JACS Au
AN OPEN ACCESS JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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Perspective

Reshaping the Landscape of the Genome: Toolkits for Precise DNA Methylation Manipulation and Beyond

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Cite This: JACS Au 2024, 4, 40−57
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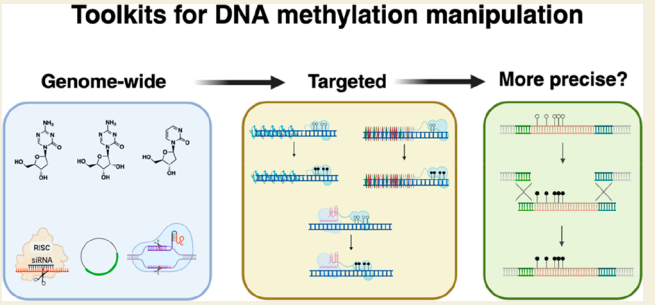
ABSTRACT: DNA methylation plays a pivotal role in various biological processes and is highly related to multiple diseases. The exact functions of DNA methylation are still puzzling due to its uneven distribution, dynamic conversion, and complex interactions with other substances. Current methods such as chemical- and enzyme-based sequencing techniques have enabled us to pinpoint DNA methylation at single-base resolution, which necessitated the manipulation of DNA methylation at comparable resolution to precisely illustrate the correlations and causal relationships between the functions of DNA methylation and its spatiotemporal patterns. Here a perspective on the past, recent process, and future of precise DNA methylation tools is provided. Specifically, genome-wide and site-specific manipulation of DNA methylation methods is discussed, with an emphasis on their principles, limitations, applications, and future developmental directions.



KEYWORDS: *DNA methylation, precise manipulation, CRISPR/Cas-based targeted methylation, homology-directed repair, genetical and pharmacological perturbation*

INTRODUCTION

DNA methylation, as a crucial biological process, modifies DNA bases by adding a methyl group, which predominantly forms 5-methylcytosine (5mC) and is commonly found at the CpG motif in mammalian cells. Methylation of cytosine is catalyzed by DNA methyltransferases (DNMTs) using *S*-adenosylmethionine (SAM) as the methyl donor and can be reverted back to unmodified cytosine.

5mC is an essential epigenetic mark imparted on the genetic code, which can modulate the cell function without altering the DNA sequence. 5mC has been reported to participate in multiple biological processes such as gene silencing,[1] genomic imprinting,[2,3] X-chromosome inactivation, etc. Furthermore, aberrant DNA methylation always exists in various diseases such as cancer,[4−6] fragile X syndrome (FXS),[7] the immunodeficiency, centromeric instability, and facile anomalies (ICF),[8] which have been used as pathogenic biomarkers and studied for therapeutic pathways. Furthermore, DNA methylation landscape is also closely connected with cell fate determination.[9]

Since the discoveries of DNA methylation in the genome,[10] enormous efforts have been unceasingly devoted to precisely interpret the functions of DNA methylation. Thanks to the invention of bisulfite conversion[11] and enzyme-based methods[12−14] together with high-throughput sequencing and nanopore sequencing techniques,[15,16] the genome-wide mapping of 5mC sites in the cellular genome at single-base resolution was successfully achieved. 5mC sequencing uncovered the distributions and dynamic changes of 5mC in the genome and connected them with corresponding phenotypes, which greatly promoted the understanding of the functions of 5mC.[1] However, it should be noted that the sequencing of 5mC can only be a snapshot for the equilibrated state of 5mC population-wide, which could not illustrate the temporal dynamics at a specific genetic locus. Moreover, casual correlations are observed between 5mC and its distribution patterns. Therefore, the speculation of 5mC functions from its sequencing results should be confirmed by manipulating 5mC at comparable resolution.

Unlike 5mC sequencing, the manipulation of 5mC proceeded much more slowly. It takes decades to achieve the regional manipulation of 5mC from genome-wide methylation, which was mainly promoted by the discoveries of programmable DNA-binding proteins.[17−19] Currently, the manipulation of 5mC at a single-base resolution is extremely difficult. Here, in this Perspective, the emerging approaches and tools for controllable DNA methylation editing are reviewed, with an

Received: October 30, 2023
Revised: November 26, 2023
Accepted: December 1, 2023
Published: December 21, 2023

https://doi.org/10.1021/jacsau.3c00671
JACS Au 2024, 4, 40−57

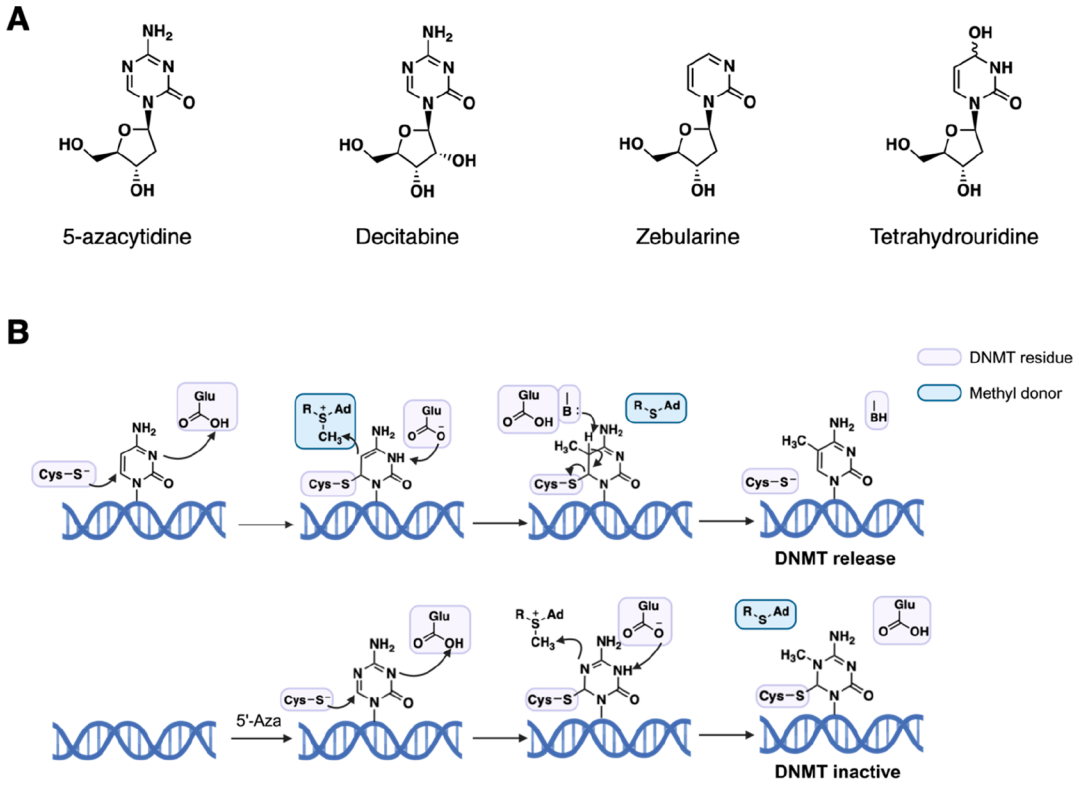


Figure 1. Representative DNMTi and effect mechanism. (A) Chemical structures of cytosine-like DNMTi. (B) Scheme of the mechanism of 5-Aza in inhibiting the activity of DNMT. In a typical reaction of DNMT with normal cytosine, the cysteine residue of DNMT will mediate a nucleophilic attack on the cytosine ring with the facilitation of another glutamic acid residue of DNMT. Then the methyl group of *S*-adenosylmethionine (SAM) (the typical methyl group donor) will be transferred on the C5 of the cytosine ring with the help of the base provided by DNMT. In the methylation process of cytosine, DNMT will be released from the covalent complex and conduct enzymatic reaction on other cytosines. However, with the addition of 5-Aza and incorporation of it into the genome, 5-Aza will irreversibly bind with DNMT, which will inhibit the activity of DNMT and send it to be digested.

emphasis on comparing their applications, effect mechanism, resolution, specificity, and potential limits. After that, future perspectives of precise DNA methylation are discussed.

