



Review

# Targeted DNA Demethylation: Vectors, Effectors and Perspectives

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**Abstract:** Aberrant DNA hypermethylation at regulatory cis-elements of particular genes is seen in a plethora of pathological conditions including cardiovascular, neurological, immunological, gastrointestinal and renal diseases, as well as in cancer, diabetes and others. Thus, approaches for experimental and therapeutic DNA demethylation have a great potential to demonstrate mechanistic importance, and even causality of epigenetic alterations, and may open novel avenues to epigenetic cures. However, existing methods based on DNA methyltransferase inhibitors that elicit genome-wide demethylation are not suitable for treatment of diseases with specific epimutations and provide a limited experimental value. Therefore, gene-specific epigenetic editing is a critical approach for epigenetic re-activation of silenced genes. Site-specific demethylation can be achieved by utilizing sequence-dependent DNA-binding molecules such as zinc finger protein array (ZFA), transcription activator-like effector (TALE) and clustered regularly interspaced short palindromic repeat-associated dead Cas9 (CRISPR/dCas9). Synthetic proteins, where these DNA-binding domains are fused with the DNA demethylases such as ten-eleven translocation (Tet) and thymine DNA glycosylase (TDG) enzymes, successfully induced or enhanced transcriptional responsiveness at targeted loci. However, a number of challenges, including the dependence on transgenesis for delivery of the fusion constructs, remain issues to be solved. In this review, we detail current and potential approaches to gene-specific DNA demethylation as a novel epigenetic editing-based therapeutic strategy.

**Keywords:** DNA demethylase; DNA-binding domains; gene targeting; epigenetic therapy; vectors



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## 1. Introduction

The exciting paradigm of epigenetics, that ‘genes are not your destiny’, has taken a novel turn with the development of targeted molecular tools to selectively modulate the epigenetic status (e.g., promoter methylation) and thus transcription of genes. The ability to take control over the epigenome and ‘edit’ it for therapeutic and experimental benefit represents an enticing but complicated endeavor. This review focuses on the rapid progress of site-specific DNA demethylation, made possible by technological innovation since the 2010s, but also traces the footsteps of DNA demethylation research since its dawn in the 1980s. The article summarizes the current knowledge on the mechanisms of DNA demethylation and details how they are or can be exploited to re-activate epigenetically silenced genes. Going beyond an overview of ‘vectored’ delivery of epigenetic modifiers, the paper introduces the concept of vector-free epigenetically acting agents, with a view to future application in epigenetic medicine.

## 2. DNA Methylation as a Key Epigenetic Mechanism of Gene Function Control

DNA methylation that alters gene expression without a change in the DNA sequence is the most widely studied epigenetic mechanism involved in the pathogenesis of various diseases [1,2]. DNA methylation is a covalent modification that occurs to cytosines in the context of cytosine-phosphate-guanosine (CpG) residues; when it occurs in key regulatory

regions of the gene (e.g., promoter), methylation suppresses the expression of the gene [3]. Virtually all cells in an organism contain all the genes of that organism's genome; epigenetic mechanisms are broadly involved in the silencing of 'unused' genes which underlies the difference in structure and function of cells [4,5]. This silencing is a powerful and inheritable mechanism of gene function control [4]. DNA methylation is a key player in epigenetic silencing of transcription [3,6]; conversely, lowered methylation of promoters or regulatory intragenic regions has been linked to enhanced transcription [5,7,8]. DNA methylation of CpG dinucleotides is catalyzed by DNMT3a and b, and DNMT1 maintains the methylation during semiconservative DNA replication [9]. In case this maintenance methylation fails for some reason, e.g., inhibition, depletion or nuclear exclusion of DNMT1, so-called "passive demethylation" occurs in dividing cells [10]. Passive demethylation is a relatively slow process and theoretically does not occur in terminally differentiated non-dividing cells [11]. Given these points, it is unlikely that passive demethylation plays a leading role in demethylation in most adult somatic cells that have completed mitosis [12], although it plays an important role when genome wide DNMT inhibitors are employed therapeutically.

The first report on biological active DNA demethylation processes independent of cell replication in vertebrates was published in 1984 [13], in which HpaII site 611 base pairs upstream from the 5' end of the of the chicken vitellogenin II gene were seen to exhibit estrogen-dependent demethylation independent of DNA synthesis, predicting the existence of a site-specific demethylase. However, no further characterization of this enzyme has been forthcoming. Numerous evidences of active demethylation have been reported since then [14–18]. Another common example of active demethylation is the demethylation of pluripotency genes in nuclear reprogramming. During normal development, genes related to pluripotency are constitutively silenced by histone and DNA methylation. For example, the proximal m5CpG of the oct4 gene regulatory site in thymocyte nuclei transplanted into *Xenopus* oocytes is immediately demethylated [19]. Demethylation usually occurs with changes in physiological cellular states. Examples include changes in cell differentiation, such as terminal differentiation, or when exposed to nuclear hormones, protein kinase C (PKC) activators, histone deacetylase inhibitors (HDACi) or changes in neural activity [20–25]. These findings indicate that active demethylation is a widespread phenomenon in both dividing and differentiated cells.

### 3. Etiological Significance of DNA Methylation

In mammals, about 60–80% of the CpG sites in the genome are modified into 5mC [7]. CpGs outside promoter CpG islands are usually highly methylated, while those within promoter CpG islands are mostly unmethylated [26,27]. However, in several diseases, the promoter CpG islands of genes 'protective' against the disease are found to be aberrantly methylated [28–30]. Epigenetic alterations in CpG islands and, more broadly, elsewhere across the epigenomic landscape have been increasingly reported, ranging from the most frequent examples, linking DNA hypermethylation to cellular carcinogenesis, to a plethora of cardiovascular, renal, hepatic, metabolic and infectious diseases and environmental exposures [31–37]. Thus, the scope of aberrant methylation in pathogenesis is becoming more and more elucidated. For example, aberrations of cell cycle checkpoints in cancer result in abnormal cell proliferation and chromosomal instability. Examples include abnormal methylation of the p53 gene [38–40], or hypermethylation of the checkpoint genes with Fork-head and Ring finger (CHFR) in cancer cells [41–49].

While methylation and transcription are usually inversely correlated, and this is typically true for promoter methylation, there are exceptional situations where an increase in methylation leads to an increase in transcription if this methylation change affects a key regulatory area of a special kind [50,51], which can happen with enhancers, or where increased methylation inhibits binding of repressive factors or methylation-sensitive transcription factors. In general, however, selective demethylation of genes occurs during gene expression enhancement, but it is not certain to what extent demethylation is a primary factor versus a secondary consequence of enhanced transcription.

Moreover, DNA demethylation may not always be sufficient to increase transcription. For example, demethylation of the IL2 promoter is necessary but not sufficient for gene activation [52]. In this case, binding of the transcription factor Oct-1 to the promoter-enhancer region of the IL2 gene is a higher priority requirement for enhanced gene expression. When Oct-1 binds to the promoter-enhancer region as a result of additional stimulation, the IL2 gene becomes more sensitive to DNA demethylation and its gene expression is more highly upregulated. When the stimulus is removed, Oct-1 remains in the promoter-enhanced region, making the second and subsequent induction of gene expression faster and stronger [52]. Thus, priming of the locus occurs to prepare for subsequent external stimuli. The transcriptional result of gene demethylation is in this case dependent on the presence of a background stimulus: if present, the newly demethylated gene becomes intrinsically upregulated, otherwise it will 'wait' for an extrinsic trigger. This leads to an important potential benefit of epigenetic editing over traditional gene overexpression or a recombinant protein administration: demethylation opens the promoter to situation-appropriate, context-dependent intrinsic (or extrinsic, if desired) stimuli.

