Reprogrammable CRISPR/Cas9-based system for inducing site-specific DNA methylation

James I. McDonald¹,*, Hamza Celik²*, Lisa E. Rois³, Gregory Fishberger³, Tolison Fowler¹, Ryan Rees³, Ashley Kramer²*, Andrew Martens², John R. Edwards¹,‡ and Grant A. Challen²,4,‡

ABSTRACT
Advances in sequencing technology allow researchers to map genome-wide changes in DNA methylation in development and disease. However, there is a lack of experimental tools to site-specifically manipulate DNA methylation to discern the functional consequences. We developed a CRISPR/Cas9 DNA methyltransferase 3A (DNMT3A) fusion to induce DNA methylation at specific loci in the genome. We induced DNA methylation at up to 50% of alleles for targeted CpG dinucleotides. DNA methylation levels peaked within 50 bp of the short guide RNA (sgRNA) binding site and between pairs of sgRNAs. We used our approach to target methylation across the entire CpG island at the CDKN2A promoter, three CpG dinucleotides at the ARF promoter, and the CpG island within the Cdkn1a promoter to decrease expression of the target gene. These tools permit mechanistic studies of DNA methylation and its role in guiding molecular processes that determine cellular fate.

KEY WORDS: CRISPR/Cas9-based system, CpG dinucleotides, DNA methylation

INTRODUCTION
DNA methylation of CpG dinucleotides is a prominent epigenetic modification of the mammalian genome that can influence gene expression, and aberrant distribution of DNA methylation is associated with a spectrum of human disorders including cancers (Egger et al., 2004). Despite intensive study, it remains unclear which CpG dinucleotides must change methylation state in order to alter transcription. Genome-wide analyses have found associations between DNA methylation and reduced gene expression that occur between DNA methylation and transcription start site (TSS) (Bell et al., 2011; Bock et al., 2012; Lou et al., 2014; VanderKraats et al., 2013). Henceforth referred to as dCas9) that lacks endonuclease activity but can still be recruited by sgRNA(s) (Jinek et al., 2012). Because the sgRNA is the DNA sequence-specific component of the system, it allows for efficient targeting of multiple regions due to the ease of design and synthesis of new sgRNAs (relative to engineering new custom proteins for each target site). A Cas9 mutant (D10A and H840A) has been used to position the DNA methyltransferase DNMT3A (Fig. 1A). To test this system we directed to genomic targets by engineered short guide RNAs (sgRNAs) (Jinek et al., 2012). Therefore, in the design of cutting-edge CRISPR (Cas9) systems, we have recently been used to target genes in mammalian cells for transcriptional activation (Perez-Pinera et al., 2013a,b; Maeder et al., 2013; Mali et al., 2013). Here, we demonstrate an easily reprogrammable CRISPR/dCas9 DNMT fusion capable of inducing site-specific DNA methylation.

METHODS & TECHNIQUES
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RESULTS
To design a flexible system to target DNA methylation, we fused dCas9 to the catalytic domain of the de novo DNA methyltransferase DNMT3A (Fig. 1A). To test this system we targeted DNA methylation to the tumor suppressor gene CDKN2A (cyclin dependent kinase 2A), which inhibits progression through the cell cycle (Liggett and Sidransky, 1998). CDKN2A is one of the most frequently hypermethylated genes in The Cancer Genome Atlas (Ciriello et al., 2013), and numerous clinical studies show a negative correlation between CDKN2A methylation and expression in colorectal cancer (Shima et al., 2011). While it is generally assumed that CDKN2A methylation induces gene silencing, it has also been suggested that DNA methylation occurs after the loss of expression (Hinshelwood et al., 2009). From a literature search, we identified 17 publications that associate CDKN2A methylation with expression and/or cancer (Fig. S1.1). Overwhelmingly, these papers studied the differentially methylated region (cancer DMR, cDMR) on the 3′ end of the CpG island that overlapped the first exon of CDKN2A (Fig. 1B).
We computationally designed three sgRNAs (g1a, g7a and g33a) to target this region and test whether DNA methylation was sufficient to induce gene silencing. We validated the ability of each sgRNA to target the \textit{CDKN2A} locus by transfecting them with active CRISPR/Cas9 and measuring the ability of Cas9 to cleave the locus (Fig. S1.2, Table S1). We then transfected HEK293T cells with the pool of three sgRNAs along with either a normal dCas9-DNMT3A catalytic domain (CD) fusion (dCas9-D3A) or one with a DNMT3AE756A mutation (dCas9-mD3A, Fig. 1A), which abolishes DNA methyltransferase activity (Reither et al., 2003). Transfection efficiencies were >80-90% for all experiments as measured by co-transfection with GFP-containing plasmids. We analyzed DNA methylation levels at 20 days post-transfection using Illumina sequencing of two amplicon regions (amplicon bisulfite sequencing,ABS). ABS results were validated using Sanger bisulfite sequencing (R²=0.83; Fig. S1.3), and DNA methylation levels at CpGs analyzed in both of two independent ABS amplicons showed strong correspondence (R²=0.98; Fig. 1C). All CpGs had >100× sequencing coverage, with a median coverage of 15,200.

