Genome-wide tracking of dCas9-methyltransferase footprints

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In normal mammalian development cytosine methylation is essential and is directed to specific regions of the genome. Despite notable advances through mapping its genome-wide distribution, studying the direct contribution of DNA methylation to gene and genome regulation has been limited by the lack of tools for its precise manipulation. Thus, combining the targeting capability of the CRISPR-Cas9 system with an epigenetic modifier has attracted interest in the scientific community. In contrast to profiling the genome-wide cleavage of a nuclease competent Cas9, tracing the global activity of a dead Cas9 (dCas9) methyltransferase fusion protein is challenging within a highly methylated genome. Here, we report the generation and use of an engineered, methylation depleted but maintenance competent mouse ES cell line and find surprisingly ubiquitous nuclear activity of dCas9-methyltransferases. Subsequent experiments in human somatic cells refine these observations and point to an important difference between genetic and epigenetic editing tools that require unique experimental considerations.
DNA methylation is widespread among organisms, with the core enzymes that catalyze the methyl group transfer being conserved for more than a billion years across plants and animals. Comparative genome-wide DNA methylation mapping has enhanced our understanding of the mammalian targets and dynamics of this modification, but many important questions regarding its precise regulatory role remain unanswered. The complex multilayered mechanisms by which DNA methylation is regulated and mitotically maintained complicate its study and the absence of tools that enable targeted manipulation has limited progress further.

However, recent advances in the field of genome editing have raised hopes that these technical limitations may finally be overcome. In particular, the CRISPR-Cas9 system for genome engineering has emerged as a powerful genomics toolbox due to its high targeting specificity and efficiency. More recently, fusion of effector domains or proteins to the catalytically inactive (dead) dCas9 protein extended the potential applications to targeted epigenome editing, including de novo methylation through dCas9-methyltransferase fusion proteins. However, several critical questions need to be explored before DNA methylation editing can be considered a reliable tool. It remains unclear what characteristics render a given locus susceptible to become ectopically methylated, i.e. how much does the transcriptional or chromatin state of a given target matter? Can canonically unmethylated regions be targeted and the methylation maintained in the absence of the inducer? For instance, recent studies suggest that directed methylation can alter target gene expression, although methylation is rapidly lost upon removal of the dCas9-effector. How dependent is the dCas9-methyltransferase on the presence of the endogenous de novo machinery? Finally, how much off-target activity arises when the dCas9-methyltransferase complex is present in the nucleus near its substrate (all cytosines)?

As for the latter, previous studies show that the nucleas active Cas9 rarely cuts at off-target sites, despite widespread engagement as shown by genome-wide mapping. However, chromatin immunoprecipitation (ChIP)-based approaches are not sensitive enough to detect transient or past interactions, which may be sufficient to induce lasting epigenetic alterations such as DNA methylation. Furthermore, high levels of DNA methylation and the presence of the endogenous de novo DNA methyltransferases (Dnmts) complicate any accurate evaluation of dCas9-methylation activity in the nucleus. Limited by these factors, current literature offers preliminary insights into the general applicability and on-target methylation efficiency of dCas9-fused methylation effectors yet lacks a general interpretation of global off-target activity. The same drawbacks have also restricted the precise measurement of methylation events and maintenance of targeted DNA methylation. Here, we present a system to measure several of these parameters and explore the effects of dCas9-methyltransferases in pluripotent cells. We observe widespread off-target activity of dCas9-methyltransferases, which occurs independently of the presence of single guide RNAs (sgRNAs) and was also apparent across multiple somatic cell types. Our results therefore provide valuable insight into the utility of epigenome editing tools that should be considered in future experimental designs.

Results

Generation of an ES cell model to track de novo methylation. To systematically assess the global effects of dCas9-fused methyltransferases, we used previously established Dnmt3a/b double knockout (DKO) embryonic stem (ES) cells and transiently repressed the maintenance methyltransferase Dnmt1 to deplete global methylation levels (as described previously). This strategy allowed us to derive a new cell line (termed DKOzero) that has significantly reduced cytosine methylation (mean global CpG methylation measured by reduced representation bisulfite sequencing (RRBS)/whole-genome bisulfite sequencing (WGBS): wild-type ES cells 0.57/0.83 and DKOzero: 0.04/0.04) but is able to maintain any emerging DNA methylation through the re-expression of endogenous Dnmt1. As a reference point, the global CpG methylation levels in the DKOzero cell line are below what has been observed for wild-type ES cells grown in 2i/LIF conditions (Supplementary Fig. 1a). Continued passaging of the DKOzero cells did not result in any notable gain of global DNA methylation, suggesting limited de novo activity of DNMT1 (Fig. 1c, Supplementary Fig. 1c). As a result, these DNA methylation depleted, maintenance competent ES cells allow us to precisely track the extent of newly added DNA methylation (without interference from the endogenous de novo enzymes) including past engagements of the catalytic domain with unmethylated cytosines that are subsequently propagated by DNMT1.