Aberrant hypermethylation occurs as a result of intrinsic deregulation or upon exposure to environmental toxicants; many environmental exposures have been linked to epigenomic effects [53,54] leading to two major challenges in the field. First, while the epidemiologic data for association of exposures with epigenomic effects are strong, proof of causality for exposure-related methylation changes at specific loci has been impossible due to a lack of experimental tools to specifically revert these alterations. Second, there are no therapeutic agents that can gene-specifically reverse 'harmful' epigenetic changes and achieve epigenetic-based treatment. These challenges offer an attractive opportunity to employ DNA demethylation as a therapeutic strategy for diseases where DNA hypermethylation is involved. Until recently, only non-specific agents, such as cytidine analogs including 5-aza-2'-deoxycytidine, have been available for removing methyl groups from CpGs based on the inhibition of DNMTs, and they have been widely used to study the effects of demethylation on gene promoters [55–58]. However, these non-specific agents lead to global demethylation of CpGs, making it impossible to define the causal effects of specific CpG aberrations [59]. Following these experimental trials, active DNA demethylation by enzymatic activity has become the focus of attention as an experimental and potential future therapeutic strategy [60], promoting interest in gene-specific (or site-specific) demethylation approaches [61].

#### 4. Biochemical Mechanisms of Active DNA Demethylation

It is increasingly recognized that active demethylation plays an important role in a wide variety of biological processes, and much attention and effort has been devoted to the elucidation of the mechanism of active demethylation, including the identification of the involved demethylase(s). DNA demethylase activity was noted for the first time in extracts of mouse erythroleukemia nuclei [62]. It was found that 5-methylcytosine (5mC) is finally replaced by C in a replication-independent manner, but this mechanism has not been elucidated in detail for a long time. Over the past years, several studies have proposed various possible mechanisms through which active DNA demethylation may take place. These include enzymatic removal of methyl groups at 5mC, a series of intermediary conversions, participation of the base excision repair (BER) and less well-known mechanisms.

##### 4.1. Enzymatic Removal of the Methyl Group of 5mC

In the initial step of the envisioned DNA demethylation process, 5mC is converted to 5-hydroxymethyl cytosine (5hmC) by the addition of atomic oxygen; 5hmC is further oxidized to 5-formylcytosine (5fC) and finally 5-carboxylcytosine (5caC) [63]. This is an important step that allows for several alternative or duplicative methods to remake normal cytosine residues autonomously or in a DNA replication-dependent manner, ultimately removing the methyl mark [64–66]. The oxidized intermediates, 5fC and 5caC, are removed and can

further regenerate unmethylated cytosine at the target site through the BER mechanism. The first half of the oxidation reactions are catalyzed by the ten-eleven translocation protein (TET) family enzymes, while the second half, removal of 5fC and 5caC, is catalyzed by thymine DNA glycosylase (TDG) [67].

The first member of TET family, TET1, was discovered in acute myeloid leukemia (AML) cells as a fusion partner for histone H3 Lys4 methyltransferase [68]. Subsequent *in vitro* studies have shown that the enzymatic activity of human TET1 includes the ability to hydrolyze 5mC [69]. TET protein can act on both fully methylated and hemimethylated DNA [70]. This ability of TETs to oxidize 5mC is particularly important in embryonic stem cells (ESCs), which must maintain the ability to self-renew and take on diverse 5mC patterns [71]. The TET protein is characterized by its distinctive core catalytic domain, and its isoforms are known to be cell type specific [72,73]. Mammals encode three TET protein orthologues (TET1, TET2, and TET3) [74]. The three TET proteins have similar C-terminal catalytic domain-containing cysteine-rich (Cys) regions and a double-stranded  $\beta$ -helix (DSBH) fold which exhibits 2-oxoglutarate (2-OG)- and Fe(II)-dependent dioxygenase activity [75].

#### 4.2. BER Through Direct Excision of 5mC Converts

Following the sequential oxidation reactions described above, TDG initiates the removal of target bases to make abasic sites [76]. Subsequently, apyrimidinic acid (AP) lyase activity nicks the DNA backbone, producing 5' phosphomonoesters and 3' sugar phosphate residues. AP endonuclease then removes the 3' sugar chain, leaving a single nucleotide gap that will eventually be filled by DNA repair polymerase or ligase [77]. Genetic and biochemical evidence suggested at first that this mechanism is used in plants [78], whereas evidence supporting the existence of similar mechanisms in the vertebrates was less compelling. In 2000, a study using extracts from chick embryos suggested that this repair mechanism may contribute to DNA demethylation in vertebrates as well [79]. This study also demonstrated the existence of 5mC glycosylase activity against hemimethylated DNA in the extract and revealed that the enzyme responsible for this activity is a homologue of human TDG. Therefore, the 5mC glycosylase activity detected in this chicken embryo extract was assumed to be due to TDG. However, the excision activity of TDG against 5mC is only 1/30 to 1/40 of that against T/G mismatch, and TDG does not have the activity to cleave N-glycosidic bonds [80]. Thus, the details of the involvement of TDG in DNA demethylation needed to be further explored. Since TDG is known to act on hydroxymethylated cytosines as well as 5fC and 5caC via its specific recognition site, though not on 5mC [80–83], it suggests a reasonable concept of the demethylation process that entails starting with a conversion of 5mC to hmC/fC/caC by TETs, followed by TDG action. This is confirmed by the observation that, in TDG-deleted cells, an accumulation of 5fC/5caC is observed [84]. Given that on average ~10% of mCs in mammals are hmCs (bisulfite-based methods, e.g., pyrosequencing, do not distinguish the two forms), this could explain the consistent but low-impact results of using TDG alone in epigenetic editing studies [85,86]. Indeed, low levels of active, replication-independent DNA demethylation were seen to be mediated by TDG [84].

The action of TDG as a demethylase, whether involving BER or not, is supported by epigenetic editing reports using solely this enzyme [85,86]. Assessment of the relative input of TETs and TDG in demethylation can be complicated by the intrinsic presence of either enzyme in the nucleus. This has been addressed in a convincing demonstration that TET and TDG interact with each other in several ways in the demethylation process: TET1 and TDG interact physically, forming stable TET1–TDG complexes [87]. Moreover, TET1CD and TDG act in concert to release 5mC so that the excision of 5mC and 5hmC from DNA requires the catalytic activities of both TET1 and TDG; finally, TET stabilizes TDG activity, and their coordinated action engages BER in a way that avoids double strand breaks [88]. Hence, TDG is not simply a sequential follower of TET, as was previously considered.

This information about TDG is not inconsistent with its previously presumed role as a demethylase following the deamination of mC by the AID (activation-induced deaminase)/APOBEC (apolipoprotein B mRNA-editing enzyme complex) family of cytidine deaminases [69,89]. Deamination-mediated demethylation could also involve the DNA damage response protein GADD45, or even methyl-binding domain protein MBD4 [19,90,91]. However, there is evidence that contradicts this premise [92,93]. It therefore appears that deaminases are unlikely to be enzymatic effectors for epigenetic editing.

#### 4.3. Potential for Enzymatic Demethylation without Excision and BER

Studies in embryonic cells allow the derivation of a mechanism in which initial conversion of mC to hmC by TET is followed by passive dilution where, as a result of replication, the unmodified C is regenerated [94,95]. It remains unclear to what extent this occurs during epigenetic editing, but it is plausible in dividing cells. It has also been reported that DNMTs that normally methylate the cytosine can, in some conditions, have a reverse effect, directly transforming mC to C [96].

AlkB homologs (ALKBH) are the other class (together with TET) of the Fe(II)/2-Oxoglutarate (2OG)-dependent dioxygenase superfamily of enzymes that have demethylating activity, and unlike TET they are capable of directly demethylating some of their substrates [97]. Besides their action on 3mC and 4mC, ALKBH act on 5mC [98], but while the outcome of the reaction for 3mC is unmethylated C, for 5mC it stops at 5caC, which then likely becomes the substrate for TDG.

Limited evidence suggests that 5caC may be converted into C directly and without excision: it was shown that both bacterial and mammalian C5-MTases can catalyze the direct decarboxylation of 5caC (but not of 5DC), yielding unmodified cytosine in the DNA [99]. It is possible that future studies will elaborate further on active enzymatic DNA demethylation. The attractiveness of this or another direct enzymatic conversion mechanism that avoids base excision lies in the lower risk of strand breaks and potential mutagenesis, which may confound translational approaches in the future.

