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SHORT REPORT



Site-specific recruitment of epigenetic factors with a modular CRISPR/Cas system

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ABSTRACT

Dissecting the complex network of epigenetic modifications requires tools that combine precise recognition of DNA sequences with the capability to modify epigenetic marks. The CRISPR/Cas system has been proven to be a valuable addition to existing methodologies that fulfill these tasks. So far, sequence-specific editing of epigenetic modifications such as DNA methylation and histone posttranslational modifications relied on direct fusions of enzymatically inactivated Cas9 (dCas9) with epigenetic effectors. Here, we report a novel, modular system that facilitates the recruitment of any GFP-tagged protein to desired genomic loci. By fusing dCas9 to a GFP-binding nanobody (GBP) we demonstrate that prevalent epigenetic modifications at mouse major satellite repeats can be erased or set *de novo* by recruiting GFP-coupled catalytic domains of TET1 and DNMT3A, respectively. Furthermore, we construct an inducible expression system that enables a temporally controlled expression of both GBP-dCas9 and the effector protein. Thus, our approach further expands the CRISPR/Cas toolbox for site-specific manipulation of epigenetic modifications with a modular and easy-to-use system.

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Introduction

Eukaryotic gene expression is controlled by a complex network of epigenetic mechanisms that include the posttranslational modification of histones as well as covalent DNA modifications.¹ Dissection of this network using knockout or overexpression studies have greatly advanced our understanding of how epigenetic modifications contribute to transcriptional regulation. However, using traditional techniques, the complex relationships and feedback circuits that interconnect epigenetic pathways make it difficult to differentiate direct consequences of epigenetic modifications on transcription from secondary effects. Site-specific manipulation of epigenetic marks therefore represents a highly desirable tool to study and understand their direct functional relevance on gene expression and genome organization.


Previously, tools that direct the enzymatic activity of epigenetic effectors to specific loci were based on zinc finger proteins (ZFPs)^{2–6} or transcription activator-like effectors (TALEs).^{6–8} However, custom design and engineering of ZFPs and TALEs is based on the

rearrangement of their modular DNA-binding domains, requiring elaborate cloning techniques and rigorous testing.^{9–12} In contrast, the RNA-guided endonuclease Cas9 of the type II CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR-associated) system recognizes specific loci via Watson-Crick base pairing between a readily exchangeable 20 bp sequence of the single guide RNA (sgRNA) and the target DNA in the direct vicinity of a PAM (protospacer adjacent motif).^{13–15} Due to this ease of use, Cas9-based approaches have been rapidly adopted for genome engineering strategies in a wide variety of cell types and organisms.^{16–20}

Importantly, engineering of a catalytically inactive variant of Cas9 (dCas9) facilitates RNA-guided genome targeting in a sequence specific manner, without cleaving the underlying DNA.^{13,21} We and others have previously demonstrated that this programmable DNA-binding platform can be harnessed for *in vivo* visualization of specific genomic regions^{22–24} as well as for determination of local chromatin composition.^{25–26} In addition, fusion of dCas9 with the methyltransferase DNMT3A enables

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targeted transcriptional repression by catalyzing *de novo* methylation at gene regulatory regions.^{27–28} *Vice versa*, dCas9 mediated targeting of ten-eleven translocation methylcytosine dioxygenase 1 (TET1) to regulatory elements results in upregulation of silenced genes via active DNA demethylation.^{29–31} Similarly, transcriptional modulation has also been reported by targeting histone acetylase³² and histone demethylase activities³³ via dCas9, highlighting the versatility of this approach.

Here, we introduce a modular CRISPR/Cas9 system, which combines the sequence specificity of dCas9 with stringent recruitment of GFP-coupled epigenetic effectors via a GFP-binding nanobody (GBP).³⁴ We show that this versatile setup can be exploited to control the levels of DNA modifications at target loci using GFP fusions of DNA methyltransferases and methylcytosine dioxygenases. Furthermore, using a bidirectional doxycycline-inducible promoter we develop a single vector system that allows the timed expression and targeted recruitment of GBP-Cas9 and GFP-fusion proteins.

