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Supplemental information

Negative autoregulation mitigates collateral RNase activity of repeat-targeting CRISPR-Cas13d in mammalian cells

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SUPPLEMENTAL FIGURES

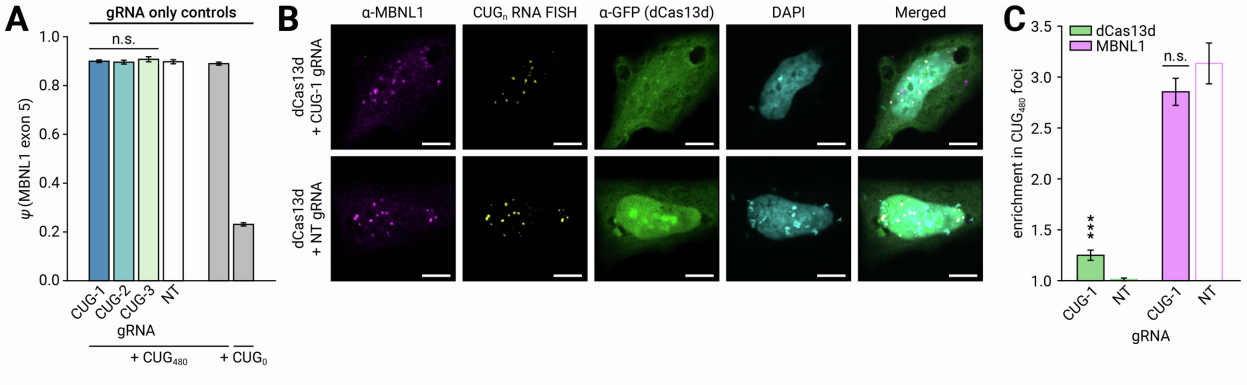

Figure S1. gRNA only controls for splicing assay and dCas13d/MBNL1 competition, related to Figure 1. A *MBNL1* exon 5 minigene splicing assay after transfection of HeLa cells with gRNA and target plasmids, in the absence of Cas13d. n=3 transfections per condition. n.s.: not significant, p>0.05, two-tailed Student's *t* test. **B** Simultaneous FISH/IF for MBNL1 (α-MBNL1 IF, magenta), CUG_{480} RNA (CAG_{10} FISH probe, yellow), and dCas13d-EGFP (α-GFP IF, green) in transfected HeLa cells. Nuclei stained with DAPI (cyan). Scale bars 10 μm. **C** Quantification of colocalization of dCas13d and MBNL1 IF signal with nuclear CUG_{480} RNA foci in FISH/IF experiment. n>15 nuclei per condition. ***p<0.001, two-sided Mann-Whitney *U* test. n.s.: not significant, p>0.05, two-sided Mann-Whitney *U* test.