## 5. Biological Effects of De-Repression of Epigenetically Silenced Genes

De-repression of epigenetically silenced genes by active, targeted DNA demethylation can have direct, exciting potential applications in a number of experimental strategies and human diseases:

### 5.1. For Instructing Cells to Produce a Protective/Therapeutic Protein

One benefit of the re-activation of epigenetically silenced genes is to confer unusual (but desirable) property to cells, e.g., production of a protein that is not typically produced by a given cell type, for example, a cytokine or immunoregulatory molecule. As an example, targeted de-methylation of nitric oxide synthase silenced in fibroblasts [85,86] confers to them an ability to produce nitric oxide (NO), which is a key component of innate immunity, including direct anti-microbial effects [100–103]. Enhancement of NO production by upregulation of NOS2 expression is a desirable therapeutic strategy [86,104]; reactivation of the enzyme's gene could lead to increased production at targeted tissue sites.

### 5.2. In Targeting Specific Monogenic Epigenetic Aberrations That Single-Handedly Cause Disease

For example, fragile X Syndrome is a form of autism linked to epigenetic silencing in the promoter of the FMR1 gene [105]; demethylation of its promoter could restore transcription, and this presumption has recently been clearly demonstrated [106].

To modify expression of cancer-related genes: silencing of tumor-suppressor promoters by DNA hypermethylation is an important mechanism of carcinogenesis [107] and could be selectively targeted.

### 5.3. In Experimental Strategies Such as Transdifferentiation and Regeneration

#### 5.3.1. Transdifferentiation

Artificial overexpression of non-typical genes in fibroblasts transforms these cells into other types [108,109]; epigenetic editing could provide heritable effect without the need for transgenesis.

#### 5.3.2. Proliferation

Specialized cells (cardiomyocytes or neurons) are non-dividing because genes responsible for cell division are switched off; epigenetic editing of cell cycle regulators could drive DNA synthesis and potentially lead to proliferation [110].

However, until recently, most of the identified DNA methylation changes are used either correlatively, or as biomarkers for various diseases: 1. diagnostic markers [111]; 2. prognostic markers [112,113]; 3. markers for optimal treatment according to disease subclasses [114]; 4. markers for monitoring treatment efficacy [115]; 5. markers to identify genes to be examined for the development of epigenetically targeted therapies [107]. These DNA hypermethylated markers have been studied most extensively in cancer [116]. However, aberrant DNA hypermethylation is being implicated in other diseases. A selection of recently published reports on non-cancer disease-linked promoter DNA hypermethylation is shown in Table 1. As shown, gene promoters are abnormally hypermethylated in many diseases, making them prime candidates for therapeutic targets. Therefore, developing strategies for site-specific CpG promoter demethylation of disease-associated genes is a critical and exciting challenge.

**Table 1.** Reports on disease associated promoter DNA hypermethylation in noncancer diseases.

| Category            | Disease                               | Gene   | Year       | References |
|---------------------|---------------------------------------|--|------------|------------|
| nervous             | Alzheimer's disease                   | HOXB6 <sup>a</sup> , ANKRD30B <sup>b</sup> , etc.            | 2019, 2020 | [117,118]  |
|                     | Parkinson's disease                   | Solute Carrier Family 7A11                                   | 2020       | [119]      |
|                     | schizophrenia                         | Solute Carrier Family 6A4                                    | 2022       | [120]      |
|                     | borderline personality disorder       | BDNF <sup>c</sup>  | 2018       | [121]      |
|                     | epilepsy                              | PABPC1 <sup>d</sup> , ARGLU1 <sup>e</sup> , etc.             | 2021       | [122]      |
|                     | depression                            | KLK8 <sup>f</sup> , NR3C1 <sup>g</sup>                       | 2021       | [123,124]  |
|                     | fragile X syndrome                    | FMR1 <sup>h</sup>  | 2019       | [105]      |
| immunological       | systemic lupus erythematosus          | NCR3 <sup>i</sup>  | 2021       | [125]      |
|                     | rheumatoid arthritis                  | SFRP2 <sup>j</sup>   | 2018       | [126]      |
|                     | systemic sclerosis                    | PARP-1 <sup>k</sup>  | 2018       | [127]      |
|                     | Sjogren's syndrome                    | IRE1α <sup>l</sup> , XBP-1 <sup>m</sup> , GRP78 <sup>n</sup> | 2018       | [128]      |
|                     | Graves' disease                       | CRHR1 <sup>o</sup> , B3GNT2 <sup>p</sup>                     | 2021       | [129]      |
|                     | type 1 diabetes                       | Cathepsin H  | 2021       | [130]      |
|                     | Crohn's disease                       | KCNJ15 <sup>q</sup>  | 2022       | [131]      |
| endocrine/metabolic | osteoporosis                          | Nrf2 <sup>r</sup>  | 2021       | [132]      |
|                     | type 2 diabetes                       | TXNIP <sup>s</sup>   | 2021       | [133]      |
|                     | hyperlipidemia                        | PPARα <sup>t</sup>   | 2022       | [134]      |
| cardiovascular      | cardiomyopathy                        | ASB1 <sup>u</sup>  | 2018       | [135]      |
| renal               | IgA nephropathy                       | Cosmc <sup>v</sup>   | 2015       | [136]      |
|                     | diabetic nephropathy                  | TRIM13 <sup>w</sup>  | 2020       | [137]      |
| pulmonary           | idiopathic pulmonary fibrosis         | PPARγ  | 2022       | [138]      |
|                     | chronic obstructive pulmonary disease | CYP4F11 <sup>x</sup> , SNRPN <sup>y</sup> , etc.             | 2014       | [139]      |
| hepatic             | hepatitis C                           | SHP-1 <sup>z</sup>   | 2021       | [140]      |

<sup>a</sup> homeobox B6, <sup>b</sup> ankyrin repeat domain 30B, <sup>c</sup> brain-derived neurotrophic factor, <sup>d</sup> poly(A) binding protein, cytoplasmic 1, <sup>e</sup> arginine and glutamate rich 1, <sup>f</sup> kallikrein-8, <sup>g</sup> Nuclear Receptor Subfamily 3 Group C Member 1, <sup>h</sup> FMRP translational regulator 1, <sup>i</sup> natural cytotoxicity triggering receptor 3, <sup>j</sup> secreted frizzled-related protein 2, <sup>k</sup> poly [ADP-ribose] polymerase 1, <sup>l</sup> Inositol-requiring enzyme 1, <sup>m</sup> X-box binding protein 1, <sup>n</sup> glucose regulated protein78, <sup>o</sup> corticotropin releasing hormone receptor 1, <sup>p</sup> UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 2, <sup>q</sup> Potassium inwardly rectifying channel, subfamily J, member 15, <sup>r</sup> nuclear factor erythroid 2-related factor 2, <sup>s</sup> Thioredoxin Interacting Protein, <sup>t</sup> peroxisome proliferator-activated receptor alpha, <sup>u</sup> ankyrin repeat and SOCS box containing 1, <sup>v</sup> core1β1, 3galactosyl transferase, <sup>w</sup> tripartite motif containing 13, <sup>x</sup> cytochrome P450, family 4, subfamily F, polypeptide 11, <sup>y</sup> Small nuclear ribonucleoprotein-associated protein N, <sup>z</sup> Src homology domain 2-containing protein tyrosine phosphatase 1.

## 6. Gene-Specific DNA Demethylation

Site-specific gene targeting DNA demethylation is accomplished by fusing the demethylases to engineered DNA-binding domains (DBDs) that tether the enzyme to the desired DNA sequence [61]. Since it was first reported simultaneously by three independent groups [85,86,141,142], the development of this innovative technology has opened the possibility of epigenetic therapeutic applications for various diseases. Here, we review the latest findings on strategies for site-specific DNA demethylation and discuss the prospects for clinical translation in the future.

### 6.1. Selection of Demethylase for Targeted DNA Demethylation

The most frequently reported DNA demethylase for gene targeting are the TET family proteins. Among these, TET1 fusion protein is most commonly used. However, there are scattered reports using TET2 and TET3, which are considered to have enzymatic activity equivalent to that of TET1, at least in vitro [75], although not in all experimental settings [141].

