Gene-Targeted DNA Methylation

Cortés-Mancera, Fabian M.; Sarno, Federica; Goubert, Désirée; Rots, Marianne G.

Published in:
DNA Methyltransferases - Role and Function

DOI:
10.1007/978-3-031-11454-0_18

Gene-Targeted DNA Methylation: Towards Long-Lasting Reprogramming of Gene Expression?

18

Fabian M. Cortés-Mancera, Federica Sarno, Désirée Goubert, and Marianne G. Rots

Abstract

DNA methylation is an essential epigenetic mark, strongly associated with gene expression regulation. Aberrant DNA methylation patterns underlie various diseases and efforts to intervene with DNA methylation signatures are of great clinical interest. Technological developments to target writers or erasers of DNA methylation to specific genomic loci by epigenetic editing resulted in successful gene expression modulation, also in in vivo models. Application of epigenetic editing in human health could have a huge impact, but clinical translation is still challenging. Despite successes for a wide variety of genes, not all genes mitotically maintain their (de)-methylation signatures after editing, and reprogramming requires further understanding of chromatin context-dependency. In addition, difficulties of current delivery systems and off-target effects are hurdles to be tackled. The present review describes findings towards effective and sustained DNA (de)methylation by epigenetic editing and discusses the need for multi-effector approaches to achieve highly efficient long-lasting reprogramming.

Keywords

Zinc finger · TALE · CRISPR-dCas9 · Epigenetic editing · DNMT · CpG methylation

18.1 Introduction

The epigenetic concept was first described by Conrad Waddington early in 1942, when he conducted experiments to understand phenotypic plasticity during embryonic development (Felsenfeld 2014). The definition has evolved over time to one of the current understandings of epigenetics as "the study of heritable changes in gene function that occur independent of changes in the primary DNA sequence"

Fabian M. Cortés-Mancera and Federica Sarno contributed equally.

F. M. Cortés-Mancera
Epigenetic Editing, Department of Pathology and Medical Biology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands

Grupo de Investigación e Innovación Biomédica, Departamento de Ciencias Aplicadas, Instituto Tecnológico Metropolitano, Medellín, Colombia
e-mail: fabiancortes@itm.edu.co

F. Sarno
Epigenetic Editing, Department of Pathology and Medical Biology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands

Dipartimento di Medicina di Precisione, Universita degli Studi della Campania "Luigi Vanvitelli" Napoli, Napoli, Italy
e-mail: f.sarno@umcg.nl

D. Goubert · M. G. Rots (✉)
Epigenetic Editing, Department of Pathology and Medical Biology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands
e-mail: m.g.rots@umcg.nl

A. Jeltsch, R. Z. Jurkowska (eds.), *DNA Methyltransferases - Role and Function*, Advances in Experimental Medicine and Biology 1389, https://doi.org/10.1007/978-3-031-11454-0_18

(Nicoglou and Merlin 2017). The heritable modifications that epigenetics refer to correspond to biochemical changes on DNA and histone proteins. These changes influence the chromatin structure and thereby the expression of genes, even when the initial trigger has gone, and without underlying DNA sequence alterations. The main covalent chemical modification on the DNA molecule itself is methylation of cytosines, mostly in the context of CpGs dinucleotides (Petryk et al. 2021). Posttranslational modifications (e.g., methylation, acetylation), mainly on histone tails, provide another class of epigenetic signatures (Huo et al. 2021).

Strong observational evidence has been obtained on how epigenetic modifications associate with gene expression. To pinpoint an actual causative role of a particular epigenetic modification at a given genomic site, epigenetic editing tools have been exponentially exploited (de Groote et al. 2012; Jurkowski et al. 2015; Nakamura et al. 2021b). Epigenetic editing refers to the technology of actively rewriting epigenetic signatures at a genomic locus of interest. Towards this end, molecular tools have been generated (Jurkowski et al. 2015) consisting of a DNA-binding platform, which can be engineered to achieve locus-specific targeting, fused to an epigenetic effector domain (see Fig. 18.1). The first programmable protein-based DNA-binding platform used for endogenous gene targeting exploited the modular zinc finger (ZF) protein transcription factors, followed by transcription activator-like effectors (TALEs), and more recently the RNA-directed clustered regulatory interspaced palindromic repeats (CRISPRs) system (Stolzenburg et al. 2016).