dCas9-cat3a displays global nuclear activity. After we established our DKOzero system, we generated a doxycycline (Dox) responsive construct that when co-introduced with the reverse trans-activator (M2rtTA) enables the inducible expression of dCas9-cat3a (dCas9 fused to the catalytic domain of Dnmt3a; Fig. 1d). After viral transduction and selection for double positive cells (M2rtTA plus the inducible construct), we derived a clonal cell line (DKOzero:cat3a) and assessed dCas9-cat3a induction by measuring GFP expression using fluorescence activated cell sorting (FACS). Based on this analysis we observed homogenous induction in the majority of cells (Supplementary Fig. 1d) with methylation analysis confirming no detectable leakiness in the absence of Dox (Supplementary Fig. 1e). However, we noticed to our surprise that the transient induction with Dox had already led to widespread gain of CpG methylation (Supplementary Fig. 1f, left), suggesting strong global activity by dCas9-cat3a. To measure the impact of dCas9–cat3a induction more precisely, we reset global methylation in the DKOzero:cat3a line through another round of transient Dnmt1 depletion as well as transient culture in 2i/LIF plus Vitamin C, a combination that rapidly reduces global DNA methylation. After again confirming the global depletion of DNA methylation (Supplementary Fig. 1f, right), we induced the reset DKOzero:cat3a cell line in the absence of a sgRNA for 0 (uninduced control), 2 and 7 days and performed WGBS (Fig. 1e, f). This data not only confirms our prior observation but also highlights how rapid and widespread the increase in DNA methylation occurs (mean CpG methylation uninduced: 0.16; 2 days: 0.28 and 7 days: 0.47). Upon closer inspection, we found that the gain of methylation is largely defined by the prior methylation state in wild-type ES cells. Specifically, genomic features that were highly methylated in wild-type ES cells, such as exons and repetitive elements, showed a notable gain upon Dox induction, as did regions of intermediate methylation (mean >0.2 and <0.8 in wild-type cells) that represent putative enhancer elements with enrichment for H3K27ac (Fig. 1g, Supplementary Fig. 1g). Alternatively, default unmethylated sites, such as CpG islands (CGIs) associated with transcription start sites, remained generally lowly methylated (Fig. 1g). A smaller subset of CGIs that were methylated in wild-type ES cells (mean >0.8, n = 2160/21,012) showed a strong gain to 0.56 mean methylation in DKOzero:cat3a cells after 7 days, which may be linked to the lack of H3K4me3 (Fig. 1h, left). Supporting this, CGIs that were unmethylated in wild-type ES cells and remained so in the induced cells (n = 13,525, mean methylation = 0.06 and 0.09 for...
wild-type and 7 day-induced DKOdCas9-cat3a, respectively) showed greater enrichment for H3K4me3. Nonetheless, a subset of hypomethylated CGIs gained a small, but significant (p < 0.01) amount of DNA methylation (Fig. 1h, right, Supplementary Fig. 1i). Finally, when we introduced the dCas9-cat3a into normally methylated, wild-type ES cells we also observed overall protection of hypomethylated CGIs (Supplementary Fig. 1j) with little overall increase due to the already high level of genomic methylation (Supplementary Fig. 1k). These results are in agreement with the previously noted association of methylated histone H3K4 tails with protection of CGIs from the de novo DNA methylation machinery23,24, which we extend here to cells with ectopic methyltransferase activity.

Ubiquitous nuclear activity is unaffected by sgRNA presence.

