Rheostatic control of Cas9-mediated DNA double strand break (DSB) generation and genome editing

John C. Rose¹, Jason J. Stephany², Cindy T. Wei¹, Douglas M. Fowler*²,⁴, and Dustin J. Maly*¹,³

Affiliations

¹Department of Chemistry
University of Washington, Seattle, Washington 98195 U.S.A.

²Department of Genome Sciences
University of Washington, Seattle, Washington 98195 U.S.A.

³Department of Biochemistry
University of Washington, Seattle, Washington 98195 U.S.A.

⁴Department of Bioengineering
University of Washington, Seattle, Washington, 98195, U.S.A.

*Correspondence:
dfowler@uw.edu (Tel: 206-221-5711)
djmal@uw.edu (Tel: 206-543-1653)
Supplemental Figures

Figure S1: Indel frequencies at early time points and comparison of the basal editing frequencies of ciCas9 and ciCas9(L22). (A) Indel frequencies resulting from activation of ciCas9 with A3 (10 μM) or A115 (10 μM) from 0 – 2 hours for time course shown in Figure 2A-B. (B) Indel frequencies resulting from activation of ciCas9(L22) with A3 (10 μM) or A115 (10 μM) from 0 – 2 hours for time course shown in Figure 2C-D. (C) Time course of indel frequencies resulting from basal activity (i.e. in the absence of BCL-xL/BH3 disruptor) of ciCas9 and ciCas9(L22). Individual replicates are shown. Lines connect means of two (n = 2) biological replicates.

Figure S2: Indel frequencies at early time points and the basal editing frequencies of ciCas9. (A) Indel frequencies resulting from activation of ciCas9 with 0 – 4 μM WEHI-539 from 0 – 2 hours for time course shown in Figure 3A & 3C. (B) Time course of indel frequencies resulting from basal activity (i.e. in the absence of BCL-xL/BH3 disruptor) of ciCas9 in the time course shown in Figure 3A & 3C. Error bars = s.e.m (n = 3 biological replicates).
Methods

ciCas9 and sgRNA expression plasmids

ciCas9, ciCas9(L22), and AAVS1 expression plasmids were cloned as described previously. Briefly, ciCas9 and ciCas9(L22) are expressed from the pcDNA5/FRT/TO vector (Life Technologies), and include an N-terminal FLAG tag and C-terminal NLS. BCL-xL (residues 4-198, Uniprot Q07817-1), BH3 peptide, and linker sequences were introduced using gBlocks (Integrated DNA Technologies) and Gibson Assembly Cloning. ciCas9 was subsequently sub-cloned into the pcDNA5/FRT/TO vector (ThermoFisher). Enhanced specificity-ciCas9, ciCas9(L22), and ciCas9(F22) were generated via restriction digest and NEBuilder HiFi Gibson Cloning (New England Biosciences).

For ciCas9, the BH3 peptide sequence used is: APPNLWAAQRYGRELRRMADELEGSDK. For ciCas9(L22), the BH3 peptide sequence used is: APPNLWAAQRYGRELRRMADELEGSDK. The sequences differ only at the 22\textsuperscript{nd} residue (underlined).

Cell culture

HEK-293T cells (293T/17, ATCC) were maintained in high glucose DMEM, 10% FBS, 4 mM L-glutamine (Life Technologies). Cells were tested and certified free of mycoplasma monthly.

Editing of genomic loci

Cells were plated in 12-well plates at the following densities: HEK-293T 3 x 10\textsuperscript{5} cells/well. The following day, cells were transfected with Turbofectin 8.0 (Origene). All transfected wells were transfected with 1.5 μL transfection reagent and 0.5 μg plasmid DNA per well. The plasmid DNA mixture consisted of 225 ng AAVS1 sgRNA, 225 ng ciCas9 or ciCas9(L22), and 50 ng pMAX-GFP plasmids as a transfection control. 24 hours after transfection, ciCas9 was activated with the indicated concentrations of A-385358, WEHI-539 (ApexBio Technology), or A-1155463 (ChemieTek). A-385358 was synthesized according to previously reported procedure\textsuperscript{18,19}. The identity of A-385358 was confirmed by 1H-NMR and mass spectrometry (Bruker Esquire Ion Trap MS). >95% purity was confirmed by analytical HPLC. Compounds were stored as 10 mM stocks in DMSO at -20°C. Final DMSO concentration in drug treated wells was 0.1%.

To harvest HEK-293T cells, plates were placed on ice at the appropriate time point, media immediately aspirated, and wells were washed with 1 mL/well ice cold DPBS. Cells were subsequently harvested via trituration with 600 μL ice cold DPBS, spun down at 1500 x g for 10 min at 4°C, decanted, and cell pellets stored at -80°C.