ZF proteins, the largest group of naturally occurring transcription factors in the human genome, consist of approximately 30 amino acid-sized modules, each recognizing 3–4 bps in the major groove of double-stranded DNA (Sgro and Blancafort 2020). Mechanistically, the alpha-helix amino acids at positions -1, 3, and 6 can be engineered to recognize the third, second, and first base pair of a $5'-3'$ target sequence. Fusing together various of these modules resulted in effective tools targeting numerous genes in preclinical research and several ZF fusions have been clinically tested for ex vivo (and were the first tested in vivo (Ledhord 2018)) gene editing purposes. Next to their use as "*molecular scissors*" (when fused to nucleases), ZFs were used in pioneering studies of gene expression modulation by fusing transcriptional activators/repressors (Artificial Transcription Factors) to target a wide variety of endogenous genes (de Groote et al. 2012). The relatively compact size and scarce immunogenicity of ZFs are a major advantage compared to other DNA-targeting proteins.

TALEs provide another class of programmable DNA-binding tools and are derived from pathogenic bacteria that naturally modulate plant gene expression (Becker and Boch 2021). TALEs consist of individual protein modules that mediate binding to the target DNA site. Subsequently, transcriptional activators/repressors, or nucleases can be fused to the TALE DNA-binding domain for targeted gene expression modulation (Jain et al. 2021).

The more recent introduction of the versatile CRISPR-Cas9 system made gene targeting readily available for any laboratory with cloning facilities. CRISPR-Cas9 is derived from the bacterial defense system that recognizes foreign DNA. The nuclease activity of Cas9 is guided to a particular target sequence in the host genome via single-guide RNA (sgRNA)-DNA base pairing (see Fig. 18.1). As the DNA-binding specificity of earlier platforms (e.g., ZFs or TALEs) is provided by the engineered DNA-binding part within the fusions, for every new target sequence a new fusion protein needs to be designed. Target specificity of CRISPR-Cas9 is provided by separate sgRNAs, which are also simpler and less expensive to design, making this system much more flexible.

All three systems have been successfully exploited for epigenetic editing through the engineering of fusion proteins with epigenetic effector domains (Epi-editors) (Sgro and Blancafort 2020). In the case of CRISPR-Cas9, the epi-editor is cloned as a fusion to Cas9 proteins lacking the endonuclease activity (deactivated Cas9, dCas9). Upon delivery into target cells, the DNA-binding platform-fusion will bind to