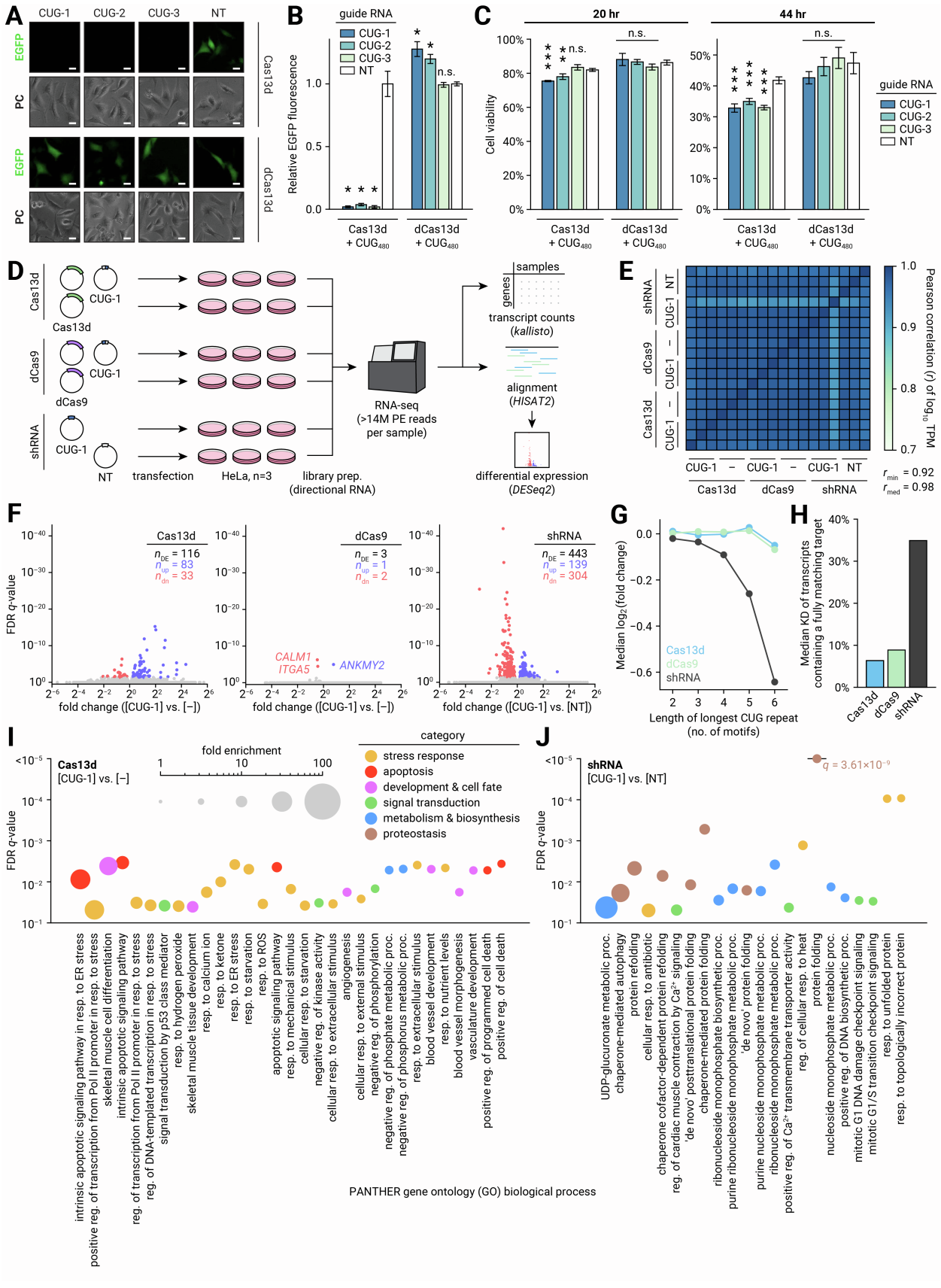
A
CUG-1
CUG-2
CUG-3
NT
EGFP
PC
Cas13d
dCas13d
B
guide RNA
CUG-1
CUG-2
CUG-3
NT
Relative EGFP fluorescence
n.s.
Cas13d + CUG480
dCas13d + CUG480
C
20 hr
44 hr
Cell viability
n.s.
guide RNA
D
Cas13d
dCas9
shRNA
CUG-1
NT
transfection
HeLa, n=3
library prep. (directional RNA)
RNA-seq (>14M PE reads per sample)
samples
genes
transcript counts (kallisto)
alignment (HISAT2)
differential expression (DESeq2)
E
shRNA
dCas9
Cas13d
Pearson correlation (r) of log10 TPM
rmin = 0.92
rmed = 0.98
F
Cas13d
nDE = 116
nup = 83
ndn = 33
dCas9
nDE = 3
nup = 1
ndn = 2
shRNA
nDE = 443
nup = 139
ndn = 304
FDR q-value
CALM1
ITGA5
ANKMY2
fold change ([CUG-1] vs. [–])
fold change ([CUG-1] vs. [NT])
G
Median log2(fold change)
Cas13d
dCas9
shRNA
Length of longest CUG repeat (no. of motifs)
H
Median KD of transcripts containing a fully matching target
I
Cas13d
[CUG-1] vs. [–]
fold enrichment
category
stress response
apoptosis
development & cell fate
signal transduction
metabolism & biosynthesis
proteostasis
FDR q-value
intrinsic apoptotic signaling pathway in resp. to ER stress
positive reg. of transcription from Pol II promoter in resp. to stress
skeletal muscle cell differentiation
intrinsic apoptotic signaling pathway
reg. of transcription from Pol II promoter in resp. to stress
reg. of DNA-templated transcription in resp. to stress
signal transduction by p53 class mediator
resp. to hydrogen peroxide
skeletal muscle tissue development
resp. to calcium ion
resp. to ketone
resp. to ER stress
resp. to starvation
resp. to ROS
apoptotic signaling pathway
resp. to mechanical stimulus
cellular resp. to starvation
negative reg. of kinase activity
cellular resp. to extracellular stimulus
angiogenesis
cellular resp. to external stimulus
negative reg. of phosphorylation
negative reg. of phosphate metabolic proc.
negative reg. of phosphorus metabolic proc.
resp. to extracellular stimulus
blood vessel development
resp. to nutrient levels
blood vessel morphogenesis
vasculature development
positive reg. of programmed cell death
positive reg. of cell death
PANTHER gene ontology (GO) biological process
J
shRNA
[CUG-1] vs. [NT]
q = 3.61×10−9
UDP-glucuronate metabolic proc.
chaperone-mediated autophagy
protein refolding
cellular resp. to antibiotic
chaperone cofactor-dependent protein refolding
reg. of cardiac muscle contraction by Ca2+ signaling
'de novo' posttranslational protein folding
chaperone-mediated protein folding
ribonucleoside monophosphate biosynthetic proc.
purine ribonucleoside monophosphate metabolic proc.
'de novo' protein folding
purine nucleoside monophosphate metabolic proc.
ribonucleoside monophosphate metabolic proc.
positive reg. of Ca2+ transmembrane transporter activity
reg. of cellular resp. to heat
protein folding
nucleoside monophosphate metabolic proc.
positive reg. of DNA biosynthetic proc.
mitotic G1 DNA damage checkpoint signaling
mitotic G1/S transition checkpoint signaling
resp. to unfolded protein
resp. to topologically incorrect protein

