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Abstract

Background. Glioblastoma (GBM) carries a poor prognosis, and new therapeutic strategies are necessary to improve outcomes for patients with this disease. Alkylating chemotherapies including temozolomide (TMZ) and lomustine (CCNU) are critical for treating GBM, but resistance mechanisms, including hypomethylation of O^6-methylguanine-DNA methyltransferase (MGMT) promoter, undermine treatment. CRISPRoff is a programmable epigenetic memory editor that can induce stable and heritable gene silencing after transient delivery, and we hypothesize that CRISPRoff could potentiate the activity of TMZ and CCNU through long-term suppression of target genes.

Methods. We transiently delivered CRISPRoff mRNA along with sgRNAs against target genes using both electroporation and lipid nanoparticles (LNPs) into established GBM cell lines, patient-derived primary GBM cultures, and orthotopic GBM xenografts. Gene repression, specificity, and stability were measured by RT-qPCR, Western blot, bisulfite sequencing, and RNA sequencing. Sensitivity to chemotherapies was measured by cell viability dose–response, microscopy, and bioluminescence imaging. Genome-wide mapping of CCNU sensitizers was performed using CRISPRi screens.

Results. CRISPRoff induced complete suppression of *MGMT* and sensitization to TMZ that was stable for over 8 months of continuous cell propagation. GBM orthotopic tumors treated with CRISPRoff against *MGMT* demonstrated sensitivity to TMZ in vivo, and CRISPRoff delivery resulted in chemosensitivity in patient-derived primary GBM. Genome-wide CRISPRi screens identified combinatorial genetic vulnerabilities (*BRIP1, FANCE*) that were targetable by multiplexed CRISPRoff to achieve sensitization to CCNU.

Conclusion. Transient delivery of a site-specific epigenetic memory can induce stable, complete, and multiplexed suppression of target genes for therapeutic application in GBM.

Key Points

1. Site-specific epigenetic memory editing using CRISPRoff induces long-term complete suppression of *MGMT* and sensitivity to TMZ in vitro and in vivo.
2. Combining genome-wide CRISPRi screens and multiplexed CRISPRoff enables a rationally designed strategy for augmenting GBM therapies.

Glioblastoma (GBM), the most common malignant primary brain tumor, carries a poor prognosis despite maximal safe resection, fractionated radiotherapy, and treatment with alkylating chemotherapy such as temozolomide (TMZ).[1–5] Epigenetic activation of the DNA repair enzyme O^6-methylguanine-DNA methyltransferase (MGMT) through

Importance of the Study

CRISPR/Cas9 genetic and epigenetic editing tools have enabled the discovery of vulnerabilities in GBM, but their use as a direct therapeutic in GBM has been limited. Given the dearth of effective therapies for patients with GBM, new treatment strategies are needed. Prior studies have used epigenetic editing tools to silence *MGMT,* resulting in the sensitization of established GBM cell lines to TMZ. In this study, we demonstrate key advantages of the epigenetic memory editor, CRISPRoff, including long-term stability of target gene repression, efficacy in GBM xenografts in vivo, efficacy in patient-derived primary GBM, as well as the application of non-viral transient delivery of the CRISPRoff system as an RNA-based therapeutic. These results are an important advancement over the current landscape of epigenetic editing strategies in neuro-oncology with potential for clinical translation.

promoter hypomethylation is a well-characterized mechanism of TMZ resistance, a feature that is predictive of response to this agent as well as prognostic for overall survival in patients.[6,7] It is estimated that ~60% of patients with GBM have hypomethylation of the *MGMT* promoter,[6] representing a significant proportion of this population that could benefit from the repair of this epigenetic aberration. In contrast to what is known for TMZ, molecular factors that predict responses to lomustine (CCNU), an alkylating chemotherapy used for the treatment of both low- and high-grade gliomas,[8–10] are not well known.

CRISPR/Cas9 and their engineered or naturally occurring variants enable interrogation of gene function and discovery of cancer vulnerabilities at the genome-wide scale.[11] One of these variants, CRISPR interference (CRISPRi), uses catalytically dead Cas9 fused to transcriptional repressors to systematically suppress the vast majority of genes in the genome.[12] However, CRISPRi requires constant, stable expression of dCas9 to induce long-term gene silencing, which limits its utility as a therapeutic platform. CRISPRoff is a programmable epigenome memory editor that can induce stable and heritable gene silencing at promoters and enhancers.[13] Through site-specific recruitment of the DNA methyltransferase domains DNMT3A and DNMT3L, as well as the KRAB repressor domain fused to dCas9, CRISPRoff can induce DNA methylation and subsequent gene silencing long-term following a transient pulse of the CRISPRoff fusion along with its cognate sgRNA.

Here, we use CRISPRoff to repress *MGMT* by targeting sgRNAs to the CpG island of the *MGMT* promoter. We demonstrate that CRISPRoff induces complete repression of *MGMT* gene expression following transient, non-viral, delivery of CRISPRoff, resulting in enhanced sensitivity to TMZ. Furthermore, both gene repression and TMZ sensitivity were stable long-term (>8 months), and TMZ sensitivity was retained in orthotopic xenografts as well as in primary patient-derived GBM cultures. To then demonstrate the applicability of CRISPRoff beyond *MGMT* repression, we identified sensitizers of CCNU using genome-wide CRISPRi screens. We then performed multiplexed repression of factors involved in DNA repair in addition to *MGMT* using CRISPRoff, demonstrating that CRISPRoff can be adapted to target multiple determinants of tumorigenesis as a therapeutically tractable approach.

Materials and Methods

Established and Primary Cell Cultures

LN18 and T98G glioblastoma cells were obtained from ATCC and genotypes were confirmed using short tandem repeat analysis. Cell cultures were routinely tested for mycoplasma using the MycoAlert PLUS Mycoplasma Detection Kit (Lonza, cat 75860-362). Cells were cultured in Dulbecco Modified Eagle Medium supplemented with 10% fetal bovine serum (Thermo Scientific, cat 11965084) and antibiotic-antimycotic (Thermo Scientific, cat 15240062). SF7996 was a gift from Joseph Costello. SF7996 cells were cultured in DMEM-F12 (Fisher Scientific, cat 11330057) supplemented with 10% fetal bovine serum (Thermo Scientific, cat 11965084) and antibiotic-antimycotic (Thermo Scientific, cat 15240062). Primary S14259, SF14346, SF14590, and SF14599 glioblastoma tumors were obtained from patient samples collected from the operating room under approved IRB #10-01318, characterized by a CLIA-certified *MGMT* promoter methylation assay,[14] and the UCSF500 targeted DNA sequencing assay.[15] Tumors were dissociated with papain and DNase (Worthington) following the manufacturer's recommendations. SF14259, SF14346, SF14590, and SF14599 cells were cultured in Neurobasal-A Medium (Gibco, cat 10888022) supplemented with B-27 (Gibco, cat 12587010), N-2 (Gibco, cat 17502-048), plasmocin prophylactic (Invivogen, cat ant-mpp), primocin (Invivogen, cat ant-pm-1), EGF at 20 ng/mL (VWR, 10781-694), and FGF at 20 ng/mL (PeproTech, cat 10777-988).

CRISPRoff mRNA and sgRNA Production

CRISPRoff mRNA was produced using in vitro transcription (IVT). For IVT reactions, a plasmid containing CRISPRoff-v2.3 with a mutated T7 promoter was designed and cloned as previously described.[13,16] The IVT templates were produced by PCR of CRISPRoff-v2.3 with the forward primer correcting the T7 mutation and reverse primer appending the polyA tail such that the final template contained the WT T7 promoter, 5′ UTR including Kozak sequence, the codon-optimized CRISPRoff-v2.3 coding sequence, 3′ UTR, and a 145 bp polyA tail. The PCR product was purified via

SPRI selection and stored at –20°C until further use. IVT reactions were performed with the HiScribe ® T7 High Yield RNA Synthesis Kit (New England Biolabs, cat E2040S) under full substitution of pseudo-UTP and in the presence of 4 mM CleanCap AG (TriLink Biotechnologies, cat N-7113-5), with the addition of RNAse Inhibitor (NEB, cat M0314L), and Yeast Inorganic Pyrophosphatase (NEB, cat M2403L). Transcribed mRNA was purified with Lithium Chloride and eluted in water. After quantification on a Nanodrop spectrophotometer, mRNA product was assessed on an Agilent 4200 Tapestation system and subsequently stored at –80°C until further use. sgRNAs were selected from existing CRISPRi/CRISPRoff databases[13,17,18] and generated as purified synthetic sgRNAs from Synthego. For LNPs, sgRNAs were chemically modified as described[19] and ordered from IDT. Protospacer sequences are in Supplementary Methods.