An increasing number of studies have reported the success of tethering TETs to DNA for targeted demethylation in recent years [143–147]. In the context of T cell differentiation, the dCas9-TET1 catalytic domain (CD) demethylates the FOXP3-Treg specific demethylated region (TSDR) of Jurkat cells, leading to a Treg-like phenotype, suggested the feasibility of dCas9-TET1-mediated Treg programming of primary T cells [148,149].

In cancer cells, dCas9-TET1 enhances the cisplatin sensitivity of A549 cells (lung carcinoma epithelial cells) by promoting the expression of nicotinamide nucleotide transhydrogenase (NNT) [150]. dCas9-TET1CD was used to demethylate the EphA7 gene: the protein correlates with life expectancy in cervical cancer, indicating the potential of dCas9-TET1CD as a therapeutic strategy for cervical cancer [151]. Leucine-rich repeat and immunoglobulin-like domain (LRIG) 1 is a negative regulator of receptor tyrosine kinases and a tumor suppressor; decreased LRIG1 expression is consistently observed in various types of cancer and is linked to poor patient prognosis [152]. dCas9-TET1CD-mediated demethylation along with VP64-mediated transcriptional activation increased endogenous LRIG1 expression in breast cancer cells, and reduced cancer cell viability [153]. TET1CD reactivated X-linked endogenous FOXP3 in breast cancer [154]. The tripartite-motif (TRIM) family proteins contribute to cancer initiation, progress, or therapy resistance, exhibiting tumor-suppressive functions: they are frequently downregulated by promoter methylation in cancerous tissues [155]. dCas9-TET1CD induced specific demethylation of TRIM58 in renal carcinoma cells (RCC) [156]. Promoter of the TMEM244 gene was demethylated to upregulate its expression in Sézary syndrome—an aggressive form of cutaneous T-cell lymphoma [157]. dCas9-TET1CD could demethylate hepatocyte nuclear factor (HNF)1A and Beta-1,4-mannosyl-glycoprotein 4-beta-N-acetylglucosaminyltransferase (MGAT3) genes in BG1 (human ovarian adenocarcinoma) cells [158], and telomeric repeat-containing RNA (TERRA) in HeLa (human cervix carcinoma) and T98G (human glioblastoma) cell lines [159].

The following reports have been published on the function of TET1 in non-tumor cells. TET1 ablation impairs cardiac differentiation of mouse embryonic stem cells and re-expression of the TET1CD rescued the differentiation defect in Tet-triple knockout mESCs [160]. Cyclin-dependent kinase-like (CDKL) 5 is associated with X-linked infantile spasm syndrome (ISSX). Halmai et al. performed artificial X chromosome inactivation (XCI) using dCas9-TET1CD targeting CDLK5 [161]. Rett syndrome is an X-linked neurodevelopmental disorder caused by loss-of-function heterozygous mutations of methyl CpG-binding protein 2 (MECP2) on the X chromosome in young females [162]. Qian et al. reported reactivation of MECP2 in human embryonic stem cells (hESCs) derived from RTT by dCas9-TET1CD [163]. These two reports suggest the potential of dCas9-TET1CD for therapeutic application in X chromosome associated diseases. dCas9-TET1CD also could re-activate Oct4 in NIH-3T3 cells [164] and beta-galactoside alpha-2,6-sialyltransferase 1 (ST6GAL1) in CHO cells [165].

A few studies signal readiness to transition epigenetic editing experiments to in vivo. Fragile X syndrome (FXS), the most common genetic form of intellectual disability in males, is caused by silencing of the FMR1 gene associated with hypermethylation in the CGG expansion mutation in the 5' UTR of FMR1. dCas9-TET1CD was used to demethylate the CGG repeats in the brain cells of mice so that FMR1 expression in edited neurons was maintained in vivo after engrafting into the mouse brain [106]. With dCas9-TET1CD system and hydrodynamic tail vein injection, Hanzawa et al. showed targeted DNA demethylation of the Fgf 21 promoter in the liver of PPAR $\alpha$ -deficient mice [166]. Russell–Silver syndrome (RSS) is a rare disorder characterized by intrauterine growth restriction (IUGR) and poor growth after birth. The patients of RSS show H19 upregulation and insulin-like growth factor (Igf) 2 downregulation. Horii et al. developed RSS model mice via demethylated H19 promoter lesion with embryonic microinjection of dCas9-TET1CD [167]. Noack et al. delivered dCas9-TET1CD targeted Dchs1, a regulator of corticogenesis, to mouse brains by electroporation and verified the biological relevance of the aberrant methylation of Dchs1 in developmental malformations and cognitive impairment [168].

On the other hand, TDG alone has been successful in demethylating and transcriptionally enhancing the NOS2 gene [85,86]. Furthermore, targeted DNA demethylation with Release of Silencing 1 (ROS1) 5mC DNA glycosylase [169] fused to the DNA-binding domain of yeast GAL4 (GBD) has been reported [170]. These reports are supplemented by studies using targeted fusions with P300 acetyltransferase, which helps support the epigenetic editing effect on gene transcription [149,171]. Thus, the selection of demethylases may be broadened in the future.

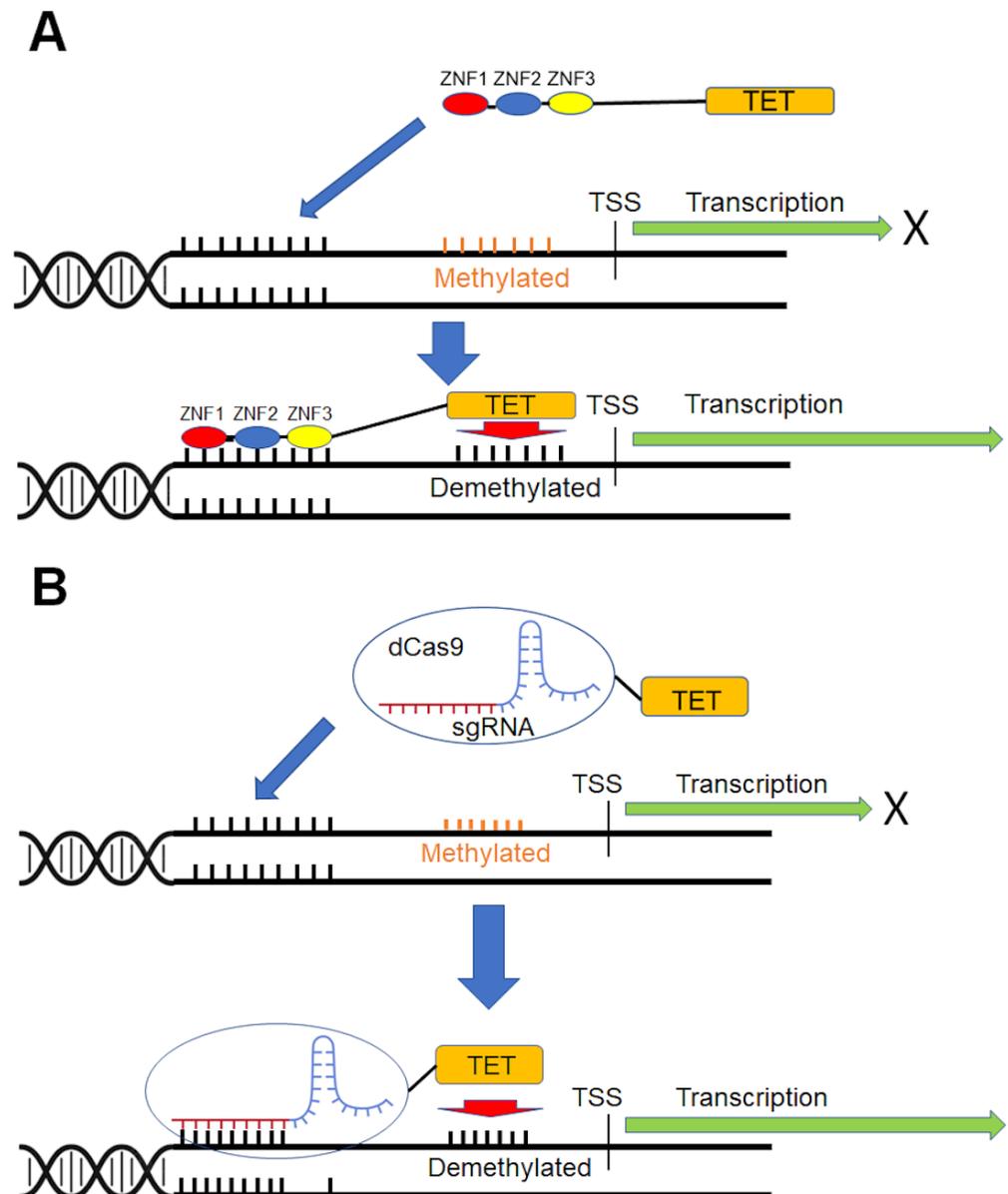
Given the multi-step nature of transformations from mC to C where TET proteins and TDG glycosylases interact in moving the atoms of the CH<sub>3</sub> group, the strategies for application of multiple demethylases for epigenetic editing form a novel area of studies that may be called 'atomic epigenetics'. When interpreting the reports of each single enzyme successfully employed for demethylation, it is important to consider that the other enzymes were intrinsically present as is natural for unmodified cells. At the same time, the paucity of studies on combinatorial use of different demethylases presents a space for future investigations.