Figure S2. CUG-targeted Cas13d suppresses EGFP expression and upregulates stress response and apoptosis pathways, related to Figure 2. A Visualization of unfused EGFP marker on Cas13d plasmid 20 hr after transfection with Cas13d, gRNA, and CUG_{480} target plasmids. PC: phase contrast. Scale bars 20 μm. **B** Quantification of EGFP expression by plate reader 20 hr after transfection. n=3 transfections per condition. Error bars indicate standard deviation. *p<0.05, two-tailed Student's *t* test. n.s.: not significant, p>0.05. **C** Resazurin cell viability assay performed 20 hr and 44 hr after transfection with Cas13d, gRNA, and CUG_{480} target plasmids. n=5 transfections per condition. Error bars indicate standard deviation. *p<0.05, **p<0.01, ***p<0.001, two-tailed Student's *t* test. n.s.: not significant, p>0.05. **D** Description of RNA-seq experiment to assess transcriptomic changes induced by Cas13d. HeLa cells were transfected with Cas13d, dCas9, or shRNA in CUG-targeting or non-targeting conditions and incubated for 3 days prior to RNA extraction, library preparation, and sequencing. n=3 transfections per condition. Data were processed using kallisto, HISAT2, and DESeq2 for alignment and differential expression (DE) analysis. **E** Heatmap of correlation coefficients of $\log_{10}$ TPM between sequencing libraries. **F** Volcano plots of DESeq2 false discovery rate (FDR)-corrected q-value vs. fold change in targeting and non-targeting conditions. DE genes (FDR q<0.05) are highlighted in red (downregulated) or blue (upregulated). **G** Plot of median fold change of transcripts in targeting and non-targeting conditions, binned by maximum CUG repeat length within the transcript in the human reference genome. **H** Median knockdown between targeting and non-targeting conditions of all transcripts containing a CUG repeat as long as or longer than the length of the Cas13d spacer (22 nt). **I** PANTHER gene ontology (GO) analysis of biological processes enriched in the DE genes between CUG-targeting and non-targeting Cas13d conditions. Enriched processes are defined as processes with a ratio of observed to expected genes >5 and FDR q<0.05. For each process, FDR q is plotted on the vertical axis and enrichment is indicated by circle area. Color indicates classification into functional categories. **J** PANTHER GO analysis of processes enriched in DE genes between CUG-targeting and non-targeting shRNA conditions.