To explore if the addition of one or more sgRNAs would
Constrain the ubiquitous nuclear activity of the dCas9-cat3a to selected target loci, we stably integrated sgRNAs into our DKO(Dnmt3a–cat3a) cell line and compared on- and off-target methylation to no guide controls. First, we tested one sgRNA against the Dazl promoter, a highly methylated, lowly expressed locus in wild-type ES cells with reduced CpG methylation in our DKO(Dnmt3a–cat3a) cell line. We independently validated the targeting efficiency of this sgRNA through the transient expression of an active Cas9 enzyme, and observed efficient cutting that mapped to the expected target site (Supplementary Fig. 2a). Interestingly, the addition of the Dazl sgRNA prior to Dox induction did not prevent the global gain of CpG methylation when compared to the no guide condition (Supplementary Fig. 2b). Moreover, while we observed a gain of CpG methylation around the sgRNA target sequence, this increase also occurred independently of the sgRNA, making it difficult to quantify the actual on-target activity for this locus (Supplementary Fig. 2c). To explore the possibility of multi-loci targeting and to see whether additional guides would help reduce the off-target activity, we next transduced our DKO(Dnmt3a–cat3a) cell line with a pool of ten sgRNAs that target representative loci with distinct methylation and expression levels in wild-type ES cells (Fig. 2a, Supplementary Fig. 3a). We used universal primers for the backbone to confirm the presence of sgRNAs via ampiclon sequencing in several clonal lines (see Methods section) and initially focused on clone 3 in which eight of the ten sgRNAs were integrated. To assess methylation at our regions of interest with high coverage, we designed a specific set of 120 bp hybrid capture probes for targeted bisulfite sequencing, tiling 20 kb on either side of the sgRNA target. This strategy provides high-resolution information of the target locus and a representative bidirectional genomic sampling surrounding it (Fig. 2a; Supplementary Fig. 3b, c; Supplementary Table 1). Given the rapid rate of methylation gain initially observed, we chose earlier time points and collected samples 1, 2 and 3 days post Dox treatment as well as an unduced (0 days) control (Fig. 2a–c). Similar to our prior observations, off-target methylation accumulated along each captured region (Fig. 2b, c; Supplementary Fig. 3c), demonstrating that even multiple sgRNAs are not sufficient to prevent dCas9-cat3a from acting off-target. Of interest, we did observe on-target methylation at the Foxbl locus, which is unmethylated and not expressed in wild-type ES cells, while other sites such as the also unmethylated but highly expressed Id1 locus did not accumulate target methylation above background (Fig. 2c, d; Supplementary Fig. 3c). It therefore appears that the on-target activity is somewhat context dependent and the expression status may have some impact on how susceptible a locus is to methylation gain in response to dCas9–cat3a recruitment. Overall, on-target vs. off-target methylation accumulated faster at only two out of six loci (Fig. 2d). This was consistent across three independent clones which showed a reproducible global gain of methylation across all captured tiles (Fig. 2e). Notably, although mean off-target methylation was comparable for all CpGs, we found variability in the affected regions between the clones (Fig. 2f), which may suggest a largely random off-target activity at accessible sites. Taken together, these results show that the presence of sgRNAs can increase on-target methylation, but has no detectable impact on reducing global dCas9–cat3a off-target activity.

Catalytic domain of Dnmt3a alone exhibits similar activity. Given these results, it was important to determine whether off-target methylation was mediated by dCas9 scanning the DNA independently of an sgRNA25 or related to the unspecific activity and ubiquitous nuclear presence of the catalytic domain of Dnmt3a (cat3a) itself. To explore this we generated two additional inducible cell lines, one expressing only the Dnmt3a catalytic domain (DKO(Dnmt3a)) and a second one expressing the full-length Dnmt3a (DKO(Dnmt3a), that includes both cat3a and its N-terminal regulatory domain; Supplementary Fig. 4a). After around two weeks of induction, DKO(Dnmt3a) cells showed near wild-type levels of CpG methylation, while DKO(Dnmt3a) and DKO(Dnmt3a–cat3a) cells reached only intermediate levels (Supplementary Fig. 4b, c). Although mechanistic details remain to be elucidated, the different remethylation behaviors may result from reduced de novo activity and/or less efficient inheritance in the absence of the N-terminal regulatory domain of Dnmt3a. Furthermore, despite the fact that in principle any cytosine is a potential target for de novo methylation, we found that only cytosines in the CpG context were methylated in DKO(Dnmt3a–cat3a) and DKO(Dnmt3a) cells (Supplementary Fig. 4d, e). In contrast, the DKO(Dnmt3a) cells showed methylation of cytosines in both the CpG and CpA context, the latter being the most frequently methylated non-CpG dinucleotide.26 This suggests a possible dependence on the Dnmt3a regulatory domain for the acquisition of non-CpG methylation.