## 6.2. Site-Specific DNA-Binding Domains

There are several types of DBDs that have been fused with the putative demethylases to tether them sequence-specifically to the promoters of interest. Aside from customizable DBDs aimed to allow single gene specificity, other naturally occurring DBDs have historically been the first used to affect a group of genes at once. For example, the Rel-homology domain (RHD) of NF- $\kappa$ B has been employed to target TDG to all NF- $\kappa$ B-dependent genes [85,86]. This and similar systems (using for example transcription factor binding domains) may be employed in epigenetic editing for re-activation of intrinsic transcription using a 'grouped' approach.

Recently, more specific and customizable DBDs have become applied. One of these is the recombinant transcription activator-like effector (TALE) [142,172]. TALE is a class of naturally occurring DNA-binding proteins found in the plant pathogen *Xanthomonas* sp. The DNA-binding domain of each TALE consists of 34-amino acid repeat modules arranged in tandem that can be rearranged according to a simple cipher to target new DNA sequences. Customized TALEs can be used for a wide variety of genome engineering applications, including transcriptional modulation and genome editing [173]. The molecular tool through which DNA demethylase is targeted to the disease-related gene promoters can be made by tethering human TET1 demethylase enzyme to a TALE repeat that has targeted DNA-binding specificities [142]. Modified TALE repeats provide an attractive platform for guiding TET1 activity. This is because assembly of individual repeat domains of known nucleotides can be used to produce large amounts of monomeric proteins that bind to virtually any target DNA sequence [174]. However, TALEs may be less suitable specifically for de-methylation, as they have difficulty binding richly methylated sequences [175].

Concomitant to the TALEs, a well-known DNA-binding protein used for targeted editing is the zinc finger protein tandem array (ZFA). The DNA recognition domain of ZFA contains 3–6 or more Cys2-His2 zinc fingers. Each zinc finger in a ZFA recognizes a 3-bp DNA sequence via a single  $\alpha$ -helix; tandem array assembly allows recognition of a longer sequence in the increments of 3, usually 9–18 bp in length [176]. ZFAs approach the DNA from the major groove [177] and bind with high specificity to DNA sequences [176,178]. Different ZFA modules are used in combination, based on their respective affinities for a particular three-base sequence, to target specific genomic regions [179]. The site-specific DNA demethylation using ZFA-TETs fusion protein is shown in Figure 1A.



**Figure 1.** ZFA-TETs fusion protein (A) and CRISPR-dCas9-TET based (B) site-specific demethylation.

Both ZFA and TALE were used as binding platforms for TET1 in site-specific DNA-demethylation studies, and both systems were equally effective in inducing transcription of targeted genes [175,180,181]. ZFAs and TALEs each have strengths and weaknesses regarding opportunities for targeting and specificity of binding; however, ZFAs provide a broader choice of specific arrays for a given target area. Novel algorithms simplify and improve the design process [182–184]. In a typical 3-finger array, 9 bp are recognized;

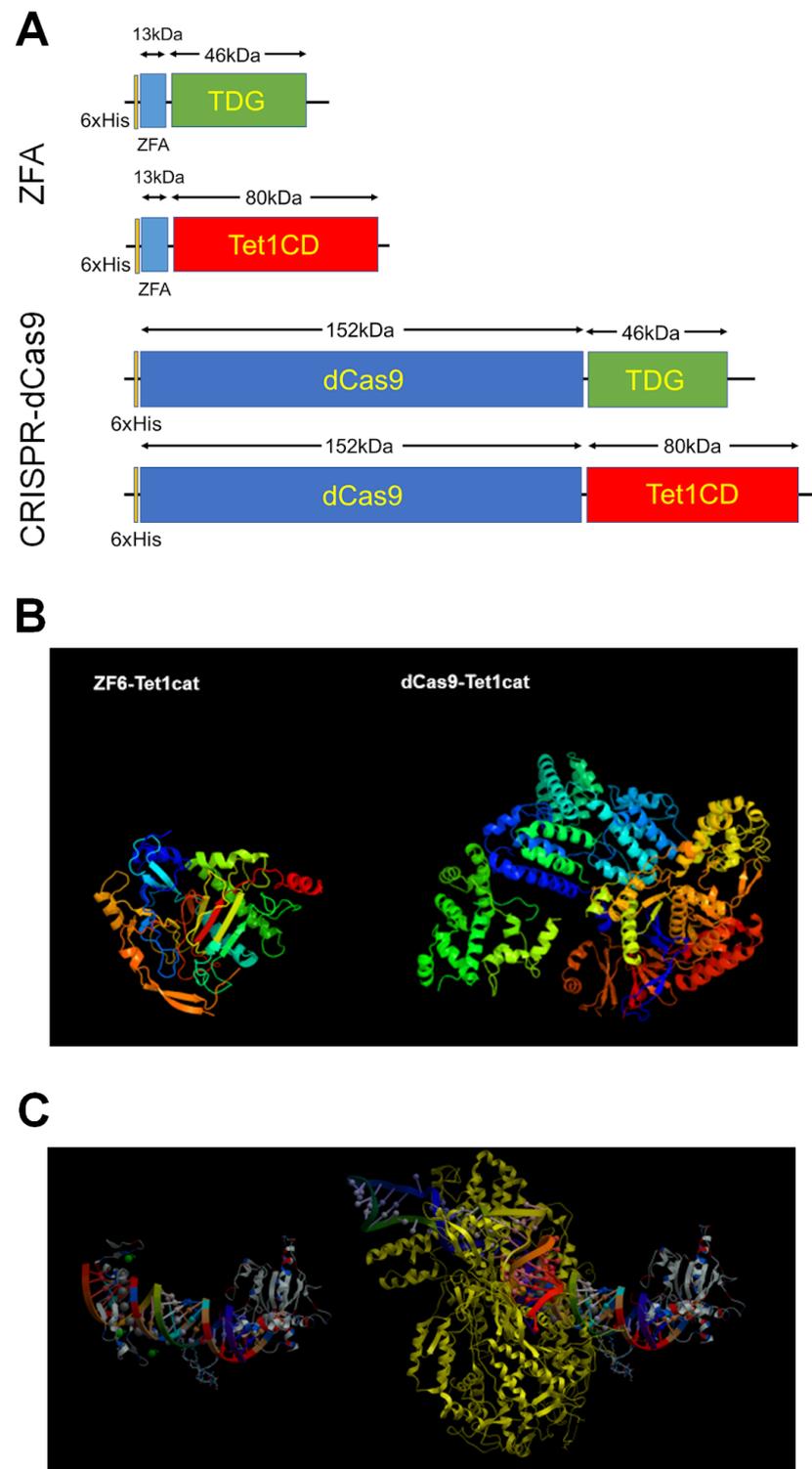
however, it is possible to design 6-mers or longer molecules to target 20–30 bp sequences for better specificity. Longer ZFAs and TALEs, however, could still suffer from some non-specific binding, as off-target binding is known to occur both for ZFs [185], and for TALE's [186,187]. To maximize single gene specificity, a 'multiple hits' approach may be used to target multiple fusion molecules to the same promoter in the near vicinity of each other, and in both strands [86], because the action of multiple demethylase molecules is cumulative, and it is unlikely that a combination of a dozen different constructs will bind in close proximity to an unrelated promoter.