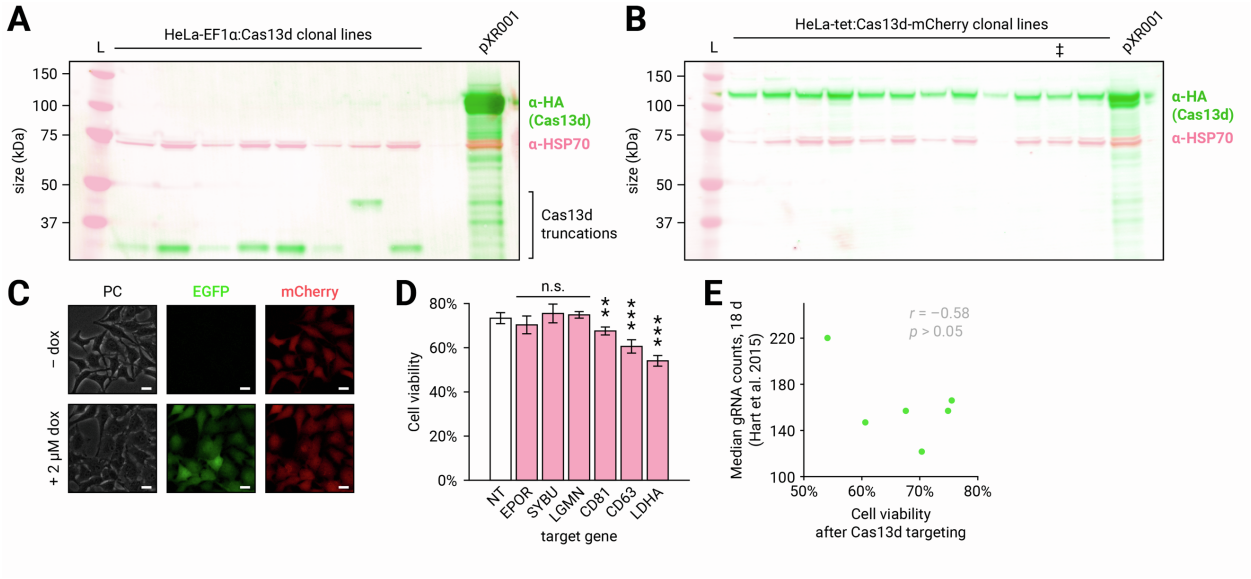

Figure S3. Development of HeLa-tet:Cas13d-mCherry cell line and cell viability upon targeting endogenous genes, related to Figure 3. A Fluorescent western blot of protein extracted from clonal HeLa cell lines after treatment with lentivirus encoding Cas13d-T2A-EGFP under the constitutive EF1α promoter. Blot stained with α-HA (green) and α-HSP70 (red) primary antibodies. Expected MW of Cas13d is 117 kDa, lower bands indicate truncations of Cas13d that retained expression of the downstream EGFP marker. L: protein ladder, pXR001: transient transfection of Cas13d plasmid in HeLa. **B** Fluorescent western blot of protein extracted from clonal HeLa cell lines after integration of constitutive mCherry and tetracycline-inducible Cas13d-T2A-EGFP. Expression induced with 2 uM doxycycline for 44 hr prior to protein extraction. Blot stained with α-HA (green) and α-HSP70 (red) primary antibodies. ‡ indicates the clone chosen for subsequent experiments. L: protein ladder, pXR001: transient transfection of Cas13d plasmid in HeLa. **C** Visualization of EGFP and mCherry before and after 44 hr doxycycline treatment by fluorescence microscopy. PC: phase contrast. Scale bars 20 μm. **D** Resazurin cell viability assay of HeLa-tet:Cas13d-mCherry cells transfected with plasmids encoding gRNAs targeting endogenous genes and induced with 2 uM doxycycline for 44 hr. n=5 transfections per condition. Error bars indicate standard deviation. *p<0.05, **p<0.01, ***p<0.001, two-tailed Student's *t* test. n.s.: not significant, p>0.05. **E** Comparison of cell viability measured 44 hr after Cas13d expression with gRNA targeting endogenous genes vs. depletion of gRNAs targeting the same genes in a CRISPR essentiality screen in HeLa (Hart et al., 2015). Pearson correlation coefficient is shown. n=5 transfections per condition. p>0.05, beta distribution c.d.f.

