Multiplex gene editing by CRISPR–Cpf1 using a single crRNA array

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Targeting of multiple genomic loci with Cas9 is limited by the need for multiple or large expression constructs. Here we show that the ability of Cpf1 to process its own CRISPR RNA (crRNA) can be used to simplify multiplexed genome editing. Using a single customized CRISPR array, we edit up to four genes in mammalian cells and three in the mouse brain, simultaneously.

Although multiplex gene editing is possible with Cas9 nuclease, it requires relatively large constructs or simultaneous delivery of multiple plasmids[1–5], both of which are problematic for multiplex screens or *in vivo* applications. By contrast, the Cpf1 nuclease requires only one Pol III promoter to drive several small crRNAs (39 nt per crRNA).

We confirmed *in vitro* that Cpf1 alone is sufficient for maturation of crRNAs[6,7] (**Fig. 1a**) using an artificial CRISPR pre-crRNA array consisting of four spacers separated by direct repeats from the CRISPR locus of *Francisella novicida* (FnCpf1) and two Cpf1 orthologs with activity in mammalian cells, *Acidaminococcus* Cpf1 (AsCpf1) and *Lachnospiraceae* Cpf1 (LbCpf1) (**Fig. 1b** and **Supplementary Fig. 1**). Small RNA-seq showed that AsCpf1 cleavage products correlate to fragments resulting from cuts at the 5′ end of direct repeat hairpins, identical to the cleavage pattern we observed in *Escherichia coli* heterologously expressing FnCpf1 CRISPR systems[6] (**Fig. 1c**).

We further validated these results by generating AsCpf1 mutants that are unable to process arrays. Guided by the crystal structure of AsCpf1 (ref. 8), we mutated five conserved amino acid residues likely to disrupt array processing (H800A, K809A, K860A, F864A, and R790A)[8]. All mutations interfered with pre-crRNA processing but not DNA cleavage activity *in vitro* (**Fig. 1d** and **Supplementary Fig. 2a**,**b**), an effect that was also observed for FnCpf1 (ref. 7). AsCpf1 recognizes specific nucleotides at the 5′ flank of the direct repeat stem loop. Substitution of these nucleotides weakened or abolished RNA cleavage (**Supplementary Fig. 3a**). Dosage tests with the five AsCpf1 mutants revealed that mutants K809A, K860A, F864A, and R790A show pre-crRNA processing when used at high concentration (**Supplementary Fig. 3b**) or for extended incubation times (**Supplementary Fig. 3c**), but H800A was inactive regardless of dose and incubation time.

We next tested whether this mutant retains DNase activity in human embryonic kidney (HEK) 293T cells using three guides. Insertion/deletion (indel) frequency at the *DNMT1* and *GRIN2b* loci were identical for wild-type and H800A AsCpf1, and only slightly higher at the *VEGFA* locus in cells transfected with wild-type AsCpf1, demonstrating that the RNA and DNA cleavage activity can be separated in mammalian cells (**Fig. 1e**).

Cpf1-mediated RNA cleavage needs to be considered when designing lentivirus vectors for simultaneous expression of nuclease and guide (**Fig. 1f**). Lentiviruses carry a (+) strand RNA copy of the DNA sequence flanked by long terminal repeats, including the pre-crRNA, allowing Cpf1 to bind and cleave at the direct repeat sequence. Hence, reversing the orientation of the direct repeat is expected to result in (+) strand lentivirus RNAs not susceptible to Cpf1-mediated cleavage. We designed a lentiviruses encoding AsCpf1 and a crRNA expression cassette. We transduced HEK293T cells with an MOI (multiplicity of infection) of <0.3 and analyzed indel frequencies in puromycin-selected cells 10 d after infection. Using guides encoded on a reversed expression cassette targeting *DNMT1*, *VEGFA*, or *GRIN2b* resulted in robust indel formation for each targeted gene (**Fig. 1g**).

We leveraged the simplicity of Cpf1 crRNA maturation to achieve multiplex genome editing in HEK293T cells using customized CRISPR arrays. We chose four guides targeting different genes (*DNMT1*, *EMX1*, *VEGFA*, and *GRIN2b*) and constructed three arrays with variant direct repeat and guide lengths for expression of pre-crRNAs (array 1, 19 nt DR with 23 nt guide; array 2, 19 nt DR with 30 nt guide; array 3, 35 nt DR with 30 nt guide; **Fig. 2a**). Indel events were detected at each targeted locus in cells transfected with array 1 or array 2. However, the crRNA targeting *EMX1* resulted in indel frequencies of <2% when expressed from array 3. Overall, array 1 performed best, with all guides showing indel levels comparable to those mediated by single crRNAs (**Fig. 2b**). Furthermore, small RNA-seq confirmed that autonomous, Cpf1-mediated pre-crRNA processing occurs in mammalian cells

