were modified to enable identification of

all off-target sites with up to 6 mismatches]. Predicted off-target sites for DTS3, DTS7, and DTS8 with

SpyCas9 sgRNAs were obtained using CRISPRseek 1.9.1 default settings for SpyCas9 (with NGG, NAG,

and NGA PAMs allowed). Batch scripts for high-performance computing running the IBM LSF

scheduling software are included in the supplemental section. Off-target sites were binned according to

the number of mismatches relative to the on-target sequence. The numbers of off-targets for each sgRNA

were counted and plotted as pie charts.

798

GUIDE-seq

We performed GUIDE-seq experiment with some modifications to the original protocol [63], as described

[65]. Briefly, in 24-well format, HEK293T cells were transfected with 150 ng of Cas9, 150 ng of sgRNA,

and 7.5 pmol of annealed GUIDE-seq oligonucleotide using Polyfect transfection reagent (Qiagen) for all

six guides (DTS3, DTS7 and DTS8 for both the NmeCas9 and SpyCas9 systems). Experiments with

DTS7 sgRNAs were repeated using Lipofectamine 3000 transfection reagent (Invitrogen) according to the

31

805   manufacturer's protocol. 48 h after transfection, genomic DNA was extracted with a DNeasy Blood and

806   Tissue kit (Qiagen) according to the manufacturer protocol. Library preparation, sequencing, and read

807   analyses were done according to protocols described previously [63, 65]. Only sites that harbored a

808   sequence with up to six or ten mismatches with the target site (for SpyCas9 or NmeCas9, respectively)

809   were considered potential off-target sites. Data were analyzed using the Bioconductor package GUIDEseq

810   version 1.1.17 (Zhu et al., 2017). For SpyCas9, default setting was used except that min.reads = 2,

811   max.mismatch = 6, allowed.mismatch.PAM = 2, PAM.pattern = "NNN$", BSgenomeName = Hsapiens,

812   txdb = TxDb.Hsapiens.UCSC.hg19.knownGene, orgAnn = org.Hs.egSYMBOL For NmeCas9, default

813   setting was used except that PAM.size = 8, PAM = "NNNNGATT", min.reads = 2,

814   allowed.mismatch.PAM = 4, PAM.pattern = "NNNNNNNN$", BSgenomeName = Hsapiens, txdb =

815   TxDb.Hsapiens.UCSC.hg19.knownGene, orgAnn = org.Hs.egSYMBOL. NmeCas9 dataset was

816   analyzed twice with max.mismatch = 6 and max.mismatch = 10 respectively. The gRNA.size was set to

817   the length of the gRNA used, and various number of 0's was added at the beginning of weights to make

818   the length of weights equal to the gRNA size. For example, for gRNA with length 24, weights =

819   c(0,0,0,0,0, 0, 0.014, 0, 0, 0.395, 0.317, 0, 0.389, 0.079, 0.445, 0.508, 0.613, 0.851, 0.732, 0.828, 0.615,

820   0.804, 0.685, 0.583) (Zhu et al., 2017). These regions are reported in Supplemental Table 2.

821   **SITE-Seq**

822   We performed the SITE-Seq assay as described previously [63]. In 50 mL conical tubes, high molecular

823   weight genomic DNA (gDNA) was extracted from HEK293T cells using the Blood and Cell Culture DNA

824   Maxi Kit (Qiagen) according to the manufacturer's protocol. sgRNAs for both NmeCas9 and SpyCas9

825   RNP assembly were transcribed from PCR-assembled DNA templates containing T7 promoters. Oligo

826   sequences used in DNA template assembly can found be in Supplemental Table 8. PCR reactions were

827   performed using Q5 Hot Start High-Fidelity 2X Master Mix (New England Biolabs) with the following

828   thermal cycling conditions: 98°C for 2 minutes, 30 cycles of 20 seconds at 98°C, 20 seconds at 52°C, 15

829   seconds at 72°C, and a final extension at 72°C for 2 minutes. sgRNAs were *in vitro* transcribed using the

830   HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs) according to manufacturer's protocol.

831   Transcription reactions were digested with 2 units RNase-free DNase I (New England Biolabs) at 37°C

832   for 10 min; the reaction was stopped by adding EDTA to a final concentration of 35 mM and incubating

833   at 75°C for 10 min. All guides were purified with RNAClean beads (Beckman Coulter) and quantified

834   with the Quant-IT Ribogreen RNA Assay kit (ThermoFisher) according to the manufacturers' protocols.