GENOME-WIDE METHYLATION

Since the discovery of DNA methylation in 1925 in *Mycobacterium tuberculosis*,[10] great efforts have been devoted to uncovering its roles in biological processes. DNA methylation was initially found in bacterial systems as an immune-like response[20] and could be replicated in the presence of methionine.[21] After that, DNA methylation was discovered in various animals, including bony fish, reptiles, and mammals, which confirmed that 5mC is a widespread DNA modification in multiple species.[22,23] Due to the fact that 5mC levels varied among different species, a thorough investigation of its functions is necessary. The technological advances of protein engineering, gene sequencing, and DNA recombination technology together with the discovery of methylation-sensitive restriction enzymes,[24,25] methyltransferase,[26,27] and demethylation-related enzymes[28−30] greatly promoted the in-depth study of DNA methylation. To control genomic 5mC, an intuitive idea is to regulate the activity of 5mC-related enzymes, the process of which could be categorized into pharmacological and genetic perturbations.

PHARMACOLOGICAL PERTURBATION

Modulation of DNA methylation can be achieved by regulating DNMTs activity through small molecules. These compounds can stably bind to the catalytic domain of DNMTs and induce their degradation, leading to demethylation in cells. Given their potential to reverse aberrant hypermethylation characteristic of various cancers, these molecules have demonstrated significant therapeutic promise. This approach, known as pharmacological perturbation, leverages molecular structures categorized into two main types: nucleoside analogs and non-nucleoside analogs. Among such interventions, 5-azacytidine (5-Aza) stands out as one of the earliest and most extensively employed agents.

5-Aza was early applied as an anticancer drug[31] and mutagen.[32] The incorporation of 5-Aza in the cellular genome was observed, and it was later found that high concentrations of 5-Aza caused inhibition of DNA, RNA, and protein synthesis.[33] Furthermore, 5-Aza was reported to significantly influence the differentiation of cultured mouse embryo cells, and Jones et al. described this effect as the result of methylation inhibition. They found that with the addition of 5-Aza or its analogs, the formation of muscle, adipocytes, and chondrocytes from the murine embryo cells was induced, which is concomitant with the inhibition of cytosine methylation in DNA and the reactivation of the *MyoD1* gene.[34] These findings drew the crucial conclusion that DNA methylation plays an important role in gene regulation and cell differentiation, which paved the way for future studies of DNA methylation.

After the discovery of the relationship between 5-Aza and its ability to induce cell differentiation and anticancer functions

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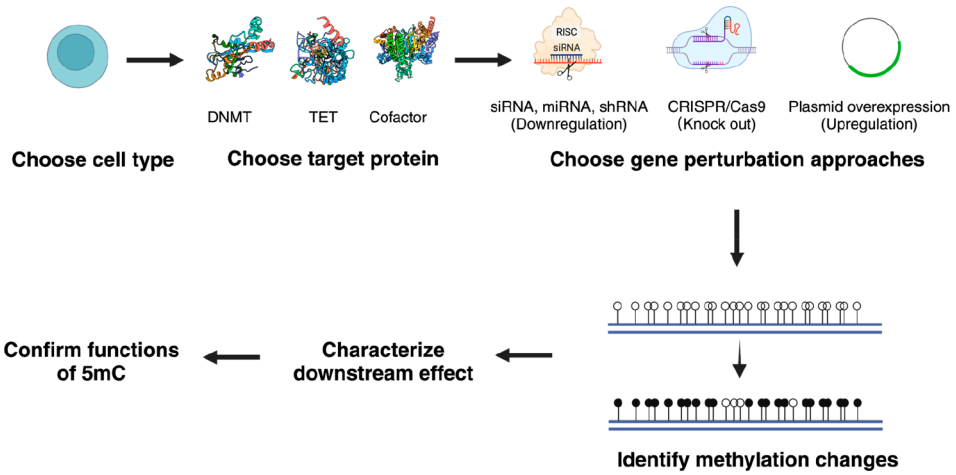

Figure 2. A typical workflow of using genetic perturbation to study the functions of 5mC.

through demethylation, a detailed study was conducted to investigate the mechanisms of demethylation by 5-Aza and the relationship between its demethylation ability and therapeutic effects. With the discoveries of prokaryotic and mammalian DNA methyltransferases, the mechanism of the demethylation activity of 5-Aza was determined. It was reported that the substitution of the 5-H of cytosine like 5-Aza would lead to the irreversibly binding of 5-Aza with DNA methyltransferases (Figure 1B), e.g., DNA methyltransferase 1 (DNMT1), which will hinder the release of the enzyme and inhibit the methylation activity.[35,36] This mechanism was further supported with experiments in mammalian and prokaryotic methyltransferase with oligonucleotides containing 5-Aza.[37−39] After that, small molecules that could inhibit the methyltransferase activity like 5-Aza and 5′-aza-2-deoxycitidine (decitabine) were categorized as DNMT inhibitors (DNMTi).

With the invention of bisulfite sequencing to precisely address the methylation landscape of specific genes, most of the tumor cells were found to contain aberrant hypermethylated CpG islands (CGIs).[40−42] These CGIs are usually located upstream of tumor suppressor genes such as E-cadherin (*CDH1*) and retinoblastoma gene (*RB1*) or DNA damage repair genes such as *hMLH1* and *MGMT*, which act as promoters to control their expression. The hypermethylation of the CGIs will cause a silencing effect and trigger carcinogenesis.[43] At the same time, there were a number of instances in which the addition of 5-Aza could lead to hypomethylation at these gene loci and reactivation of the functions of the tumor suppressor gene, which gave a preliminary illustration of the therapeutic effects of 5-Aza treatment.

The intersection of the demethylation and the antitumor activity of 5-Aza and its analogs has underscored the importance of DNA methylation in carcinogenesis and contributed to the development of novel treatment modalities based on the epigenetic regulation of 5mC. Till now, the Food and Drug Administration (FDA) has approved the usage of 5-Aza and decitabine for the treatment of several forms of myelodysplastic syndromes (MDS), acute myeloid leukemia (AML), and chronic myelomonocytic leukemia (CMML).[44] Even though the usage of 5-Aza and decitabine could induce hypomethylation with high efficiency and reverse the tumor effect, the broad applications of such cytosine analogs are still facing huge challenges. The covalent trapping of DNMT by 5-Aza and decitabine will lead to high cytotoxicity.[45] Furthermore, the addition of these molecules will result in genome-wide demethylation, the severe off-target effect of which remains the possibilities of activating endogenous retroviral elements and causing the expression of oncogenic genes, causing unpredictable side-effects.[46,47] The poor chemical and/or metabolic stability of 5-Aza and decitabine also limits their use.[48,49] Therefore, many different structures of DNMTi with cytosine analogs or non-nucleoside compounds were constructed to address the problems for lower cytotoxicity, higher stability, selectivity to DNMT isoforms, etc.[50−53]

GENETIC PERTURBATIONS

In addition to the use of small molecules to control the activity of DNMTs and enable global loss of methylation, an alternative strategy involves direct manipulation of the endogenous levels of methylation-regulating enzymes. This strategy, known as genetic perturbation, is classified into two categories based on targeting enzymes: DNMTs and demethylation-related enzymes. The modulation of the gene expression levels mainly involves upregulation, downregulation, or the removal of these functional enzymes, which will greatly influence the DNA methylation dynamics in cells. Upon identification of methylation alternations at specific loci or across the entire genome following genetic perturbation, and their correlation with concurrent changes in biological processes, the functions roles of 5mC can be elucidated (Figure 2).