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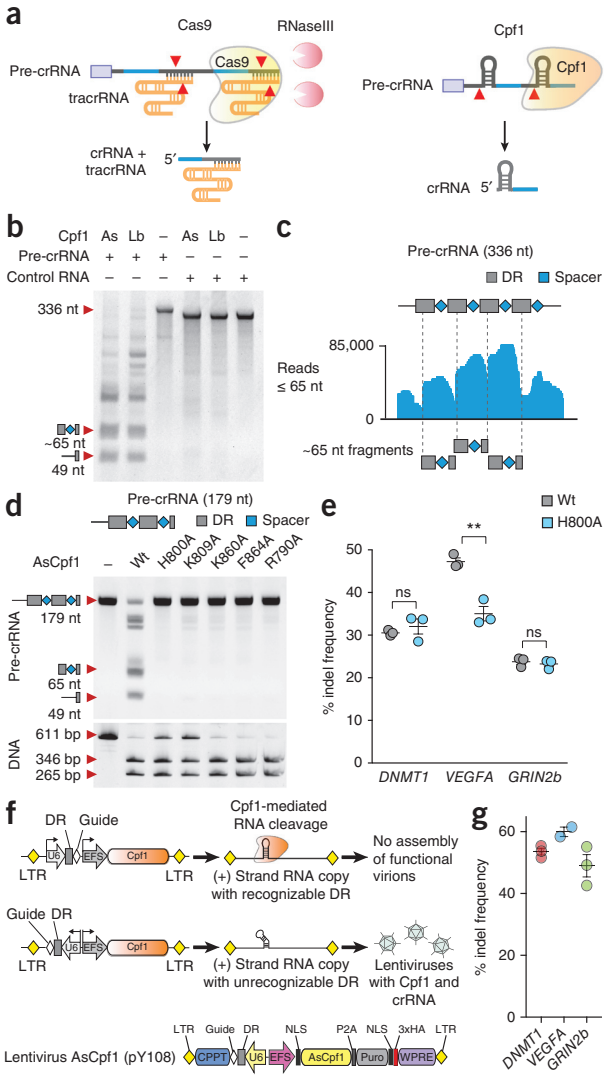


Figure 1 Cpf1-mediated processing of pre-crRNA is independent of DNA cleavage. (**a**) Schematic of pre-crRNA processing for Cas9 and Cpf1. Cleavage sites indicated with red triangles. Trans-activating crRNA (tracrRNA). (**b**) *In vitro* processing of FnCpf1 pre-crRNA transcript (80 nM) with purified AsCpf1 or LbCpf1 protein (~320 nM), cropped gel image. (For full gel, see **Supplementary Fig. 1**.) (**c**) RNA-seq analysis of FnCpf1 pre-crRNA cleavage products, as shown in **b**. A high fraction of sequence reads smaller than 65 nt are cleavage products of spacers flanked by direct repeat sequences, cropped gel images. (**d**) Pre-crRNA (top) and DNA cleavage (bottom) mediated by AsCpf1 point mutants. H800A, K809A, K860A, F864A, and R790A fail to process pre-crRNA but retain DNA cleavage activity *in vitro*. 330 nM pre-crRNA was cleaved with 500 nM Cpf1 in 15 min and 25 nM DNA was cleaved with 165 nM Cpf1 in 30 min. (For full gels, see **Supplementary Fig. 2**.) (**e**) Indel frequencies mediated by AsCpf1H800A are comparable to wt AsCpf1, bars are mean of 3 technical replicates from one experiment, error bars are s.e.m. (Student *t*-test; n.s., not significant; $^{**}P = 0.003$). (**f**) Schematic of lentivirus Cpf1 construct with the U6::direct repeat cassette in different orientations (top and middle), (+)-strand RNA copy with recognizable direct repeats are susceptible to Cpf1-mediated degradation, preventing functional virion formation. Schematic of AsCpf1 (pY108) construct (bottom). (**g**) Indel frequencies analyzed by SURVEYOR nuclease assay after puromycin selection 10 d after transduction with lentivirus AsCpf1 in HEK cells. Horizontal bars are mean of 2 or 3 individual infections; error bars are mean ± s.e.m. U6, Pol III promoter; CMV, cytomegalovirus promoter; NLS, nuclear localization signal; HA, hemagglutinin tag; DR, direct repeat sequence; P2A, porcine teschovirus-1 2A self-cleaving peptide; LTR, long terminal repeat; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element.