Results and discussion

We first set out to assess, whether GFP can be recruited to a defined genomic locus via dCas9. To this end, we constructed a GBP-dCas9-mRFP construct, which enabled us to simultaneously visualize dCas9 and GFP localization (Fig. 1A). Since heterochromatic chromocenters (CCs) are distinct subnuclear regions, which can be readily distinguished and are characterized by well-defined epigenetic marks,^{35–36} we decided to tether GBP-dCas9-mRFP to these loci via a major satellite specific sgRNA (MaSgRNA).

Transient co-transfection of mouse embryonic stem cells (ESCs wt J1) with GBP-dCas9-mRFP and MaSgRNA resulted in a specific enrichment of GBP-dCas9-mRFP at CCs. Importantly, when we additionally transfected a GFP-encoding plasmid, we observed co-localization of GFP with GBP-dCas9-mRFP at CCs (Fig. 1B and C). This experiment confirms the functionality of the GBP-dCas9-mRFP construct in facilitating the recruitment of GFP to target loci.

Next, we aimed to target GFP-tagged epigenetic effector proteins to chromocenters via GBP-dCas9-mRFP. To test the feasibility of such an approach we used the catalytic domains of the methylcytosine dioxygenase TET1 and the *de novo* methyltransferase DNMT3A coupled to GFP (GFP-TET1CD and GFP-

DNMT3ACD, respectively). DNMT3A catalyzes the methylation of cytosine generating 5-methylcytosine (5mC), a repressive epigenetic mark enriched at CCs.³⁶ In contrast, TET1 oxidizes 5mC to 5-hydroxymethylcytosine (5hmC), a DNA modification generally found in euchromatin and depleted at the heterochromatic CCs.³⁷

Similar to GFP alone, GFP-TET1CD was successfully recruited to CCs in cells, which co-expressed GBP-dCas9-mRFP and MaSgRNA. Notably, TET1CD recruitment to the highly methylated CCs in wt ESCs, resulted in an ectopic enrichment of 5hmC at these sites (Fig. 2A). To test the feasibility of GFP-DNMT3ACD recruitment to CCs we used DNMT triple knockout (TKO) cells,³⁸ which are virtually devoid of genomic DNA methylation. In TKO cells, which co-expressed GBP-dCas9-mRFP and MaSgRNA GFP-DNMT3ACD was successfully recruited to CCs, leading to a dramatic increase of 5mC at CCs (Fig. 2B). Taken together, these data show that GBP-dCas9-mRFP is capable of directing the enzymatic activity of epigenetic factors to targeted genomic regions.

Besides targeted recruitment, control over the timing of site-specific epigenetic editing is crucial for the dissection of direct functional consequences resulting from local epigenetic perturbations. To this end, we constructed an inducible system for GBP-dCas9-mRFP and GFP/GFP-effector expression. We used an Epstein-Barr virus (EBV) derived episomal expression plasmid (pRTS), which harbors a doxycycline-inducible bi-directional promoter and additionally encodes a tet-transcriptional (tTR) repressor as well as a tet-transcriptional activator (rtTA).^{39–40} Conditional transcriptional activation is achieved in the presence of doxycycline (Dox) by the rtTA, whereas tTR mediates active repression in Dox-free conditions. This single vector system allows tight control over the timing and expression level of GBP-dCas9-mRFP as well as a GFP-fusion protein (pRTS-GBP-dCas9-mRFP; Fig. 2C). Moreover, using a bidirectional promoter ensures that both proteins are expressed at comparable levels reducing the amount of freely diffusing GFP-fusion protein, thereby minimizing off-target effects. We first tested the inducible system in mouse myoblast (C2C12) using a pRTS-GBP-dCas9-mRFP, additionally harboring either GFP alone or GFP-TET1CD. Both GFP and GFP-TET1CD were expressed and specifically recruited to CCs in a strictly sgRNA- and Dox-dependent manner (Fig. 3A and B;