GENETIC PERTURBATION OF METHYLATION-RELATED ENZYMES

After the discovery of the mammalian cytosine-5 methyltransferase DNMT1, the other four DNMTs were identified, which are DNMT2, DNMT3A, DNMT3B, and DNMT3L. Among the five DNMTs, DNMT1, DNMT3A, and DNMT3B are canonical DNMTs, which show catalytic activity on genomic DNA. DNMT3A and DNMT3B are reported to be the de novo methyltransferase, and DNMT1 is served as a maintenance methyltransferase.[54,55] Downregulation, upregulation, or silencing of these three enzymes was frequently used to manipulate the DNA methylation levels in the genome, allowing elucidation of their distinct functions in biological processes. For example, the reduction of DNMT1 levels in

https://doi.org/10.1021/jacsau.3c00671
JACS Au 2024, 4, 40−57

mice will cause substantial genome-wide hypomethylation in all tissues, which were shown to be runted at birth and frequently displayed the development of aggressive T cell lymphomas.[56] This indicated a close relationship between hypomethylation and carcinogenesis. Furthermore, it was reported that the Arg882 mutation of DNMT3A, which will interfere with oligomerization and reduce the activity of DNMT3A, resulted in the aberrant methylation in the gene body and intergenic regions when transduced into murine bone marrow cells.[57] In the decitabine-treated colon cancer cell line HCT116, the genes whose body methylation positively correlated with their expression, exhibited low methylation and gene expression when DNMT3B was knocked out. This shows that DNMT3B is essential for the establishment of gene body methylation and could be the target to manipulate the methylation level at this locus. The RNAi knockdown of DNMT3B in human embryonic stem cells (hESCs) resulted in hypomethylation in the X chromosome and pericentromeric regions, which altered the timing of neuronal differentiation and maturation.[58] In a more comprehensive study, Meissner et al. carefully studied the methylation changes after the inactivation of all three canonical DNMTs in hESCs. Using the whole genome bisulfite sequencing (WGBS) method, they categorized the differentially methylated regions (DMRs) between wild-type cells with knockout cells into four classes based on the specificity of DNMT3A and DNMT3B. They found that redundant targets that require either DNMT3A or DNMT3B were enriched for intergenic regions, introns, CpG island shores, and promoters with intermediate and low CpG density, whereas DNMT3A/3B-specific targets and targets that require both DNMT3A and DNMT3B were mainly enriched for CGIs. This study indicates the selectivity of the targets of DNMT3A and DNMT3B is closely related to the region's CpG density.[59]

Apart from the three canonical DNMTs, the regulation of DNMT3L in mammalian cells is also able to induce methylation changes in cells. DNMT3L is a truncated version of DNMT3 and lacks the catalytic activity of DNMT3A and DNMT3B.[60] However, two DNMT3L molecules could form a tetrameric complex with DNMT3A, which is essential for the efficiency of DNA methylation.[61] It has been reported that DNMT3L deletion in mouse embryonic stem cells (mESCs) will prevent the methylation of imprinted regions without alternation of global genome methylation levels.[62] In another study, Bestor et al. reported that the deletion of DNMT3L will inhibit the de novo methylation of retrotransposons and cause the retrotransposons reactivation and meiotic catastrophe in male germ cells.[63] Another important protein is UHRF1 (ubiquitin-like, containing PHD and RING finger domain 1), which is reported to closely interact with DNMT1 and help the recruitment of DNMT1 to hemimethylated DNA to ensure maintenance fidelity. Deletion of UHRF1 in mESCs resulted in global losses of DNA methylation, including the minor satellite, LINE-1, and intracisternal A particle (IAP) element.[64]

GENETIC PERTURBATION OF DEMETHYLATION-RELATED ENZYMES

In addition to the genetic perturbation of DNMTs, the manipulation of demethylation-related enzymes can also regulate the DNA methylation level. The demethylation process in mammalian cells can be divided into passive and active demethylation. Passive demethylation indicates the replication-dependent dilution of genomic 5mC. Active demethylation is mainly accomplished by TET dioxygenase-mediated oxidation of 5mC to 5-hydroxylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC).[65−67] Following thymine DNA glycosylase (TDG)-mediated excision of 5fC and 5caC and base excision repair (BER) could finally reverse the 5mC into unmodified cytosine.[29] There are three main isoforms of TET in eukaryotic cells, named TET1, TET2, and TET3. Therefore, the genetic perturbation of these three enzymes could interrupt the demethylation process and alter the methylation level. For example, the homozygous TET1 gene-trap mouse ($Tet1^{Gt/Gt}$) primordial germ cells (PGCs) showed increased methylation in several TET1-binding sites like Sycp1, MaeI, and Sycp3, which are related to cell cycle and meiosis-related processes.[68] Another study showed that the knockout of TET1 in male mice exhibited hypermethylation of DMRs of imprinted genes of E13.5 and sperm, which originated from its PGCs.[69] Ren et al. deleted TET2 in mESCs and used TET-assisted bisulfite sequencing to map the 5mC and 5hmC at base resolution. They found that with the deletion of TET2, hypermethylation of 5mC accompanied by loss of 5hmC an enhancer was induced, which delayed the gene induction such as *Slit3* and *Lmo3* during differentiation into neural progenitor cells (NPCs).[70] Xu et al. discovered that the TET3 KO mouse zygotes inhibited the demethylation of paternal *Oct4* and *Nanog* genes and impeded their expression in early embryos.[71] The triple knockout of TET1, TET2, and TET3 in mESCs resulted in a bimodal distribution of DNA methylation levels, with the hypermethylation regions mainly enriched in DNase I-hypersensitive sites (DHSs), enhancer regions, and bivalent promoters, and hypomethylated sites not enriched for any genomic features.[72]

Apart from directly regulating the activity of the three TET enzymes, the regulation of the cofactors and substrates of TETs could be an alternative way to control DNA methylation levels in cells. TET-mediated oxidation of 5mC requires α-ketoglutarate (α-KG) as a substrate, which is dependent on the activity of isocitrate dehydrogenase 1 (IDH1), IDH2, and IDH3.[73] The IDH1/2 was reported to frequently exhibit mutations in AML patients. The mutations of IDH1/2 will lead to the neomorphic enzyme activity and generate the oncometabolite 2-hydroxyglutarate (2HG), which will compete with α-KG and inhibit the activity of TET2.[74] The IDH1/2 mutation was reported to result in aberrant promoter hypermethylation and impair the myeloid differentiation.[75]

It should be noted that apart from the oxidation ability of TETs, they are also capable of influencing the DNA methylation dynamics based on the catalytic-activity-independent ability, which originates from their complex binding patterns. TET1 has been reported to bind with polycomb repressive complex 2 (PRC2),[76] and TET2 could recruit histone deacetylase 2 (HDAC2).[77] Therefore, dissecting the catalytic-activity-dependent and catalytic-activity-independent functions of TETs is essential to more comprehensively manipulate the DNA methylation levels based on their genetic perturbation.

LIMITATIONS OF GENETIC PERTURBATION AND PHARMACOLOGICAL PERTURBATION

The aforementioned methods by genetic or pharmacological perturbation of cellular DNA methylation levels will mainly induce the methylation change at a genome-wide scale. Though some approaches caused only regional effects, the

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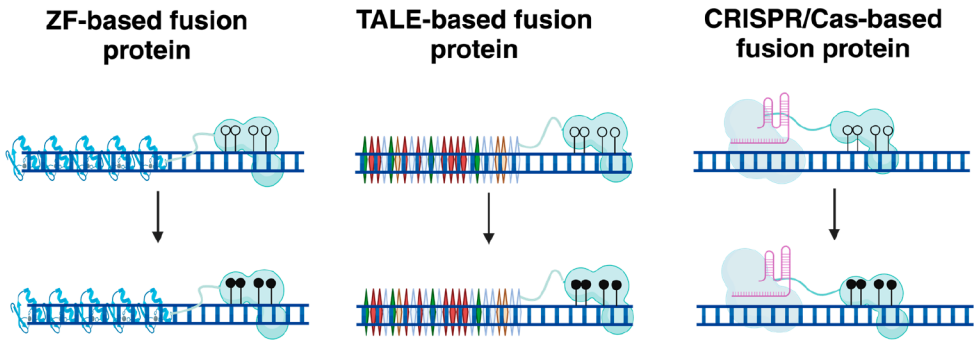

Figure 3. Scheme of the construction of fusion proteins for targeted DNA methylation manipulation. Shown is an example of targeted methylation. In accordance with the types of programmable DNBPs, fusion proteins can be categorized into those based on ZF, TALE, and CRISPR/Cas9 fusion proteins.

consequences of these manipulations were cell-type- and genomic-context-dependent, which were unpredictable. These genome-wide manipulations of DNA methylation failed to precisely interpret the functions of DNA methylation due to the generation of pleiotropic effects.[78] Furthermore, epigenetic therapy based on these approaches would cause cytotoxicity, distinct responses of different cells, and unknown side-effects. Therefore, the development of precise DNA methylation toolkits is highly urged.

TARGETED DNA METHYLATION BY FUSION PROTEINS

Targeted methylation is mainly facilitated by the discoveries of multiple programmable DNA binding proteins (DNBPs).[17−19] Based on the discoveries of DNMTs and TETs to catalyze the methylation and demethylation processes, an intuitive way to enable targeted manipulation of DNA methylation is by fusing DNBPs with DNMTs or TETs. In such a fusion protein system, the addressability of DNBPs could help recruit the complex to desired sites, and the linked DNMTs or TETs could catalyze the methylation or demethylation of nearby genomic DNA to accomplish the targeted DNA methylation or demethylation. Based on different DNBPs, fusion proteins can be classified into three main categories: zinc finger (ZF) proteins, transcription activator-like effectors (TALEs), and CRISPR/Cas proteins (Figure 3).