(**Fig. 2c**). Using arrays with guides in different orders resulted in similar indel frequencies, suggesting that positioning within an array is not crucial for activity (**Supplementary Fig. 4a,b**).

To confirm that multiplex editing occurs within single cells, we generated AsCpf1-P2A-GFP constructs to enable fluorescence-activated cell sorting (FACS) of transduced single cells (**Fig. 2d**) and clonal expansion. We used next-generation deep sequencing (NGS) to compare edited loci within clonal colonies derived from cells transfected with either pooled single guides or array 1. Focusing on targeted genes edited at every locus (indels ≥95%) shows that multiplex editing occurs more frequently in colonies transfected with array 1 (6.4% all targets, 12.8% three targets, 48.7% two targets) than in pooled transfection (2.4% all targets, 3.6% three targets, 11.9% two targets) (**Fig. 2e**).

We next tested multiplex genome editing in neurons using AsCpf1. We designed a gene-delivery system based on adeno-associated viral vectors (AAVs) for expression of AsCpf1. We generated a dual vector system in which AsCpf1 and the CRISPR-Cpf1 array were cloned separately (**Fig. 2f**). We constructed a U6-promoter-driven Cpf1 array targeting the neuronal genes *Mecp2*, *Nlgn3*, and *Drd1*. This plasmid also included a green fluorescent protein (GFP), fused to KASH nuclear transmembrane domain[9], in order to enable FACS of targeted cell nuclei[10].

We first transduced mouse primary cortical neurons *in vitro* and observed robust expression of AsCpf1 and GFP-KASH 1 week after viral delivery. A SURVEYOR nuclease assay run on purified neuronal DNA confirmed indel formations in all three targeted genes (**Supplementary Fig. 5**). Next, we tested whether AsCpf1 could be expressed in the brains of living mice for multiplex genome editing *in vivo*. We stereotactically injected our dual vector system in a 1:1 ratio into the hippocampal dentate gyrus (DG) of adult male mice. Four weeks after viral delivery we observed robust expression of AsCpf1 and GFP-KASH in the DG (**Fig. 2g,h**). Consistent with previous studies[10,11], we observed ~75% co-transduction efficiency of the dual viral vectors (**Supplementary Fig. 5c**). We isolated targeted DG cell nuclei by FACS (**Supplementary Fig. 6**) and quantified indel formation using NGS. We found indels in all three targeted loci with ~23%, ~38%, and ~51% indel formation in *Mecp2*, *Nlgn3*, and *Drd1*, respectively (**Supplementary Fig. 5d,e**). We quantified the effectiveness of biallelic disruption of the autosomal gene *Drd1* and found that ~47% of all sorted nuclei (i.e., ~87% of all *Drd1*-edited cells) harbored biallelic modifications (**Fig. 2i**). Next, we quantified the multiplex targeting efficiency in single neuronal nuclei. Our results show that ~17% of all transduced neurons were modified in all three targeted loci (**Fig. 2j**). Taken together, our results demonstrate the effectiveness of AAV-mediated delivery of AsCpf1 into the mammalian brain and simultaneous multi-gene targeting *in vivo* using a single array transcript.

Taken together, these data highlight the utility of Cpf1 array processing in designing simplified systems for *in vivo* multiplex gene editing. This system should simplify guide RNA delivery for many genome editing applications in which targeting of multiple genes is desirable.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Accession codes. SRA: PRJNA354073