Figure S1A; Figure S2A and B). Similar to co-transfections performed in wt ESCs, GFP-TET1CD recruitment resulted in a drastic increase of hmC at myoblast CCs (Fig. 3B). Comparably, transfection of pRTS-GBP-dCas9-mRFP harboring GFP-DNMT3ACD into TKO cells led to a specific, Dox-dependent enrichment of 5mC at CCs (Fig. 3C; Figure S1B; Figure S2C).

In summary, we demonstrated that our modular system can be used to edit prevalent epigenetic

marks such as DNA methylation at heterochromatic chromocenters in a timely controlled manner. While we used repetitive target sequences, which allow a fast and simple readout of efficient effector recruitment by microscopy, it is in principle possible to apply our system for the epigenetic modification of single target loci. Using multiple gene/target specific sgRNAs in parallel will also enable the modification of multiple loci at once or the

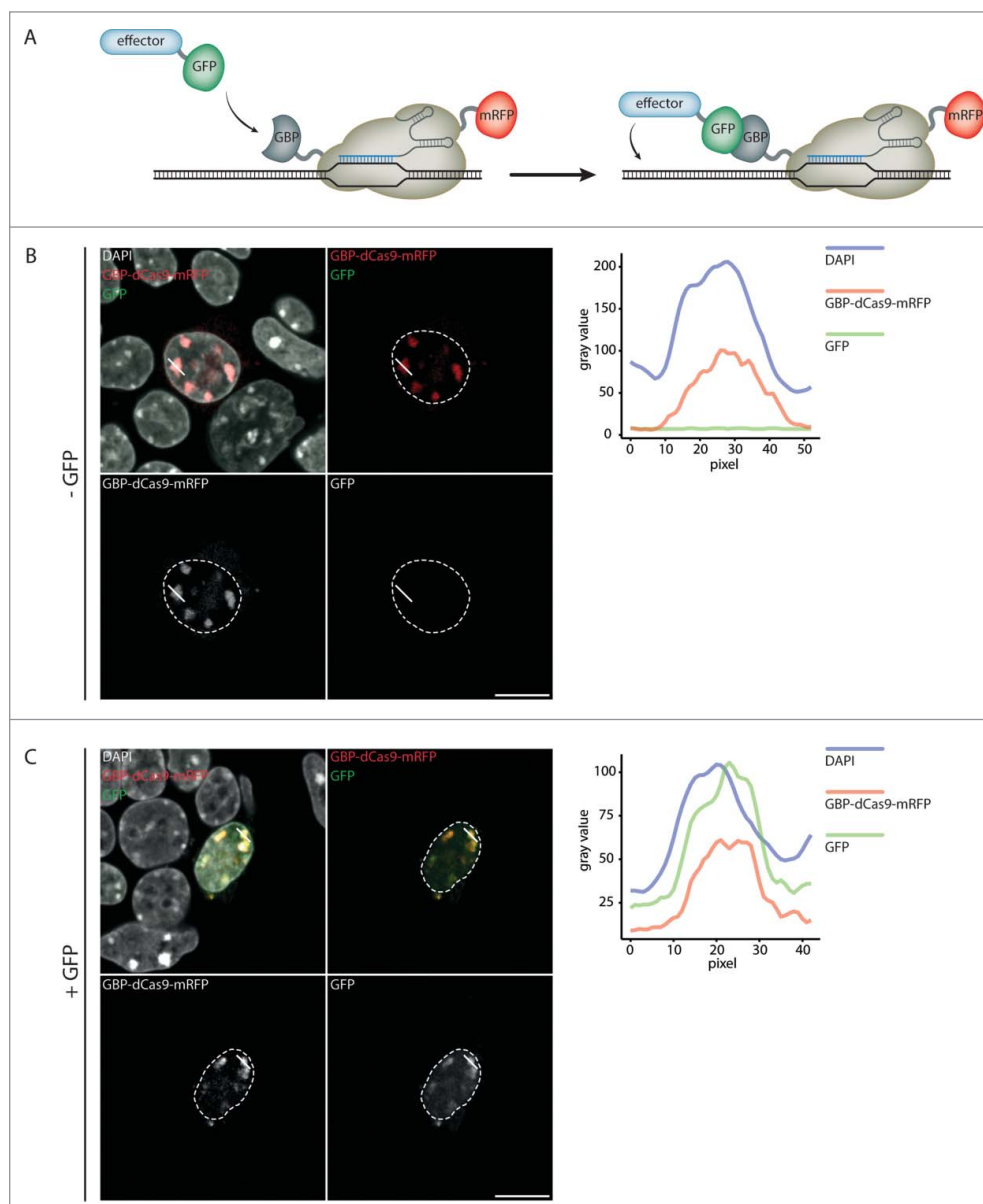


Figure 1. Targeted recruitment of GFP to major satellites. (A) Schematic outline of dCas9-mediated effector recruitment. GBP-dCas9-mRFP is guided to a desired locus by a sgRNA and interacts with a GFP-coupled epigenetic effector via GBP. Subsequently, the effector modifies the underlying DNA. (B-C) Representative confocal images of ESCs, co-transfected with GBP-dCas9-mRFP and major satellite specific sgRNA. GBP-dCas9-mRFP specifically localizes at CCs and recruits GFP, when it is additionally co-transfected (C). Line plots represent the signal intensity of the different channels along the indicated chromocenter (solid white line). White dashed lines indicate the nuclear border. Scale bar: 10 μ m.