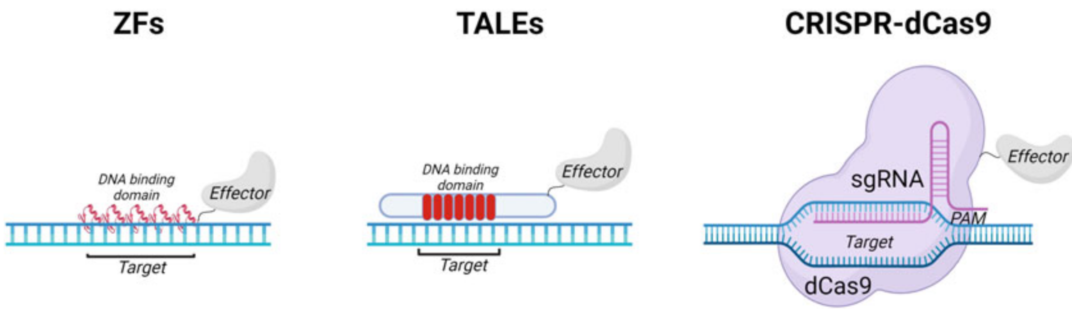

Fig. 18.1 Schematic representation of modular systems used in epigenetic editing. Epigenetic effector domains are recruited to the target DNA sequence by a DNA-binding platform: *ZFs* zinc finger proteins, *TALEs* transcription activator-like effectors or *CRISPR-dCas* the Clustered Regulatory InterSpaced Palindromic Repeat platform with *dCas9* deactivated Cas9 protein, *sgRNA* single-guide RNA, *PAM* proto-spacer adjacent motif. Figure made in https://biorender.com

the target sequence and exert its (enzymatic) activity. Initially, the assumed inaccessibility of heterochromatic genes, the unclear causative role of epigenetic marks on gene expression, as well as the unknown stability of edited marks was thought to hamper successful expression modulation of (silenced) genes. Pioneering studies and the general acceptance of CRISPR as a straightforward DNA-targeting approach, set the stage for the broad application of epigenetic editing as a research tool, e.g., to assess causative roles of epigenetic marks (Wang et al. 2021; Policarpi et al. 2021) and as potential therapeutic approach (Sgro and Blancafort 2020; Nakamura et al. 2021b).

The first well studied epigenetic mechanism is DNA methylation, predominately occurring on cytosine in the context of CpG (5mC), although methylation in non-CpG context has also been described (Ehrlich 2019). This epigenetic modification is important in stable (re)programming of expression patterns during development and cell differentiation, genome integrity and X chromosome inactivation, in health and disease (Ehrlich 2019; Petryk et al. 2021). In promoter regions, CpG dinucleotides often cluster in so-called CpG islands (CGIs), and more than half of the human gene promoters contain a CGI. These CpG-rich promoters are usually unmethylated, with a few exceptions, including tissue-specific methylation during development (Greenberg and Bourc'his 2019). Gene promoters with high levels of DNA methylation are generally transcriptionally inactive, while hypermethylated gene bodies generally associate with actively transcribed genes (Jeziorska et al. 2017).

DNA methyltransferase enzymes (DNMTs) generate this epigenetic mark. Specifically, DNMT1 is responsible for the methylation maintenance process coupled to DNA replication targeting hemimethylated strands (Petryk et al. 2021). DNMT3A and DNMT3B are capable of establishing new methylation patterns on previously unmodified cytosines, mainly in the CpG context. DNMT3L does not possess enzymatic activity but works as a coactivator of DNMT3A or 3B (Petryk et al. 2021). On the other hand, a family of enzymes called ten-eleven translocation proteins (TET1, TET2, and TET3) (Wu et al. 2018) possess dioxygenases activity that can convert methylated cytosine to 5-hydroxymethylcytosine (5hmC), followed by 5-formylcytosine (5fC) formation, and then 5-carboxylcytosine (5caC). Finally, 5fC and 5caC are removed by thymine DNA glycosylase (TDG), and cytosine is reestablished by base excision repair (BER) mechanism (Onodera et al. 2021).

Thanks to the programmable protein-based DNA-binding platforms, targeting (de)-methylation at specific loci is achievable and can be applied in a huge variety of physiological and pathological contexts. A better understanding of factors that promote on-target epigenetic effects, and induce the desired long-lasting transcriptional states will facilitate further breakthroughs and the clinical application of epigenetic editing. Here, we will discuss findings on the use of epigenetic editing in exploring causative roles of DNA methylation and gene expression, with a specific focus on in vivo models and on the understanding of achieving long-lasting effects on gene expression levels.

