Supplementary Discussion

Detection limit of existing methods for identifying Cas9-induced genome-wide off-target effects

Various methods have been described for identifying the genome-wide off-target effects of CRISPR-Cas9 nucleases. These approaches include GUIDE-seq[1], Digenome-seq[2], integration-deficient lentivirus capture[3, 4], high-throughput genome-wide translocation sequencing[5], and breaks labeling, enrichment on streptavidin and next-generation sequencing (**BLESS**)[6, 7]. Some of these methods possess the capability to identify sites modified at or below frequencies of 0.1%. However, to our knowledge, no method described to date has been shown to identify sites that are modified with lower frequencies in cells, and it can be challenging to confirm whether alterations below 0.1% frequency are *bona fide* nuclease-induced changes due to the error rate associated with existing deep sequencing methodologies. As the sensitivities of genome-wide methods for detecting off-targets improve, it will be interesting to see whether these methods might unveil any lower frequency off-target sites for wild-type SpCas9, and whether our HF variants might also reduce mutagenesis at these sites as well.

Practical considerations for targeting sites of interest with SpCas9-HF1

SpCas9-HF1 may not be compatible with the common practice of using a G at the 5' end of the gRNA that is mismatched to the protospacer sequence. Our testing of four sgRNAs bearing a 5' G mismatched to its target site showed three of the four had diminished activities with SpCas9-HF1 compared to wild-type SpCas9 (**Extended Data Fig. 7b**), perhaps reflecting the ability of SpCas9-HF1 to better discriminate against a partially mismatched site.

The SpCas9-HF1 variant might also be combined with other mutations that have been shown to alter Cas9 function. For example, we previously described an SpCas9 mutant bearing three amino acid substitutions (D1135V/R1335Q/T1337R, also known as the SpCas9-VQR variant) that recognizes sites with NGAN PAMs (with relative efficiencies for NGAG>NGAT=NGAA>NGAC)[8] and we have also recently identified a quadruple SpCas9 mutant (D1135V/G1218R/R1335Q/T1337R, which we refer to as the SpCas9-VRQR variant) that has improved activities relative to the VQR variant on sites with NGAH (H = A, C, or T) PAMs (**Extended Data Fig. 8a**). We introduced the four mutations (N497A/R661A/Q695A/Q926A) from SpCas9-HF1 into SpCas9-VQR and SpCas9-VRQR to create SpCas9-VQR-HF1 and SpCas9-VRQR-HF1, respectively. Both HF versions of these nucleases show on-target activities comparable (i.e., 70% or more) to their non-HF counterparts with five of eight sgRNAs targeted to the EGFP reporter gene and with seven of eight sgRNAs targeted to endogenous human gene sites (**Extended Data Figs. 8b-8d**). It will be of interest in future experiments to characterize the genome-wide specificities of these variants with altered PAM recognition.

SpCas9-HF1 retains robust on-target activities, inducing mutagenesis at frequencies comparable to that observed with wild-type SpCas9 with ~85% of sgRNAs we tested. It will be important in future studies to define the parameters that distinguish sgRNAs active with SpCas9-HF1 from the minority that are inactive, as is currently being done in ongoing studies to understand properties of sgRNAs that function efficiently with wild-type SpCas9[9-12].

Potential mechanisms to explain improved specificity of the SpCas9-HF1 variant

Based on available crystal structures of SpCas9[13-15], we initially targeted alanine substitutions to residues N497, R661, Q695, and Q926 as a way to decrease non-specific binding to the target DNA strand. In general, our results showed that increasing the number of alanine substitutions decreased the modification rates observed with mismatched sgRNAs (a surrogate for mismatched off-target sites) in our initial EGFP modification screen using a single target site (**Fig. 1b**). However, our results also show that certain combinations of the same number of these substitutions led to larger decreases in activities with mismatched sgRNAs than others. For example, among the four different doubly substituted SpCas9 variants (**Fig. 1b**), Q695A/Q926A showed the lowest frequencies of modification when using mismatched sgRNAs. The addition of R661A to Q695A/Q926A led to a complete loss of activities with mismatched

sgRNAs, while the addition of N497A to Q695A/Q926A led to a smaller loss relative to R661A (**Fig. 1b**). N497 and R661 make hydrogen bonds to the same DNA phosphate group, suggesting that R661 may play an additional role that somehow affects SpCas9 activity with mismatched sgRNAs. While N497, R661, and Q926 all sit in the minor groove of the sgRNA:DNA complex[13, 14], R661 and Q926 lie deeper in the minor groove and therefore one can speculate that these residues might be able to stabilize a mispaired sgRNA:DNA duplex. Q695, which is solvent exposed, also appears to be in a position where it might be able to stabilize the target DNA in a mispaired sgRNA:DNA duplex.