Over the 20-day time course we observed an increase in DNA methylation at the \textit{CDKN2A} target locus that ranged from 20-43% at its peak on day three. Background methylation from transfection with dCas9-mD3a was consistently less than 1.5%, while background methylation from an off-target sgRNA, which controls for DNMT3A-CD overexpression, was less than 14% on day three. Induced DNA methylation levels were highest over a set of eight CpGs directly between the g33a and g7a sgRNA target sites (Fig. 1C). Increases in CHG and CHH methylation were minimal (Fig. S1.4). DNA methylation decreased rapidly after passaging the cells on day four, but stabilized 20 days post-transfection at 6-10% (Fig. 1D; Fig. S1.5). Despite the literature support for a negative

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**Fig. 1. Site-specific induction of DNA methylation using a CRISPR-Cas9 DNMT fusion.** (A) dCas9-DNMT3A CD fusion constructs. The E756A mutation inactivates the DNMT3A-CD. NLS, nuclear localization signal; FLAG, FLAG tag domain. (B) UCSC genome browser view showing the locations of the three \textit{CDKN2A} sgRNA (g1a, g7a and g33a). The three sgRNA were validated to ensure they targeted this locus (Fig. S1.2). cDMR indicates the region from the literature where methylation changes are associated with expression changes (Fig. S1.1). (C) Induced DNA methylation at the \textit{CDKN2A} promoter three days post-transfection. Colors correspond to the red and blue ABS regions in B. Three CpGs were independently measured in both amplicons. sgRNA target sites are indicated above the graphs. Pool sgRNA indicates g1a, g7a, g33a were used simultaneously. Sanger sequencing validation is presented in Fig. S1.3, and non-CpG methylation data is presented in Fig. S1.4. (D) Time course of the percent methylation data for the CpG marked with an asterisk in C. Additional CpGs are shown in Fig. S1.5. (E) Methylation induced by a pair of sgRNA decreases with increasing intervening distance. Distance is calculated relative to the 3’ end of the g33a sgRNA. Diamonds indicate the location each CpG monitored for methylation; whose color corresponds to appropriate line in the graph. Additional data from individual and paired sgRNA is presented in Fig. S1.6. (F) \textit{CDKN2A} expression for samples with induced methylation. Expression is normalized to day one for each respective sample. Error bars=mean±s.e.m. (n=1 performed in technical triplicate).
correlation between expression and DNA methylation in this region, we did not observe a measurable effect on CDKN2A gene expression by RT-qPCR (Fig. 1F). This suggests that a limited increase in methylation in the region 100-400 bp downstream of the CDKN2A TSS is insufficient to trigger gene silencing.