Electroporation Delivery of CRISPRoff mRNA and sgRNAs

Electroporation of CRISPRoff and sgRNAs targeting *MGMT*, *BRIP1*, and *FANCE* in LN18, T98G, and SF7996 were performed using the Lonza SE Cell Line 4D X Kit L (Lonza, cat V4XC-1024). Cells were subcultured 2–3 days pre-nucleofection and electroporated with 1.5 µg CRISPRoff mRNA, combined with equimolar pools of sgRNA described in Supplementary Methods. Electroporation efficiency was confirmed with GFP mRNA. For primary patient-derived GBM cultures grown in tumorsphere conditions, electroporation of 1.5 µg CRISPRoff mRNA and pooled MGMT sgRNA targets (sgMGMT_1B:sgMGMT_3A :sgMGMT_3B), at 333 ng equimolar each, was performed in SF14259, SF14346, SF14590, and SF14599 using the Maxcyte ATx Electroporation System, OC-25X3, using the THP-1 protocol.

Generation of Monoclonal CRISPRoff Cell Populations

Monoclonal CRISPRoff electroporated cells were isolated 4–15 days post-electroporation by limiting dilution. A cell suspension was prepared at a concentration of 5 and 10 cells/mL in 10 mL of conditioned medium and seeded onto respective flat-bottom 96-well plates. Cells were expanded for 30–40 days. Upon colony formation, cells were washed with PBS, trypsinized, and transferred to larger culture dishes for further expansion.

CRISPR/Cas9 Deletion of MGMT

A pool of 3 sgRNAs targeting exon 3 of *MGMT* was obtained from the Synthego GKOv2 kit; protospacer sequences in Supplementary Methods. RNP's were assembled using a 9:1 sgMGMT to Cas9 ratio and electroporated into LN18 and T98G using the DS-126 program on the Lonza 4D Nucleofector. Cas9 deletion was confirmed using amplicon next-generation sequencing. Genomic DNA was harvested from 100 000 cells per sample for each clone using the Nucleospin Blood kit (Machery-Nagel, cat 740951.50). A total of 5 ng of DNA, 10 µM of forward and reverse primers, and Q5 High-Fidelity 2× Master Mix (New England Biolabs, cat M0492S) were used for PCR reactions. PCR products were purified using the DNA Clean & Concentrator-5 kit (Zymo Research, cat D4013), and 500 ng of each sample was submitted for NGS Amplicon-EZ analysis (Genewiz).

Cell viability Drug Dose–Responses

GBM cells were seeded at 1000–5000 cells/well as 6 technical replicates per dose on a 96-well plate. TMZ stock solutions were prepared at 100 mM (Sigma-Aldrich, cat T2577-100MG). CCNU stock solutions were prepared at 10 mM (Selleckchem, cat S1840). TMZ or CCNU were added to cell suspensions with 1:300 serial dilutions. O^6-Benzylguanine (Sigma-Aldrich cat B2292) was added concurrently at 50 µM with TMZ for co-treatment experiments. Cell Titer Glo was performed per the manufacturer's recommendations (Promega, cat G7571) 7 days following the drug. Luminescence was measured using GloMax 96 Microplate Luminometer (Promega, cat E6521). IC50 values were obtained using GraphPad Prism, nonlinear fit on [inhibitor] versus response, 3 parameters (unless otherwise stated), using values normalized to the mean of the vehicle-only condition within each genotype, or scrambled controls in the case of O^6-Benzylguanine co-treatment.

CRISPRoff and sgRNA Delivery Using LNPs

The preparation of L-10 (cKK-E12), L-25 (Lipid A9), L-36 (Lipid CL1) LNP/mRNA/sgRNA complexes was performed as previously described.[20] Briefly, stock solutions (10 mg/mL) of cKK-E12 (Cayman Chemicals, cat#: 36700) and Lipid A9 (Cayman Chemicals, cat#: 37667) were obtained in solution. DOPE (Avanti Polar Lipids, cat#: 850725), cholesterol (Sigma-Aldrich, cat#: C8667), and DMG-PEG (Avanti Polar Lipids, cat#: 880151) were dissolved individually in ethanol at the same concentration (10 mg/mL). The cholesterol solution was warmed to dissolve any crystals formed during cold storage. Subsequently, cationic lipids (L-10/L-25/L-36), DOPE, cholesterol, and DMG-PEG were mixed in molar ratios of 34.5:24.7:39.6:1.2, respectively. RNAs (1 µg/µL CRISPRoff mRNA, sgRNA) were dissolved in 25 mM citrate buffer (pH 4) and combined with the lipid solution at a 1:3 volume ratio (organic phase: aqueous phase). The ratio of CRISPRoff mRNA to sgRNA was either 1:1 or 1:2 by mass. The resulting LNP-mRNA complexes were gently vortexed and incubated at room temperature for 5–10 minutes for immediate use or stored at 4°C prior to use within 6 hours. LNP complexes were then added to GBM cells and analyzed as described in Supplementary Methods.

Genetic Rescue of MGMT Suppression

GBM cells were transfected with 3×FLAG-MGMT (Gift from Harish Vasudevan) or pMax-GFP (Lonza) cDNA expression plasmids by transfection with L-10 (cKK-E12) LNP as previously described,[20] using a 1:3 volume ratio of LNP:DNA solution. Cells were harvested for initiation of

TMZ dose–response assays, RNA, and protein analyses 48 hours following transfection.

Real-Time qPCR

RNA was harvested from 100 000 cells per sample, in triplicates, for each condition, using the Quick-RNA Purification Kit, Microprep (Zymo Research, cat R1051). A total of 500 ng to 1 µg of RNA was used for the reverse transcriptase reaction using the Iscript cDNA Synthesis Kit (Bio-Rad, cat 1708891). Real-time PCR products were detected using the PowerUp SYBR Green Master Mix for qPCR (Applied Biosystems, A25777) and human primers targeting MGMT[21] and ribosomal RPLP0 as a control. Primer sequences in Supplementary Methods.

Protein Expression Analysis by Western Blot

Protein was harvested from 1 to 2 million cells per sample and processed as described in Supplementary Methods. MGMT (Cell Signaling Technology, cat 58121S) and β-Tubulin (Cell Signaling Technology, cat 86298S) antibodies were used at 1:1000 dilution. MGMT antibody was used to detect both endogenous and ectopic MGMT.

Targeted Bisulfite Next-Generation Sequencing

DNA was harvested from 100 000 cells per sample using the Machery-Nagel Blood, Mini Kit for DNA from blood (Machery-Nagel, cat 740951.50). Bisulfite conversion was performed using 100 ng of extracted DNA with the EZ DNA Methylation-Gold Kit (Zymo Research, cat D5005). PCR amplification of bisulfite-converted DNA was performed using the Takara EpiTaq-HS (for bisulfite-treated DNA) kit (Takara, cat R110A) and primer sequences 5′-attatttttgtgataggaaaaggta-3′ and 5′-ctaaaacaatctacacatcc-3′ targeting a 149 bp region located 209 bp upstream of *MGMT* Exon 1. Gel electrophoresis was performed on the PCR products, followed by gel DNA extraction using the Zymoclean Gel DNA Recovery Kit (Zymo Research, cat D4007). Targeted amplicon sequencing was performed by Genewiz (Azenta Life Sciences) using the Amplicon-EZ protocol, targeting >50 000 reads per sample using a 2 × 250 bp paired end strategy on illumina MiSeq. Reads were aligned to the human *MGMT* promoter sequence using the bisulfite-aware aligner Bismark (v0.24)[22] with the non-directional protocol. The proportion of reads representing methylated cytosines was quantified for each CpG position.