dCas9-cat3a induces off-target methylation in somatic cells. While our inducible ES cell lines have the advantage of homogenous induction and temporal control, most prior studies have selected transient transfection as the method of choice for introducing dCas9 epigenetic effectors. Therefore, we decided to compare the consequences of a transient transfection strategy13 in our methylation depleted, maintenance competent DKOzero system (Supplementary Fig. 5a) as well as in two frequently utilized human somatic cell lines (293T; human embryonic kidney and MCF7; human breast adenocarcinoma, Fig. 3a). Consistent with the results of the inducible line, we found a global gain of CpG methylation (and lack of non-CpG methylation) when transiently transfecting our DKOzero line (Supplementary Fig. 5a–c). Importantly, no gain of methylation was observed when the cells were transfected with a control construct encoding a mutated (E756A), catalytically inactive version of cat3a (dCas9–cat3a–ANV13), we utilized a previously described BACH2 sgRNA and observe on-target methylation that is comparable to their published results13 (Fig. 3b, c). Of note, BACH2 is either not or only marginally expressed in both of our somatic cell lines (FPKM 293T: 1.11, FPKM MCF7: <1). This low expression may render the locus more amenable to targeted methylation, similar to the Foxbl locus in the mouse ES cells (Fig. 2b). Despite successful on-target methylation, we observed an extensive accumulation at off-target sites (Fig. 3d, e). Notably, both cell lines are globally methylated—unlike our DKOzero line—and therefore make it more difficult to determine the exact scale of the off-target activity. When we compared 200 bp tiles between the dCas9–cat3a–ANV control and the dCas9–cat3a, 91% (MCF7) and 94% (293T) of the significantly different tiles (adj. p-value <0.05 (t-test) and difference in methylation >0.1) overlapped with otherwise lowly methylated CGIs (Fig. 3d–f), with targets varying between 293T and MCF7 cells (Supplementary Fig. 5f). Biological replicates showed a similar behavior, with 50% (293T Rep1) and 70% (293T Rep2) significant tiles being shared targets, indicating that dCas9–cat3a is acting genome-wide with no obvious sequence preference (Supplementary Fig. 5g). To more closely inspect the gain of methylation, we performed read-level analysis and find that for both on- and off-target sites only half of the reads show any methylation (Supplementary Fig. 5h, i), suggesting either a mixed population of cells or that the de novo methylation is added to only one strand generating hemi-methylated DNA. In agreement with previous reports we...
observed a decrease in the BACH2 target methylation within a few days post-transfection (Supplementary Fig. 5j). Similarly, most off-target methylation decreased within 7 days post-transfection (Supplementary Fig. 5k), which is somewhat surprising given the low expression levels of TET enzymes (Supplementary Fig. 6a). This makes an active mechanism less likely and raises important questions regarding the stability of DNA methylation when it is added out of its normal context.

dCas9-cat3a activity has limited impact on gene expression. To further investigate what renders loci more or less susceptible to off-target methylation, and considering that the majority of hypermethylated CGIs were located in promoter regions (Supplementary Fig. 6a), we wanted to explore how gained promoter methylation is linked to associated gene expression. We collected matched RNA samples from our 293T cells before and after transfection and performed RNA-sequencing which confirmed the high abundance of cat3a transcripts that should originate from the exogenous dCas9–cat3a, stable levels of DNMT1 and background levels of DNMT3B (Supplementary Fig. 6b). Importantly, the dCas9–cat3a complex predominantly added methylation to promoters of either silenced or lowly expressed genes (Supplementary Fig. 6c, d) and was only retained at a subset of lowly transcribed genes after 7 days post-transfection (Supplementary Fig. 6c, Supplementary Data 1). Despite the gain of methylation, the vast majority of hypermethylated targets were not associated with significant changes in gene expression in the steady state (Supplementary Fig. 6e). In line with the fact that DNA methylation rarely acts as a primary silencing mechanism, our results suggest that silencing actively expressed genes using...