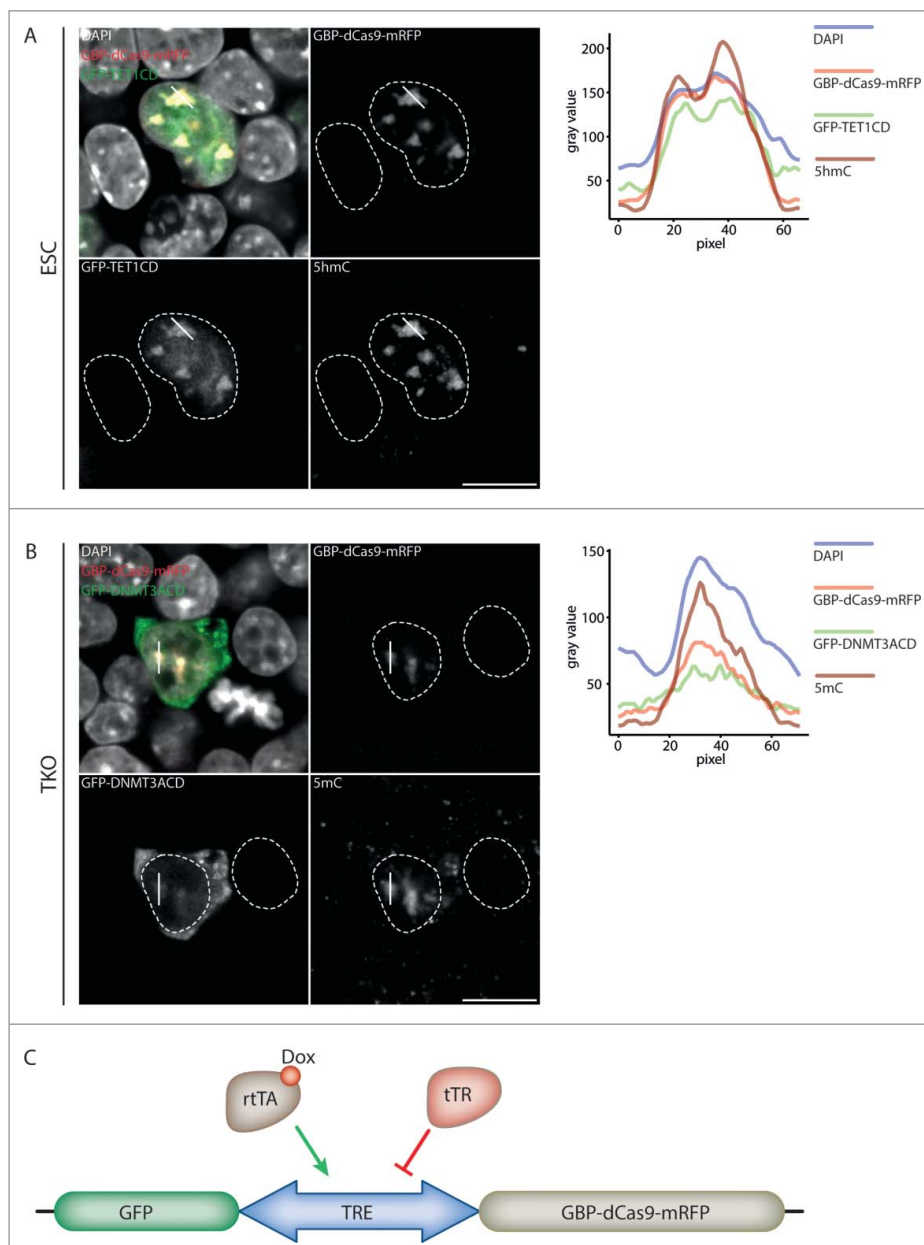


Figure 2. Targeted recruitment of GFP-tagged effector proteins. (A) GBP-dCas9-mRFP recruits GFP-TET1CD to chromocenters. Recruited GFP-TET1CD oxidizes 5mC to 5hmC at CCs in transfected ESCs. In untransfected cells, no 5hmC signal was detected. (B) When targeted to CCs, GFP-DNMT3ACD mediates *de novo* DNA methylation in TKO cells, which was not observed in untransfected control cells. Line plots represent the signal intensity of the different channels along the indicated chromocenter (solid white line). White dashed lines indicate the nuclear border. Scale bar: 10 μ m. (C) Schematic representation of the inducible vector system. A bi-directional promoter drives the expression of GBP-dCas9-mRFP as well as GFP. The vector additionally encodes a transcriptional repressor (tTR) and a transcriptional activator (rtTA). In the absence of doxycycline (Dox), tTR binds to a tetracycline response element (TRE) within the promoter sequence and represses transcription. Upon addition of Dox to the culture medium, rtTA replaces tTR and induces gene transcription.

epigenetic editing of a single copy locus.²² The bi-directional, inducible system offers the added advantage to titrate of the amount of epigenetic modifier and Cas9 protein simultaneously, greatly reducing the possibility of off-target effects. Furthermore, our GBP-based approach for dCas9-assisted targeted

recruitment can be combined with virtually any GFP-tagged protein. Considering the widespread use of GFP-fusions in cell lines and animal models, this system will help to facilitate the systematic dissection of biologic processes in basic and biomedical research.⁴⁵