RNA Sequencing

RNA was harvested using the Quick-RNA Purification Kit, Microprep (Zymo Research, cat R1051) following the manufacturer's recommendations. Purified total RNA was submitted for RNA-sequencing to Azenta Life Sciences, using the 2 × 150 bp paired end protocol on an illumina NovaSeq. Paired reads were aligned to the human genome reference assembly GRCh38 using hisat2 (v2.2.0)[23] using default parameters, and gene level quantification was performed using featureCounts (v2.0.6).[24] Differential expression analysis was performed using DESeq2[25] with the Wald test. Individual replicates are listed in Supplementary Methods. The significance threshold was set as adjusted $P < .01$. Gene ontology analysis of differentially expressed genes (adjusted $P < .01$, |fold change >1) was performed using Enrichr against the MSigDB Hallmark gene sets.[26]

Cell Viability Dose–Response and Growth Assays of Patient-Derived Primary GBM

After 14 days post CRISPRoff-sgMGMT electroporation, patient-derived SF14259 and SF14346 were seeded at 100 000 cells/well onto 6-well Matrigel-coated plates (Corning, cat 356237), and dosed with 0, 50, and 100 µM TMZ. Patient-derived SF14590 and SF14599 were seeded at 30 000 cells/well onto 24-well Matrigel-coated plates (Corning, cat 356237), and dosed with 0, 50, and 100 µM TMZ. Drug dosing was performed with media changes every 3 days, and brightfield light microscopy was performed on the ECHO Revolve 4× objective after 15–35 days, corresponding to when the vehicle-treated controls reached near-confluency. Imaging-based assays were preferred over plate reader-based drug dose–responses in primary GBM's due to wide variability in their growth rates and the ability to track 3D growth. Quantification is described in Supplementary Methods. Cell cycle analysis was performed by 70% ethanol fixation followed by propidium iodide staining (Cell Signaling Technology, cat 4087S) analyzed on the Attune NxT Flow Cytometer 48 hours after treatment with the drug. Analysis of apoptosis was performed using the Caspase-Glo 3/7 3D Assay kit (Promega cat G8981) according to the manufacturer's recommendations 7 days following TMZ addition. Significance was calculated using a 2-sided Student's *t*-test compared to sgScrambled controls.

Crystal Violet Assay of Cell Growth

After 113 days following CRISPRoff-sgMGMT electroporation of patient-derived SF7996, 100 000 cells were seeded onto 6-well plates and added with 0, 100, or 500 µM TMZ in growth media. TMZ was re-dosed every 3 days with media changes. After 7 days, drug-containing growth media was aspirated from each well, washed with PBS, fixed in methanol for 30 minutes, and stained with crystal violet overnight. The following day, crystal violet was removed from each well, followed by 3 washes using Millipore water, and plates were dried face-down and then imaged on a Zeiss Stemi 508 at 2× magnification. Quantification described in Supplementary Methods.

CRISPRi Screens

CRISPRi screens were performed as described previously.[27] LN18 or T98G cells stably expressing CRISPRi components (dCas9-Zim3) were transduced with lentivirus supernatant containing the dual sgRNA V3 CRISPRi library, which targets 20 528 genes and 1025 sgNTC. Screens

were performed in triplicate cultures with coverage of at least 500× cells per target gene. sgRNA-expressing cells were selected using puromycin (1 μm/mL) for 48 hours and transferred to puromycin-free growth media for 24 hours. Initial (T0) cell populations were then frozen in 10% DMSO and processed for genomic DNA alongside endpoint (LN18: T13, T98G: T16) cell populations, which corresponded to 8–10 population doublings in vehicle conditions. Triplicate screens were also performed in the presence of CCNU at concentrations corresponding to LD50 in test cultures (LN18: 10 μM, T98G: 40 μM) added at each passage ($n = 4$). Genomic DNA was harvested using the NucleoSpin Blood L Kit (Machery-Nagel, #740954.20) for each cell population, and sgRNA cassettes were amplified using 22 cycles of PCR using NEBNext Ultra II Q5 PCR MasterMix (New England Biolabs, #M0544L). Sequencing was performed on a NovaSeqX (Illumina) using custom sequencing primers. Analysis was performed using ScreenPro2 (https://github.com/ArcInstitute/ScreenPro2). sgRNAs with fewer than 50 mean read alignments at T0 were omitted. Pseudocount of 0.1 was added to counts of 0. Growth phenotype was defined as log2(sgRNA count Tend / sgRNA count T0). Drug phenotype was calculated similarly to growth phenotype, using Tend reads from cells treated with the drug. The drug/vehicle ratio was defined as log2(sgRNA count Tend [drug] / sgRNA count Tend [vehicle]). Statistical significance was quantified using a 2-sided Student's *t*-test comparing replicate distributions of library-normalized counts for each sgRNA between conditions or time points. Gene set enrichment analysis was performed using FGSEA[28] to query ranked lists of screen phenotypes against the Reactome database. Hit calling was performed by calculating a combined score (product of z-standardized phenotype × negative log10(*P*-value)) and setting a threshold corresponding to an empirical false discovery rate of 2% for non-targeting control hits.[27]

Orthotopic Intracranial Tumor Models

Experiments were approved by the University of California San Francisco Institutional Animal Care and Use Committee. Firefly luciferase-expressing LN18 cells with or without *MGMT* repressed by CRISPRoff were intracerebrally injected into the right caudate putamen of female athymic (nu/nu, homozygous), 5–6 week old mice from Envigo, as previously described.[29] 3 μL of tumor cell suspension (2 million cells) was injected into the right caudate putamen at a rate of 1 μL/min. Mice were treated with 0.5 mg/kg/day of TMZ for 5 days at days 6–10 by oral gavage. Tumor size was measured using bioluminescent imaging by intraperitoneal injection of 150 mg/kg luciferin (D-luciferin potassium salt, Gold Biotechnology) on an IVIS Lumina imaging station and Living Image software (Caliper Life Sciences).

Immunohistochemistry

Tumors were micro-dissected 30 minutes following the final dose of TMZ and fixed in 4% PFA for 24 hours, followed by 70% ethanol. Tissues were processed and analyzed as described in supplementary methods. Staining was performed with MGMT antibody (17195-1-AP, Proteintech 1:100) or cleaved caspase-3 (Asp175; CC3) antibody (9661, Cell Signaling, 1:100). Quantification of IHC positive cells was performed using QuPath.[30] Tumor ROIs were selected manually, followed by cell detection using the Universal StarDist method and positive cell discrimination with the ANN_MLP object classifier.

Results

Epigenetic Editing of MGMT in GBM Cells

To induce epigenetic silencing of *MGMT* in GBM, we first designed 3 independent sgRNAs targeting the CpG island of the *MGMT* promoter (Figure 1A and B). We used electroporation of individual sgRNAs or a pool of all 3 sgRNAs targeting *MGMT* in conjunction with mRNA encoding CRISPRoff v2.3, comprised of a catalytically inactive Cas9 (dCas9) fused to DNMT3A, DNMT3L, and the KRAB repressor domain.[13] This transient delivery of CRISPRoff mRNA and sgRNA resulted in 97% decreases in *MGMT* transcript levels using either individual sgRNAs or sgRNA pools, across 2 GBM cell lines (LN18, T98G), when measured 5 to 8 days following CRISPRoff delivery (Figure 1C–E). Repression of *MGMT* mRNA was accompanied by loss of *MGMT* protein (Figure 1F–H). In clonally isolated populations of GBM cells following transient delivery of CRISPRoff, complete silencing of *MGMT* expression was achieved for both individual and pooled sgRNA conditions (up to 99.99% suppression in the case of sgMGMT-3A), when measured between 40 and 62 days following electroporation to allow for monoclonal expansion (Figure 1C–H).

To then assess the specificity of CRISPRoff epigenetic editing, we performed targeted bisulfite sequencing of the *MGMT* promoter, which demonstrated site-specific increases of CpG DNA methylation at the expected positions based on sgRNA targeted location (Figure 1I). We then performed bulk RNA sequencing (RNA-seq), which showed that among significantly downregulated genes (adjusted P value < .01), *MGMT* was consistently the most downregulated gene at both intermediate (40–64 days) and late (134–209 days) time points across the entire transcriptome (Figure 1J, Table S1), demonstrating the specificity of CRISPRoff targeting. Comparison of differentially expressed genes between intermediate and late timepoints for each sgRNA demonstrated that aside from *MGMT* itself, very few genes (none for sgMGMT-1B, 2 for sgMGMT-3B) were significantly downregulated at both time points (Figure S1A). Gene ontology analysis of differentially expressed genes at the intermediate time points revealed downregulation of interferon or TNFα pathways, but these pathway alterations were not sustained at the late timepoints (Figure S1B). In sum, these data support a low incidence of off-target effects.