Alternatively, a recent mechanistic study suggests that SpCas9 may adopt a partially active conformation, capable of binding and interacting with a subsequence of the target DNA but not cleaving it, prior to adopting a final cleavage-active conformation[16]. According to this model, conformational changes in the HNH domain are communicated to the RuvC domain through a two helix linker (that contains Q926[16]), thereby activating the RuvC domain for cleavage. Mutations in the two-helix linker that disrupt the helix also block RuvC cleavage activity, while alanine substitutions in the linker restore RuvC activity[16]. Q926 lies near the N-terminus of the second helix in the two-helix linker, and we therefore speculate that Q926A might alter the stability of this helix (alanines generally stabilize alpha helices[17, 18]). Therefore, it is possible that SpCas9-HF1, at least partially through alanine substitution at Q926, alters the cleavage activation mechanism when the sgRNA-SpCas9-HF1 complex is bound to mismatched off-target sequences.

References:

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Supplementary Sequences – a subset of plasmids used in this study

Name	Addgene ID	Description
JDS246	43861	CMV-T7-humanSpCas9-NLS-3xFLAG
VP12	72247	CMV-T7-humanSpCas9-HF1(N497A, R661A, Q695A, Q926A)-NLS-3xFLAG
MSP2135	72248	CMV-T7-humanSpCas9-HF2(N497A, R661A, Q695A, Q926A, D1135E)-NLS-3xFLAG
MSP2133	72249	CMV-T7-humanSpCas9-HF4(Y450A, N497A, R661A, Q695A, Q926A)-NLS-3xFLAG
MSP469	65771	CMV-T7-humanSpCas9-VQR(D1135V, R1335Q, T1337R)-NLS-3xFLAG
MSP2440	72250	CMV-T7-humanSpCas9-VQR-HF1(N497A, R661A, Q695A, Q926A, D1135V, R1335Q, T1337R)-NLS-3xFLAG
BPK2797	72251	CMV-T7-humanSpCas9-VRQR(D1135V, G1218R, R1335Q, T1337R)-NLS-3xFLAG
MSP2443	72252	CMV-T7-humanSpCas9-VRQR-HF1(N497A, R661A, Q695A, Q926A, D1135V, G1218R, R1335Q, T1337R)-NLS-3xFLAG
BPK1520	65777	U6-BsmBIcassette-Sp-sgRNA

JDS246: CMV-T7-humanSpCas9-NLS-3xFLAG
Human codon optimized *S. pyogenes* Cas9 colored in blue, NLS underlined, 3xFLAG tag in **bold**

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VP12: CMV-T7-humanSpCas9-HF1(N497A, R661A, Q695A, Q926A)-NLS-3xFLAG
Human codon optimized *S. pyogenes* Cas9 colored in blue, modified codons in red, NLS underlined, 3xFLAG tag in **bold**

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MSP2135: CMV-T7-humanSpCas9-HF2(N497A, R661A, Q695A, Q926A, D1135E)-NLS-3xFLAG
Human codon optimized *S. pyogenes* Cas9 colored in blue, modified codons in red, NLS underlined,
3xFLAG tag in **bold**

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<u>MSP2133</u>: CMV-T7-humanSpCas9-HF4(Y450A, N497A, R661A, Q695A, Q926A)-NLS-3xFLAG
Human codon optimized *S. pyogenes* Cas9 colored in blue, modified codons in red, NLS <u>underlined</u>,
3xFLAG tag in **bold**
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<u>MSP469</u>: CMV-T7-humanSpCas9-VQR(D1135V, R1335Q, T1337R)-NLS-3xFLAG
Human codon optimized *S. pyogenes* Cas9 colored in blue, modified codons in red, NLS <u>underlined</u>,
3xFLAG tag in **bold**
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MSP2440: CMV-T7-humanSpCas9-VQR-HF1(N497A, R661A, Q695A, Q926A, D1135V, R1335Q, T1337R)-NLS-3xFLAG

Human codon optimized *S. pyogenes* Cas9 colored in blue, modified codons in red, NLS underlined, 3xFLAG tag in **bold**

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BPK2797: CMV-T7-humanSpCas9-VRQR(D1135V, G1218R, R1335Q, T1337R)-NLS-3xFLAG
Human codon optimized *S. pyogenes* Cas9 colored in blue, modified codons in red, NLS underlined, 3xFLAG tag in **bold**

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MSP2443: CMV-T7-humanSpCas9-VRQR-HF1(N497A, R661A, Q695A, Q926A, D1135V, G1218R, R1335Q, T1337R)-NLS-3xFLAG
Human codon optimized *S. pyogenes* Cas9 colored in blue, modified codons in red, NLS underlined, 3xFLAG tag in **bold**

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BPK1520: U6-BsmBIcassette-Sp-sgRNA
U6 promoter in green, BsmBI sites underlined, *S. pyogenes* sgRNA colored in purple, U6 terminator underlined

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