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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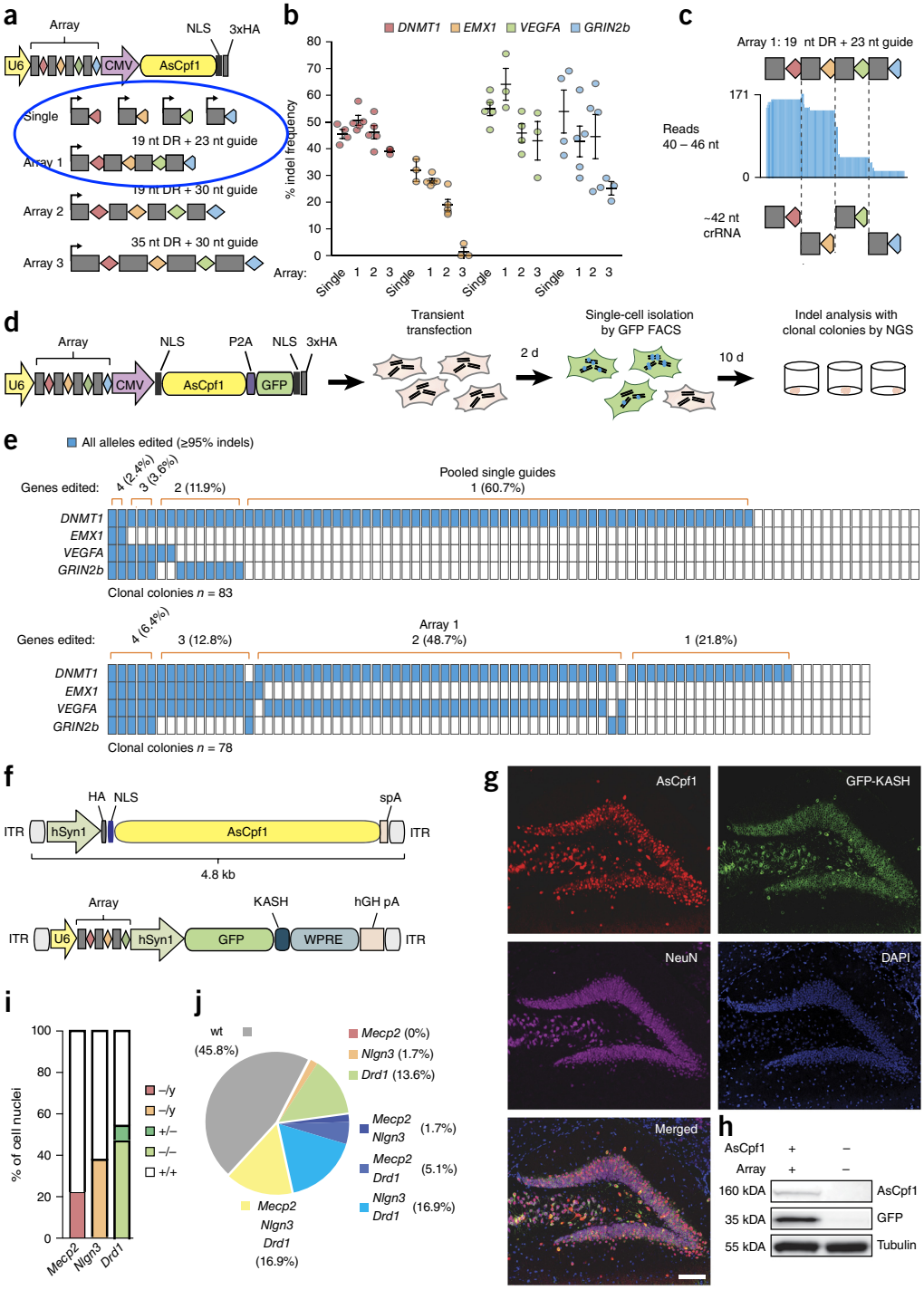


Figure 2 Cpf1-mediated multiplex gene editing in mammalian cells and mouse brain. (**a**) Schematic of multiplex gene editing with AsCpf1, using a single plasmid approach. (**b**) Genome editing at four different genomic loci mediated by AsCpf1 with different versions of artificial CRISPR arrays (array 1, crRNAs in their mature form (19-nt DR with 23-nt guide); array 2, crRNAs are in an intermediate form (19-nt DR with 30-nt guide); array 3 crRNAs are in their unprocessed form (35-nt DR with 30-nt guides)). Indels were analyzed by SURVEYOR nuclease assay 3 d after transfection. Horizontal bars are the means of two individual experiments with three to five technical replicates; error bars are mean ± s.e.m. (**c**) Small RNA-seq reads from HEK cells transfected with AsCpf1 and array 1 show fragments corresponding to mature crRNA for each of the four guides. (**d**) Schematic for analysis of indel events in clonal colonies 48 h after transient transfection. (**e**) Quantification of indel events measured by NGS in clonal colonies from HEK cells transiently transfected with pooled single-guide-RNA plasmids or plasmid carrying array 1. Colonies were expanded for 10 d after sorting. Each column represents one clonal colony; blue rectangles indicate target genes with all alleles edited. (**f**) Schematic of AAV vector design for multiplex gene editing. Bottom: gray rectangles, direct repeat; diamonds, spacer (red: *Mecp2*, orange: *Nlgn3*, green: *Drd1*). (**g**) Immunostaining of dorsal DG 4 weeks after stereotactic AAV injection (representative image of $n = 4$ mice). Brain sections were co-stained with anti-HA (red), anti-GFP (green) and anti-NeuN (magenta) antibodies. Nuclei were labeled with DAPI (blue). Scale bar, 100 um. (**h**) Western blot analysis of DG expressing HA-AsCpf1 and GFP-KASH (representative blot from $n = 4$ mice). (**i**) Fraction of mono- (–/+), bi- (–/–) or maternal (–/y) allele editing for *Drd1* (autosomal), *Mecp2* and *Nlgn3* (x-chromosomal). (**j**) Analysis of multiplexing efficiency in individual cells. ITR, inverted terminal repeat; spA, synthetic polyadenylation signal; hSyn1, human synapsin 1 promoter; KASH ANC1, Syne homology nuclear transmembrane domain; hGH pA, human growth hormone polyadenylation signal.