Indel quantification with high-throughput sequencing

Genomic DNA was isolated using the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer’s instructions with the following modification: proteininase K digestion
was extended to 1h at 56 °C. 20 cycles of primary PCR to amplify the region of interest was performed using 2 μL of DNeasy eluate (~100-300 ng template) in a 5 μL Kapa HiFi HotStart polymerase reaction (Kapa Biosystems, for primers see Supplementary Data Set 1). The PCR reaction was diluted with 45 μL DNase free water (Ambion). Illumina adapters and indexing sequences were added via 25 cycles of secondary PCR with 3 μL of diluted primary PCR product in a 10 μL Taq polymerase reaction (New England Biosciences, for primers see Supplementary Data Set 1). The final amplicons were run on a TBE-agarose gel (0.7%), and the product band was excised and extracted using the Freeze and Squeeze Kit according to the manufacturer’s instructions (Bio-Rad). Gel purified amplicons were quantified by qPCR using the Illumina Library Quantification Kit (Kapa Biosystems). Then, up to 300 indexed amplicons were pooled and sequenced on a MiSeq (MiSeq 150 V3 kit, Illumina, for primers see Supplementary Data Set 1).

After demultiplexing of reads (bcl2fastq, Illumina), indels were quantified with a custom Python script, which is freely available upon request. Briefly, 8-mer sequences were identified in the reference sequence located 20 base pairs up- and downstream of the target sequence. Sequence distal to these 8-mers was trimmed. Reads lacking these 8-mers were discarded. The trimmed reads were then evaluated for indels using the Python difflib package. Indels were defined as trimmed reads which differed in length from the trimmed reference and for which an insertion or deletion operation spanning or within 1 bp of the predicted Cas9 cleavage site was present.

**DSB-ddPCR**

DSB-ddPCR was carried out as previously described\(^7\). A step-by-step protocol is available in Protocol Exchange [https://www.nature.com/protocolexchange/protocols/6021/](https://www.nature.com/protocolexchange/protocols/6021/) <download protocol exchange paper and site>. Briefly, two amplicons were designed for digital droplet PCR (ddPCR), one including the sgRNA binding/Cas9 cut site, the other a proximal control uncut site. Dual-quenched probes were used for the AAVS1 loci (Integrated DNA Technologies). Droplets were created using Droplet generating oil for probes, DG8 cartridges, DG8 Gaskets and the QX200™ Droplet generator (Bio-Rad), amplification was performed using ddPCR Supermix for Probes (Bio-Rad). The ddPCR Supermix amplification reactions were set up according to the manufacturer’s specifications (Bio-Rad). 1μL of DNeasy eluate (~50-150 ng), obtained as described above, was added to a 20 μL amplification reaction with final primer and probe concentrations of 900 nM and 250 nM respectively. The AAVS1 locus required addition of Smal and AvrII restriction enzymes (New England Biolabs) to the master mix for better separation of target and template amplicon probe signals (see Bio-Rad instructions). The reaction was divided into droplets for amplification following the manufacturer’s protocol (Bio-Rad). Droplets were transferred to a 96 well PCR plate and heat-sealed using PX1 PCR plate sealer (Bio-Rad). Droplets were amplified using the following cycling conditions: 95 °C x 10 minutes, 40 cycles (94 °C x 30 seconds, 60 °C x 60 seconds), 98 °C x 10 minutes. Following thermal cycling, droplets were individually scanned using the QX200™
Droplet Digital™ PCR system (Bio-Rad). Positive and negative droplets in each fluorescent channel (VIC/FAM) were distinguished on the basis of fluorescence amplitude using a global threshold, set by the minimal, intrinsic fluorescence signal resulting from imperfect quenching of the fluorogenic probes (negative droplets) as compared to the strong fluorescence signal from cleaved probes in droplets with amplified template(s). Cleaved control DNA was created by digesting genomic DNA using the restriction enzyme BSU36I for the AAVS1 locus (New England Biolabs) according to the manufacturer’s instructions. The DSB frequency in the control and experimental samples was calculated as:

\[
\frac{[\text{target}^- \cdot \text{template}^+]}{[\text{target}^- \cdot \text{template}^+] + [\text{target}^+ \cdot \text{template}^+]}
\]

To generate standard curves, restriction enzyme digested control DNA was mixed with uncleaved control DNA in defined amounts, DSB frequency was quantified using the ddPCR protocol and a linear regression was performed. For the time course experiments, absolute DSB frequencies were calculated by fitting raw frequencies to standard curves.