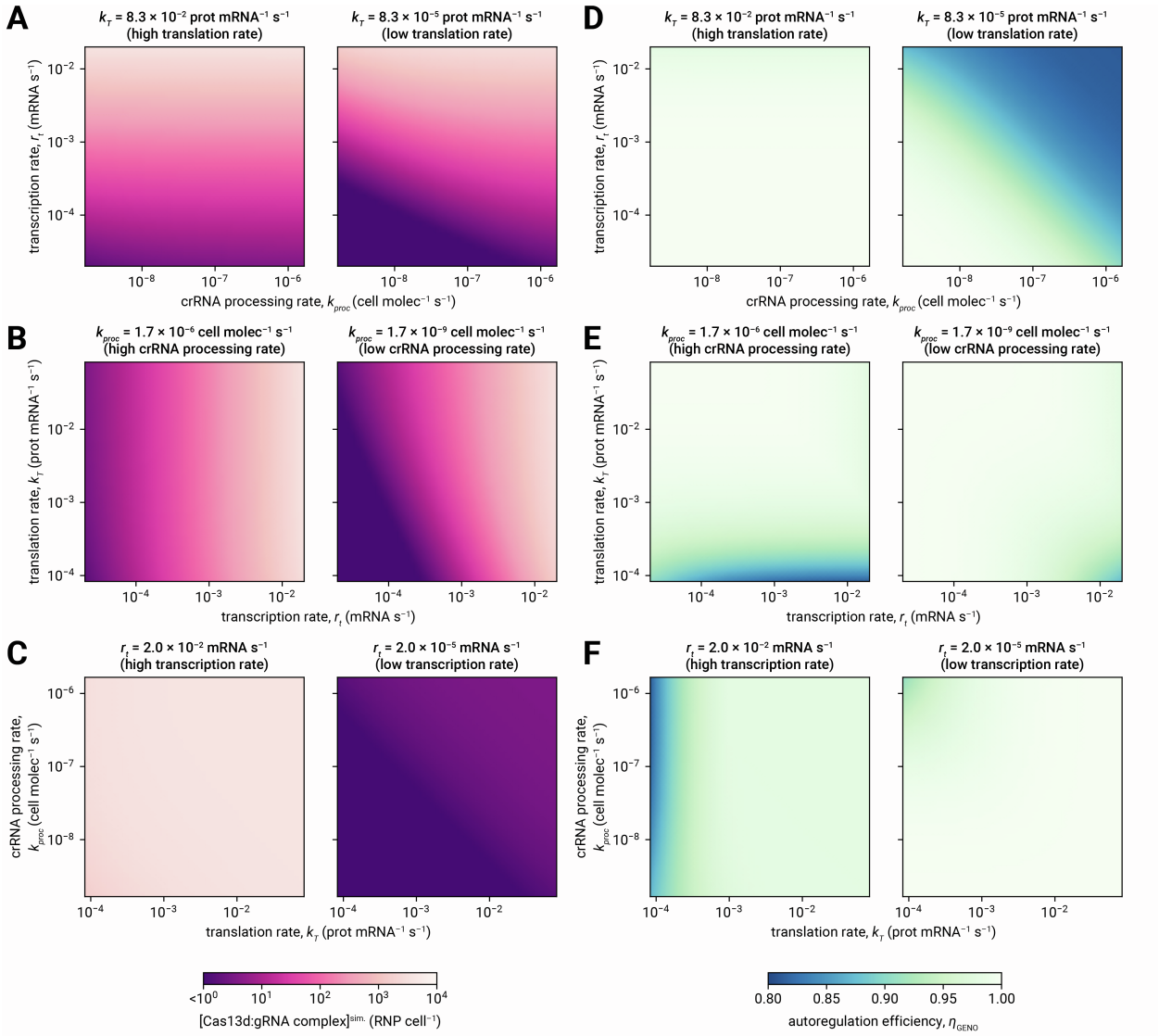

Figure S4. Prediction of Cas13d binary complex concentration and autoregulation efficiency from simulation of GENO dynamics, related to Figure 4. A Equilibrium Cas13d binary complex concentration as a function of transcription rate and crRNA processing rate, for high (left) and low (right) translation rate. **B** Equilibrium binary complex concentration as a function of translation rate and transcription rate, for high (left) and low (right) crRNA processing rate. **C** Equilibrium binary complex concentration as a function of crRNA processing rate and translation rate, for high (left) and low (right) transcription rate. **D** Autoregulation efficiency (η_{GENO}, defined in Data S1) as a function of transcription rate and crRNA processing rate, for high (left) and low (right) translation rate. **E** η_{GENO} as a function of translation rate and transcription