Fig. 2 Targeted bisulfite sequencing surrounding multiple sgRNA targets. a Schematic describing the experimental design for the transduction of DKO-dCas9-cat3a cells with multiple sgRNA constructs. Cells were collected at 0, 1, 2 and 3 days after Dox treatment. Extracted DNA was hybridized to capture probes for enrichment of target loci. See Methods section for more details. b Top: tracks displaying methylation levels for wild-type (WT) ES cells and DKO-dCas9-cat3a at 0 and 3 days of Dox treatment. The target site for the Foxb1 sgRNA is displayed with 20 kb on either side, captured by our custom probes. Below: loess-smoothed methylation levels for 0, 1, 2 and 3 days of Dox treatment for the full region shown at the top, as well as a zoomed in region displaying the 2 kb surrounding the targeted locus. The sgRNA position is indicated as a red bar in the top part and red arrowhead in the bottom zoom in. c Same as in b, but for the Id3 locus. d The difference in methylation after 3 days of dCas9-cat3a expression for the on-target locus (200 bp downstream of the sgRNA) as well as all other captured 200 bp tiles with mean methylation level +/- 0.05 of the on-target locus in WT ES cells (collectively termed “off-target”). Error bars display standard deviation for three clones. e Mean methylation for all CpGs within six 40 kb target regions that are covered in three independent clones over the Dox time course. Error bars display standard deviation for the three clones. f Venn diagram showing the overlap between the top 100 most highly methylated 200 bp tiles for the three independent clones...
dCas9-cat3a is not trivial and may require recruitment of additional repressive effectors.\textsuperscript{10,28}

Discussion

Since the emergence of the CRISPR-Cas9 system for genome editing, it has evolved into a versatile toolbox for numerous applications, including the targeted manipulation of the epigenome using dCas9-coupled epigenetic effector proteins. Initial proof-of-concept studies have highlighted the general applicability of the system, thereby raising the possibility for functional dissection of individual epigenetic features and their direct impact on gene regulation.\textsuperscript{8} However, this requires precise activity and to date a careful evaluation of the global on- vs. off-target activity has not been reported. Our study offers a unique tool (the date a careful evaluation of the global on- vs. off-target activity, occurs regardless of

[Fig. 3 Global off-target CpG methylation after transient dCas9-cat3a transfection. a] 293T human embryonic kidney and MCF7 human breast cancer cells were transfected with pdCas9-DNMT3A-EGFP (Addgene #71666) or pdCas9-DNMT3A-EGFP (ANV) (Addgene #71685) with an sgRNA targeting BACH2. Cells were collected 3 days post-transfection (p.t.) and RRBS was performed. b Top: loess-smoothed CpG methylation for 293T cells (WGBS) highlighting the location of the BACH2 target within a CpG island. Bottom: the on-target smoothed methylation levels (RRBS) for 293T cells transfected with dCas9-cat3a or dCas9-cat3a-ANV 3 days p.t. The bold line indicates the mean methylation level with standard deviation shown by shaded fill. The position and orientation of the Bach2 sgRNA is indicated with a red arrow. c same as in b but for MCF7 cells. d Smooth scatter plot displaying mean methylation over 200 bp tiles in dCas9-cat3a and dCas9-cat3a-ANV transfected 293T cells 3 days p.t. with the Bach2 sgRNA (total tiles: 219,949). Red dots indicate tiles with significant changes in methylation (adj. p-value < 0.05 (t-test) and difference >10%, significant tiles: 32,665). The BACH2 locus is significant and highlighted as a white dot. e Same as in d but for MCF7 cells (total tiles: 200,157; significant tiles: 10,881). f The proportion of significantly differentially methylated 200 bp tiles that are located within CpG islands (CGIs) for 293T and MCF7 cells. g Methylation levels for CGIs that have <10% methylation in WT cells shown for dCas9–cat3a–ANV and dCas9–cat3a transfected 293T/MCF7 cells.]