835   Individual RNPs were prepared by incubating each sgRNA at 95°C for 2 minutes, then allowed to slowly

836   come to room temperature over 5 minutes. Each sgRNA was then combined with its respective Cas9 in a

837   3:1 sgRNA:Cas9 molar ratio and incubated at 37°C for 10 minutes in cleavage reaction buffer (20 mM

838   HEPES, pH 7.4, 150 mM KCl, 10 mM MgCl2, 5% glycerol). In 96-well format, 10 μg of gDNA was

839   treated with 0.2 pmol, 0.8 pmol, 3.2 pmol, and 12.8 pmol of each RNP in 50 μL total volume in cleavage

840   reaction buffer, in triplicate. Negative control reactions were assembled in parallel and did not include

841   any RNP. gDNA was treated with RNPs for 4 hours at 37°C. Library preparation and sequencing were

842   done according to protocols described previously [63] using the Illumina NextSeq platform, and ~3

843   million reads were obtained for each sample. Any SITE-Seq sites without off-target motifs located within

844   1 nt of the cut-site were considered false-positives and discarded.

845

846   **Targeted deep sequencing analysis**

847   To measure indel frequencies, targeted deep sequencing analyses were done as previously described [65].

848   Briefly, we used two-step PCR amplification to produce DNA fragments for each on-target and off-target

849   site. In the first step, we used locus-specific primers bearing universal overhangs with complementary ends

850   to the TruSeq adaptor sequences (Supplemental Table 7). DNA was amplified with Phusion High Fidelity

851   DNA Polymerase (New England Biolabs) using annealing temperatures of 60°C, 64°C or 68°C, depending

852   on the primer pair. In the second step, the purified PCR products were amplified with a universal forward

853   primer and an indexed reverse primer to reconstitute the TruSeq adaptors (Supplemental Table 7). Input

854   DNA was PCR-amplified with Phusion High Fidelity DNA Polymerase (98°C, 15s; 61°C, 25s; 72°C, 18s;

855   9 cycles) and equal amounts of the products from each treatment group were mixed and run on a 2.5%

33

856   agarose gel. Full-size products (~250bp in length) were gel-extracted. The purified library was deep

857   sequenced using a paired-end 150bp MiSeq run.

858   MiSeq data analysis was performed using a suite of Unix-based software tools. First, the quality of

859   paired-end sequencing reads (R1 and R2 fastq files) was assessed using FastQC

860   (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Raw paired-end reads were combined

861   using paired end read merger (PEAR) [94] to generate single merged high-quality full-length reads. Reads

862   were then filtered by quality [using Filter FASTQ [95]] to remove those with a mean PHRED quality

863   score under 30 and a minimum per base score under 24. Each group of reads was then aligned to a

864   corresponding reference sequence using BWA (version 0.7.5) and SAMtools (version 0.1.19). To

865   determine indel frequency, size and distribution, all edited reads from each experimental replicate were

866   combined and aligned, as described above. Indel types and frequencies were then cataloged in a text

867   output format at each base using bam-readcount (https://github.com/genome/bam-readcount). For each

868   treatment group, the average background indel frequencies (based on indel type, position and frequency)

869   of the triplicate negative control group were subtracted to obtain the nuclease-dependent indel

870   frequencies. Indels at each base were marked, summarized and plotted using GraphPad Prism. Deep

871   sequencing data and the results of statistical tests are reported in Supplemental Table 3.

872   SITE-Seq cell-based validation was performed as previously described with minor modifications

873   [63]. In brief, SITE-Seq sites were amplified from ~1,000-4,000 template copies per replicate and

874   sequencing data from Cas9-treated samples were combined to minimize any variability due to uneven

875   coverage across replicates. Cas9 cleavage sites were registered from the SITE-Seq data, and mutant reads

876   were defined as any non-reference variant calls within 20 bp of the cut site. Sites with low sequencing

877   coverage (< 1,000 reads in the combined, Cas9-treated samples or <200 reads in the reference samples)

878   or >2% variant calls in the reference samples were discarded. Sites were tallied as cellular off-targets if

879   they accumulated > 0.5% mutant reads in the combined, Cas9-treated samples. This threshold

880   corresponded to sites that showed unambiguous editing when DNA repair patterns were visually

881   inspected.