CRISPRoff Repression of MGMT is Stable Long Term

A distinct advantage of CRISPRoff-based targeting of regulatory elements is the potential for long-term stability

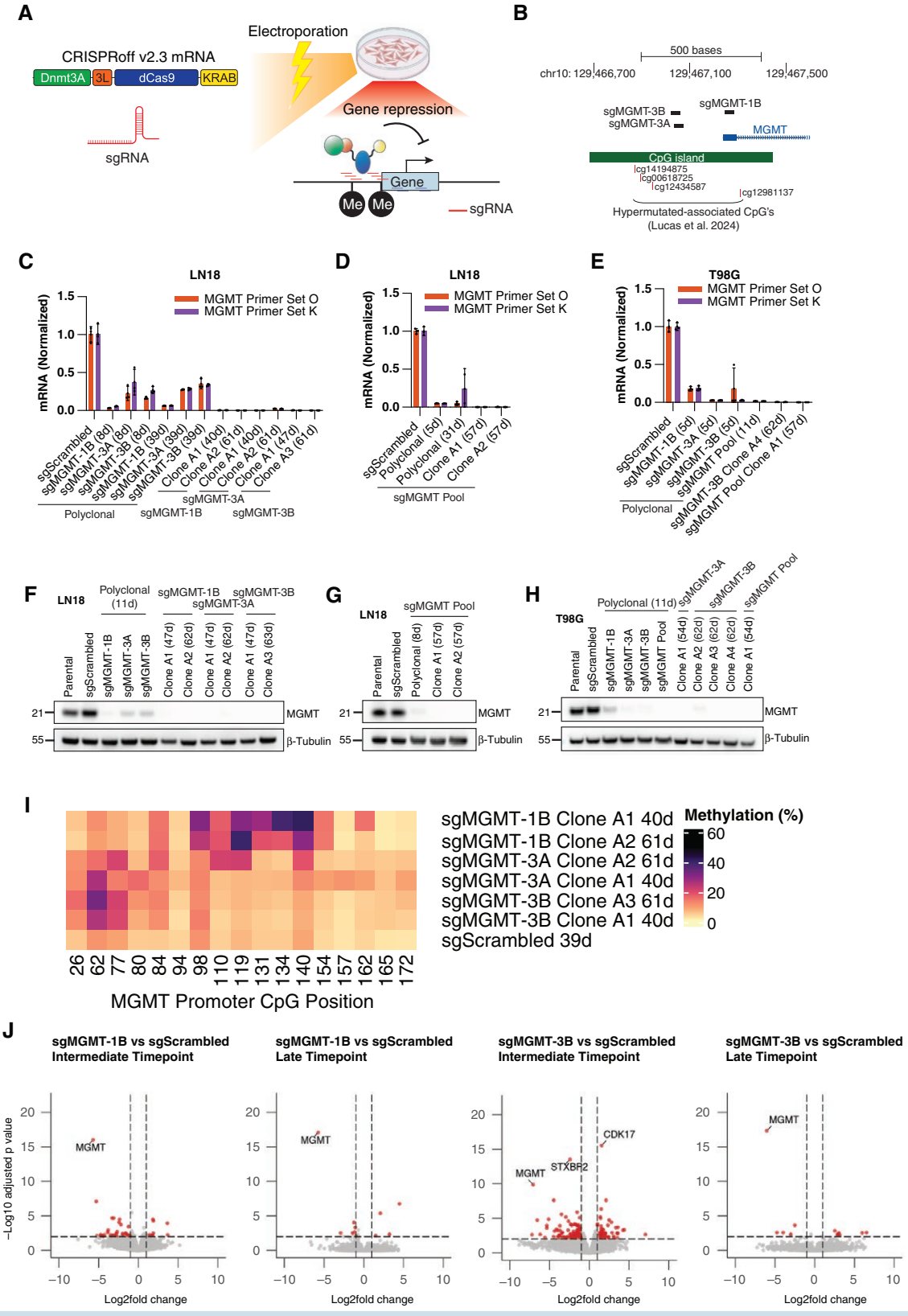

Figure 1. CRISPRoff potentially and specifically represses *MGMT* in GBM cells. (A) Schematic of CRISPRoff mRNA and sgRNA delivery using electroporation into GBM cells, leading to gene repression through DNMT3A/3L and KRAB domains. (B) Genomic locus of the *MGMT* promoter with protospacer locations of the 3 independent sgRNAs targeting the CpG island, as well as 4 CpG sites significantly associated with

a hypermutated subtype of glioma with improved survival reported in.[31] (C) RT-qPCR of *MGMT* mRNA normalized to *RPLP0* housekeeping gene, in polyclonal and monoclonal LN18 cells at the indicated number of days following delivery of CRISPRoff and the indicated sgRNA. Primer Set K and O represent 2 independent qPCR primer pairs from (Kreth et al. 2011) and Origene, respectively. (D) RT-qPCR of *MGMT* mRNA as in (C) for LN18 cells delivered with a pool of all 3 sgRNAs against *MGMT*. (E) RT-qPCR of *MGMT* mRNA in polyclonal and monoclonal T98G cells following delivery of CRISPRoff and indicated sgRNA(s). (F) Western blot against MGMT protein in polyclonal and monoclonal LN18 cells at the indicated number of days following delivery of CRISPRoff and the indicated sgRNA. (G) Western blot against MGMT protein as in (F) for LN18 cells delivered with a pool of all 3 sgRNAs against *MGMT*. (H) Western blot against MGMT protein as in (F) for T98G cells. (I) Targeted bisulfite sequencing of the *MGMT* promoter, assessing 17 CpG sites in LN18 monoclonal cells following delivery of CRISPRoff and the indicated sgRNA. (J) RNA-seq volcano plots comparing CRISPRoff targeting of *MGMT* promoter across 2 independent sgRNAs (sgMGMT-1B, -3B) in intermediate (40 – 64d) and late (134–209d) time points.

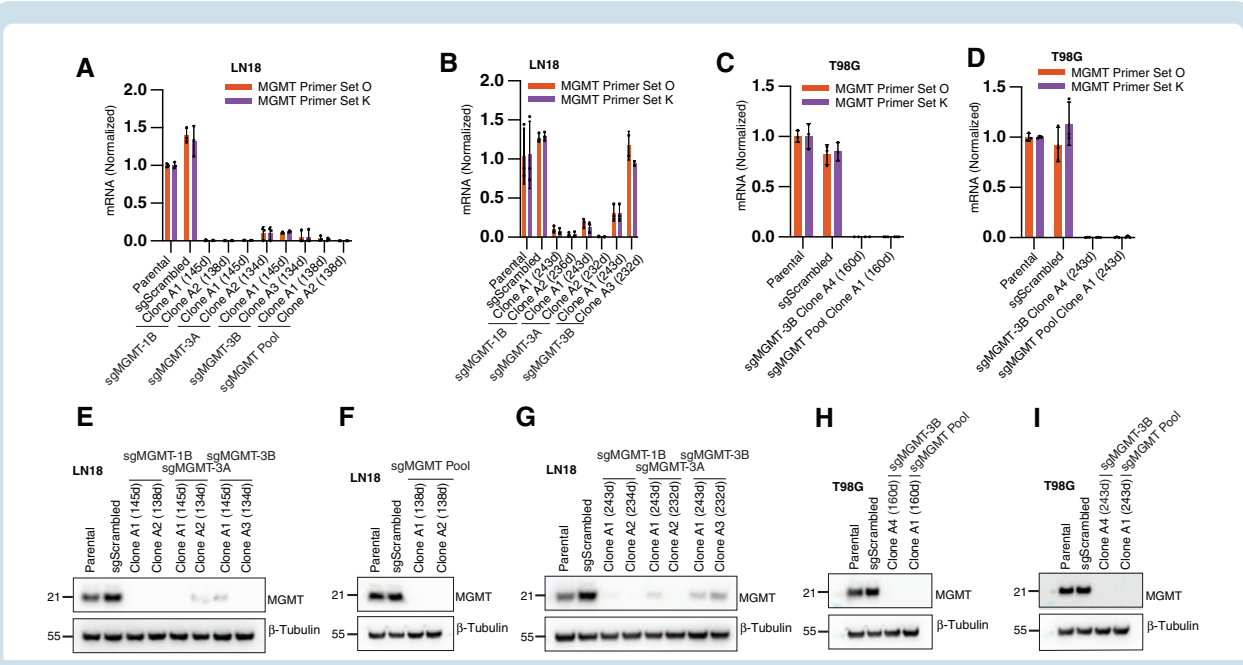

Figure 2. CRISPRoff repression of *MGMT* exhibits long-term stability. (A) RT-qPCR of *MGMT* mRNA normalized to *RPLP0* housekeeping gene, in monoclonal LN18 cells at the indicated number of days following delivery of CRISPRoff and the indicated sgRNA(s). (B) RT-qPCR of *MGMT* mRNA as in (A) for LN18 cells continuously passaged long term. (C-D) RT-qPCR of *MGMT* mRNA for T98G monoclonal populations continuously passaged long term following delivery of CRISPRoff and the indicated sgRNA(s). (E) Western blot against MGMT protein in monoclonal LN18 cells at the indicated number of days following delivery of CRISPRoff and the indicated sgRNA. (F) Western blot against MGMT protein as in (E) for LN18 cells delivered with a pool of all 3 sgRNAs against *MGMT*. (G) Western blot against MGMT protein in monoclonal LN18 cells continuously passaged long term following delivery of CRISPRoff and the indicated sgRNA. (**H-I**) Western blot against MGMT protein in T98G monoclonal populations continuously passaged long term following delivery of CRISPRoff and the indicated sgRNA(s).

and heritability of gene silencing after a transient pulse of CRISPRoff activity. To assess the stability of CRISPRoff silencing of *MGMT*, we continuously passaged clonally isolated populations of LN18 and T98G GBM cells up to 8.1 months after transient electroporation of CRISPRoff mRNA with sgRNAs targeting *MGMT*. At 134 days as well as 243 days, *MGMT* repression was observed as high as 99.65%, in the case of sgMGMT Pool Clone A1 in T98G cells (Figure 2A-D). *MGMT* repression was corroborated at the protein level (Figure 2E-I). Of the 8 clones isolated from LN18 cultures subjected to CRISPRoff delivery, only one clone had reactivated *MGMT* expression to near baseline levels of *MGMT* expression, but not until 232 days following transient CRISPRoff electroporation (sgMGMT-3B Clone A3; Figure 2 B and G).