Spatially, the induced DNA methylation spiked near the sgRNA target sites and dropped quickly toward background levels at surrounding CpG sites. Analysis of DNA methylation induced by single sgRNAs indicates that methylation occurs primarily within 50 bp of the sgRNA binding site (Fig. S1.6). Higher DNA methylation levels were often observed 3′ of the sgRNA binding site (Fig. S1.6). Our initial data from the three pooled sgRNAs suggested that CpG methylation was higher between pairs of sgRNAs. To investigate this effect, we transfected pairs of sgRNA with varying intervening distances and monitored methylation of six clustered CpGs between the sgRNA pairs using ABS (Fig. 1E). DNA methylation of the three CpGs (cluster 1) within 20 bp of the fixed sgRNA (g33a) did not change with addition of a second sgRNA 77 bp or further away. However, the methylation level increased from 30-40% to 42-53% when sgRNAs were paired within 80 bp and both sgRNAs were within 50 bp of CpG cluster 2 (Fig. 1E). This suggests the DNMT3A-CD activity at the target locus is additive.

We next tested whether we could use our approach to methylate an entire CpG-island (CGI). We designed 17 sgRNAs (Fig. 2A; Table S1) to target DNA methylation across the CDKN2A CGI, which spans the TSS. We applied three combinations of sgRNAs (Set 1, 2, All) to test whether inducing DNA methylation of the entire CGI could decrease gene expression (Fig. 2A). ABS analysis of eight amplicons (minimum per CpG sequencing depth of 100) showed that the DNA methylation level increased to an average of 22% across the entire region with a peak of 54% (Fig. 2B). As an off-target negative control, we used three sgRNAs targeted to the ARF promoter located 20 kb away. The average background methylation at CDKN2A after treatment with off-target ARF sgRNAs was 9% (Fig. 2B). The other two sgRNA sets (Set 2 and All) induced similar increases of methylation across the CGI overlapping the TSS of CDKN2A (Fig. S2).

Analysis of CDKN2A expression by RT-qPCR in all three sgRNA targeting experiments (Set 1, 2, All) indicated an average 39% decrease in CDKN2A mRNA expression after targeting with dCas9-D3A (Fig. 2C). Cells transfected with dCas9-mD3A showed a 16-26% reduction in CDKN2A expression, likely due to CRISPR inhibition (Fig. 2C). Across the three replicates (Set 1, 2, All) expression decreased by an average of 17% in dCas9-D3A relative to dCas9-mD3A (P<0.01 paired-t-test). This indicated that DNA methylation decreases gene expression at the CDKN2A promoter in a context dependent manner.

![Fig. 2. DNA methylation decreases gene expression at the CDKN2A promoter in a context dependent manner. (A) Locations of the 17 sgRNAs used in Sets 1 and 2 and regions sequenced in B. sgRNA coordinates are in Table S1. (B) Percent methylation is plotted for regions designated in A. Set 1 sgRNA target sites are indicated above the graphs. Data for All sgRNA, Set 2 sgRNA, and off-target SgRNA is presented in Fig. S2. (C) Methylation induced by CDKN2A-targeted sgRNA decreases gene expression. Relative expression of CDKN2A is normalized to a mock-treated control. Error bars=mean±s.e.m. (All, n=2; Set 1, n=1; Set 2, n=1; all performed in technical triplicate, paired t-test).](http://bio.biologists.org/Downloaded from http://bio.biologists.org/)
methyltransferase directly decreased CDKN2A expression, but targeting of the entire CGI was required to trigger this effect. Our results are consistent with other studies that find a similar reduction in gene expression after inducing methylation at the CDKN2A promoter using ZFP- and TALE-based systems (Bernstein et al., 2015; Cui et al., 2015).

To verify the effect of our system with a separate locus, we designed three inwardly-directed (5′ to 3′) sgRNAs that bracketed three CpG sites in the ARF promoter located 150-170 bp downstream of the TSS (Fig. 3A). ARF-targeted sgRNAs increased the DNA methylation level to 27-30% at these three CpG sites with less than 15% methylation induced in adjacent sites (Fig. 3B). Induced methylation of the ARF promoter was associated with a 19% decrease in its expression (Fig. 3C).