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AUTHOR CONTRIBUTIONS

B.Z., M.H., J.v.d.O., and F.Z. conceived this study and designed the experiments. B.Z., M.H., P.M., I.F., J.K., E.M.D., N.W., S.R.C., O.O.A., J.S.G., W.Y.W. and D.A.S. conducted the experiments. K.S., J.v.d.O., and F.Z. supervised this project. B.Z., M.H., J.v.d.O., and F.Z. wrote the manuscript with input from all authors.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Cpf1 protein purification. Humanized Cpf1 were cloned into a bacterial expression vector (6-His-MBP-TEV-Cpf1, a pET-based vector kindly given to us by Doug Daniels). Two liters of Terrific Broth growth media with 100 µg/mL ampicillin was inoculated with 10 mL of an overnight culture of Rosetta (DE3) pLyseS (EMD Millipore) cells containing the Cpf1 expression construct. Growth media plus inoculant was grown at 37 °C until the cell density reached 0.2 OD_{600}, then the temperature was decreased to 21 °C. Growth was continued until OD_{600} reached 0.6 when a final concentration of 500 µM IPTG was added to induce MBP-Cpf1 expression. The culture was induced for 14–18 h before harvesting cells and freezing at −80 °C until purification. Cell paste was resuspended in 200 mL of lysis buffer (50 mM HEPES pH 7, 2 M NaCl, 5 mM $MgCl_2$, 20 mM imidazole) supplemented with protease inhibitors (Roche cOmplete, EDTA-free) and lysozyme. Once homogenized, cells were lysed by sonication (Branson Sonifier 450), then centrifuged at 10,000*g* for 1 h to clear the lysate. The lysate was filtered through 0.22-µm filters (Millipore, Stericup) and applied to a nickel column (HisTrap FF, 5 mL), washed, and then eluted with a gradient of imidazole. Fractions containing protein of the expected size were pooled, TEV protease (Sigma) was added, and the sample was dialyzed overnight into TEV buffer (500 mM NaCl, 50 mM HEPES pH 7, 5 mM $MgCl_2$, 2 mM DTT). After dialysis, TEV cleavage was confirmed by SDS-PAGE, and the sample was concentrated to 500 µL before loading on a gel filtration column (HiLoad 16/600 Superdex 200) via FPLC (fast protein liquid chromatography, AKTA Pure). Fractions from gel filtration were analyzed by SDS-PAGE; fractions containing Cpf1 were pooled and concentrated to 200 µL (50 mM Tris-HCl pH7.5, 2 mM DTT, 5% glycerol, 500 mM NaCl) and either used directly for biochemical assays or frozen at −80 °C for storage.

***In vitro* synthesis of pre-crRNA arrays.** Pre-crRNA arrays were synthesized using the HiScribe T7 High Yield RNA Synthesis Kit (NEB). PCR fragments coding for arrays, with a short T7-priming sequence on the 5′ end, were used as templates for *in vitro* transcription reaction (**Supplementary Table 1**). T7 transcription was performed for 4 h and then RNA was purified using the MEGAclear Transcription Clean-Up Kit (Ambion).

***In vitro* cleavage assay.** *In vitro* cleavage was performed with purified recombinant proteins for AsCpf1 and LbCpf1. Cpf1 protein and *in vitro*-transcribed pre-crRNA arrays were incubated at 37 °C in cleavage buffer (20 mM Tris HCl, 50 mM KCl supplemented with RNase Inhibitor Murine (NEB)) for 5 min to 1 h, as indicated in figure legends. Each cleavage reaction contained 20–630 nM of Cpf1 protein and 165 or 330 nM of synthetized pre-crRNA array, as indicated in figure legends. For DNA cleavage, 25 nM of target was cleaved with 165 nM Cpf1 and 340 nM crRNA for 30 min at 37 °C. Reactions were stopped with proteinase K (Qiagen), heat denaturation and run on 10% TBE-Urea polyacrylamide gels. Gels were stained with SYBR Gold DNA stain (Life Technologies) for 10 min and imaged with a Gel Doc EZ gel imaging system (Bio-Rad).