CRISPRoff Repression of MGMT Sensitizes GBM Cells to TMZ Chemotherapy

We then asked whether CRISPRoff targeting of the *MGMT* promoter could sensitize GBM cells to TMZ, an alkylating chemotherapy whose efficacy is known to be correlated with *MGMT* promoter methylation.[6] Compared to the delivery of CRISPRoff with scrambled control sgRNAs, CRISPRoff against *MGMT* through transient mRNA/sgRNA electroporation was capable of inducing over 100-fold increased sensitivity to TMZ in polyclonal populations of LN18 or T98G cells 4–5 days following delivery (Figure 3A). While TMZ sensitivity was retained 32d following delivery in polyclonal populations (Figure 3A), a much greater extent of TMZ sensitization was observed in

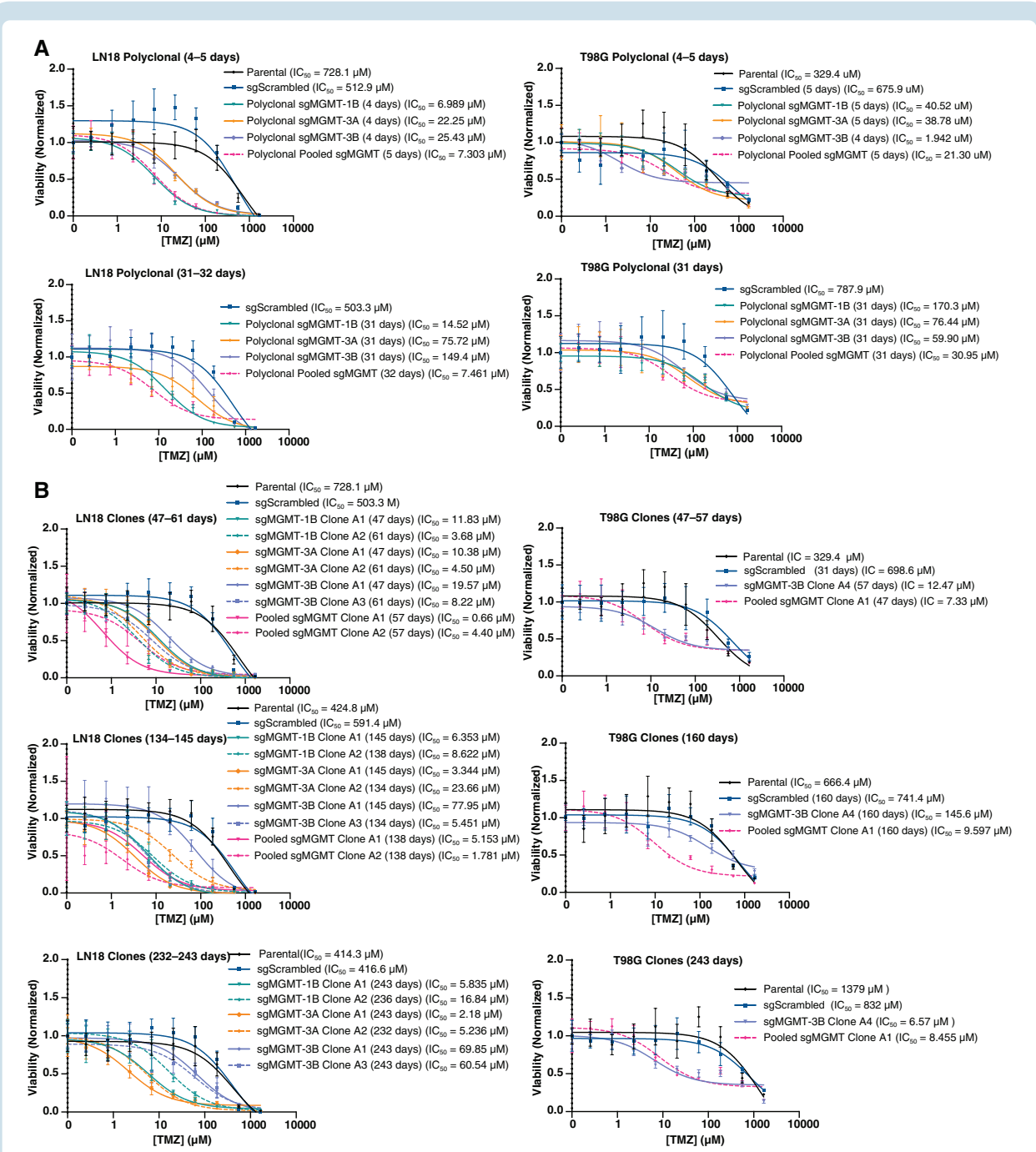

Figure 3. CRISPRoff repression of *MGMT* sensitizes GBM cells to TMZ long term. (A) Cell viability dose–response curves for temozolomide (TMZ) sensitivity in LN18 (left) and T98G (right) polyclonal populations at the indicated number of days following delivery of CRISPRoff and the indicated sgRNA(s). IC50 is calculated for each condition. (B) Cell viability dose–response curves for temozolomide (TMZ) sensitivity in LN18 (left) and T98G (right) intermediate and long-term monoclonal populations at the indicated number of days following delivery of CRISPRoff and the indicated sgRNA(s). IC50 is calculated for each condition using 3 parameter nonlinear fit on [inhibitor] versus response.

monoclonal populations, in which we observed up to 136-fold increased sensitivity to TMZ in the case of individual sgRNA-1B and 762-fold in the case of pooling 3 sgRNAs against *MGMT* (Figure 3B), suggesting that CRISPRoff suppression is highly potent at the individual clonal level. Consistent with its ability to induce long-term gene repression (Figure 2), CRISPRoff against *MGMT* also induced sensitivity to TMZ in GBM cells at 8.1 months following transient CRISPRoff electroporation, up to 191-fold in the case of individual sgRNA-3A (Figure 3B). To confirm the specific role of MGMT in TMZ sensitivity, we ectopically expressed MGMT cDNA in clonal populations of GBM cells harboring MGMT silenced by CRISPRoff, which resulted in complete rescue of TMZ resistance (Figure S2A-C).