18.2 Locus-Specific DNA Methylation Editing

18.2.1 Targeted DNA Methylation

Targeting DNA methyltransferases (MTase) to given genomic loci by epigenetic editing provides unique tools to investigate the causal role of DNA methylation in the modulation of gene expression (see Fig. 18.2), and to exploit this mechanism to combat diseases (Sgro and Blancafort 2020). The first proof of concept of targeted DNA methylation inhibiting gene expression was reported by Xu and Bestor in 1997, who constructed a fusion protein consisting of an engineered ZF and the prokaryotic DNA MTase *M.SssI* to induce DNA methylation on a p21 synthetic oligonucleotide promoter target (Xu and Bestor 1997). Several subsequent studies of targeted DNA methylation using human or bacterial DNA methyltransferases confirmed that induction of DNA methylation results in transcriptional repression in an exogenous system or non-mammalian genomes reviewed by us earlier (Stolzenburg et al. 2016). Genome-wide studies, however, pointed out that not all genes are equally permissive to methylation-induced gene silencing (Galonska et al. 2018; Broche et al. 2021). Moreover, cell heterogeneity, with even unexpected gene expression upregulation in response to DNA methylation editing, is incompletely understood (Vizoso and Van Rheenen 2021).

In 2012 and 2013, the endogenous repression of human genes by targeted DNA methylation was reported for the first time in two independent publications, targeting the vascular endothelial cell growth factor A (*VEGF-A*) promoter (Siddique et al. 2013), and *SOX2* and *MASPIN* oncogenes (Rivenbark et al. 2012). These studies used designed ZF proteins fused to the catalytic domain of the murine or human DNA methyltransferase 3A, respectively. The former report also demonstrated a twofold enhanced methylation activity by the fusion of DNMT3A and DNMT3L single chain dual effector (ZN-DNMT3A-3L) compared to ZN-DNMT3A alone (28 versus 14%, respectively). The increase is explained by the ability of the non-enzymatic DNMT3L to not only enhance the activity of other DNMTs, but also to recruit endogenous DNMTs (O'Geen et al. 2019). This synergy between DNMT3A and 3L was confirmed by various subsequent studies (Stepper et al. 2017, O'Geen et al. 2019, Tarjan et al. 2019; Nakamura et al. 2021a). Although the DNTM3A/3L fusion was frequently used in editing studies (Saunderson et al. 2017; Shayevitch et al. 2018; Hofacker et al. 2020), effective gene repression was also obtained by targeting DNMT3A catalytic domain (DNMT3A-CD) only (Bernstein et al. 2015; Vojta et al. 2016; McDonald et al. 2016; Qu et al. 2018; Josipovic et al. 2019; Tiane et al. 2021), or DNMT3A full length (Liu et al. 2016). Even targeting DNMT3L alone was sufficient to induce gene repression (O'Geen et al. 2019; Nakamura et al. 2021b), although not in all contexts (Amabile et al. 2016). Compared to the effective targeting of the long isoform (DNMT3A1) or the short isoform (DNMT3A2) using the dCas9-SunTag system (see Fig. 18.3), transient targeting of multiple copies of the catalytic domain (DNMT3A-CD) alone, resulted in no significant methylation or gene expression changes on *HOXA5* (Huang et al. 2017), indicating context-dependent effects. Compared to dCas9-DNMT3A, DNMT3B exhibited a lower methylation activity when targeted to the endogenous urokinase (*uPA*) promoter in HEK293T cells. Also for DNMT1, although DNMT1 recruitment had been shown to induce DNA methylation (Van et al. 2021), Lin and coworkers could not demonstrate changes in

