Supplemental information

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

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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.
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 µM 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 µM 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 ($\eta_{\text{GENO}}$, defined in Data S1) as a function of transcription rate and crRNA processing rate, for high (left) and low (right) translation rate.  

E $\eta_{\text{GENO}}$ as a function of translation rate and transcription rate, for high (left) and low (right) crRNA processing rate.  

F $\eta_{\text{GENO}}$ as a function of crRNA processing rate and translation rate, for high (left) and low (right) transcription rate.
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   | GCAGCAGCAGCAGCAGCAGCAGCAGCAG |
| CUG-2   | CAGCAGCAGCAGCAGCAGCAGCAGCAGC |
| CUG-3   | AGCAGCAGCAGCAGCAGCAGCAGCAGCA |
| DMPK-1  | CTGGAGCGGTTTGAAGTTCAGG        |
| DMPK-2  | GTCTACAAGGACACCTTCGAGCC       |
| DMPK-3  | GTCCGTAGCTGTCAGCGAGT          |
| DMPK-4  | GACAAGACAAATACCCGAGGA         |
| DMPK-5  | CGGAGTCGACAGATTTCTAGG         |
| DMPK-6  | CACTGTGCAGCACCAGATAGGG        |
| DMPK-7  | AACTCCATCGCTCCTGCAGA          |
| DMPK-8  | TCCTCCAGGTGTCTATACAGGC        |
| MS2-1   | CTAATGAACCAGGGGGAGATCTGC      |
| MS2-2   | TAGCCAATGCTGTACCTTGAGG        |
| MS2-3   | GTTTTCTAGAGTCGACCTGCAG        |
| puro-1  | GTTCCGTAACTCGCTCAATGTC        |
| puro-2  | CAAACACTGCACCTTCAACTC         |
| puro-3  | CACCATCATCTGCAACCCCATAC       |
| LDHA    | GACTTGGCGAGTGAACCTTGCTC       |
| CD63    | GCCTGCAAGGAGAAGATCTTGCTC      |
| CD81    | CACGTCGCTTCCTCAACTTGATCTC     |
| LGMN    | TGCCATGCTACCAGATCTTCC         |
| SYBU    | CAGAAAGAGGTGACAGTGAGC         |
| EPOR    | TGACTCTGGCATCTCAACTGAC        |
Table S2. List of transcripts in the human reference genome (hg19) by longest CUG<sub>n</sub> 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     | CAAAGTGAGGAGGACCCAGTACC               |
| RG6_R     | GCGCATGAAACTCCTTGATGAC                |
| mCherry_F | GACTACTTGAAGCTGCTTTCC                 |
| mCherry_R | CGCAGCTTCACCTTTGTAGAT                 |
| GAPDH_F   | GGTGAAGGTCGGTGTGAACG                  |
| GAPDH_R   | CTCGCTCCTGGAAGATGGTG                 |
| CUG480_F  | CGATCTCTGCTGCTTACTC                   |
| CUG480_R  | GTCGGAGGACGAGGTCAATAAA                |
SUPPLEMENTAL ITEMS

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