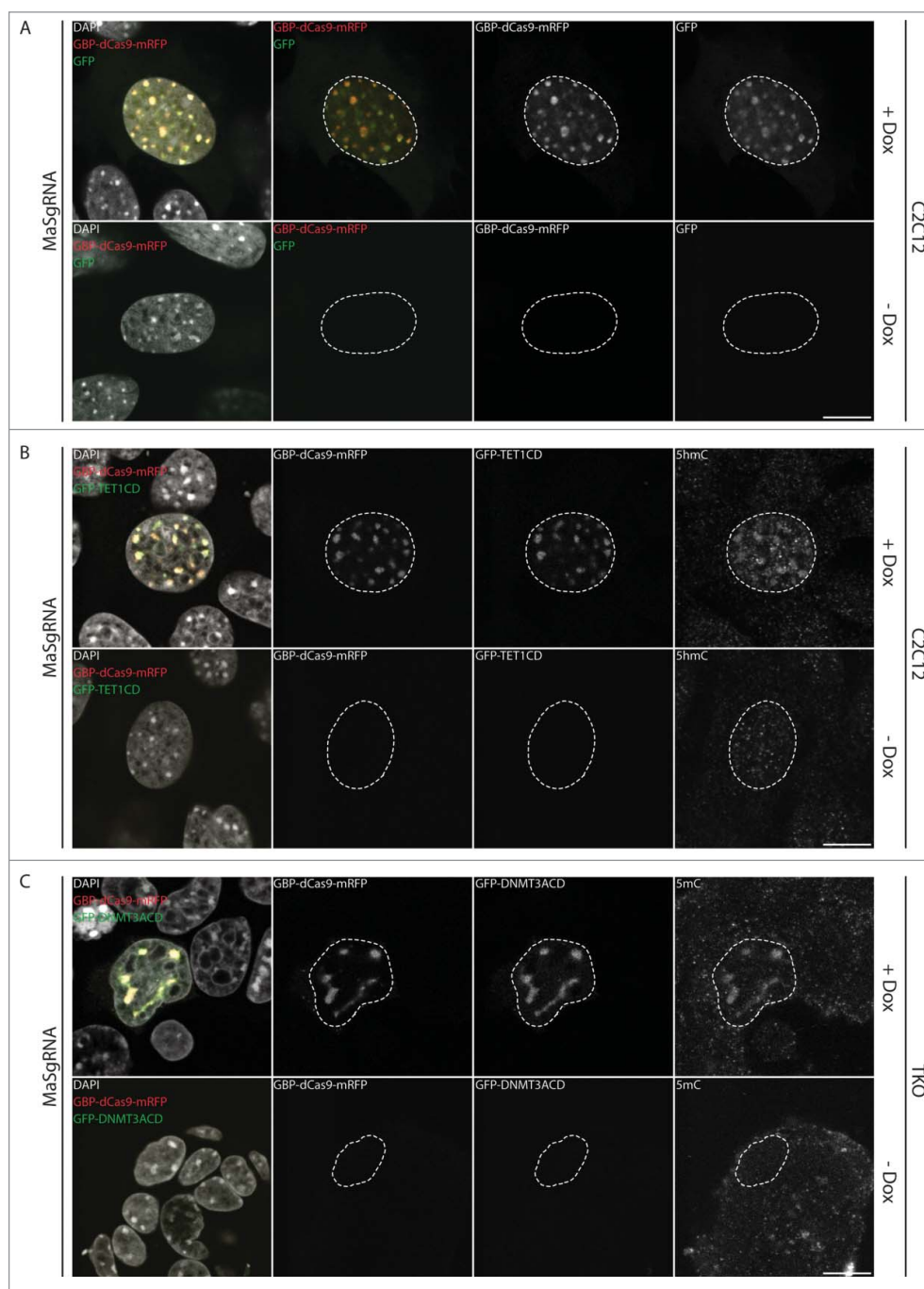


Figure 3. Doxycycline-dependent, coordinated expression of GBP-dCas9-mRFP and GFP-tagged effectors. Cells were co-transfected with MaSgRNA and pRTS-GBP-dCas9-mRFP, additionally encoding GFP (A), GFP-TET1CD (B) or GFP-DNMT3ACD (C). Upon induction with doxycycline both GBP-dCas9-mRFP and the corresponding GFP-tagged effector are expressed, resulting in the oxidation of 5mC (5 hmC; B) or *de novo* methylation of CCs (5 mC; C). White dashed lines indicate the nuclear border. Scale bar: 10 μ m.