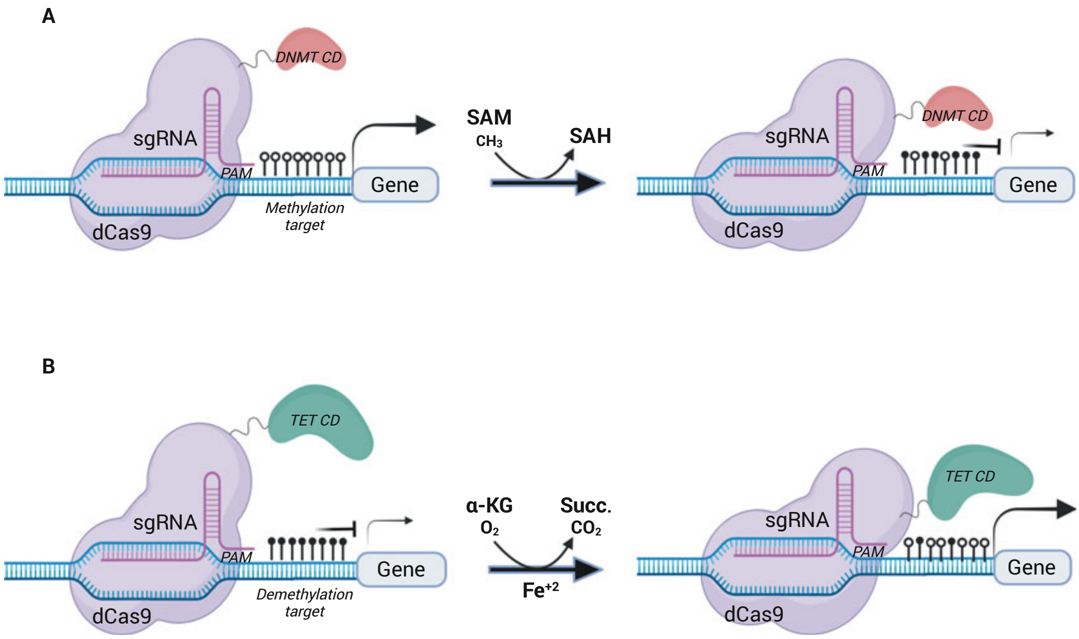

Fig. 18.2 Gene expression regulation via CRISPR-dCas9 targeting (de)methylation. (**a**) Representation of dCas9-DNMT (DNA methyltransferase) writing methylation at the target promoter region to induce gene expression downregulation. (**b**) Representation of dCas9-TET (Ten-eleven translocation methylcytosine dioxygenase) oxidizing (erasing) the methyl group at 5mC to induce gene re-expression. SAM *S*-adenosylmethionine, SAH *S*-adenosylhomocysteine, α-KG alpha-ketoglutarate, Succ succinate. Figure made in https://biorender.com

methylation levels in cells transfected to express dCas9-DNMT1 (Lin et al. 2018), suggesting that DNMT1 is less suitable for methylation editing.

The higher activity of DNMT3A was, however, also associated with off-target methylation. Although off-target effects can be sgRNA-driven (Zhang et al. 2015; McDonald et al. 2016), some studies indicate sgRNA-independent off-targeting (Lin et al. 2018; Galonska et al. 2018; Hofacker et al. 2020) via effector overexpression and/or interactions with endogenous de novo methylation enzymes. In this respect, Galonska and coworkers confirmed that increasing the pool of transduced sgRNAs spanning multi-loci regions to achieve simultaneous dCas9 recruitment did not reduce off-target effects (Galonska et al. 2018). Some reports described that increasing the efficiency of inducing local methylation (e.g., by dCas9-SunTag) improved the specificity (Huang et al. 2017; Pflueger et al. 2018). However, Hofacker and coworkers did not confirm improved specificity for the SunTag system when targeting *ISG15*, using the endogenous *VEGFA* promoter as an off-target reporter. Transfection of dCas9-DNMT3A-DNMT3L (dC) or dCas9-SunTag-DNMT3A/DNMT3L resulted in similar *ISG15* methylation levels (around 80%), while off-target *VEGF-A* methylation was higher for dCas9-SunTag (53%) versus dC (36%) (Hofacker et al. 2020). Therefore, constructs carrying different single mutations affecting DNA binding (K766E, K844E, R887E and R831E variants) were evaluated to improve methylation targeting specificity. Compared to wild-type dCas9-SunTag-DNMT3A/DNMT3L, residual on-target methylation activity of mutated effectors remained high (56 to 77% on *ISG15*), while methylation on *VEGFA* dramatically decreased. The R831E mutant provided the highest specificity with approximately 5% off-target methylation at the *VEGFA* promotor versus around 50% for the