Experimental Setup (transfection vs. transduction) and across multiple cell types. This extensive off-target activity is perhaps not surprising considering that the amount of free nuclear dCas9-cat3a is much higher than the target bound complex as each cell has only two alleles for a single copy locus that match the sgRNA. In the future, it would be interesting to explore whether redirecting the complex to inert genomic loci, such as abundant silent repeat elements or other ectopic targets would have an impact on reducing off-target activity by skewing its nuclear activity sufficiently to maximize the on-to-off-target ratio. Furthermore, it remains to be seen whether other epigenetic effector fusions show similar global effects, although regulators of de novo DNA methylation may be particularly prone, as even transient interactions result in the addition of methyl groups that subsequently be mitotically inherited through ubiquitously expressed DNMT1. Although we and others also highlight the transient nature of transfection-based methylation gain\textsuperscript{10,13}, which will require further studies to delineate the underlying mechanism of failed, incomplete maintenance or active clearance. Nonetheless, sustained dCas9-cat3a activity may result in more permanent changes and it is worth considering the possibility that in some cases the aberrantly methylated loci could create a selective advantage that results in an undesired clonal expansion. Moreover, while we observed only limited effects on gene expression, we envision that these may be more striking in a system where cells are induced to change identity and have to activate pre-existing silent regulators. In summary, our findings and the
possible implications highlight the need to further investigate these valuable tools as well as remind us to consider the ubiquitous activity of dCas9-fused epigenetic effector proteins in experimental designs and interpretations.

**Methods**

dCas9-cat3a plasmid design and construction. We modified the dCas9 sequence of the on-target regions of P2A plasmid (Addgene #47106) to include a BsaI restriction site followed by EcoRI and a Kozak sequence (N-termus), as well as a BsiWI restriction site, P2A-GFP, followed by EcoRI and BsaI restriction sites (C-termus). The C-terminal Nuclear Localization Sequence (NLS) was left intact. Following BsaI digest, the dCas9-BsiWi-P2A-GFP fragment was subcloned into the FUW backbone (Addgene #20723). The BsiWI restriction site was used to clone in the catalytic domain of Dnmt3a (cat3a, last 301 amino acids of Dnmt3a Isoform 1), with addition of a two amino acid linker (GS) between dCas9 and cat3a.

LentiGuide-Puro cloning and transduction. The LentiGuide-Puro plasmid (Addgene #52963) was used for downstream sgRNA integration. sgRNAs were subcloned using Gibson Assembly (see also: ResearchGate DOI: 10.13140/RG.2.1.1941.1461). Lentiviral particles were produced and the rest dKOΔCas9-cat3a line was transduced with either a single sgRNA (Dazl) or a pool of 10 sgRNAs (Dazl, Dazl_2, Foxb1, Foxa2, H19, Fbxo40, Id3, Prdm14, Dppa3, and Pou5f1) for the hybrid capture experiment.

**sgRNA sequences and coordinates.** Mouse

Dazl: 5′-GAGGGTGCGCTGGGCCC-3′ (mm9, chr17: 50432925–50432944)

Dazl_2: 5′-GAGGGGGCAGCTAGTGGA-3′ (mm9, chr17: 50432789–50432808)

Foxb1: 5′-GAGGGGTAGAAGGGGACAIGTC-3′ (mm9, chr17: 69606961–69606980)

Foxa2: 5′-CTTTTGTTGTGCAATGCTTC-3′ (mm9, chr17:14782875–14782873)

H19: 5′-GAGGCCATGTTGACCAAGAG-3′ (mm9, chr7: 14976398–149762717)

Fbxo40: 5′-GTGATCCCTCAGGAGG-3′ (mm9, chr16: 36969271–36969290)

Id3: 5′-GGGGCCCTGAGGAACTTGGTC-3′ (mm9, chr4: 135699940–135699959)

Prdm14: 5′-GAGAGATACTAGCTTGAGG-3′ (mm9, chr1: 13108873–13108890)

Dppa3: 5′-GAGGACAGTCTGAGGAGGTA-3′ (mm9, chr6: 35642849–35642868)

Human

Bach2: 5′-GAATGGACAGATTGAGGTT-3′ (hg19, chr6:19006602–19006621)

**Confirming gRNA targeting efficiency.** To confirm the gRNA integration and targeting efficiency we nucleofected cells with pSpCas9(BB)-2A-GFP (Addgene #49348) and sorted for GFP 3 days post-nucleofection. Individual clones were picked and the targeting locus (Dazl) was amplified by sequencing.