To further validate our design rules, we attempted to induce DNA methylation in a different system in a regulated fashion with a minimalistic combination of sgRNAs. dCas9-D3A and dCas9-mD3A were cloned into a doxycycline-inducible lentivirus where expression of the fusion protein is linked to mCherry via internal ribosome entry site (IRES), and targeted to the Cdkn1a locus with two rationally-designed sgRNAs. We designed two sgRNAs targeting the CpG island located in the Cdkn1a promoter (Fig. 4A) and cloned them into lentiviral vectors (in which sgRNAs and GFP are expressed constitutively from two different promoters) (Fig. S4.1) and transduced 32D cells (mouse IL3-dependent myeloid progenitor cell line with an unmethylated Cdkn1a promoter and active Cdkn1a expression). Additional negative controls were generated by transducing 32D cells with sgRNAs only, dCas9-D3A only, or sgRNAs plus dCas9-mD3A. Cells were then FACS-sorted by GFP and/or mCherry to establish stable cell lines. Analysis of the stable cell lines induced by doxycycline showed successful expression of dCas9-fusions and sgRNAs via mCherry and GFP signal (Fig. S4.2). In the absence of doxycycline, the expression of fusion constructs was silenced (Fig. S4.2).

Stable cells were induced with doxycycline for eight days. Cdkn1a promoter DNA methylation and gene expression were then evaluated by bisulfite sequencing and RT-qPCR respectively. Cells transduced with dCas9-D3A together with sgRNAs showed an increase in Cdkn1a promoter DNA methylation by over 25% across the entire target region (Fig. 4B), and a reduction in Cdkn1a expression by approximately 40% (Fig. 4D). There was no difference in Cdkn1a promoter DNA methylation or expression between any control groups (Fig. 4B-D). Cells transduced with dCas9-D3A together with sgRNAs showed a growth advantage, consistent with an increase in proliferation due to repression of a cell cycle inhibitor (Fig. 4E). As with targeting of the human CDKN2A locus, these data suggested that induced DNA methylation pioneered by dCas9-D3A targeted with a minimal combination of sgRNAs can influence gene expression and generate subsequent downstream functional phenotypes.

We reverse cloned viral integrants to identify clones that contained either one of the two guides, or both sgRNAs together with dCas9-D3A. No induced promoter DNA methylation or reduction in gene expression was observed in clones expressing only a single sgRNA (Fig. 5A,B). In contrast, Cdkn1a promoter methylation was increased and its expression decreased in clones that expressed both sgRNAs (Fig. 5A,B). To confirm the presence of both sgRNAs were required to execute induced DNA methylation, clones that contained only a single sgRNA were transduced with their complementary sgRNA. Following this, after eight-days of induction, Cdkn1a DNA methylation increased across the promoter by up to 33% compared to the parental clone that contained only a single sgRNA (Fig. 5C). Cdkn1a expression was reduced by 40-50% in the complemented clones (Fig. 5D).
data indicate that targeting with multiple sgRNAs results in a synergism and induces robust DNA methylation. We also examined the stability of the induced DNA methylation by withdrawing doxycycline for eight days. *Cdkn1a* promoter DNA methylation and expression profiles remained largely unchanged (Fig. 5E,F) even though expression of dCas9-D3A was silenced (Fig. S4.2).

To assess non-specific activity in this system, we initially attempted to analyze off-target genomic areas with high homology to predicted binding regions of both *Cdkn1a* sgRNAs. However, no regions with high homology were found as these sgRNAs were designed based on their minimal off-target binding and the presence of a PAM-site. As an alternative, we measured the global DNA methylation levels between cells that contained fusion proteins (dCas9-D3A or dCas9-mD3A) in the presence of both sgRNAs using multiple distinct sgRNA combinations in the case of *Cdkn1a* or at the clonal levels as observed for *Cdkn1a*. Moreover, the reduction in *Cdkn1a* expression clearly had functional consequences (increased proliferation) for the transduced cells. Though modest, the expression decreases caused by induced methylation are consistent with previously published results using ZFP and TALE fusions (Bernstein et al., 2015; Cui et al., 2015).