Pre-crRNA array design and cloning. Guide sequences targeting human genes are listed in **Supplementary Table 2**. crRNAs were designed as four oligos (IDT) consisting of direct repeats, each one followed by a crRNA (**Supplementary Table 3**). The oligos favored a one-directional annealing through their sticky-end design. The oligonucleotides (final concentration 10 µM) were annealed in 10×T4 ligase buffer (final concentration 1×; NEB) and T4 PNK (5 units; NEB). Thermocycler conditions were adjusted to 37 °C for 30 min, 95 °C for 5 min followed by a −5 °C/min ramp down to 25 °C. The annealed oligonucleotides were diluted 1:10 (final concentration 1 µM) and ligated into *BsmB*I-cut pcDNA-huAsCpf1-U6 (pY26), using T7 DNA ligase (Enzymatics), in room temperature for 30 min. The constructs were transformed into STBL3 bacteria and plated on ampicillin-containing (100 g/ml) agar plates. Single colonies were grown in standard LB media (Broad Facilities) for 16 h. Plasmid DNA was harvested from bacteria according to QIAquick Spin Miniprep protocol (QIAGEN).

Cell culture and transfection. Human embryonic kidney 293T (HEK293T) cell line (Life Technologies) were maintained in Dulbecco's modified Eagle's Medium (DMEM) + GLUTAMAX (Gibco) supplemented with 10% FBS (HyClone) at 37 °C with 5% CO_2 incubation. HEK293FT cells were seeded onto 24-well plates (Corning) 24 h before transfection. Cells were transfected using Lipofectamine 2000 (Life Technologies) at 70–80% confluency following the manufacturer's recommended protocol. For each well of a 24-well plate, a total of 500 ng plasmid DNA was used; each well represents one technical replicate.

Surveyor nuclease assay for genome modification. HEK293T cells were transfected with DNA, as described above. Cells were incubated at 37 °C for 72 h after transfection before genomic DNA extraction. Genomic DNA was extracted using the QuickExtract DNA Extraction Solution (Epicentre) following the manufacturer's protocol. Briefly, pelleted cells were resuspended in QuickExtract solution and incubated at 65 °C for 15 min, 68 °C for 15 min, and 98 °C for 10 min. The genomic region flanking the CRISPR target site for each gene was PCR amplified (primers listed in **Supplementary Table 4**), and products were purified using QIAQuick PCR purification Kit (Qiagen) following the manufacturer's protocol. 200 ng total of the purified PCR products were mixed with 1 µl 10× Taq DNA Polymerase PCR buffer (Enzymatics) and ultrapure water to a final volume of 10 µl, and subjected to a re-annealing process to enable heteroduplex formation: 95 °C for 10 min, 95 °C to 85 °C ramping at −2 °C/s, 85 °C to 25 °C at −0.25 °C/s, and 25 °C hold for 1 min. After re-annealing, products were treated with Surveyor nuclease and Surveyor enhancer S (IDT) following the manufacturer's recommended protocol, and analyzed on 10% Novex TBE polyacrylamide gels (Life Technologies). Gels were stained with SYBR Gold DNA stain (Life Technologies) for 10 min and imaged with a Gel Doc gel imaging system (Bio-Rad). Quantification was based on relative band intensities. Indel percentage was determined by the formula, $100 \times (1 - (1 - (b + c)/(a + b + c))1/2)$, where a is the integrated intensity of the undigested PCR product, and b and c are the integrated intensities of each cleavage product.

Small RNA extraction from cells. HEK293T cells were harvested 48 h after transfection and the total RNA was extracted with the miRNeasy mini kit (Qiagen) according to manufacturer's conditions. rRNA was removed using the bacterial Ribo-Zero rRNA removal kit (Illumina).

NGS analysis of *in vitro* and *in vivo* cleavage pattern. RNA-seq libraries were prepared using a derivative of a previously described method[12]. Briefly, after PNK treatment in the absence and presence of ATP (enrichment of 5′OH and 3′P, respectively) RNA cleavage products were poly-A tailed with *E. coli* Poly(A) Polymerase (NEB), ligated to 5′ RNA adapters using T4 RNA ligase I (NEB) and reverse transcribed with AffinityScript Multiple Temperature Reverse Transcriptase (Agilent Technologies). cDNA was amplified by a fusion PCR method to attach the Illumina P5 adapters as well as unique sample-specific barcodes to the target amplicons[13]. PCR products were purified by gel-extraction using QiaQuick PCR purification Kit (Qiagen) following the manufacturer's recommended protocol. DNA samples from single nuclei were pre-amplified with SURVEYOR primers (**Supplementary Table 4**) and nested-PCR was performed with NGS primers (**Supplementary Table 5**) before Illumina barcodes were added. Finally, barcoded and purified DNA samples were quantified by Qubit 2.0 Fluorometer (Life Technologies) and pooled in an equimolar ratio. Sequencing libraries were then sequenced with the Illumina MiSeq Personal Sequencer (Life Technologies).