ZF-BASED TARGETED DNA METHYLATION MANIPULATION

The recognition pattern between DNBPs and DNA sequences could be divided into protein−DNA interactions and RNA-DNA base pairing interactions. Zinc finger (ZF) and transcriptional activator-like effector (TALE) are the two most widely used DNBPs based on protein−DNA interactions. ZF proteins are eukaryotic transcription factors (TFs) that are responsible for DNA sequence identification. With each finger recognizing 3 to 4 bases, tandem linkage of several different zinc fingers could be used to specifically bind to a desired genomic locus.[79,80] Fusing ZF with nucleases like FokI has opened the era of genome editing technology.[81,82] Similarly, fusing ZF with DNMTs or TETs has been reported to facilitate targeted methylation/demethylation in multiple loci in genomic DNA. Previous in vitro studies showed that 5mC could be well-targeted by the fusion of 4-bp recognizing DNMTs such as M.*Hha*I, M.*Hpa*II, and M.SssI with zinc-finger proteins.[83,84] In 2003, Kladde et al. fused 2-bp recognizing CviPI (GC-specificity) and SssI (CG specificity) with ZF proteins Zif268 and Zip53 and delivered the expression plasmid in yeast, which enabled significant and specific methylation enhancement at nearby ZF-binding sites.[85] Using a similar strategy by fusing mitochondrial-specific ZF proteins with human DNMT3A and a nuclear export signal (NES) to prevent the selective localization of ZF proteins in the nucleus, specific methylation of cytosine near T8893G mutation of mitochondrial DNA could be achieved.[86] Apart from these proof-of-concept studies, Jeltsch et al. fused the catalytic domains (CDs) of mouse de novo methyltransferase DNMT3A with engineered Cys_2His_2 ZF B1 and 6F6, which targeted the IE175k promotor. They found that the transfection of plasmid encoding the fusion protein into HEK293T cells resulted in a significant increase of methylation near the ZF-binding site at the IE175k promotor on the reporter plasmid, and concomitant repression of the reporter IE175-luciferase gene.[87] They further showed that with the infection of HSV-1 virus of COS-7 cells, only the transfection of plasmid encoding active DNMTs-ZF fusion led to the methylation of IE175k promoter in viral DNA and repression of HSV-1 propagation. This study is the first example of the use of ZF-DNMTs fusion to enable gene silencing by targeted methylation. In practical use, ZF proteins targeting the promoter of *SOX2* and *Maspin* gene with DNMT3A were used for methylation-directed downregulation of these genes in cancer cells, which is shown to be inheritable and accompanied by phenotypic reprogramming such as cell proliferation.[88] The aforementioned fusion of ZF proteins and DNMTs showed successful targeted DNA methylation and downstream effects, such as gene downregulation. However, the fusion proteins were shown to methylate undesired DNA sequences due to the activity of DNMTs in the absence of ZF proteins and the intrinsic off-target effect of ZF proteins.[89] To reduce such an off-target effect, the monomeric DNMT M.*Hha*I and M.SssI were split into two fragments and separately fused with ZF proteins. By optimizing the orientation of DNMT fragments to each other and to DNA, introducing mutations in DNMTs to reduce the interactions between two fragments, and choosing the proper linker length and spacing between ZF-binding sites and desired effect sites, targeted methylation could be achieved with minimal off-target methylation.[90] However, such a new construct was just tested on the plasmid and verified methylation by methylation-sensitive endonucleases. Its effect in the native genomic context and the regional and off-target effects of the methyltransferase activity should be further studied.

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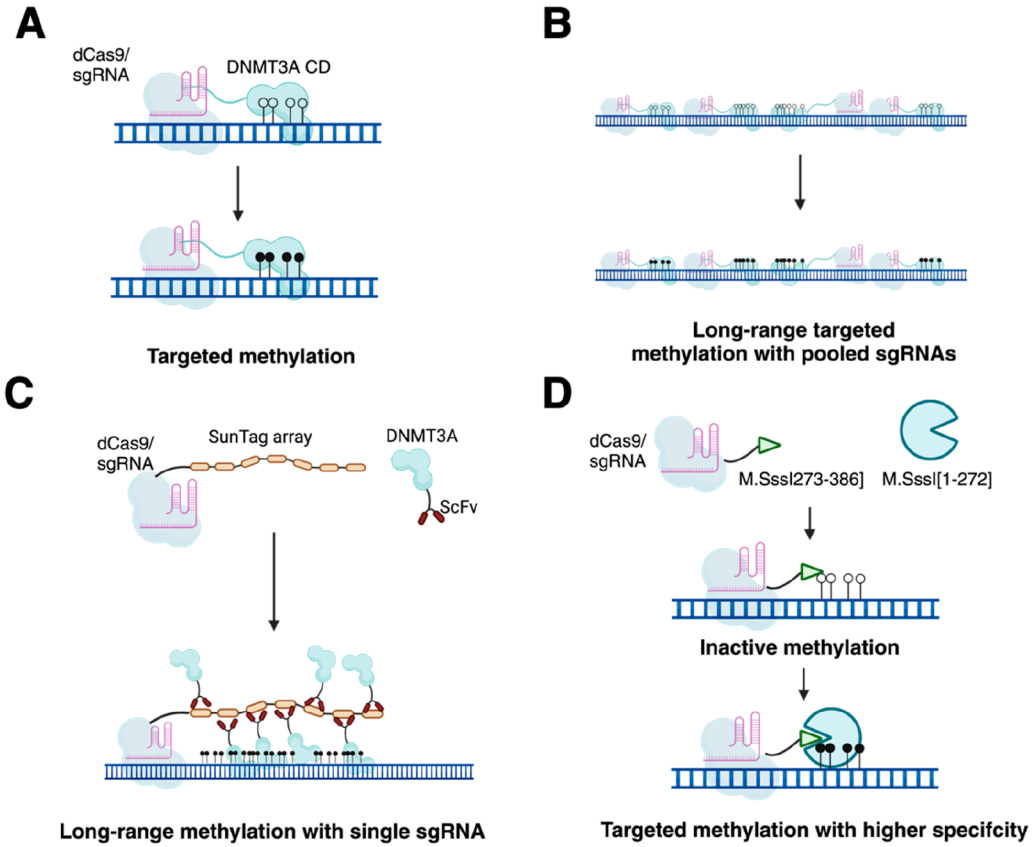

Figure 4. Scheme of dCas9-based fusion proteins for targeted DNA methylation. (A) The common construction of dCas9-based fusion proteins, which involves linking the dCas9 with the catalytic domain of de novo methyltransferase like DNMT3A to enable targeted DNA methylation.[104,106] Reproduced with permission from ref 104. Copyright 2016 Oxford University Press. (B) Scheme of multiplexed sgRNA-targeting for long-range targeted methylation. Pooled sgRNAs are used for multilocus targeting and enabling long-range targeted methylation.[104] Reproduced with permission from ref 104. Copyright 2016 Oxford University Press. (C) Scheme of targeted methylation using the dCas-SunTag system. To enable long-range methylation with only a single sgRNA, dCas9 is fused with a SunTag array, which is an amplifier based on the array of multiple antibody epitopes. DNMT3A is fused with scFv, which can recognize the SunTag unit, and the in situ enrichment of multiple copies of DNMT3A-scFv to broaden the effective region.[108,111] Reproduced with permission from ref 108. Copyright 2017 Springer Nature. (D) Scheme of reduction of off-target effect by split-methyltransferase. dCas9 is fused with C-terminus of methyltransferase M.SssI (M.SssI[273−386]) and only the simultaneous enrichment of dCas9-M.SssI[273−386] and M.SssI[1−272] at targeted sites can reactivate M.SssI, which will theoretically increase the specificity of the system.[110] Reproduced with permission from ref 110. Copyright 2017 Springer Nature.

The integration of ZF proteins with DNMTs represents a significant advancement from genome editing to epigenome editing. These engineered proteins have been tailored to target specific genomic loci, yielding inheritable methylation with different efficacies and inducing gene expression changes, such as the silencing of viral genes and the downregulation of cancer-related genes. Ongoing research aims to expand these techniques and minimize off-target effects.