34

882

List of Abbreviations

884 **AAV:** adeno-associated virus;
885 **BLESS**: breaks labelling, enrichment on streptavidin and next-generation sequencing;
886 **BLISS:** breaks labeling *in situ* and sequencing;
887 **bp:** base pair;
888 **Cas:** CRISPR-associated;
889 **Circle-seq:** circularization for *in vitro* reporting of cleavage effects by sequencing;
890 **CjeCas9:** *Campylobacter jejuni* Cas9;
891 **CMV:** cytomegalovirus;
892 **CRISPR**: clustered, regularly interspaced, short palindromic repeats;
893 **crRNAs:** CRISPR RNAs;
894 **dCas9:** "dead" Cas9;
895 **Digenome-seq**: digested genome sequencing;
896 **DSB:** double-strand breaks;
897 **dsODN:** double-stranded oligodeoxynucleotide;
898 **DTS:** dual target site;
899 **EF1α:** elongation factor-1α;
900 **GeoCas9:** *Geobacillus stearothermophilus*;
901 **GUIDE-seq:** genome-wide unbiased identification of double strand breaks enabled by sequencing;
902 **HDR:** homology-directed repair;
903 **HTGTS:** high-throughput genome-wide translocation sequencing;
904 **IDLV:** integrase-defective lentiviral vector;
905 **mESC:** mouse embryonic stem cell;
906 **NHEJ:** non-homologous end joining;
907 **NLS:** nuclear localization signal;
908 **NmeCas9:** *Neisseria meningitidis* (strain 8013) Cas9;
909 **NTS:** NmeCas9 target site;
910 **PAM:** protospacer adjacent motif;
911 **RNP:** ribonucleoprotein;
912 **SauCas9:** *Staphylococcus aureus* Cas9;
913 **sgRNA:** single-guide RNA;
914 **SITE-Seq:** selective enrichment and identification of tagged genomic DNA ends by sequencing;
915 **SpyCas9:** *Streptococcus pyogenes* Cas9;
916 **T7E1:** T7 Endonuclease 1;
917 **tracrRNA:** *trans*-acting CRISPR RNA;
918 **tru-sgRNAs:** truncated sgRNAs.
919

Declarations

921 *Ethics approval and consent to participate.* Not applicable.

922 *Consent for publication.* Not applicable.

942

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1188   **Figures**

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1190   **Figure 1.** NmeCas9 expression and activity in human (HEK293T) cells. (*A*) Western blot detection of HA-tagged

1191   NmeCas9 in transiently transfected HEK293T cells. Lane 1: Cells transfected with SpyCas9 plasmid under the

1192   control of the CMV promoter. Lane 2: Cells transfected with NmeCas9 plasmid under the control of the elongation

1193   factor-1α (EF1α) promoter. Lane 3: Cells expressing NmeCas9 and a non-targeting sgRNA (nt-sgRNA), which lacks

1194   a complementary site in the human genome. Lane 4: Cells expressing NmeCas9 and a sgRNA targeting

1195   chromosomal site NTS3. Upper panel: Anti-HA western blot. Lower panel: Anti-GAPDH western blot as a loading

1196   control. (*B*) NmeCas9 targeting co-transfected split-GFP reporter with ps9, ps24 and ps25 sites. Plasmid cleavage by

1197   SpyCas9 is used as a positive control, and a reporter without a guide-complementary site (No ps: no protospacer) is

1198   used as a negative control to define background levels of recombination leading to GFP+ cells. (*C*) NmeCas9

1199   programmed independently with different sgRNAs targeting eleven genomic sites flanked by an N_4GATT PAM,

1200   detected by T7E1 analysis. (*D*) Quantitation of editing efficiencies from three independent biological replicates

1201   performed on different days. Error bars indicate ± standard error of the mean (± s.e.m.). (*E*) Genomic lesions with

1202   NmeCas9 programmed independently with different guides in different cell lines and using different methods of

1203   delivery.