rate, for high (left) and low (right) crRNA processing rate. **F** η_{GENO} as a function of crRNA processing rate and translation rate, for high (left) and low (right) transcription rate.

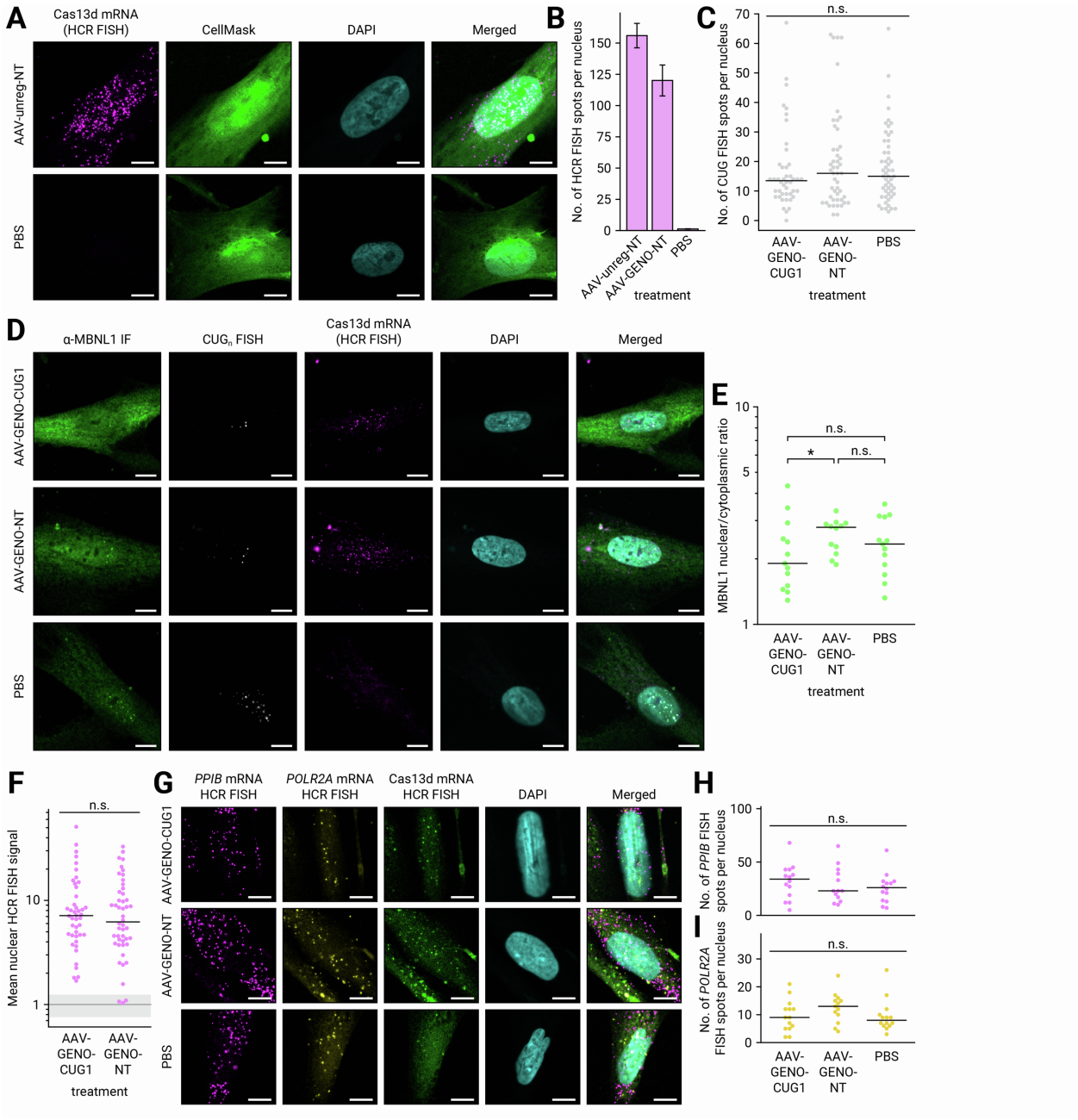
A
Cas13d mRNA (HCR FISH)
CellMask
DAPI
Merged
AAV-unreg-NT
PBS
B
No. of HCR FISH spots per nucleus
AAV-unreg-NT
AAV-GENO-NT
PBS
treatment
C
n.s.
No. of CUG FISH spots per nucleus
AAV-GENO-CUG1
AAV-GENO-NT
PBS
treatment
D
α-MBNL1 IF
CUGn FISH
Cas13d mRNA (HCR FISH)
DAPI
Merged
AAV-GENO-CUG1
AAV-GENO-NT
PBS
E
n.s.
*
n.s.
MBNL1 nuclear/cytoplasmic ratio
AAV-GENO-CUG1
AAV-GENO-NT
PBS
treatment
F
n.s.
Mean nuclear HCR FISH signal
AAV-GENO-CUG1
AAV-GENO-NT
treatment
G
PPIB mRNA HCR FISH
POLR2A mRNA HCR FISH
Cas13d mRNA HCR FISH
DAPI
Merged
AAV-GENO-CUG1
AAV-GENO-NT
PBS
H
n.s.
No. of PPIB FISH spots per nucleus
I
n.s.
No. of POLR2A FISH spots per nucleus
AAV-GENO-CUG1
AAV-GENO-NT
PBS