TALE-BASED TARGETED DNA METHYLATION MANIPULATION

Although great progress has been made in targeted DNA methylation by fusion of ZF proteins with DNMTs, the context-dependent activity of multi-ZF proteins has made the selection of a combination of different fingers labor-intensive.[91,92] An alternative DNBP based on the protein−DNA interaction is TALE, which was first discovered in *Xanthomonas* and could be designed to theoretically target any DNA sequence of interest. The TALEs were composed of multiple repeats, and each repeat could specifically recognize a single nucleotide based on the two amino acids at position 12−13 named the repeat-variable diresidue (RVD). The RVD code was deciphered as the following pattern: NI, HD, NG, and NN specifically recognize A, C, T, and G/A bases.[93,94] Thus, several tandem repeats of TALE units with different RVDs could be constructed to specifically bind with any desired genomic DNA sequence and fused with endonuclease to enable targeted gene editing.[95]

As TALEs are shown to be more modular than ZF proteins due to their simpler binding mode with DNA, they have been used as the second generation of DNBPs to facilitate targeted DNA methylation editing. TALEs were fused with TET1 to target *KLF4*, *RHOXF2*, and *HBB* promoters in K562, HEK293T, and HeLa cells. Each gene was designed for multiple TALEs targeting different regions, and the position-dependent demethylation capacity was characterized by bisulfite sequencing. It was found that TALEs-TET1 fusion exhibited the strongest demethylation effect at CpG dyads within 30 bp of binding sites. The substantial upregulation of these endogenous genes was induced by the demethylation effect, and the demethylated CpGs became remethylated as the TALEs-TET1-encoding plasmid was lost during cell passage.[96] Apart from conducting targeted demethylation using TALEs, the C-termini of DNMT3A and DNMT3L were fused with

https://doi.org/10.1021/jacsau.3c00671
JACS Au 2024, 4, 40−57

TALEs to achieve targeted methylation in human fibroblast cells at the *CDKN2A* promoter, which controls the expression of cell cycle inhibitor p16. By minimizing the direct repeats of TALE DBD and decreasing the number of repeats of TALEs, the TALE-DNMT constructs were successfully recombined into the primary human cells using lentivirus transduction. After transduction, the average 5mC level across the *CDKN2A* CGI was increased by about 10% with a 30−50% increase at several CpG dyads. The downregulation of p16 and the increase in cell proliferation were observed. The same effect was also validated in primary human coronary artery smooth muscle cells.[97] In addition to directly fusing TALEs with DNMTs or TETs, Zhou et al. reported a light-controlled targeted DNA methylation/demethylation system by separately fusing TALEs with CIB1 and DNMT3A-CD or TET1-CD with CRY2. The CRY2 and CIB1 will be dimerized into protein pair cryptochrome-2 under the irradiation of blue light, which will bring the DNMTs/TETs to the TALE-binding sites. Thus, targeted DNA methylation manipulation is blue-light inducible. The TALEs were designed to target a DMR of *Ascl1* promoter in murine dorsal root ganglion and striated cells. The methylation state at the locus was successfully altered and was followed by the change in the level of gene expression.[98]

The discovery of TALEs offers a more modular alternative due to a clear and simple DNA binding mechanism, where each repeat unit can recognize a single nucleotide, offering a high level of specificity. TALEs have been successfully fused with enzymes such as DNMTs or TETs to achieve targeted methylation or demethylation at specific genomic loci, demonstrating their effectiveness in regulating gene expression in various cell types. Moreover, advances in protein engineering have augmented these fusion proteins with additional functionalities such as light sensitivity, granting controlled epigenetic modifications in spatial and temporal manner.

CRISPR/Cas-BASED TARGETED METHYLATION MANIPULATION

For every new target site, ZFs and TALEs will require the design, synthesis, and screening of new protein sequences, which is expensive and labor-intensive. The difficulties were addressed after the discovery of the clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated (Cas) system in *Escherichia coli* in 1987.[18] CRISPR/Cas systems were initially identified as the adaptive immune system existing in various prokaryotic genomes.[99] Later, two classes and five types of CRISPR/Cas systems were discovered, among which the Type II system from *Streptococcus pyrogenes* is the most widely used and studied one.[100] The specific recognition of the DNA sequence by Cas proteins is based on the base complementarity between their associated accessory guide RNA (gRNA) and target DNA sequence, which is different from the binding mode of ZFs and TALEs. In the *S. pyrogenes* system, the associated guide RNA is composed of CRISPR RNA (crRNA) and transactivating CRISPR RNA (tracrRNA), which could be fused into a chimeric single-guide RNA (sgRNA).[101] If the target sequence is recognized, the spCas9 will cleave the target DNA strand to generate a double-stranded break (DSB), which will trigger the repair by cells via nonhomologous end joining (NEHJ) for gene disruption or homology-directed repair (HDR) for precise insertion, deletion, or replacement of gene. Therefore, the CRISPR/Cas9 system has been widely used for gene editing.[102,103] Furthermore, the facile recognition of the target sequence by RNA-DNA interactions allowed CRISPR/Cas9 a potential DBD to enable targeted DNA methylation or demethylation. By disrupting the HNH and RuvC-like nuclease domains, the nuclease-deactivated Cas9 (dCas9) could be produced and used as an addressable protein to recruit DNMTs or TETs for methylation manipulation at targeted sites.[101]

In 2016, Vojta et al. first used the fusion of DNMT3A-CD with Cas9 to target IL6ST and BACH2 promoters in HEK293T cells and showed a peak methylation increase at a 25−35 bp window that was centered 27 bp downstream from the PAM sequence (Figure 4A). The fusion proteins induced the repression of target genes by both DNA methylation and CRISPR interference effect. They also found that simultaneously using a multiplexed sgRNA pool could increase the level of DNA methylation in a large part of the two promoters (Figure 4B). However, the usage of pooled sgRNAs showed lower levels of methylation increase at some CpG dyads compared with single sgRNA, which indicated that the steric hindrance between Cas9 should be considered when using multiple sgRNAs within a small region.[104] A similar strategy was used by the fusion of DNMT3A-CD with dCas9 with a different NLS-FLAG linker to target the CDKN2A CGI in HEK293T cells. The pooled sgRNAs were used to methylate the whole CGI of CDKN2A, which is required to trigger repression of the gene. They further cloned the fusion protein which targets *Cdkn1a* promoter in a doxycycline-inducible lentiviral vector and transduced 32D cells. After 8 days of transduction and doxycycline induction, the methylation level of the whole *Cdkn1a* promoter was increased by over 25% with the reduction of expression by ∼40%.[105] Liu et al. used Cas9-DNMT3A fusion to methylate the *Gapdh-Snrpn*-GFP reporter in mESCs, and found methylation at the *Snrpn* promoter with little methylation spreading to *Gapdh* and inactivation of the GFP reporter. They also used the fusion protein to induce the targeted methylation at two CTCF anchor sites, which blocked the looping functions of CTCF, removed the insulation of harboring superenhancer, and increased gene expression in the adjacent loop.[106] Stepper et al. adopted the chimeric dCas9-DNMT3A-DNMT3L to increase the efficacy of targeted methylation. They found that the fusion of DNMT3A-3L outperformed DNMT3A with 3.8× (EpCAM promoter in SKOV-3 cells), 4.9× (CXCR promoter in HEK293 cells), and 4.6× (TFRC promoter in HEK293 cells) high methylation efficiency. However, such fusion proteins caused significant methylation spreading even to as far as 1 kb away, which is attributed to the multimerization of DNMT3A/DNMT3L complexes.[107] To enable the targeted methylation in a large area, dCas9 protein was fused with repetitive peptide epitopes (SunTag) to recruit single-chain variable fragment (scFv)-fused DNMT3A. Using only one sgRNA (Figure 4C), the large area of *HOXA5* (>4.5 kb) was methylated with high efficiency, and the expression was repressed. The influence on the global methylome and transcriptome was minimal, which was confirmed by reduced representation bisulfite sequencing (RRBS) and RNA-seq.[108]