**Hybrid capture and Amplicon-Seq.** The rest dKOΔCas9-cat3a line was transduced with a pool of 10 sgRNAs (see also section on sgRNA cloning, sequences and coordinates). Upon Puromycin selection (2μg/ml), several colonies were picked, individually propagated and then induced for 0, 1 day, 2 days and 3 days with doxycycline (2μg/ml). Genomic DNA was isolated, followed by Amplicon-Seq to confirm the sgRNA insertion in each clone. To do so, a fragment spanning the sgRNA integration site within the LentiGuide plasmid was amplified from the gDNA of each clonal line (FP: 5′-CTTGAGCTAGAAGGAAATTTCTTCG-3′, RP: 5′-GTTATACCTGAGAAGGCGGACG-3′). Amplicons were repaired using the End-It DNA End-Repair Kit (Epicentre), extended using Klenow fragment (N-terminal), as well as a BsiWI fragment was subcloned in the FUW backbone (Addgene #20723). The BsiWI restriction site was used to clone in the catalytic domain of Dnmt3a (cat3a, last 301 amino acids of Dnmt3a Isoform 1), with addition of a two amino acid linker (GS) between dCas9 and cat3a.

**Cell culture.** ES cells were cultured in regular media containing 15% FBS, 1% PEN-STREP, 1% glutamine, 1% NEAA, and 10°/μL U LIF. For ES cell maintenance, dishes were coated with 0.2% gelatin and irradiated DW mouse embryonic fibroblasts (MEFs) were plated as a confluent layer of feeder cells. ES cells were seeded in a density of 50,000 cells per well of a six-well plate and were split every 3–4 days.

Doxycycline and Puromycin were used at a concentration of 2μg/ml. MCF7 cells (ATCC HTB-22) were grown in DMEM F-12 with 10% FBS and 1% Penicillin-Streptomycin. 293Ts (ATCC CRL-3216) were grown in DMEM supplemented with 10% FBS, 1% glutamine and 1% Penicillin-Streptomycin.

**dKOzero cell line generation.** For the generation of our dKOΔCas9,14 we transduced the original DKO line19 with a previously described Dnmt1 shRNA cassette (termed Tko-like cells)20.31. After several passages the TKO-like cells expressing the shRNA was transiently transduced with Cre recombinase (Addgene #24593) to remove the shRNA-GFP construct, followed by sorting for GFP negative cells. Several clones were picked and propagated individually. To further enable any downstream Puromycin selection, the dKOΔCas9,14 line was modified by disrupting its existing Puromycin resistance cassette (sgRNA: 5′-GAAGCTCTCTCT-CAGGGGAGT-3′). Individual clones were picked and dKOΔCas9,14 clone 15.1 was chosen for all further experiments.

**dKOΔCas9-cat3a cell line generation.** For the generation of the inducible DKOΔCas9,14-cat3a line, 20,000 dKOΔCas9-2a3a cells were transduced with FUW-dCas9-cat3a and FUW-M2rtTA (Addgene #20342) and treated with doxycycline to allow GFP based FACs and isolation of individual GFP-positive colonies. Cells were taken off doxycycline for one passage prior to LentiGuide-Puro (Addgene #52963) transduction, (integration of Dazl sgRNA, sequence: 5′-GGAGGTGGTTTCGCGCCC-3′). Upon transduction, cells were selected with Puromycin (one passage afterwards). After which the cas9-cat3a was induced for two passages by genomic DNA extraction and whole-genome bisulfite sequencing (WGBS). To reset the dKOΔCas9,14-cat3a line, cells were passaged in 2i/Vitamin C52 and Dnmt1 was transiently knocked down using the shRNA described above. For the time course experiments, 0, 1 or 7 days were used for DNA extraction and WGBS. Homogenous induction was confirmed by inducing cells for three days with or without doxycycline, followed by FACs analysis.

**dKOΔcat3a, DKOΔDnmt3a and WTΔCas9-cat3a generation.** The full-length Dnmt3a Isoform 1 or its catalytic domain (the last 301 amino acids) were fused to P2A-GFP, followed by subcloning into the FUW backbone (Addgene #20723). The dKOΔDnmt3a cells were transduced with FUW-M2rtTA (Addgene #20342) and FUW–Dnmt3a-GFP or FUW–cat3a–GFP, respectively and treated with doxycycline for about two weeks to allow GFP based FACs and isolation of individual GFP-positive colonies. For the generation of the inducible dCas9-cat3a wild-type line, V6.5 wild-type ES cells were transduced with FUW-M2rtTA and FUW–dCas9–cat3a, selected and induced as described above. Each cell line was induced with doxycycline for two passages (7 days) prior to DNA extraction and bisulfite sequencing. For the WTΔCas9–cat3a line, a non-induced (no doxycycline) sample was also collected for comparison.