Figure S5. Detection of diffraction-limited spots in Cas13d HCR FISH and CUG_n FISH images of AAV-treated DM1 myoblasts, related to Figure 5. A Representative images at 40x magnification of DM1 myoblasts stained for Cas13d mRNA (HCR FISH, magenta) after treatment with AAV for 6 days. Cytoplasm (CellMask, green) and nuclei (DAPI, cyan) are also labeled. Scale bars 10 μm. **B** Mean number of Cas13d HCR FISH spots detected in nuclei after AAV treatment. Error bars indicate SEM. n>43 nuclei per condition, 21 images per condition. **C** Number of CUG_n FISH spots (RNA foci) detected in each nucleus after AAV treatment. Dots represent individual nuclei, black line indicates median. n>43 nuclei per condition, 21 images per condition. n.s.: not significant, p>0.05, two-sided Mann-Whitney *U* test. **D** Representative images at 40x magnification of DM1 myoblasts labeled with α-MBNL1 IF (green), CUG_n FISH (greyscale), and Cas13d HCR FISH (magenta) after treatment with AAV for 6 days. Nuclei (DAPI, cyan) are also labeled. Scale bars 10 μm. **E** Nuclear-to-cytoplasmic ratio of MBNL1, calculated from α-MBNL1 IF for each cell. Dots represent individual cells, black line indicates median. n>11 cells per condition, 8 images per condition. *p<0.05, one-sided Mann-Whitney *U* test. n.s.: not significant, p>0.05. **F** Mean nuclear intensity of Cas13d HCR FISH across nuclei in GENO-regulated targeting and non-targeting conditions. Dots represent individual nuclei, black line indicates median. n>43 nuclei per condition, 21 images per condition. n.s.: not significant, p>0.05, two-sided Mann-Whitney *U* test. Grey line indicates mean baseline nuclear FISH signal in PBS-treated myoblasts and grey shaded region indicates standard deviation, n=53 nuclei, 21 images. **G** Representative images at 40x magnification of DM1 myoblasts labeled with HCR FISH for mRNAs of *PPIB* (magenta), *POLR2A* (yellow), and Cas13d (green) after treatment with AAV for 6 days. Nuclei (DAPI, cyan) are also labeled. Scale bars 10 μm. **H** Number of *PPIB* HCR FISH spots detected in each nucleus after AAV treatment. Dots represent individual nuclei, black line indicates median. n>12 nuclei per condition, 6 images per condition. n.s.: not significant, p>0.05, two-sided Mann-Whitney *U* test. **I** Number of *POLR2A* HCR FISH spots detected in each nucleus after AAV treatment. Dots represent individual nuclei, black line indicates median. n>12 nuclei per condition, 6 images per condition. n.s.: not significant, p>0.05, two-sided Mann-Whitney *U* test.