To prevent the multimerization effect of DNMT3A and endogenous DNMT3L, prokaryotic methyltransferase MQ1 was fused with dCas9 to enable targeted DNA methylation. Due to the high activity of the native MQ1 enzyme, the methylation could not be controlled and extended to nearby regions. Therefore, the nonspecific activity of MQ1 was reduced by introducing mutation of Q147 amino acid into lysine to produce $MQ1^{Q147L}$, which was shown to exhibit

https://doi.org/10.1021/jacsau.3c00671
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Table 1. Summary of Targeted Methylation Based on Fusion Proteins

construct	delivery	target site	cell line	efficiency[a]	effective range	off target effect	effect	ref
ZF-97-DNMT3ACD	Lentiviral transduction	*Maspin*	SUM159	10%-60%	~500 bp	-	Downregulation of Maspin	144
TALE-DNMT3ACD 3L	Lentiviral transduction	*CDKN2A*	Primary human fibroblasts	~10%	~600 bp	At nearby sites (BS-PCR sequencing)	Repression of *p*16	145
dCas9-DNMT3A(4 gRNAs)	Plasmid transfection	*GRN* promoter	HEK293T	~15%	~200 bp	-	*GRN* repression	146
			Hep3B	40%-50% upregulation	~200 bp	-		
dCas9-SunTag + scFv-DNMT3A	Plasmid transfection	*UNC5C* promoter	HeLa	~13%	>500 bp	Little (ChIP-seq, BS-seq of off-target site and TSC-bs-seq)	Reduce CTCF and NRF binding	111
		CCDC85C promoter		~47%				
		SHB intron		44%				
dCas9-BFP-DNMT3A	Plasmid transfection	*u*PA promoter	HEK293T	~10%-65%	~70 bp	Substantial (WGBS)	Repression of *u*PA and TGFBR3	147
		TGFBR3 promoter		~10%-80%	~100 bp			
dCas9-BFP-DNMT3B		*u*PA promoter		Little	-			
		TGFBR3 promoter		~15%-60%	~100 bp			
dCas9-DNMT3A	Lentiviral transduction	*SNCA* intron	hiPSC-derived neurons from a PD patient	~20%-70%	~400 bp	Minimal (immunoassay of whole 5mC ratio)	Reduction of SNCA expression	148
dCas9-DNMT3ACD	Plasmid transfection	*BACH2* promoter	HEK293T	~30%	~100 bp	-	Reduction of BACH2 and IL6ST expression	104
		IL6ST promoter		~40%	~100 bp			
dCas9-DNMT3ACD 3L	Plasmid transfection	*EpCAM* promoter	SKOV-3	~30%	~800 bp	Mild (methylation changes of predicted off-target sites)	Reduction of EpCAM, TFRC and CXCR4 expression	107
		TFRC promoter	HEK293	~25%				
		CXCR4 promoter		~30%				
dCas9-SunTag + scFv-DNMT3A1 (long version of DNMT3A)	Lentiviral transduction	First exon of *HOXA5*	HEK293T	~80%	>4 kb	Minimal (RRBS and RNA-seq)	Reduction of HXOA5 expression	108
dCas9-MQ1^{Q147L}	Plasmid transfection	Near TSS of *HOXA5*	HEK293T	~10%-60%	~30 bp-50 bp	Undetected (RRBS and BS-PCR of predicted off-target sites)	Reduction of HXOA5 expression	109
		RUNX1	K562	~10%-65%	~200 bp		Decrease CTCF binding	

[a]Average or the range of upregulation of the 5mC ratio.

https://doi.org/10.1021/jacsau.3c00671
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Table 2. Summary of Targeted DNA Demethylation Based on Fusion Proteins

construct	delivery	target site	cell line	efficiency[a]	effective range	off target effect	effect	ref
TALE-TET1CD	Plasmid transfection	*KLF4* intron	K562	~10%-30%	~30 bp	-	-	149
TALE-DNMT3ACD 3L		*RHOXF2* promoter	Primary human fibroblasts	~10%-20%	~150 bp		Increase of RHOXF2 expression	
ZF-TET1		*KLF4* intron	HEK293T	~10%-60%	~200 bp		-	
dCas9-TET1CD	Retroviral/lentiviral transduction	*FOXP3* CNS2	Mouse primary T cells	~10%-90%	~400 bp	-	-	150
dCas9-TET1CD (sgRNA2.0[b]) + MS2-TET1CD	Plasmid transfection	*RANKL*	HEK293FT	~15%	~500 bp	Undetected (with top 10 potential off-target gene loci)	Upregulation of the transcription of *RANKL* mRNA	113
dCas9-TET1CD	Plasmid transfection	*BRCA1* promoter	HeLa	~15%	-	Undetected in LINE-1 sequence	Upregulation of transcription of BRCA1	112
dCas9-SunTag + scFv-GFP-TET1CD	Plasmid transfection	STAT3-binding site of *GFAP*DMR of *H19*	ESCs	~80%	~200 bp	Minimal (BS-seq of off-target sites and RNA-seq)	Upregulation of GFAP expression	114
				~15%-90%	~1 kb		Upregulation of H19 expression	
dCas9-TET1	Lentiviral transduction	5′ UTR of *FMR1*	FX52 iPSCs	60%-100%	>500bp	Minor (anti Cas9 ChIP-BS-seq)	Increase of *FMR1* expression	151
dCas9-TET1CD	Lentiviral transduction	Snrpn	mESCs	~10%-80%	-	Minimal (dCas9 ChIP and bisulfite sequencing analysis)	Upregulation of downstream gene (GFP)	106

[a]Average or the range of downregulation of methylation. [b]sgRNA2.0: two MS RNA elements inserted in the canonical sgRNA.

https://doi.org/10.1021/jacsau.3c00671
JACS Au 2024, 4, 40−57

locus-specific activity when fused with dCas9. They used dCas9-MQ1^{Q147L} to methylate *HOXA4*, *HOXA5*, and *EYA4* and found that the specific increase of methylation was achieved with high efficiency and a width of 30−50 bp, which is more precise than the dCas9-DNMT3A CD counterpart.[109] In another study, Xiong et al. used the split-methyltransferase as well as the mutations of the methyltransferase strategy to lower the off-target effect. They separately transfected M.SssI[1−272]- and dCas9-M.SssI[273−286]-encoding plasmids into cells and achieved targeted methylation with high precision and efficiency (Figure 4D).[110]

In addition to targeted methylation based on dCas9, targeted demethylation could also be accomplished using dCas9-TETs as fusion proteins. The plasmid transfection and lentiviral transduction methods of delivering dCas9-TET1 were reported to enable targeted DNA methylation and corresponding gene activation.[106,112] Apart from directly tethering TETs with dCas9, Xu et al. reported the usage of bacteriophage MS2 RNA-inserted modified sgRNA (sgRNA2.0) with MS2 coat protein-TET1CD. When the sgRNA2.0 and dCas9 target to a specific region, the MS2 RNA in sgRNA2.0 could recruit TET1CD to enable targeted DNA demethylation.[113] Similarly, the SunTag system was also applied in the dCas9-TET system by introducing dCas9-SunTag to enrich scFv-TET1CD in targeted genomic regions for site-specific DNA demethylation. This tool showed higher efficiency compared with the TALE and ZF system.[114]

Current DNA methylation editing relies on constitutive expression of the programmable methylation/demethylation effectors to maintain the inheritance of DNA methylation for transcription regulation in many target genes. Those recombinant effectors are limited to generating stable and heritable alterations in effective gene regulation. Recent work demonstrated that the fusion of endogenous retroviruses (REV) silencing machinery such as the Krupprl-associated box (KRAB) and epigenetic factors DNMT3A, DNMT3L to three different DNBPs (ZNF, TALE, dCas9) resulted in efficient and long-lasting gene silencing upon simultaneous delivery.[115] Combined transient expression of these effectors induced long-lasting, significant, and highly specific gene silencing, in which KRAB exerted a strong repressive effect and DNMTs permitted inheritable and stable repression by 5mC hypermethylation.

The invention of dCas9 offers an excellent platform for targeted DNA methylation and demethylation. However, several challenges still remain. First, the steric hindrance between dCas9 and the protection of the sgRNA-binding sequence from methylation will reduce the versatility of such tools.[104] Second, the binding of dCas9 at the targeted region could compete with other endogenous proteins such as TFs, which will confound the interpretation of the causal relationship between DNA methylation and the change of gene expression. This interference activity has been widely used in the field of CRISPR interference (CRISPRi) for site-specific reduction of transcriptional activity.[116] It has also been reported that a single dCas9-binding activity could be used for nonenzymatic targeted DNA demethylation, which further indicated the blocking effect of dCas9.[117,118] Third, the base complementarity between sgRNA and targeted DNA is not strict, and several papers have reported that the mismatched pairing between sgRNA and the genomic region could also be cleaved by Cas9.[119−121] And the whole genome dCas9 chromatin immunoprecipitation and high-throughput sequencing (ChIP-seq) with different sgRNAs confirmed the ubiquitous binding mode in the genomic DNA.[122] This nonspecific binding of dCas9 brings the concern of off-target DNA methylation/demethylation when applied *in vivo*.