RNA-sequencing analysis. The prepared cDNA libraries were pooled and sequenced on a MiSeq (Illumina). Pooled sequencing reads were assigned to their respective samples on the basis of their corresponding barcodes and aligned to the proper CRISPR array template sequence using BWA 3. Interval lists were generated using the paired-end alignment coordinates and the intervals were used to extract entire transcript sequences using Galaxy tools (https://usegalaxy.org/)[14]. The extracted transcript sequences were analyzed using Geneious 9.

AAV DNA constructs. The AAV hSyn1-HA-NLS-AsCpf1-spA vector was generated by PCR amplifying the AsCpf1 encoding sequence using forward PCR primer including HA-NLS (5′-aacacaggaccggtgccaccatgtacccatacgat gttccagattacgcttcgccgaagaaaaagcgcaaggtcgaagcgtccacacagttcgagggctttaccaa

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cctgtatcaggtgagc-3′) and reverse PCR primer including a short poly A signal (spA)(5′-gcggccgcacacaaaaaaccaacacacagatctaatgaaaataaagatcttttattgaattc ttagttgcgcagctcctggatgtaggccagcc-3′)[10], and cloning of the resulting PCR template into AAV backbone under control of the human *Synapsin* 1 promoter (hSyn1). For the generation of AAV U6-DR(*Sap*I)-hSyn1-GFP-KASH-hGH (not shown) and U6-*Mecp2-Nlgn3-Drd1* array-hSyn1-GFP-KASH-hGH vectors, gene blocks (Integrated DNA Technologies) encoding U6-DR(*Sap*I) and U6-*Mecp2-Nlgn3-Drd1* array, respectively, have been cloned into AAV hSyn-GFP-KASH-hGH backbone[10]. All constructs were verified by Sanger sequencing.

Production of AAV vectors. AAV1 particles in DMEM culture medium were produced as described previously[11]. Briefly, HEK293FT cells were transfected with transgene plasmid, AAV1 serotype plasmid and pDF6 helper plasmid using polyethyleneimine (PEI). DMEM culture medium containing low-titer AAV1 particles was collected after 48 h and sterile filtered. For high-titer AAV1/2 production, HEK293FT cells were transfected with AAV1 and AAV2 serotype plasmids in equal ratios, transgene plasmid and pDF6 helper plasmid. 48 h after transfection, cells were harvested and high-titer AAV1/2 virus was purified on heparin affinity column[11]. The titer of AAV vectors was determined by real-time quantitative PCR (qPCR) using probe and primers specific for the hSyn1 promoter sequence (Integrated DNA Technologies).

Primary cortical neuron culture. Mice used to obtain neurons for tissue cultures were euthanized according to the protocol approved by the Broad's Institutional Animal Care and Use Committee (IACUC). Primary neurons were prepared from postnatal day P0.5 mouse brains and plated on laminin/poly-D-lysine-coated coverslips (VWR). Briefly, cortices were dissected in ice-cold HBSS (Sigma) containing 50 μg/ml penicillin/streptomycin (Thermo Fisher) and incubated for 10 min at 37 °C with HBSS containing 125 Units papain (Worthington Biochemical) and 400 Units DNase I (Sigma). After enzymatic digestion, the tissues were washed twice in HBSS and gently triturated with a fire-polished Pasteur pipette. Cells were then transferred into neuronal growth medium (Neurobasal A medium, supplemented with B27, Glutamax (Life Technologies) and penicillin/streptomycin) and grown at 37 °C and 5% CO_2. For inhibition of glia cell proliferation, cytosine-beta-D-arabinofuranoside (AraC, Sigma) at a final concentration of 10 μM was added to the culture medium after 48 h and replaced by fresh culture medium after 72 h. For AAV1 transduction, cultured neurons were infected with low-titer AAV1 as described previously[11]. One week after transduction, neurons were harvested for isolating genomic DNA (QuickExtract DNA extraction buffer (Epicentre)), or fixed in 4% paraformaldehyde (PFA) for immunofluorescence staining.