The effects of the induced methylation also appeared to be context dependent. While methylation of the entire CGI at the *CDKN2A* promoter repressed gene expression, inducing DNA methylation of a region 100–400 bp downstream of the *CDKN2A* TSS alone was insufficient to affect expression despite the frequent observation of a negative correlation between methylation and expression in this region. This indicates the importance of the flexibility to target multiple regions offered by our CRISPR/dCas9 DNMT fusion system.

Although approaches for targeted DNA methylation have been previously described, our method is advantageous for several reasons including; (1) the ease of designing new sgRNAs for targeting, (2) the flexibility to target multiple regions offered by our CRISPR/dCas9 DNMT fusion system.

**DISCUSSION**

We provide an outline for using a modified CRISPR/dCas9 system to evaluate the functional relevance of DNA methylation at specific CpGs and described guidelines for its use. DNA methylation induction occurs within ~50 bp of a sgRNA target site and is strongest between two adjacent and inwardly directed sgRNA binding sites. Based on our design criteria, we designed sets of sgRNAs that induced methylation at the human *CDKN2A* and *ARF* promoters, and the mouse *Cdkn1a* promoters with similar efficiency. Induced methylation was sufficient to decrease expression of all three genes. Methylation increases and changes in expression were highly significant and reproducible either by using multiple distinct sgRNA combinations in the case of *CDKN2A* or at the clonal levels as observed for *Cdkn1a*. Moreover, the reduction in *Cdkn1a* expression clearly had functional consequences (increased proliferation) for the transduced cells. Though modest, the expression decreases caused by induced methylation are consistent with previously published results using ZFP and TALE fusions (Bernstein et al., 2015; Cui et al., 2015).

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**METHODS & TECHNIQUES**

**MATERIALS AND METHODS**

dCas9 fusion protein design and construction

The catalytic domain (CD) of human DNMT3A (amino acids 598 to 912 of NP_783328.1) both with and without the E756A mutation was cloned between the Nhel and AgeI sites of pCMV_dCas9_VP64 (Addgene plasmid #49015, Cambridge, MA) with a NLS and FLAG tag linker. For lentivirus plasmids, TetO-FUW-OSKM (Addgene plasmid #20321), was digested with EcoRI and a multiple cloning site containing EcoRI, XbaI, Nhel, AgeI, PspXI, Ascl and EcoRI was cloned into this plasmid to generate TetO-FUW plasmid. An IRES-mCherry insert was amplified by PCR and cloned into AgeI- and Ascl-digested TetO-FUW to generate TetO-FUW-IRES-mCherry. dCas9-D3A or dCas9-mD3A was cloned into this plasmid using XbaI and AgeI sites to generate TetO-dCas9-D3A or TetO-dCas9-mD3A. In the final plasmid, expression of both fusion proteins is controlled from the tetracycline operator (tetOP) and a minimal CMV promoter. The full amino acid sequence of the dCas9-3a or dCas9-3aA protein is shown in Fig. S4. Plasmids were validated by Sanger sequencing and prepared for transfection using a Qiagen Maxprep kit. All plasmids are available in Addgene, and detailed protocol information is available at http://epigenomics.wustl.edu/epigenomeEditing and http://www.challlenlab.com.

**sgRNA design**

Target sequences were entered into the MIT sgRNA design software (http://crispr.mit.edu/), the BROAD sgRNA design tool (http://www.broadinstitute.org/mgi/public/analysis-tools/sgrna-design-v1) (Doench et al., 2014), and the sgRNAcas9 tool (version 2.0.10) (Xie et al., 2014). The intersection of sgRNA target sites produced by all tools was taken for further analysis. sgRNA sequences that failed the BROAD test (score<0.2) were excluded. sgRNA sequences were selected based on high BROAD scores and location relative to other sgRNAs. sgRNA coordinates and sequences are in Tables S1 and S2. Oligonucleotides corresponding to the target sites were annealed and cloned into MLM3636 (Addgene plasmid #43860). For lentivirus plasmids, U6-sgRNA coding sequences were amplified from pMML3636 by PCR and cloned into an EcoRI and Clal linearized pLVTHM lentiviral backbone (Addgene plasmid #12247) generating pL-sgRNA1 and pL-sgRNA2 in which GFP is expressed from EF1a promoter.