In summary, fusion proteins based on CRIPSR/Cas systems, especially the Type II system from *S. pyrogenes*, offer a more straightforward approach and greatly simplify the targeting process. The repurposed dCas9 variant for targeted DNA methylation/demethylation has resulted in significant advances in methylation manipulation at various gene loci alongside gene expression regulation. The transition from protein-based to sgRNA-based addressability has broadened the possibilities for engineering more efficient and specific epigenetic modifications, as exemplified by the incorporation of the SunTag system. To achieve persistent and stable gene expression control, additional effectors like the KRAB repression domain can be integrated to reinforce the regulatory impact and achieve persistent and stable gene expression control. However, challenges such as potential off-target effects, competition with endogenous proteins, and the versatility of tools remain areas for future research and optimization.

REMARKS ON TARGETED DNA METHYLATION MANIPULATION BY FUSION PROTEINS

The method of fusion proteins offers a fabulous way to tune the DNA methylation level in a locus-specific manner, which facilitates a deeper understanding of the functions of DNA methylation and epigenetic therapeutics based on DNA methylation manipulation. In the process of facilitating site-specific methylation editing, these fusion proteins used different building blocks to enable targeted DNA methylation/demethylation in various cell types and genomic contexts with different effective ranges and efficiency (Tables 1 and 2). At the same time, these toolkits also introduce certain inherent limitations that cannot be overlooked. First, when employing different fusion proteins to target distinct loci within the genome, the efficiency and effective range exhibited significant variability. This could be attributed to differences in protein sizes, linker lengths, histone modifications, 3D genome structures, and endogenous bound proteins within various genomic contexts. The unpredictive downstream effect made fusion proteins difficult to use as a versatile approach to study DNA methylation across the genome. Second, the DNMTs and TETs have been reported to interact with multiple endogenous proteins such as HDAC1 and OGT1, respectively.[123,124] The simultaneous actions with other proteins, which are independent of DNA methylation, can complicate the interpretation of the true functions of 5mC.

In addition to the above, TET enzymes are able to convert 5mC into 5hmC, 5fC, and 5caC. Notably, various studies have reported the high enrichment of 5hmC in specific cell types like mouse cerebellar Purkinje neurons, where it constitutes approximately 40% of the 5mC content.[125] Using isotope-labeled methyl donor SAM to study the turnover of oxidized 5mC has indicated the stability of 5hmC.[126] Overall, 5hmC, as a stable epigenetic marker, exhibited functions distinct from those of 5mC within the cellular genome. Furthermore, research suggests that the genetic[127−129] or pharmacological perturbation[130,131] of TET enzymes could lead to significant alternations of 5hmC levels across different genomic loci, resulting in subsequent biological effects independent of 5mC.

https://doi.org/10.1021/jacsau.3c00671
JACS Au 2024, 4, 40−57

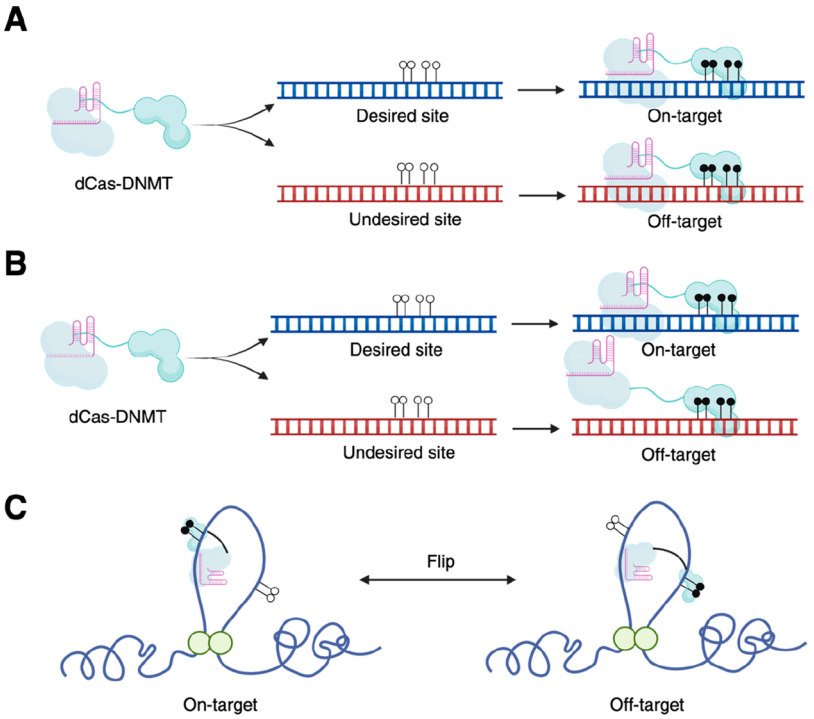

Figure 5. Scheme of the generation of off-target effects of the fusion protein. Shown is the taking targeted methylation by dCas9-based fusion proteins as an example. (A) Scheme of DNBP-based off-target effect. In the dCas9 system, the mismatch between sgRNA and target DNA could be tolerated, which will recruit dCas9-DNMT fusion protein to undesired sites, which will result in the off-target effect. (B) Scheme of effector protein-based off-target effect. The effector proteins such as DNMTs could exhibit enzymatic activity at undesired sites which generates off-target efficiency. (C) Scheme of off-target effect generated from genome high-order structures. 3D genome structures could bring distantly located genomic regions into close proximity, and the flexibility of the linker will enable the flipping of effector proteins between on-target sites and off-target sites, which generates the off-target effect.

Given these findings, it is crucial to delineate the subsequent methylation status resulting from 5mC demethylation or specific formation of 5hmC in the context of methylation manipulation, particularly when employing TET-mediated demethylation strategies. For instance, when employing dCas9-TET fusion proteins for targeted demethylation, it is essential to utilize advanced sequencing techniques such as TET-assisted bisulfite sequencing (TAB-seq)[132] and oxidative bisulfite sequencing (oxBS-seq)[133] to map of 5mC loss and 5hmC generation concurrently. This comprehensive strategy will provide a deeper understanding of the epigenetic landscape and facilitate a more nuanced understanding of the genuine regulatory mechanism at play.

Compared with ZF and TALEs, Cas9 is easier to design and more cost-effective because it needs only the synthesis of sgRNA with different crRNA sequences to enable specificity to different targets. For methylation of large areas, Cas9 can be multiplexed to simultaneously target multiple genes or regulatory elements, offering a scalable approach for targeted methylation on a large genomic scale. Furthermore, for targeted demethylation, Cas9 exhibited less sensitivity to 5mC than TALEs, which enabled stronger binding at targeted sites and higher demethylation efficiency.[120,134] Notably, in some cases, ZFs and TALEs showed higher specificity than Cas9, and the sizes of ZFs (40 kDa for 4 fingers) and TALEs (105 kDa for 17.5 repeats) are smaller than Cas9 (160 kDa), which facilitate their delivery.[135]

It should be emphasized that many of the ZF-, TALE-, and dCas9-based fusion proteins showed detectable off-target binding activities in the genome, the reasons for which were multifaceted. First, the off-target binding of DNBPs could recruit effector proteins to undesired sites and result in off-target effects (Figure 5A). Previous studies have underscored the detectable off-target cleavage activities of ZFN, TALEN, and Cas9.[119,120,122,136−140] However, the off-target effect was shown to be marginal or even undetectable when fused with effector proteins.[106,108,109] This could be ascribed to the unstable binding of DNBPs at undesired sites, which makes it hard for the effector proteins to perform their enzymatic activities. Second, the bound DNMTs and TETs could change the methylation level without the binding of their linked DNBPs (Figure 5B). A study confirmed this off-target effect by tracking the methylation footprints of dCas9-DNMT3ACD in DNMT3A/3B-knockout mESCs with transiently repressed DNMT1. They found the appearance of global off-target activity regardless of the presence of sgRNA.[141] Third, 3D genome structures, such as topologically associated domains (TADs) and enhancer-promotor interactions, may bring distantly located genomic regions into proximity, potentially enabling the on-target dCas9 to methylate/demethylate an adjacent region[142,143] (Figure 5C).