**Transfection experiments.** MCF7 and 293T cells were transfected with pcDNA3–DNMT3A–EGFP (Addgene #17666) or pcDNA3–DNMT3A–EGFP–ANV (Addgene #17685) including the Bach2 sgRNA sequence (sequence: 5′-GAGG CTGTTGGCTGCGCCGGCCC-3′) using FUGENE HD Transfection Reagent (Promega). Cells were sorted for GFP 3 days post-transfection if transfection efficiency was below 90% (based on visual inspection). DKOΔCas9 cells were transfected with pcDNA3–DNMT3A–EGFP (Addgene #17666) or pcDNA3–DNMT3A–EGFP–ANV (Addgene #17685) including the Dazl sgRNA (sequence: 5′-GAGG CTGTTGGCTGCGCCGGCCC-3′) using Xfect mouse ES cell Transfection reagent (Clontech). 293T cells were partially replenated 3 days post-transfection for later collection time points at day 7 and day 15. Upon DNA extraction, the DNA was digested with DpnII for 2 h at 37 °C to digest all residual plasmid DNA. This was followed by a 40 x AMPure XP bead (Beckman Coulter) cleanup to select for high molecular/non-digested DNA.

**RNA sequencing.** RNA-seq libraries were prepared using the TrueSeq RNA Sample Prep v2 HS Protocol (Illumina), followed by 50 bp paired-end sequencing on the NextSeq 500 Sequencing System (Illumina).

**Reduced representation bisulfite sequencing.** Genomic DNA was quantified using a Qubit 2.0 Fluorometer, and quality-assessed on an Agilent 2200 TapeStation D1000 ScreenTape. RRBS was performed on 20 ng of each sample using the NuGen Ovation® RRBS Methyl-Seq System following the manufacturer’s recommendations except that barcoded adapter-ligated samples were pooled in groups of 100.
were then aligned to the mm9 build of the mouse genome using BSmap32. DNA bp were clipped from the WGBS raw sequencing reads at either end. The reads sequenced with 100 bp paired-end reads on an Illumina HiSeq 2500 sequencer. 10 then conducted using the EZ DNA Methylation-Gold kit (Zymo Research), eluting according to manufacturer's recommendations unless specified. The libraries were sequenced with 100 bp paired-end reads on an Illumina HiSeq 2500 sequencer. 10 bp were clipped from the WGBS raw sequencing reads at each end. The reads were then aligned to the mm9 build of the mouse genome using BSmap32. DNA methylation calling for individual CpG sites was performed using mcall from the msir package in R (V1.3.1) with span set to 0.4. Violin plots were created using the vioplot toolkit using standard parameters. Boxplots and heatmaps were created using R.

**Data processing and analysis.** For all methylation analysis, only CpGs covered by at least 5 reads were considered (unless otherwise stated). For genomic methylation plots, loess smoothing and standard deviation calculations were derived using the mss package in R (V1.3.1) with span set to 0.4. Violin plots were created using the R package `vioplot` using standard parameters. Boxplots and heatmaps were created using R.

For all genomic feature comparisons, CpGs were intersected using BedTools v2.25.0 with: CpG islands, as previously described or exons, introns, CGI shores, short interspersed nuclear repeats (SINEs), long interspersed nuclear repeats (LINEs), long terminal repeats (LTRs) or Intron-class A-type (IAP) elements downloaded from the UCSC Genome Browser (mm9 or hg19 depending on data).

To detect CpAs.

To compare CGI methylation to H3K4me3 methylation, BedTools was used to detect CpAs.

To compare CGI methylation to H3K4me3 methylation, BedTools was used to detect CpAs.

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Author contributions

C.G. and A.M. designed and conceived the study, C.G. generated the constructs, cell lines and performed the experiments, J.C. performed the data analysis, C.G., J.C., and A.M. interpreted the data, C.G. and A.L.M. did the full-length overexpression, J.D. and D.C. helped with the derivation of the inducible lines, E.K.S., H.G., A.W.M., and A.G. generated the DNA methylation profiling libraries, C.G. and M.J.Z. designed the hybrid capture probe set, S.K. and B.T. performed the sequencing and initial data processing, K. C. provided support with analysis strategy. T.C. and H.R.S. provided support with interpretation of the data. C.G., A.M., and J.C. wrote the manuscript.

Additional information

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