In the last decade, the clustered regularly interspaced short palindromic repeat-associated 'dead' Cas9 (CRISPR/dCas9) module has received significant attention as a site-specific epigenome editing tool [166,188]. The CRISPR module can be used as a site-specific demethylation tool by combining the specific DNA-binding ability of inactivated 'dead' cas9 (dCas9) with demethylase, such as TET1 or TDG. Quite a few targeted demethylation studies have been published using CRISPR-dCas9 systems [143–168,189–192]. Generally, CRISPR-dCas9-TET1CD fusion proteins are paired with sgRNAs that have programmable 20 nucleotide sequences homologous to the target loci (Figure 1B).

The first report on site-specific demethylation using the CRISPR-dCas9-TET1 system was published in 2016 [192]. In that study, the demethylation and transcription of RANKL, MAGEB2 and MMP2 were promoted by the delivery of dCas9-TET1 constructs to cultured cells (HEK293, SH-SY5Y, HeLe) using plasmid vectors. They also modified the sgRNA by inserting bacteriophage MS2 RNA elements into the conventional sgRNA to improve the efficiency of delivery of the fused proteins to their targeted binding regions. Several other attempts have been made to amplify the response to associated effector modules in the CRISPR system. For example, Taghbalout et al. developed the Casilio-DNA Methylation Editing (ME) platform to amplify the efficacy of effector modules working in the targeted promoter loci [146,193]. Nguyen et al. introduced the Sun Tag linker to the system to recruit multiple copies of VP64, a strong non-epigenetic transcriptional activation domain, at each locus of interest, which improved the efficiency of fused TET1 in inducing demethylation and transcription [190].

The characteristics of the three DNA-binding modalities are summarized in Table 2.

Comparing ZFA with CRISPR, both have their own strengths and weaknesses. ZFA, being a protein, is more stable *in vivo* than a guide RNA. As shown in Figure 2, ZFAs are much smaller than dCAS9, for which the size and structural complexity represent a significant problem. ZFAs can permeate into cells and further into their nuclei, spontaneously delivering a fused enzymatic payload [194], whereas for dCAS9, additional measures for internalization and translocation are required. At the same time, gRNA DBD promises a better specificity of the binding; re-designing a ZFA-based construct to target a new gene requires re-expression and repurification of the entire fusion protein, whereas, with dCAS9-demethylases, only a replacement of gRNA is required to re-target; finally, the emerging wealth of tools for CRISPR editing creates a favorable landscape for the development of new applications. Nevertheless, the current enthusiasm for CRISPR technology in gene editing cannot be directly translated to vector-free epigenetic editing; the benefits of ZFA over dCAS9 (spontaneous internalization, small size, absence of the need to co-transfect gRNAs and protect them from the aggressive *in vivo* airway milieu) make it a valuable approach that remains a useful tool for epigenetic editing [195,196].



**Figure 2.** (A). Structure and size comparison of ZFA-TDG and ZFA-TET1 constructs. (B). Anticipated 3D structural drawings of ZFA-TET1CD and dCAS9-TET1CD constructs. The images were generated with PHYRE2 Protein Fold Recognition Server. (<http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index> accessed on 20 April 2023). (C). Sequence-accurate to-scale 3D rendering of ZFA-TDGCD and dCAS9-TDGCD bound to a target gene promoter. The image was derived from ICM Browser using custom PDB files.

**Table 2.** Comparison of three DNA-binding domains for vector-free demethylation.

|                               | ZFA  | TALE   | CRISPR/dCas9  |
|-------------------------------|--|--|---|
| Components                    | Zinc finger domain   | TALE DNA-binding domain  | crRNA/tracrRNA or sgRNA, dCas9  |
| Size                          | ~1 kb + demethylase  | ~2 kb + demethylase  | 4.2 kb (dCas9) + 0.1 kb (sgRNA)   |
| Working mechanism             | DNA/protein recognition  | DNA/protein recognition  | DNA/RNA recognition   |
| Length of the target sequence | 9–36 bp  | 30–40 bp per TALE pair   | 20–22 bp  |
| Target recognition efficiency | High   | High   | Very High   |
| Off target effects            | Low for long arrays  | Unknown  | Lowest  |
| Multiplexing                  | Possible   | Difficult  | Possible  |
| Cloning                       | Essential  | Essential  | Not essential   |
| Advantages                    | Small protein size (<1 kDa) facilitates packaging and purification   | High specificity with each module recognizing 1bp; no need to engineer linkage between repeats | Enable multiplexing   |
|                               | When administered as a naked protein, spontaneously internalize into cells and translocate into nuclei, delivering the fused enzymatic payload |  | Only a replacement of sgRNA is required to retarget new genes   |
| Limitations                   | Re-designing the entire protein is required to re-target   | Re-designing is required to target new genes   | Large protein size (~150 kDa) makes packaging into vectors or nanovesicles difficult  |
|                               | Cloning methods that require additional linker sequence to fuse modules together add variability   | Repetitive sequences may induce undesirable recombination events                               | When administered as a naked protein, additional measures for cellular internalization and nuclear translocation are required |
|                               |  | Has difficulty binding methylated DNA  | sgRNA may be unstable under in vivo and in vitro biological conditions, may require packaging/protection                      |

Finally, some older domains may become re-employed to support the transition of epigenetic editing from virally vectored to protein-only tools. [197].

## 7. In Vivo Delivery

Most of the previous findings on site-targeted DNA demethylation have been based on in vitro experiments. As one of the few exceptions, Lei et al. introduced the CRISPR system into mice by microinjection of the dCas9-DNA methyltransferase MQ1 construct on a lentivirus vector into zygotes [198]. Hanzawa et al. also expressed plasmid-based CRISPR/dCas9 in mouse livers using the hydrodynamic tail vein injection [166]. These studies are highly relevant because they demonstrate that the approach of site-specific DNA demethylation can potentially work in vivo. However, laboratory techniques such as zygotes microinjection and hydrodynamic tail vein injection have problems in clinical application, such as high procedural difficulty and inability to ensure accurate organ-specific delivery. Methods such as microinjection and electroporation are more suitable in vitro [199,200].

Since in vivo knowledge of site-specific DNA demethylation is so limited, an overview of in vivo site-specific demethylation will depend on what is known about targeted nucleases genome editing, which has similarities [198]. Here, we summarized current strategies of three main viral vectors and other non-viral technologies.

### 7.1. Viral Delivery

Viral transduction benefits from the ability of virions to incorporate their genome into the host for replication; thus, viruses are used as vectors to encode proteins and deliver them to target cells.

### 7.1.1. Adenoviral Vectors (AdVs)

First-generation adenovirus vectors (AdVs) were designed to substitute a transgene for the E1 (3.15 kb) and/or E3 (3.1 kb) regions. [201]. The first-generation adenovirus vectors could transduce a wide variety of target cells, achieved high levels of gene expression and were sufficient for applications where transient high activity was required and an immune response to the vector or transgene was negligible. However, the first generation of adenoviral vectors had to overcome several safety concerns. At high 'multiplicities of infection' (MOI), certain host gene products with E1-like activity may be expressed, thereby unnecessarily inducing host cell proliferation [202]. Leakage of adenoviral DNA replication accumulating cytotoxic late gene products has a direct cytotoxic effect on transduced cells and triggers a host cellular immune response [203,204]. To overcome these adverse effects, the second generation of AdVs were developed with extended genome packaging capacity by removing two or more initial genes, such as E2 and E4 [205]. Several clinical trials of genome editing nucleases have been conducted using such AdVs. For systemic delivery of ZFAs, the expression unit coding the ZFAs is inserted into a serotype 5 AdV pseudotyped with serotype 35 fiber (AdV5/35) to repair autologous CD4+ helper T cells from HIV infected patients [206]. Despite improvements compared to the first generation, the problem of immune side effect *in vivo* has not been completely resolved in the second generation AdVs, and therefore they are not yet optimal vectors for therapeutic approaches [207]. A further evolution of AdVs is the high-capacity AdV (HCAdV), which removes all virus-specific genetic information, leaving only the inverse terminal repeat sequence (ITR) and packing signal. Compared to common AdVs, HCAdVs have the following advantages: they suppress both innate and adaptive immune responses, and their total packaging capacity has been expanded to a maximum of 36 kb, facilitating delivery of large TALE expression cassettes or the CRISPR/dCas system [208].