SUPPLEMENTAL TABLES

Table S1. List of spacer sequences for RfxCas13d gRNAs used in this study, related to STAR Methods.

gRNA ID	Spacer
NT	CGAGGGCGACTTAACCTTAGGT
CUG-1	GCAGCAGCAGCAGCAGCAGCAG
CUG-2	CAGCAGCAGCAGCAGCAGCAGC
CUG-3	AGCAGCAGCAGCAGCAGCAGCA
DMPK-1	CTCGGAGCGGTTGTGAACTGGC
DMPK-2	GGCTACAAGGACCCTTCGAGCC
DMPK-3	GTCCTGTAGCCTGTCAGCGAGT
DMPK-4	GACAGACAATAAATACCGAGGA
DMPK-5	CGGAGTCGAAGACAGTTCTAGG
DMPK-6	CACTTTGCGAACCAACGATAGG
DMPK-7	AACTCCATCCGCTCCTGCAACT
DMPK-8	TCCTCCAGGTGTCTATACACGC
MS2-1	CTAATGAACCCGGGAATACTGC
MS2-2	CTAGGCAATTAGGTACCTTAGG
MS2-3	GTTTTCTAGAGTCGACCTGCAG
puro-1	GTTCCGTAACTCGCTCAATGTG
puro-2	CAAACACTGCACCTGCTTCAAC
puro-3	CACCATCATCTGCAACCCATAC
LDHA	GACTTGGCAGATGAACTTGCTC
CD63	GCCTGCAAGGAGAACTATTGTC
CD81	CACGTCGCCTTCAACTGTAATC
LGMN	TGCCATGCCTACCAGATCATTC
SYBU	CAGAAAGAGGTGACAGTGAGAC
EPOR	TGACTCTGGCATCTCAACTGAC

Table S2. List of transcripts in the human reference genome (hg19) by longest CUG_n repeat length, related to Figure S2.

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Table S3. Mean transcripts per million (TPM) for selected genes in non-transgenic HeLa cells in our RNA-seq study, related to Figure 3.

Gene	Average TPM in HeLa (RNA-seq)
LDHA	1211.99628
CD63	354.456528
CD81	112.262125
LGMN	40.980735
SYBU	12.224917
EPOR	3.8507395

Table S4. Primer sequences used in this study, related to STAR Methods.

Name	Sequence
RG6_F	CAAAGTGGAGGACCCAGTACC
RG6_R	GCGCATGAACTCCTTGATGAC
mCherry_F	GACTACTTGAAGCTGTCCTTCC
mCherry_R	CGCAGCTTCACCTTGTAGAT
GAPDH_F	GGTGAAGGTCGGTGTGAACG
GAPDH_R	CTCGCTCCTGGAAGATGGTG
CUG480_F	CGATCTCTGCCTGCTTACTC
CUG480_R	GTCGGAGGACGAGGTCAATAAA

SUPPLEMENTAL ITEMS

Data S1. Dynamical model of Cas13d gRNA excision for negative-autoregulatory optimization (GENO), related to Figure 4.