Stereotactic injection of AAV1/2 into the mouse brain. The Broad's Institutional Animal Care and Use Committee (IACUC) approved all animal procedures described here. Craniotomy was performed on adult (12–16 weeks) male C57BL/6N mice according to approved procedures, and 1 μl of 1:1 AAV mixture (AAV hSyn1-HA-NLS-AsCpf1-spA: 2.25×10^{12} Vg/ml; AAV U6-*Mecp2-Nlgn3-Drd1* array-hSyn1-GFP-KASH-hGH: 9.7×10^{12} Vg/ml) was injected into the dorsal dentate gyrus (anterior/posterior: −1.7; mediolateral: +/−0.6; dorsal/ventral: −2.15). The pipette was held in place for 3–5 min after injection to prevent leakage. After injection, the incision was sutured and post-operative analgesics were administered according to approved IACUC protocol for 3 d following surgery.

Purification of cell nuclei from intact brain tissue. Cell nuclei from AAV1/2-injected hippocampal tissue were purified as described previously[10]. Briefly, dissected tissue was homogenized in ice-cold homogenization buffer (HB) (320 mM sucrose, 5 mM $CaCl_2$, 3 mM $Mg(Ac)_2$, 10 mM Tris pH7.8, 0.1 mM EDTA, 0.1% NP40, 0.1 mM PMSF, 1 mM β-mercaptoethanol) using 2 ml type A and B Dounce homogenizer (Sigma). For gradient centrifugation, OptiPrep density gradient medium (Sigma) was used. Samples were centrifuged at 10,100*g* (7,500 r.p.m.) for 30 min at 4 °C (Beckman Coulter, SW28 rotor). Cell nuclei pellets were resuspended in 65 mM β-glycerophosphate (pH 7.0), 2 mM $MgCl_2$, 25 mM KCl, 340 mM sucrose, and 5% glycerol. Number and quality of purified nuclei was examined using bright-field microscopy.

FACS of cell nuclei. Purified cell nuclei were co-labeled with Vybrant DyeCycle Ruby Stain (1:500, Life Technologies) and sorted using a Beckman Coulter MoFlo Astrios EQ cell sorter (Broad Institute Flow Cytometry Core). Single and population (250–500 nuclei) GFP-KASH$^+$ and GFP-KASH$^-$ nuclei were collected in 96-well plates containing 5 μl of QuickExtract DNA extraction buffer (Epicentre) and spun down at 2,000*g* for 2 min. Each 96-well plate included two empty wells as negative control.

Western blot analysis. AAV-injected dentate gyrus tissues were lysed in 100 μl of ice-cold RIPA buffer (Cell Signaling Technologies) containing 0.1% SDS and protease inhibitors (Roche, Sigma) and sonicated in a Bioruptor sonicator (Diagenode) for 1 min. Protein concentration was determined using the BCA Protein Assay Kit (Pierce Biotechnology, Inc.). Protein samples were separated under reducing conditions on 4–15% Tris-HCl gels (Bio-Rad) and analyzed by western blotting using primary antibodies: mouse anti-HA (Cell Signaling Technologies 1:500), mouse anti-GFP (Roche, 1:500), rabbit anti-Tubulin (Cell Signaling Technologies, 1:10,000) followed by secondary anti-mouse and anti-rabbit HRP antibodies (Sigma-Aldrich, 1:10,000). Blots were imaged with Amersham Imager 600.

Immunofluorescent staining. 4 weeks after viral delivery, mice were transcardially perfused with PBS followed by PFA according to approved IACUC protocol. 30 μm free-floating sections (Leica, VT1000S) were boiled for 2 min in sodium citrate buffer (10 mM tri-sodium citrate dehydrate, 0.05% Tween20, pH 6.0) and cooled down at RT for 20 min. Sections were blocked with 4% normal goat serum (NGS) in TBST (137 mM NaCl, 20 mM Tris pH 7.6, 0.2% Tween-20) for 1 h. Primary antibodies were diluted in TBST with 4% NGS and sections were incubated overnight at 4 °C. After three washes in TBST, samples were incubated with secondary antibodies for 1 h at RT. After washing three times with TBST, sections were mounted using VECTASHIELD HardSet Mounting Medium including DAPI and visualized with confocal microscope (Zeiss LSM 710, Ax10 ImagerZ2, Zen 2012 Software). Following primary antibodies were used: mouse anti-NeuN (Millipore, 1:400); chicken anti-GFP (Aves Labs, 1:200–1:400); rabbit anti-HA (Cell Signaling Technologies, 1:100). Anti-HA signaling was amplified using biotinylated anti-rabbit (1:200) followed by streptavidin AlexaFluor 568 (1:500) (Life Technologies). Anti-chicken AlexaFluor488 and anti-mouse AlexaFluor647 secondary antibodies (Life Technologies) were used at 1:1,000.

Randomization and blinding. Neither randomization nor blinding were used in these experiments.

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