One drawback of AdVs is that the level of coxsackie-adenovirus receptor (CAR), the receptor for Ad on cells, determines the delivery efficiency [209]. For example, AR expression in cancer cells is said to be negatively correlated with tumor grade, which means that high delivery efficiency may not be achieved when targeting cancer cells in the advanced clinical stage [210]. Therefore, the study of AdVs that could specifically act on cancer cells is taking on a new and important role in anti-tumor research.

### 7.1.2. Adeno-Associated Viruses Vectors (AAVs)

Adeno-associated viral vectors (AAVs) are delivery vectors derived from icosahedral, non-enveloped viruses of the family Parvoviridae, genus Dependovirus, and are commonly used in genome editing technology because of their ability to be incorporated site-specifically and because of their low immunogenicity. The pathogenicity of AAV has not been confirmed so far. AAVs can deliver their genomes to mitotic and non-mitotic cells and can survive outside the chromosome without delivering their genome to the host cell [211]. A major drawback of the AAV vector system is its relatively small genome size (approximately 4.7 kb), which limits the size of the foreign genes that it can carry. However, with careful design, the sequences for expressing ZFA and TALE expression cassettes can be successfully encapsulated in the AAV vector [207]. Multiple studies on AAV/ZFA-mediated genome editing have shown positive results in experiments with animal models *in vivo* [212–214]. For example, in a study on hemophilia B using a humanized mouse model, the concentration of human factor IX in plasma recovered to 23% of normal on average by administering ZFA-carried AAV to the model mice [215].

Since the size of a typical spCas9 is about 4.2 kb, packaging it using an AAV with a cargo size of about 4.5 kb poses a challenge [215]. To solve this problem, a smaller saCas9 (~3.2 kb) was developed from *Staphylococcus aureus* species [216]. To date, AAV is established as a delivery vehicle for the CRISPR/Cas system and has been used to target brain cells, as well as skeletal and cardiac muscle cells [217,218].

On another topic, AAV has been reported to be a promising vaccine vector. Taking advantage of this property, attempts are being made to develop a COVID-19 vaccine

based on the AAV vector [219,220]. The AAV vaccines reportedly have advantages over first-generation vaccines targeting the same spike protein, such as thermostability, high efficiency, safety and single-dose vaccination. This research demonstrates the potential and benefits of an AAV as a gene transfer vector.

### 7.1.3. Lentiviral Vectors (LVs)

Lentiviruses are viruses belonging to the retroviridae family and are characterized by the stable insertion of viral genomic information into the host genome using viral RT (reverse transcriptase) and IN (integrase) [221]. Lentivirus-based vectors have attractive advantages as gene transfer vectors, including the following [222]:

- (i) sustained gene transfer through stable integration of the vector into the host genome.
- (ii) ability to infect both dividing and non-dividing cells.
- (iii) broad tissue and cell orientation.
- (iv) no expression of viral proteins in the host cells after vector transduction.
- (v) ability to deliver complex gene elements.
- (vi) a more secure integrated site profile.
- (vii) relatively easy vector manipulation and production.

However, multiple patient deaths in early clinical trials have prevented lentiviral use as delivery vectors in humans [223,224]. Therefore, safer integrase-deficient lentiviral vectors (IDLV) have been developed in recent years. For example, Lombardo et al. used IDLV as a delivery vector for ZFA-based genome editing in human stem cells [225]. However, the efficiency of IDLV may depend on the target tissue [224]. For example, IDLV is less efficient at transferring genes into mouse hepatocytes than a conventional lentiviral vector, but it is highly efficient at transferring genes into murine muscle [224,226].

The characteristics of the three vectors mentioned above are summarized in Table 3.

**Table 3.** The characteristics of the three gene transfer vectors.

|                                   | AdVs *                                  | AAVs **                      | LVs ***             |
|-----------------------------------|---|------------------------------|---------------------|
| Cell affinity                     | Inefficient for some types of cells     | Dependent on viral serotypes | Broad               |
| Infection into non-dividing cells | +                                       | +                            | +                   |
| Transient/Stable expression       | Stable expression by genome integration | Transient, episomal          | Transient, episomal |
| Maximum titer                     | Very high                               | High                         | High                |
| In vivo immunogenicity            | High                                    | Very low                     | Low                 |

\* adenoviral vectors. \*\* adeno-associated viruses vectors. \*\*\* lentiviral vectors.

Although viral vectors are by far the most efficient tools for gene transfer, many issues preclude their in vivo and especially clinical use. Therefore, while the transduction/transfection-based approach to embedding epigenetically acting fusion proteins into recipient cells has served a useful purpose in the early stages of epigenetic editing, with experimentation almost exclusively being performed in vitro, it is linked to numerous problems when it comes to translating it into in vivo, and especially into human applications. The viral vector is a foreign invader; thus, the body may mount an immune response [227,228]; a repeat administration or, in case of a pre-existing immunity to the viral vector, even the first administration will be ineffective for an immune subject [227].

Especially with retroviral vectors, genes incorporated into chromosomes pose a risk of oncogenesis due to random gene transfer [229–231]. Moreover, insertional mutagenesis, viral persistence, accumulation of proto-oncogenic lesions, immunogenicity, cellular toxicity, risks related to immune surveillance and other problems common to gene therapy will similarly affect epigenetic therapy [232–235]. Finally, viral transfection can affect transcriptional activity of host genes, which creates the potential for misinterpretation of

gene expression data generated from transfected cells [236] and impairs usefulness for experimentation on epigenetic control of transcription, even *in vitro* but especially *in vivo*.

### 7.2. Non-Viral Vectors

Non-viral vectors are classified into several subtypes based on their raw materials, sizes or production methods. For example, by raw material they can be classified into lipid-based vectors, polymer-based vectors, etc. Lipid-based vectors are further classified into lipid nanoparticles (LNPs) and liposomes, etc., depending on their manufacturing method and size. Liposomes, polymersomes, lipoplexes, polyplexes and dendrimers, are commonly used as gene delivery vectors and can enclose the mRNA encoding the fusion chimeras [237–240]. However, the low transfection efficiency and low gene expression rate compared to viral vectors have been obstacles to the clinical application of non-viral vectors. Considering this, various improvements of non-viral vectors have been attempted [241]. One example of these improvements is cationic liposomes that have been validated for their high transfection efficiency and biocompatibility [242]. Positively charged gene particles packaged in cationic lipids or cationic polymers are expected to be attracted to the negative charges of the cell membrane, thus increasing the efficiency of cellular uptake [243]. Studies on non-viral nanoparticle-mediated *in vivo* delivery are increasing in number, especially for the delivery of relatively small molecules such as RNAi [244–246]. However, there are few reports on non-viral vector-mediated *in vivo* delivery of megamolecules for genome editing. As an example, Han et al. recently reported using nanoparticles to deliver antithrombin-targeted CRISPR/Cas9 to the liver of a hemophiliac model mouse [247]. Several groups have now reported LNP formulations for successful expression in lungs following inhalation [248–252], indicating that the approach may be ready for therapeutic efforts in pulmonary disease. Specifically, in [253], more efficient delivery to fibrotic than healthy lungs was reported, reached over 12% of epithelial cells and 10% endothelial cells in fibrotic lungs following intratracheal delivery.