In summary, when opting for fusion proteins as a strategy for targeted methylation regulation, a comprehensive assessment and validation of their efficiency, effective region, off-target effects, and impact on endogenous proteins are essential considerations. Optimization of the system can be achieved through the implementation of design strategies such as altering linker lengths, incorporating the SunTag system, introducing split-effector proteins, etc.

https://doi.org/10.1021/jacsau.3c00671
JACS Au 2024, 4, 40−57

SITE-SPECIFIC METHYLATION USING EXOGENOUS DNA

Apart from fusion proteins, the introduction of exogenous DNA combined with the gene editing toolbox is also capable of achieving site-specific DNA methylation. These inserted exogenous DNA could disrupt the methylation-blocking machines or copy the 5mC in the parental DNA donor into genomic DNA.

INTEGRATION OF CpG-FREE DNA

It has been reported that the hypomethylated state of CGIs in normal cells was mainly attributed to protection by Cys-X-X-Cys (CXXC) ZF protein or CXXC-containing TET1. Furthermore, CXXC ZF protein (CFP1) will recruit SET1 complex to CGIs, which catalyzes the H3K4me3 and inhibits the methylation by preventing the recruitment of de novo methyltransferase.[152−154] Owing to the localization to CpG clusters by the CXXC domain of these proteins, the integration of CpG-free DNA into hypomethylated CGIs could potentially disrupt the methylation-blocking machinery and trigger de novo methylation near the inserted sites. Takahashi et al. reported that the insertion of CpG-free DNA into the CGI of the DNA mismatch repair gene MLH1 induced the allele-specific de novo methylation spanning the entire MLH1 CGI in hESCs. After the removal of the CpG-free cassette, the nascent hypermethylation state was unchanged after more than 20 passages. The de novo methylation triggered by CpG-free DNA integration was also observed in other CGIs such as HSP90AB1 and AARS2. It should be noted that no de novo methylation at MLH1 CGI was observed when integration of the same CpG-free DNA in other cell line such as human fibroblast cells (HFCs), mesenchymal stem cells (MSCs), and HeLa cells; such discrepancy was likely attributed to the lower level of DNMT3B in these cell lines. Furthermore, the genesis of de novo methylation is based on both the interruption of the methylation-blocking machinery and the suppression of the transcriptional activity. Therefore, the cell type and target regions should be carefully considered when using this method.

INTEGRATION OF EXOGENOUSLY METHYLATED DNA

Different from interrupting the methylation-blocking machinery, Cali et al. integrated the in vitro methylated exogenous ssDNA/dsDNA repair template into the cellular genome via a homology-directed repair (HDR) pathway. This strategy is named Homology Assisted-Repair Dependent Epigenetic eNgineering (HARDEN).[155] They used HARDEN to methylate the C9orf72 promoter in HEK293T cells and observed enhanced methylation levels. They found that the dsDNA template could increase the methylation level compared with its ssDNA counterpart, and longer dsDNA templates also improved the methylation efficiency and stability. The genome-wide methylation of HARDEN and dCas9-DNMT3A was further studied. Higher methylation efficiency and specificity of HARDEN compared with dCas9-DNMT3A were confirmed by genome-wide methylation analysis. Another locus APP was further targeted for methylation using HARDEN, and the enrichment of methylation for DNA with the methylated repair template was observed, which illustrated the generalizability of HARDEN.

CONCLUSIONS AND FUTURE PERSPECTIVES

To accurately elucidate the functions of 5mC, the programmed reading, writing, and erasing of 5mC at specific loci or even individual cytosine sites are required. Over the past few decades, the sequencing of 5mC has made significant advancement, processing from quantitative measurements to genome-wide single-base resolution at the single-cell level.[156] However, the bottom-up manipulation of DNA methylation has been less developed. Here we reviewed the toolkits designed for the manipulation of DNA methylation. These manipulation strategies primarily revolve around the control of 5mC-related proteins or their targeted recruitment to specific sites. Regulating 5mC-related proteins by pharmacological or genetic perturbation results in genome-wide regulation with inevitable pleiotropic effects. Overall, these methods have been instrumental in providing initial insights into the roles of 5mC in biological processes and in identifying potential targets for further methylation manipulation studies.

Following the direct modulation of protein levels, the invention of programmable DNBPs facilitated targeted DNA methylation manipulation and exhibits great promise for targeted DNA methylation. Currently, various fusion proteins based on the ZF, TALE, and CRISPR/Cas system have been utilized to enable targeted methylation/demethylation at different genomic loci and in different cell lines. However, many limitations exist and need to be addressed. First and foremost, these fusion proteins usually result in regionally targeted editing in uncontrollable effective scales and an unpredicted editing efficiency of individual cytosine affected by local chromatin structure. Though the engineering of effector proteins,[107,109] linkers,[157] and changing different expression approaches[108,111,147] are available ways for optimization of these parameters, the final results are highly dependent on the cell type and local chromatin landscape of targeted regions. Second, the off-target effect remains to be considered. Many studies claimed minimal or even undetectable off-target sites. However, due to the high methylation level of the CpG motif in the genome, the upregulation of methylation at undesired loci could be underestimated.[141] A large proportion of the off-target sites were RNA-guided, which could be mitigated by using Cas proteins with high-fidelity,[158−160] engineering the sgRNA structures,[161,162] modifications of sgRNA,[163−165] etc. It should be noted that engineering Cas proteins and sgRNAs could tolerate the decrease of the cleavage activity of the ribonucleoprotein, which may provide broader choices for methylation manipulation. Off-target effects originating from other factors such as the background activities of effector proteins or higher-order genome structures should be mainly addressed by mitigating the nonspecific activities by mutation and carefully screening the sgRNA-binding sites, respectively. Third, the large sizes of fusion proteins especially for dCas9-based platforms make them hard to be delivered into patient's cells by especially nonpathogenic adeno-associated viruses (AAVs). The fusion of effector proteins with deactivated RNA-guided nuclease with small volume[166,167] or split-Cas9,[168,169] or using novel delivery methods[170,171] could help to facilitate the delivery of fusion proteins.

Emerging pieces of evidence have shown that CpGs are not independent but display a synergetic effect that the methylation of a given CpG site is influenced by the status of surrounding methylated CpGs.[172] The availability of whole-genome-wide bisulfite sequencing (WGBS) allows us to

https://doi.org/10.1021/jacsau.3c00671
JACS Au 2024, 4, 40−57

examine the collaboration roles of 5mC on a genomic scale. However, the underlying mechanism, especially the dynamic behavior in local genomic loci, remains elusive due to the lack of facile methods for site-specific manipulation of 5mC at specific genomic loci. HDR-based DNA methylation offers a potential way to accomplish DNA methylation with a higher resolution and efficiency. Compared with DNBPs-facilitated DNA methylation that undergoes regional editing with uncontrollable methylation/demethylation efficiency for each targeted site, HDR-based DNA methylation can achieve accurate 100% methylation for each individual 5mC once the methylated ssDNA/dsDNA donor was directly recombined to target sites. Moreover, HDR-based manipulation is capable of site-specific non-CpG (CpH, H = A/C/T) methylation, which will facilitate speculation of the functions of CpH methylation. To date, HDR-based 5mC manipulation can only perform global, context-specific, or distal methylation through recombination of a methyltransferase treated or methylated primer amplified DNA donor. This limited manipulation was attributed to a lack of methods to site-specifically synthesize methylated long donor ssDNA/dsDNA with arbitrary 5mC patterns. Therefore, it is highly demanded to synthesize hundreds of nucleotides long ssDNA with site-specific DNA modifications to precisely depict the dynamic behavior of 5mC maintenance in fundamental genomic elements such as promoter, enhancer, boundary elements, etc.

Last but not least, the challenges related to off-target effects and editing accuracy in existing toolkits for precise DNA methylation manipulation should not be underestimated. Considerable efforts need to be devoted to developing DNA methylation manipulation methods that offer single-base resolution with high efficiency and minimal off-target effects for various genomic contexts and cell types to gain a deeper understanding of DNA methylation. In the future, the methods of improving HDR efficiency should be adopted to enable therapeutic trials based on precise methylation manipulation.[173−175] As a stable epigenetic modification, DNA methylation exhibits the property of self-perpetuation that can be inherited and remain stable during the lifetime of the cell. This advantage permits sustained targeted manipulation, which holds great potential for epigenetic therapeutic applications.

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The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors would like to thank the National Natural Science Foundation of China (21890731, 21821001, 21971248, 32270627, and 21890730) and the National Basic Research Plan of China (2018YFA0208900) for financial support. Figures were created with Biorender.com.

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