**Cell culture, lentiviral production and doxycycline induction**

HEK293T cells were acquired from ATCC (CRL-3216, Manassas, VA) and grown in DMEM supplemented with 10% FBS (Gibco, Waltham, MA), 1× Penicillin/Streptomycin (Gibco), and 2mM GlutaMax (Gibco). For transfection experiments, 3×10⁵ HEK293T cells were plated in a 60 mm dish. The next day, the cells were transfected with Lipofectamine LTX (Thermo Fisher Scientific, Waltham, MA). The Lipofectamine/DNA ratio was 3.5, with a total of 5.5 μg of plasmid DNA. The mass of Cas9-DNMT3A-CD fusion plasmid was equal to the total mass of the pMD2.G plasmid. Since HEK293T cells incorporate either all plasmids or none, 0.5 or 0.7 μg of pMaxGFP was co-transfected in order to indicate the transfection efficiency. The plasmid DNA was first diluted in Gibco OptiMEM, then Lipofectamine LTX was added to the growth media. Every other day, a half-media change with 0.25 μg/ml doxycycline (Sigma, D9891-100G) was re-plated in fresh media for expansion. For the induction of fusion proteins in transduced 32D cells, 0.25 μg/ml doxycycline (Sigma, D9891-100G) was added to the growth media. Every other day, a half-media change with (0.5 μg/ml) doxycycline was performed.

**Sanger bisulfite sequencing**

Genomic DNA (gDNA) was isolated using the PureLink Gemonic DNA Mini Kit (Invitrogen) or the Zymo Research (Irvine, CA) Quick gDNA Mini Kit. Genomic DNA was digested with EcoRI and a multiple cloning site containing EcoRI, XbaI, Nhel, AgeI, PspXI, Ascl and EcoRI was cloned into this plasmid to generate TetO-FUW plasmid. An IRES-mCherry insert was amplified by PCR and cloned into AgeI- and Ascl-digested TetO-FUW to generate TetO-FUW-IRES-mCherry. dCas9-D3A or dCas9-mD3A was cloned into this plasmid using XbaI and AgeI sites to generate TetO-dCas9-D3A or TetO-dCas9-mD3A. In the final plasmid, expression of both fusion proteins is controlled from the tetracycline operator (tetOP) and a minimal CMV promoter. The full amino acid sequence of the dCas9-3a or dCas9-3aA protein is shown in Fig. S4. Plasmids were validated by Sanger sequencing and prepared for transfection using a Qiagen Maxprep kit. All plasmids are available in Addgene, and detailed protocol information is available at http://epigenomics.wustl.edu/epigenomeEditing and http://www.challlenlab.com.
MiniPrep kit and quantified with the Qubit dsDNA broad range assay (Thermo Fisher Scientific, Waltham, MA). gDNA was bisulfite converted with the Zymo Research EZ DNA methylation kit or the Epitech Bisulfite Kit (Qiagen) according to the manufacturer’s instructions. All samples underwent bisulfite conversion with a high efficiency of at least 98% as determined by conversion of unmethylated, non-CpG cytosines. For **CDKN2A**, the target regions were amplified with the Qiagen PyroMark PCR kit and CDKN2A_B primers in Table S3. PCR products were cloned into the Promega pGEM-T Easy plasmid and transformed into NEB 10β competent cells. PCR products from individual colonies were sequenced by Sanger.

A nested PCR strategy was used to amplify **Cdkn1a** target genomic DNA site. In primary PCR reaction, 20 ng of total bisulfite converted DNA was used as a template with the primer pair F1 (GTTTAGATTTTTAGGGGAGGG) and R1 (CAAAAACTAAAAAAATAACTACCATCC). PCR was performed using Taq DNA polymerase (Invitrogen) using following cycling conditions: (1) 94°C for 3 min, (2) 95°C for 45 s, (3) 58°C for 58°C, (4) 72°C for 45 s and (5) 72°C for 10 min. Steps 2-4 were repeated for 40 cycles. For the second part of the nested PCR, 1.0 µl of the primary PCR reaction was used as a template and the same PCR conditions were used with primer pairs F1 and R2 (TCTCTAAAATTTCCCACTTACTATACC). The PCR amplicons were then gel extracted and cloned into pCR4-TOPO TA vector (Invitrogen) and resulting plasmids were transformed into TOP10 competent cells. PCR products from individual colonies were sequenced by Sanger.

