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Abasic CRISPR RNAs inherently harness fidelity of SpCas9 for genome editing

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CRISPR-Cas9, an RNA-guided adaptive immune system in bacteria that restricts foreign DNAs, inadvertently cleaves numerous off-targets in genome editing. However, the CRISPR-Cas9 system is stably maintained in *Streptococcus pyogenes (S. pyogenes)*, from which CRISPR-Cas9 genome editing technology was originally derived, without inducing detrimental off-target self-cleavage, suggesting that current applications may not fully reflect its natural behavior. To investigate this, we analyzed 481 CRISPR RNA (crRNA) sequences from 107 *S. pyogenes* strains and identified numerous potential off-target sites not only in PAM-proximal but also in PAM-distal regions that could compromise cellular viability. Based on sequencing analyses of crRNAs, we also discovered abasic modifications at the 5′ end of crRNAs, including oxidized dinucleotide overhangs, which naturally attenuate off-target cleavage by activated SpCas9 during bacteriophage infection. By mimicking the natural mechanism, we engineered abasic crRNA modifications (ØXØ) at the 5′ end, enhancing target specificity by limiting base pairing and extending crRNA length to regulate SpCas9 activity; SpCas9 becomes intolerant to mismatches at both 5′ end and protospacer adjacent motif (PAM) proximal regions, validated by using targeted sequencing, CIRCLE-seq, and random mismatch target libraries. Inspired by native strategies of abasic crRNAs, our study delineated ØXØ to improve SpCas9 fidelity, applicable to therapeutic treatment.