Material and methods

Cell culture and transient transfections

J1 ESCs and TKO cells were cultivated at 37°C and 5% CO₂ on gelatin-coated dishes in Dulbecco's modified Eagle's medium (DMEM, Sigma), supplemented with 16% fetal bovine serum (FBS, Biochrom), 0.1 mM

β -mercaptoethanol (Invitrogen), 2 mM L-glutamine, 1x MEM non-essential amino acids, 100 U/ml penicillin, 100 μ g/ml streptomycin (PAA Laboratories GmbH), 1000 U/ml recombinant mouse LIF (Millipore), 1 μ M PD032501 and 3 μ M CHIR99021 (Axon Medchem). C2C12 cells were cultured at 37°C and 5% CO₂ in DMEM, supplemented with 20% FBS, 2 mM

L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin. For conditional transcription activation, the culture medium was additionally supplemented with 1.5 μ g/ml doxycycline for 24 hours. Transient transfections were performed using Lipofectamine® 3000 (Thermo Fisher Scientific) according to the manufacturer's instructions and cells were analyzed 24 – 48 hrs post-transfection.

Plasmid generation

For generating the GBP-dCas9-mRFP expression plasmid, the GBP and mRFP coding sequences were amplified from pGFPbinderImR⁴¹ and cloned into pCAG-dCas9²³ via XbaI and AsiSI/NotI, respectively. The expression constructs for GFP-TET1CD and MaSgRNA were described previously.^{23,42} GFP-DNMT3ACD was constructed by amplifying the catalytic domain of DNMT3A from pCAG-GMT3a.⁴³ Subsequently TET1CD was exchanged by DNMT3ACD using AsiSI and NotI restriction enzymes. The doxycycline-inducible expression system is based on the pRTS plasmid described previously.³⁹ The GBP-dCas9-mRFP coding sequence was cloned into pRTS via SfiI, whereas TET1CD and DNMT3ACD, respectively, were inserted upstream of GFP via SmaI.

Constructs generated for this study are available via Addgene.

Immunofluorescence staining and microscopy

Immunofluorescence staining was performed as described previously.⁴⁴ Briefly, cells were grown on coverslips (thickness 1.5H, 170 μ m \pm 5 μ m; Marienfeld Superior) and transfected with the respective expression plasmids. 24 – 48 hours after transfection, cells were washed with phosphate buffered saline (PBS), fixed with 3.7% formaldehyde for 10 min and permeabilized with 0.5% Triton X-100 in PBS. For 5 mC and 5 hmC detection, DNA was first denatured with 1 N HCl for 15 min and then neutralized with 150 mM TRIS-HCl (pH 8.5). Subsequently, cells were transferred into blocking buffer (0.02% Tween and 2% bovine serum albumin in PBS) for 1 hour. Both primary and secondary antibodies were diluted in blocking buffer and cells were incubated in a dark, humidified chamber for 1 hour at room temperature. Nuclei were counterstained with 0.2 μ g/ml DAPI in PBS or SiR-DNA (Spirochrome). Coverslips were mounted with antifade medium (Vectashield, Vector

Laboratories) and sealed with colorless nail polish. Primary antibodies used in this study were: GFP- and RFP-booster conjugated to Atto 488 and Atto 593, respectively (1:200; Chromotek), anti-5hmC (1:250; Active Motif) and anti-5 mC (1:500, Diagenode). The secondary antibodies were: anti-rabbit IgG conjugated to Alexa 647, anti-mouse IgG conjugated to Alexa 647 (1:400; Thermo Fisher Scientific) and anti-mouse IgG conjugated to Alexa 405 (1:400; Invitrogen).

Confocal images were acquired with a Leica TCS SP5 microscope equipped with a Plan Apo 63x/1.4 NA oil immersion objective. Image processing and assembly of figures was performed with FIJI and Photoshop CS5.1 (Adobe), respectively.

Abbreviations

CC	Chromocenter
DNMT3A	DNA methyltransferase 3A
GBP	GFP-binding protein.
TET1	ten-eleven translocation methylcytosine dioxygenase 1
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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