Amplonc bisulfite sequencing data were checked for quality using fastQC, adaptor and poor quality sequence (quality less than 20) was trimmed using fqtrim, and the trimmed sequences were mapped to the target sequences using Bismark (Krueger and Andrews, 2011).

**ELISA methylation analysis**

For quantification of total 5-mC level, the ELISA-based MethylFlash Methylated DNA Quantification Kit (Colorimetric; all from Epigentek, distributed by BioCat GmbH, Heidelberg, Germany) were utilized to quantify the amount of 5-mC in the DNA of transduced 32D cell lines. Quantification was performed in technical and biological duplicates according to the manufacturer’s instructions. 100 ng of total DNA isolated from each transfected cells was used for 5-mC quantification. For absolute 5-mC quantifications, a standard curve was generated by plotting the various concentrations of the positive control provided with the kit against the optical density (OD) at 450 nm.

**Expression analysis**

For human cells, RNA was extracted with the Zymo Research Quick mRNA Miniprep kit. RNA concentration was measured with the Qubit.
RNA BR kit. RNA integrity was determined by visualizing rRNA bands using agarose gel electrophoresis. Reverse transcription was performed using the Bio-Rad iScript Reverse Transcription kit. Quantitative reverse-transcription polymerase chain reaction (RT-qPCR) was performed with the Bio-Rad iQ® Universal SYBR Green reagent on an Applied Biosystems Viaa7 instrument. The thermocycler protocol was the following: (1) 95°C, 20 s; (2) 95°C, 3 s; (3) 60°C, 20 s; for 40 cycles. qPCR primers are listed in Table S4. A melt curve was performed to indicate there was no off-target amplification. Data was analyzed as described by Helleman et al. (2007) using the geometric mean of ACTB, GAPDH, and RPLP0 as an internal control. The ΔΔCt method was used to calculate fold change compared to the ΔCt method.

For mouse cells, total RNA was isolated from 5×10⁶ FACSort-sorted cells using NucleoSpin RNA XS (Macherey-Nagel) and reverse transcription was performed with TaqMan master mix (Applied Biosystems), 18S rRNA probe (VIC-MGB; Applied Biosystems), and Cdbn1a gene probe (FAM-MGB; Mm04205640_g1, Applied Biosystems) on a StepOnePlus Real-Time PCR System (Life Technologies). Samples were normalized to expression of 18S and fold change determined by the ΔΔCt method.

Western blot
32D cells transduced with dCas9-D3A or dCas9-mD3A with or without sgRNAs were grown in the presence of doxycycline for 12 days, collected and washed with ice cold PBS twice. Cells were then lysed in complete RIPA buffer containing protease inhibitors (Santa Cruz Biotechnology). 20 μg of protein lysates were separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes (Millipore). Membranes were subsequently probed to detect fusion proteins using primary antibodies recognizing Cas9 (Active Motif) or β-actin (Santa Cruz) and detection was performed using horseradish-peroxidase-conjugated secondary mouse antibody (Santa Cruz) and chemiluminescence (Millipore).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

J.I.M and H.C were equally responsible for cell culture, methylation and expression experiments, data analysis, and writing the manuscript. L.E.R. performed methylation analyses and assisted with writing the manuscript. T.F. performed methylation analyses, F.E. and R.R. performed cloning of target loci for sequencing analysis. A.K. helped with cell culture and off-target analysis. A.M. designed the cloning of dCas9-D3A and dCas9-mD3A fusion proteins. J.R.E. and G.A.C supervised the work, edited and approved the manuscript.

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Supplementary information

Supplementary information available online at http://bio.biologists.org/lookup/suppl?tnumber=10.1242/bio.019067/-/DC1

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