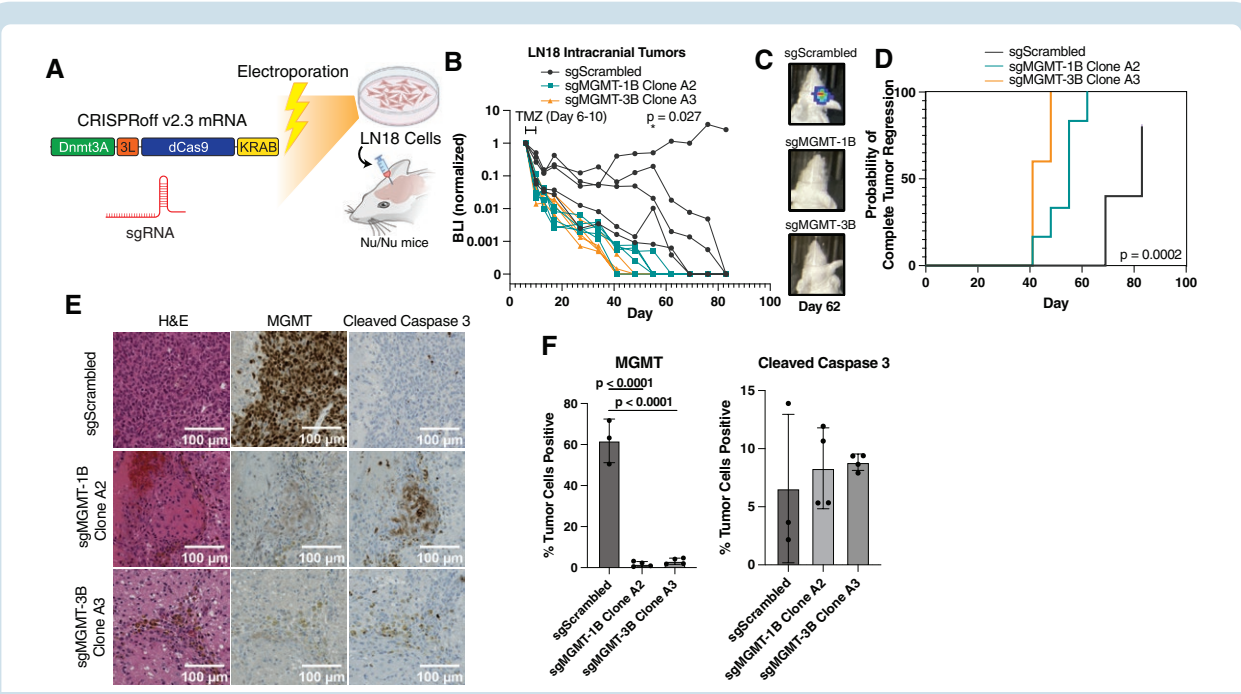

Figure 4. CRISPRoff repression of *MGMT* sensitizes GBM xenografts to TMZ in vivo. (A) Schematic of CRISPRoff mRNA and sgRNA delivery using electroporation into GBM cells, followed by intracranial transplantation of monoclonal populations (2 million cells per animal, $n = 5$ animals per condition) into immunocompromised mice. Animals were treated with TMZ between days 6–10 at 500 µg/kg/day by oral gavage. (B) Bioluminescent imaging (BLI) of orthotopic xenografts of CRISPRoff modified LN18 cells. *P* value = 2-way ANOVA comparing across all groups at day 55. (C) Representative BLI images for orthotopic xenografts for each CRISPRoff modified LN18 tumor at the specified timepoint post-transplantation. (D) Time to complete tumor regression, as measured by BLI, for orthotopic tumors following 5d TMZ treatment between days 6–10. *P* value = log-rank test comparing all time-to-event curves across the entire duration of the experiment. (E) H&E and immunohistochemistry against MGMT or cleaved caspase 3 from orthotopic tumors 30 minutes following the final dose of TMZ (day 10). Representative images are shown for $n = 3-4$ tumors per condition. (F) Quantification of IHC against MGMT or cleaved caspase 3. *P* value = 2 tailed Student's *t*-test.

Furthermore, treatment of GBM cells with a chemical inhibitor of MGMT, O^6-Benzylguanine, induced TMZ sensitivity in control populations but only minimally in sgMGMT cells (consistent with the minimal residual MGMT levels in these monoclonal populations [Figure S2A]), overall supporting that drug sensitivity was through the specific loss of MGMT (Figure S2D).

To then test whether alternative non-viral modes of delivery could be used to induce CRISPRoff-based gene silencing and TMZ sensitization, we co-packaged CRISPRoff mRNA and sgRNA targeting *MGMT* using 3 different lipid nanoparticles (LNPs) and 2 mRNA:sgRNA mass ratios. All LNP combinations were sufficient to induce potent *MGMT* repression and TMZ sensitization (Figure S3A–D).

We then compared CRISPRoff repression with Cas9 deletion of *MGMT*. A pool of 3 sgRNAs spanning exon 3 of *MGMT* was selected to disrupt the methyltransferase and DNA binding domains of MGMT (Figure S4A). This sgRNA pool was co-electroporated with Cas9 protein into GBM cells, and clonally isolated and expanded populations of LN18 and T98G cells were confirmed to have *MGMT* deletions (Figure S4 B and C). Compared to clonal populations of CRISPRoff silenced cells, Cas9 deletion of *MGMT* resulted in comparable or inferior sensitization to TMZ (Figure S4D), demonstrating an advantage of complete gene silencing achievable with CRISPRoff.

CRISPRoff Repression of MGMT Sensitizes GBM Xenografts to TMZ In Vivo

Given the long-term silencing of *MGMT* and associated TMZ sensitivity achieved by CRISPRoff, we asked whether a transient pulse of CRISPRoff against *MGMT* could sensitize GBM xenografts to TMZ in vivo. After electroporation of CRISPRoff mRNA and individual sgRNAs targeting *MGMT* in luciferase-labeled LN18 cells, clonally isolated populations were expanded and transplanted intracranially at equal cell numbers into (Nu/Nu) athymic mice (Figure 4A). Intracranial xenografts were allowed to engraft for 5 days and then treated with TMZ for 5 days. Bioluminescence imaging (BLI) revealed increased rates of tumor regression for xenografts treated with sgMGMT compared to scrambled sgRNA controls ($P = .027$, 2-way ANOVA; Figure 4 B and C). Analysis of time to complete tumor regression with TMZ treatment, as measured by BLI, demonstrated significantly faster complete tumor regression for *MGMT*-targeted tumors compared to controls ($P = .0002$, log-rank test; Figure 4D). Immunohistochemistry of orthotopic xenografts immediately following completion of TMZ treatment demonstrated the absence of MGMT expression in the CRISPRoff-sgMGMT tumors compared to sgScrambled (Figure 4 E–F, Figure S5). The apoptosis marker cleaved caspase 3 showed variable activity with a trend toward increased apoptosis in sgMGMT, although the analysis was