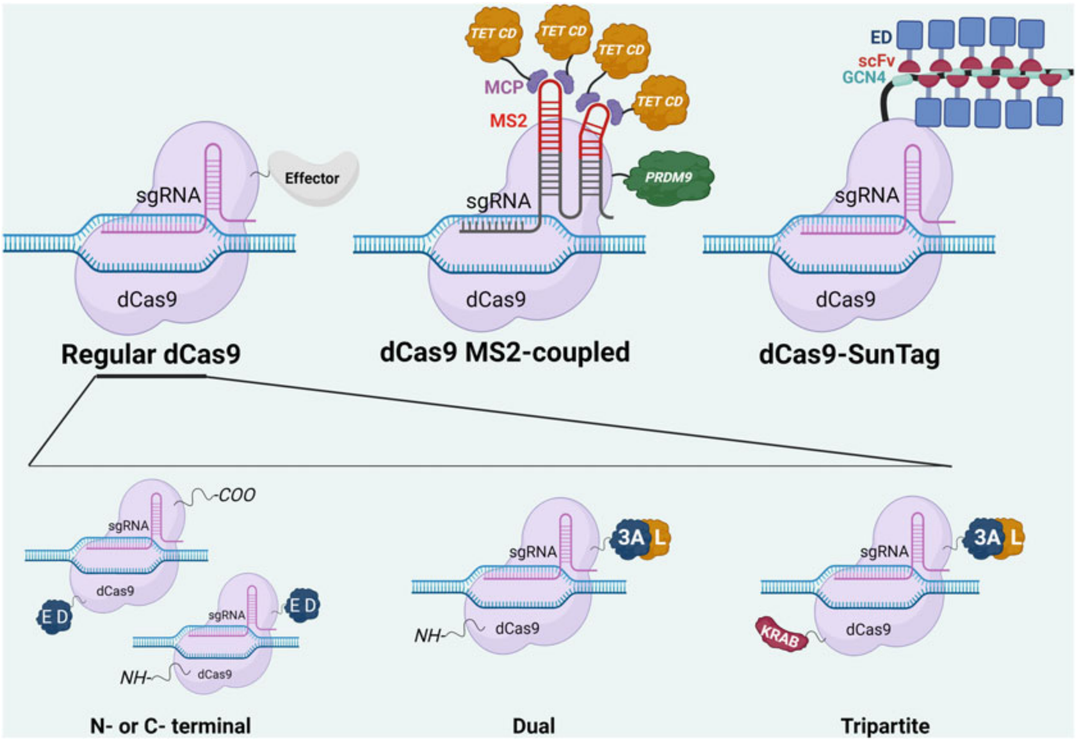

Fig. 18.3 Representation of enhanced CRISPR-dCas9 tools. At the top, three commonly used dCas9 tools. ***dCas9 MS2-coupled***: sgRNA is engineered to harbor RNA motifs (**MS2**) that can be recognized by RNA-binding proteins (**MCP**) fused to epigenetic effector domains such as TETCD to synergize with, for example, dCas9-PRDM9 writing H3K4me3 (Cano-Rodriguez et al. 2016). ***dCas9-SunTag***: dCas9 is fused to **GCN4** repeats that can recruit effector domains (**ED**) fused to a GCN4 recognizing single chain antibody (**scFv**) (Pflueger et al. 2018). At the bottom, options in effector configuration diversity (N/C-terminal orientation, different numbers/combinations) are shown. Figure made in https://biorender.com

wild-type enzyme, as confirmed using a genome-wide approach.