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1205   **Figure 2.** NmeCas9 guide length requirements in mammalian cells. (*A*) Split-GFP activity profile of NmeCas9

1206   cleavage with ps9 sgRNAs bearing spacers of varying lengths (18-24 nts) along with 5'-terminal G residues to enable

1207   transcription. Bars represent mean values ± s.e.m. from three independent biological replicates performed on

1208   different days. (*B*) T7EI analysis of editing efficiencies at the NTS33 genomic target site (with an N$_4$GATT PAM)

1209   with sgRNAs bearing spacers of varying lengths (13-25 nts) with 1-2 5'-terminal G residues. (*C*) Quantitation of

1210   lesion efficiencies (of experiment in *B*) from three independent biological replicates performed on different days.

1211   Error bars indicate ± standard error of the mean (± s.e.m.). (*D*) As in (*B*), but targeting the NTS32 genomic site (with

1212   an N$_4$GCTT PAM). (*E*) Quantitation of lesion efficiencies (of experiment in *D*) from three independent biological

1213   replicates performed on different days. Error bars indicate ± standard error of the mean (± s.e.m.).

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1215   **Figure 3.** Characterization of functional PAM sequences in human (HEK293T) cells. (*A*) Split-GFP activity profile

1216   of NmeCas9 cleavage with ps9 sgRNA, with the target site flanked by different PAM sequences. Bars represent

1217   mean values ± s.e.m. from three independent biological replicates performed on different days. (*B*) T7E1 analysis of

1218   editing efficiencies at seven genomic sites flanked by PAM variants, as indicated. Products resulting from NmeCas9

1219   genome editing are denoted by the red dots. (*C*) Quantitation of data from (*B*), as well as an additional site (NTS31;

1220   N$_4$GACA PAM) that was not successfully edited. Bars represent mean values ± s.e.m. from three independent

1221   biological replicates performed on different days.

1222

1223   **Figure 4.** NmeCas9 and SpyCas9 have comparable editing efficiencies in human (HEK293T) cells when targeting

1224   the same chromosomal sites. (*A*) Western blot analysis of NmeCas9 and SpyCas9. HEK293T cells were transfected

1225   with the indicated Cas9 ortholog cloned in the same plasmid backbone, and fused to the same HA epitope tags and

1226   NLSs. Top panel: anti-HA western blot (EP, empty sgRNA plasmid). Bottom panel: anti-GAPDH western blot, used

1227   as a loading control. Mobilities of protein markers are indicated. (*B*) T7E1 analysis of three previously validated

1228   SpyCas9 guides targeting the *AAVS1* locus, in comparison with NmeCas9 guides targeting nearby *AAVS1* sites (mean

1229   ± s.e.m., *n* = 3). (*C*) Representative T7EI analyses comparing editing efficiencies at the dual target sites DTS1,

1230   DTS3, DTS7, DTS8, and NTS7, using the indicated Cas9/sgRNA combinations. (*D*) Quantitation of data from (*C*)

1231   (mean ± s.e.m., *n* = 3).

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1232

1233　**Figure 5.** Bioinformatic and empirical comparison of NmeCas9 and SpyCas9 off-target sites within the human

1234　genome. (*A*) Genome-wide computational (CRISPRseek) predictions of off-target sites for NmeCas9 (with N_4GN_3

1235　PAMs) and SpyCas9 (with NGG, NGA, and NAG PAMs) with DTS3, DTS7 and DTS8 sgRNAs. Predicted off-

1236　target sites were binned based on the number of mismatches (up to six) with the guide sequences. (*B*) GUIDE-Seq

1237　analysis of off-target sites in HEK293T cells with sgRNAs targeting DTS3, DTS7 and DTS8, using either SpyCas9

1238　or NmeCas9, and with up to 6 mismatches to the sgRNAs. The numbers of detected off-target sites are indicated at

1239　the top of each bar. (*C*) Numbers of independent GUIDE-Seq reads for the on- and off-target sites for all six