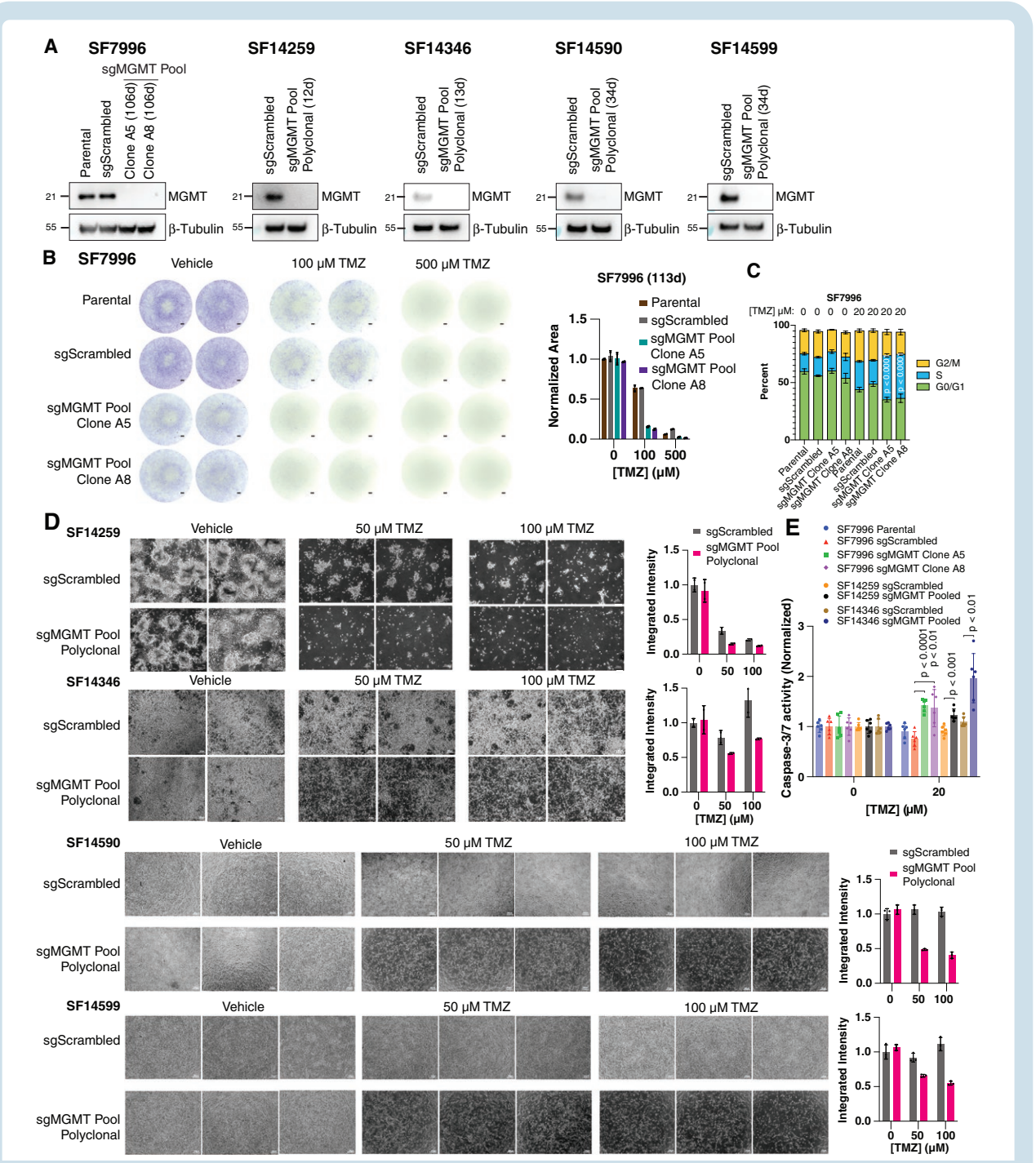

Figure 5. CRISPRoff targeting of MGMT in primary GBM. (A) Western blots against MGMT protein in monoclonal (SF7996) or polyclonal (SF14259, SF14346, SF14590, SF14599) populations of patient-derived primary GBM cultures at the indicated number of days following delivery of CRISPRoff and the indicated sgRNA(s). (B) Crystal violet assay of cell viability 7 days following cell seeding and TMZ treatment (replenished every 3 days) at the indicated concentrations for SF7996 primary GBM. Quantification of cell growth (right). Scale bar = 1000 μm. (C) Propidium iodide cell cycle analysis of SF7996 cells treated with TMZ. *P* value = 2 tailed Student's *t*-test for S-phase compared to sgScrambled 20 μM TMZ condition, $n = 3$ biological replicates. (D) Brightfield light micrographs of CRISPRoff-modified primary GBM cultures (SF14259, SF14346, SF14590, SF14599) 15–35 days following cell seeding, corresponding to the time at which vehicle-treated cells reached confluency. TMZ was replenished every 3 days during culture. Integrated intensity from live cells was quantified (right) for each cell type. (E) Apoptosis assay of primary GBM cultures 7 days following cell seeding and TMZ treatment. *P* value = 2 tailed Student's *t*-test compared to cognate sgScrambled.

limited by heterogenous tumor regression at the time of immunohistochemistry.

Epigenetic Editing of MGMT in Primary GBM

To test whether CRISPRoff could be applied to epigenetic editing of patient tumors, we established 4 primary GBM cultures in defined serum-free conditions from surgical resections of patients with newly diagnosed (SF14259, SF14346, SF14590) or recurrent (SF14599) GBM with unmethylated *MGMT* promoters determined by CLIA certified targeted bisulfite sequencing clinical assays (Table S2). Glioma stem cells capable of propagating in serum-free conditions were enriched through growth in tumorsphere culture conditions (methods). Delivery of CRISPRoff mRNA and sgRNAs against *MGMT* promoter by electroporation resulted in complete loss of *MGMT* protein in all primary GBM cultures, including in clonal populations 106 days following CRISPRoff delivery in primary GBM SF7996, which was derived prior to this study[32] (Figure 5A). In primary GBM cultures, CRISPRoff targeting of *MGMT* resulted in sensitization to TMZ treatment as reflected by diminished cell growth, S phase cell cycle arrest, and induction of apoptosis (Figure 5B-E), even at TMZ concentrations that were otherwise ineffective against proliferation in control conditions (eg, SF14590 and SF14599).

Multiplexed Targeting of Sensitizers of CCNU Identified by Genome-Wide CRISPRi Screens

Thus far we have demonstrated highly effective long-term silencing of a single target gene that modifies sensitivity to TMZ chemotherapy in GBM in vitro and in vivo. To generalize the versatility of the CRISPRoff system in targeting mediators of treatment response, we investigated the genetic determinants of resistance and sensitivity to CCNU, a bifunctional alkylating chemotherapy that induces DNA interstrand crosslinks and is used in the treatment of low- and high-grade gliomas, especially in the recurrent setting.[8–10] We performed genome-wide CRISPR interference (CRISPRi) screens across 2 cell lines (LN18, T98G) using an optimized dual sgRNA library[17,27] in triplicates to identify sensitizers of CCNU-mediated cell depletion (Figure 6A, Figure S6, Table S3). These CRISPRi screens were performed in the presence or absence of CCNU, and we ranked gene targets based on their degree of enrichment or depletion in the CCNU conditions compared to the vehicle conditions (Figure 6B). Gene set enrichment analysis (GSEA) demonstrated that the Fanconia Anemia pathway, DNA double-strand break processing, and homologous recombination gene sets were among the most significant sensitizers of CCNU-mediate cell killing (Figure 6C).

We then asked whether multiplexed CRISPRoff targeting of the top sensitizers of CCNU identified from CRISPRi screens could enhance the efficacy of CCNU in GBM. sgRNAs against *BRIP1, FANCE*, and *MGMT* were co-electroporated with mRNA encoding CRISPRoff. Pairwise and triplex gene targeting achieved complete silencing of target genes (Figure 6 D and E). CCNU drug dose–response assays for GBM cell viability demonstrated similar modest enhanced sensitivity to CCNU when *BRIP1, FANCE,* or *MGMT* were targeted individually by CRISPRoff, potentially a reflection of the baseline increased sensitivity of GBM cells to CCNU compared to TMZ (Figure 6F,G). However, multiplex targeting of CCNU sensitizers resulted in further increased sensitivity to CCNU (eg, 6.7 fold lower IC50 for *BRIP1, FANCE,* and *MGMT* triplex targeting in T98G [IC50 3.29 μM; 95% CI: 2.25–4.89 μM] compared to sgScrambled [IC50 66.97 μM; 95% CI: 31.47–181.3 μM]). In the absence of CCNU, *BRIP1* or *FANCE* CRISPRoff also resulted in decreased viability of GBM cells (Figure S7). However, multiplex targeting of CCNU sensitizers resulted in modest increases in viability in the absence of CCNU, suggesting genetic interactions that are beyond the scope of this report. These findings underscore that the presence of the drug is essential to achieve the sensitizing effects of this multiplexed approach.

Discussion

Here, we adapted an epigenetic editing platform, CRISPRoff, to site-specifically induce repression of critical genes for tumorigenesis and treatment resistance in GBM. We demonstrate that transient delivery of CRISPRoff against *MGMT* can induce long-term suppression of *MGMT* expression and function in GBM cells, GBM primary cultures, as well as in GBM orthotopic tumors. Furthermore, we used genome-wide CRISPRi screens to systematically identify sensitizers for alkylating chemotherapy, and then we targeted them using multiplexed CRISPRoff to further increase chemosensitivity in GBM. The results of these CRISPRi screens demonstrated major roles for the Fanconia Anemia and homologous recombination DNA repair pathways as vulnerabilities that could potentiate the efficacy of CCNU. This is consistent with the known mechanism of nitrosourea chemotherapeutics such as CCNU in the formation of interstrand crosslinks, which are cytotoxic and require coordination of numerous DNA damage response genes for repair.[33] Nonetheless, genes without established roles in DNA damage (eg, *NRL, EMC4*) were also identified by our screens and represent novel targets for future investigation.

While other groups have used DNMT3-based epigenetic editors to silence *MGMT* in cell cultures,[34–37] our study represents several major advancements by demonstrating long-term stability of target gene repression, efficacy in GBM xenografts in vivo, efficacy in primary GBM cultures from recently derived surgical resections, the use of non-viral transient delivery strategies, and demonstration of multiplexed gene targeting using rationally informed targets from unbiased genome-wide screens.