Applications of programmable nuclease complexes are often hampered by the inability of the complex to reach the target tissue or, if it does reach the target, to pass through the cell membrane and then into the nucleus, and to exert therapeutic activity *in vivo*. Similar challenges can be expected to occur with site-specific demethylases.

### 7.3. Direct Delivery of Epigenetically Acting Fusion Proteins

Transition from viral vectors to the use of non-viral engineered packaging systems for epigenetically acting constructs could be a step forward in the field. It is important to consider, however, that the very need for genomic integration/transgenesis is linked to some of the side effects and challenges listed above for viral vectors. Lack of control on the site of insertion, problematic control of expression (both the duration of expression and its extent) including inability to stop the action if needed, immunogenicity of the mRNA, design challenges and finally susceptibility to degradation by nucleases are commonly seen with mRNA therapies and vaccines [254]. Thus, development of novel, safe and effective approaches, e.g., based on protein-only ‘biologics’ that act on DNA methylation, is enticing.

Key challenges that may be anticipated here will include ascertaining internalization of such fusion protein constructs into recipient cells and nuclei, and maintaining their intactness in an *in vivo* environment. Proteins are usually less lipophilic, causing problems in the cell membrane permeation process, but a ZFA protein complex can, surprisingly, penetrate cell membranes due to the positive charge of Cys2-His2 zinc finger domains, and Gaj et al. showed that the direct delivery of ZFA-tethered nuclease protein can disrupt the CCR5 gene in both HEK-293 HDF cells, and human CD4+ T cells [255]. Unlike the naturally cell-penetrating ZFAs, for TALEs and CRISPR/Cas9, conjugation of cell-penetrating peptides (CPPs) is needed to facilitate cell entry [256]. In recent years, various CPPs, such as TAT peptide (YGRKKRRQRRR), have been developed [257].

Another problem with direct delivery, especially for *in vivo* applications, is protein stability. In the *in vivo* environment, unprotected proteins will come into direct contact

with degrading enzymes. Therefore, it is important to not only expedite delivery, but also to increase the stability of the protein or otherwise protect it, e.g., by encapsulation into vesicles. One method utilized plasma membrane-derived extracellular microvesicles (ARMMs; arrestin domain-containing protein 1-mediated microvesicles) [258,259] for delivery of CRISPR-Cas9/guide RNA complex, which can be employed for epigenetic editing. For ZFAs, strategies to keep them stable have also been proposed, such as modifying the lysine residues necessary for degradation [260]. Finally, the LNP packaging discussed above can also be employed to package not only mRNA, but also the purified epigenetically acting constructs, provided their size allows.

At the moment, non-vectored (ribo)protein-only epigenetic editing is the cutting edge of the field.

## 8. Conclusions

The number of publications on targeted DNA demethylation has increased at an accelerating rate in recent years, reflecting the high level of interest in this field. Most of them are in vitro (Table 4) or ex vivo [106] studies using plasmid or viral vectors as a carrier, but, recently, in vivo experiments have also been reported [143,166]. Although neither of the gene delivery strategies are immediately applicable to clinical research, further expansion of in vivo applications of the targeted DNA demethylation is expected to follow in the future.

**Table 4.** Selected publications for targeted DNA demethylation/gene reactivation.

| DBDs                              | Enzymes                  | Targeted Gene  | Research Materials  | Publication Year | Ref   |
|-----------------------------------|--------------------------|--|---|------------------|-------|
| TALE                              | TET1                     | <i>RHOXF2</i> <sup>a</sup>   | HeLa cells  | 2013             | [142] |
| TALE                              | TET1                     | <i>Ascl1</i> <sup>b</sup>  | Neural stem cells   | 2017             | [261] |
| ZFA                               | TDG                      | <i>Nos2</i> <sup>c</sup>   | NIH-3T3 cells   | 2013             | [86]  |
| ZFA                               | TET2                     | <i>C13ORF18</i> <sup>d</sup> , <i>CCNA1</i> <sup>e</sup> ,<br><i>TFPI2</i> <sup>f</sup> , <i>SERPINB5</i> <sup>g</sup>   | HeLa cells, SiHa cells, CaSki cells, C33a cells                       | 2016             | [262] |
| ZFA                               | TET1                     | <i>FWA</i> <sup>h</sup>  | Arabidopsis   | 2016             | [263] |
| CRISPR                            | TET1                     | <i>RANKL</i> <sup>i</sup> , <i>MAGEB2</i> <sup>j</sup> ,<br><i>MMP2</i> <sup>k</sup>   | HEK293FT cells  | 2016             | [192] |
| CRISPR                            | TET1                     | <i>H19</i> <sup>l</sup> , <i>RHOXF2</i> , <i>CARD9</i> <sup>m</sup> ,<br><i>SH3BP2</i> <sup>n</sup> , <i>CNKSR1</i> <sup>o</sup> ,<br><i>GFAP</i> <sup>p</sup> | ESC, cancer cell lines, primary neural precursor cells, mouse fetuses | 2016             | [143] |
| CRISPR                            | TET1                     | <i>BRCA1</i> <sup>q</sup>  | HeLa cells, MCF7 cells  | 2016             | [264] |
| CRISPR                            | TET1                     | <i>ST6GAL1</i> <sup>r</sup>  | CHO cells   | 2018             | [165] |
| CRISPR                            | TET1                     | <i>Oct4</i> <sup>s</sup>   | NIH-3T3 cells   | 2022             | [164] |
| CRISPR                            | TET1                     | <i>FOXP3</i> <sup>t</sup>  | HCC202 cells, HEK293T cells   | 2022             | [154] |
| CRISPR                            | TET1                     | <i>PPH</i> <sup>u</sup>  | Arabidopsis   | 2022             | [265] |
| Rel-homology domain (RHD) of NFκB | TDG                      | <i>Nos2</i>  | NIH-3T3 cells   | 2012             | [85]  |
| DNA-binding domain of yeast GAL4  | ROS1 5mC DNA glycosylase | Targeted reporter gene   | HEK293 cells  | 2013             | [170] |

Proteins coded by the genes

<sup>a</sup> Rhox homeobox family member 2, <sup>b</sup> Achaete-scute homolog 1, <sup>c</sup> Nitric oxide synthase 2, <sup>d</sup> Chromosome 13 Open Reading Frame 18, <sup>e</sup> Cyclin-A1, <sup>f</sup> Tissue factor pathway inhibitor 2, <sup>g</sup> Maspin, <sup>h</sup> homeodomain-containing transcription factor that controls flowering, <sup>i</sup> Receptor activator of nuclear factor kappa-B ligand, <sup>j</sup> Melanoma-associated antigen B2, <sup>k</sup> Matrix metalloproteinase 2, <sup>l</sup> long noncoding RNA, <sup>m</sup> Caspase recruitment domain-containing protein 9, <sup>n</sup> SH3 domain-binding protein 2, <sup>o</sup> Connector enhancer of kinase suppressor of ras 1, <sup>p</sup> Glial fibrillary acidic protein, <sup>q</sup> Breast cancer type 1 susceptibility protein, <sup>r</sup> Beta-galactoside alpha-2,6-sialyltransferase 1, <sup>s</sup> octamer-binding transcription factor 4, <sup>t</sup> Forkhead box P3, <sup>u</sup> Pheophytin pheophorbide hydrolase.

Selected targeted DNA demethylation/gene reactivation-related papers are summarized in Table 4.

Targeted DNA demethylation in key regulatory areas of genes allows re-activation of epigenetically silenced transcripts for experimental and therapeutic purposes. This technology can be broadly useful in biomedical research, and its current advances aim to overcome the key challenges of delivery, efficiency and gene-specificity of epigenetic effects.

Summary of benefits:

- Opens the gene to context-dependent, situation-appropriate stimuli
- Potentially heritable (at least mitotically) effects in tissues
- Mechanistic studies in epigenetics (environmental epigenetics, immunotoxicology)
- Vector-free methods will allow studying causality of immunoregulatory genes
- Forms a new class of ‘biologic’ drugs

Summary of challenges:

- Not every gene can likely be targeted
- Gene-specificity/off-target effects
- Non-viral delivery challenges
- Cell-specific delivery

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