1240　Cas9/sgRNA combinations from (*B*) (SpyCas9, red; NmeCas9, green), binned by the number of mismatches with

1241　the corresponding guide. (*D*) Targeted deep sequencing analysis of lesion efficiencies at on- and off-target sites from

1242　(*A*) or (*B*) with SpyCas9 (left, red) or NmeCas9 (right, green). Data for off-target sites are in grey. For SpyCas9, all

1243　off-target sites were chosen from (*B*) based on the highest GUIDE-Seq read counts for each guide (Supplemental

1244　Table 3). For NmeCas9, in addition to those candidate off-target sites obtained from GUIDE-Seq (*C*), we also

1245　assayed one or two potential off-target sites (designated with the "-CS" suffix) predicted by CRISPRseek as the

1246　closest near-cognate matches with permissive PAMs. Data are mean values ± s.e.m. from three biological replicates

1247　performed on different days.

1248

1249　**Figure 6.** Off-target analyses for additional NmeCas9 sgRNAs, targeting sites with consensus and variant PAMs. (*A*)

1250　Number of GUIDE-Seq reads for the on-target sites, with the PAM sequences for each site indicated underneath. (*B*)

1251　Number of GUIDE-Seq-detected off-target sites using the Bioconductor package GUIDEseq version 1.1.17 [79]

1252　with default settings except that PAM.size = 8, PAM = "NNNNGATT", min.reads = 2, max.mismatch = 6,

1253　allowed.mismatch.PAM = 4, PAM.pattern = "NNNNNNNN$", BSgenomeName = Hsapiens, txdb =

1254　TxDb.Hsapiens.UCSC.hg19.knownGene, orgAnn = org.Hs.egSYMBOL gRNA.size was set to length of the gRNA

1255　used, and various number of 0's were added at the beginning of weights to make the length of weights equal to the

1256　gRNA size. For example, for gRNA with length 24, weights = c(0,0,0,0,0, 0, 0.014, 0, 0, 0.395, 0.317, 0, 0.389,

1257　0.079, 0.445, 0.508, 0.613, 0.851, 0.732, 0.828, 0.615, 0.804, 0.685, 0.583) for all sixteen sgRNAs used in (*A*). (*C*)

1258　Lesion efficiencies for the on-target sites as measured by PCR and high-throughput sequencing. Data are mean

1259　values ± s.e.m. from three biological replicates performed on different days. (*D*) NmeCas9 lesion efficiencies at the

1260   NTS1C (left) and NTS25 (right) on-target sites, and at the off-target sites detected by GUIDE-Seq from (*B*), as

1261   measured by PCR and high-throughput sequencing. Data are mean values ± s.e.m. from three biological replicates

1262   performed on different days. (*E*) Schematic diagrams of NmeCas9 sgRNA/DNA R-loops for the NTS1C (left) and

1263   NTS25 (right) sgRNAs, at the GUIDE-Seq-detected on- and off-target sites. Black, DNA residues; boxed nts, PAM;

1264   red line, NmeCas9 cleavage site; cyan and purple, mismatch/wobble and complementary nts (respectively) in the

1265   NmeCas9 sgRNA guide region; green, NmeCas9 sgRNA repeat nts. (*F*) Comparison of NmeCas9 and SpyCas9

1266   biochemical off-target sites using SITE-Seq analysis.

1267

1268   **Figure 7.** Guide truncation can suppress off-target editing by NmeCas9. (*A*) Lesion efficiencies at the NTS1C (on-

1269   target, red) and NTS1C-OT1 (off-target, orange) genomic sites, after editing by NmeCas9 and NTS1C sgRNAs of

1270   varying lengths, as measured by PCR and high-throughput sequencing. Data are mean values ± s.e.m. from three

1271   biological replicates performed on different days. (*B*) As in (*A*), but using sgRNAs perfectly complementary to the

1272   NTS1C-OT1 genomic site.

1273

51

Figure 1

Figure 2

A

B

% lesion: 16   19   19   19   19   16   14   0   0   0   16   16   12   0   0   0   0

C

D

% lesion: 14   14   19   21   0   0   0   0

E

Figure 3

A

B

C

Figure 4

A

B

C

DTS1 | DTS3 | DTS7

DTS8 | NTS7

D

Figure 5

Figure 6

Figure 7