The therapeutic application of epigenetic editing has several advantages over existing gene therapy approaches. RNA interference gene silencing can be limited by partial knockdown, unpredictable efficiencies, and significant off-target effects due to partial complementarity with unintended transcripts.[38,39] Oligonucleotide therapeutics such as aptamers or antisense oligonucleotides, which can bind to and degrade target mRNA transcripts, have shown potential for the treatment of genetic diseases,[40,41] but their efficacy in cancer has been limited.[42] CRISPR/Cas9 gene editing permanently modifies the DNA sequence, resulting

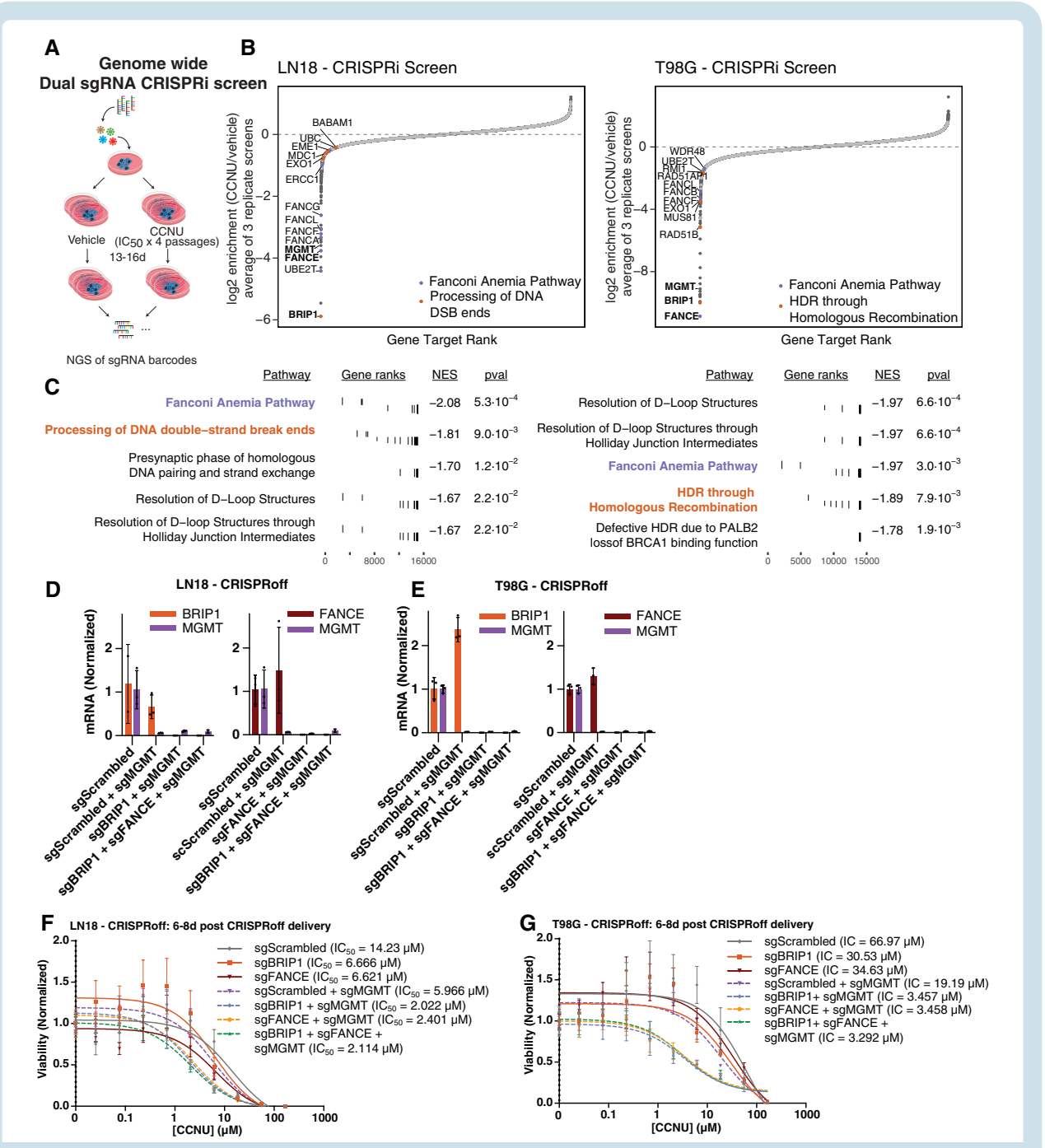

Figure 6. Multiplex targeting of CCNU sensitizers identified through Genome-wide CRISPRi screens. (A) Schematic of genome-wide CRISPRi screens in GBM cells stably expressing dCas9-Zim3. Dual sgRNA libraries were transduced via lentivirus. Parallel screens were performed in the presence of vehicle or CCNU in LN18 as well as T98G cells. Phenotypes of CRISPRi suppression of target genes were quantified using targeted sequencing of integrated sgRNA barcodes. (B) Waterfall plot of all gene targets from genome-wide CRISPRi screens in LN18 (left) and T98G (right) ranking the log2 ratio of sgRNA barcodes in the CCNU vs. vehicle conditions at the endpoint of each screen. Phenotypes are the average across 3 replicate screens. Light gray = non-targeting sgRNA. Dark gray = gene targeting sgRNA. (C) Gene set enrichment analyses of CCNU screens, ranked by the topmost negative enrichment terms. (D) RT-qPCR of *BRIP1* or *MGMT* mRNA normalized to *RPLP0* housekeeping gene, in polyclonal LN18 cells following delivery of CRISPRoff mRNA and the indicated combination of sgRNAs. (E) as in (D) but for T98G. (F) Cell viability dose–response curves for CCNU sensitivity in LN18 (left) and T98G (G) polyclonal populations following delivery of CRISPRoff and the indicated combinations of sgRNAs. IC50 is calculated for each condition.

in irreversible modifications whose functional consequences can be challenging to predict.[43–45] Consistent with this observation, we found that Cas9 knockout of MGMT led to heterogeneous TMZ sensitivity phenotypes, which could be a result of hypomorphic MGMT variants generated by variable DNA repair following Cas9 mutagenesis. In a therapeutic setting, epigenetic modification using modalities such as CRISPRoff can be specific, durable long term, and yet can be reversed,[13,46] representing favorable properties for clinical translation. Of note, the CpG loci of *MGMT* targeted by CRISPRoff in this report colocalize with 4 CpG sites that were recently reported to be significantly associated with a hypermutated subtype of glioma with improved survival[31] (Figure 1B), supporting the rationale for direct epigenetic targeting of these loci as a therapeutic approach. Nevertheless, the observation that not all GBM cells were substantially sensitized to TMZ by this approach points to orthogonal resistance mechanisms, such as increased activity of telomerase reverse transcriptase (TERT), which can lead to sustained cell proliferation despite TMZ treatment.[47] Another limitation of CRISPRoff epigenetic editing is reliance on optimal sgRNA design for the efficient and selective knockdown, which may be variable across genomic loci such as at repetitive elements. Furthermore, in vivo development of these systems, including broadening the range of putative target genes and utilizing lipid nanoparticle-based delivery, which we demonstrate to be highly effective in GBM cultures, will be required to maximize their potential in neuro-oncology.

Supplementary material

Supplementary material is available online at *Neuro-Oncology* (https://academic.oup.com/neuro-oncology).

Keywords

CRISPR | CCNU | epigenetic editing | glioblastoma | temozolomide

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Conflicts of interest statement

L.A.G has filed patents on CRISPR tools and CRISPR functional genomics. L.A.G. is a co-founder of Chroma Medicine and serves on the scientific advisory board of Myllia Biotechnology. A.M. is a cofounder of Site Tx, Arsenal Biosciences, Spotlight Therapeutics and Survey Genomics, serves on the boards of directors at Site Tx, Spotlight Therapeutics and Survey Genomics, is a member of the scientific advisory boards of Site Tx, Arsenal Biosciences, Cellanome, Spotlight Therapeutics, Survey Genomics, NewLimit, Amgen, and Tenaya, owns stock in Arsenal Biosciences, Site Tx, Cellanome, Spotlight Therapeutics, NewLimit, Survey Genomics, Tenaya and Lightcast and has received fees from Site Tx, Arsenal Biosciences, Cellanome, Spotlight Therapeutics, NewLimit, Gilead, Pfizer, 23andMe, PACT Pharma, Juno Therapeutics, Tenaya, Lightcast, Trizell, Vertex, Merck, Amgen, Genentech, GLG, ClearView Healthcare, AlphaSights, Rupert Case Management, Bernstein and ALDA. A.M. is an investor in and informal advisor to Offline Ventures and a client of EPIQ. The Marson laboratory has received research support from the Parker Institute for Cancer Immunotherapy, the Emerson Collective, Arc Institute, Juno Therapeutics, Epinomics, Sanofi, GlaxoSmithKline, Gilead and Anthem and reagents from Genscript and Illumina.

Authorship statement

All authors contributed to the inception, design, implementation, analysis, supervision, or writing of this manuscript. S.J.L., M.S.B., L.A.G., N.M., D.R.R., and K.L. designed the studies. Experiments were performed by K.L., C.Z., A.H., S.S., I.W., L.G., R.S., J.Pak., K.F., T.O., and S.J.L.. Analyses were performed by K.L, C.Z., and J.Phillips.. Supervision of this study was conducted by S.J.L., M.S.B., L.A.G., N.M., D.R.R., H.N.V., J.Fd.G., T.O., and A.M.. The manuscript was prepared by S.J.L., K.L., and M.S.B. with input from all authors.

Data availability

Raw data will be made available upon reasonable request. RNA-sequencing data have been deposited in SRA accession PRJNA1153036.

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