Other strategies to reduce off-target methylation include the usage of the prokaryotic MTase *M.SssI* variant MQ1^{Q147L} that does not recruit endogenous mammalian DNA methyltransferases, and demonstrated less off-target effects compared to wild type at endogenous loci (Lei et al. 2017). Alternatively, a split version of the *M.SssI* MTase was shown to generate efficient targeted DNA methylation, with less off-target effects when compared to dCas9 fused to full-length *M.SssI* (Xiong et al. 2017). Recently, Ślaska-Kiss and colleagues studied *M.SssI* variants fused to zinc fingers or dCas9, and demonstrated in *E. coli* cells that methylation specificity on plasmids was predominantly influenced by mutations affecting catalytic activity rather than DNA-binding affinity of the MTase domain (Slaska-Kiss et al. 2021).

To further improve the toolbox of targeted methylation, spatiotemporal control has been exploited to enhance site specificity by cloning light-inducible protein pairs to DNA-binding modules and to a DNA methyltransferase. Indeed, Lo and coworkers engineered DNMT3A-CRY2-EGFP and TALE-CINB1-mCherrry constructs to control *Ascl1* promoter methylation changes by exploiting the optogenetic blue light inducible dimerizing of cryptochrome-2 (CRY2) and its interacting protein (CIB1). Upon blue light exposure, DNMT3A-CRY2 paired to TALE-CINB1 and effectively induced highly specific DNA methylation and subsequent decrease in gene expression (Lo et al. 2017).

18.2.2 Targeted DNA Demethylation

To exploit the reversibility of DNA methylation in a gene-targeted manner, Ten–eleven translocation (TET) dioxygenase enzymes offer unique tools for DNA demethylation (see Fig. 18.2). Using the ZF or TALE platforms, the first TET-editing reports compared the potency of the three different TET domains (Chen et al. 2014), and demonstrated the improved efficacy of the catalytic domain (CD) over full length (Maeder et al. 2013), in inducing active DNA demethylation and subsequent transcriptional upregulation. Using CRISPR-dCas9, effective demethylation was further demonstrated for various genes (Choudhury et al. 2016; Amabile et al. 2016; Xu et al. 2016; Okada et al. 2017), and the approach was rapidly translated to in vivo models as described hereafter (Liu et al. 2016; Morita et al. 2016; Xu et al. 2018; Ou et al. 2019; Wang et al. 2019; Horii et al. 2020; Hanzawa et al. 2020).

The SunTag system (Morita et al. 2016) and MS2 elements inserted into sgRNAs (Xu et al. 2016) were shown to enhance the effect of targeted demethylation via dCas9-TET (see Fig. 18.3). Also combining TET demethylation activity with VP64 activation showed promise, as demonstrated for *CDKL5*, a gene causative for an infantile epilepsy in human neuronal-like cells (Halmai et al. 2020). As known from literature, a significant number of X-linked genes escape from X chromosome inactivation and are associated with a distinct epigenetic signature like reduced DNA promoter methylation. Halmai and coworkers created such escape by removing DNA methylation on the promoter of the *CDKL5* promoter. The dCas-TET1 targeting caused a significant reactivation of the inactive allele (Halmai et al. 2020), which was further improved by dCas9-TET1 and dCas9-VP64 co-treatment resulting in reactivation of the inactive allele to levels of >60% of the active allele. This artificial escape study confirmed earlier observations of synergism between TET and transcriptional activation domains, such as VPR (VP64-p65-Rta). Interestingly, despite a more effective demethylation by TET alone compared to the combination, a synergism with respect to increased re-expression of *Hnf1a* was observed (Josipovic et al. 2019).

Targeted demethylation of DNA can also be induced using the plant-derived ROS effector (Devesa-Guerra et al. 2020) or Thymidine DNA Glycosylase (Gregory et al. 2013) or even by dCas9 alone or with an inactive enzyme as recently demonstrated (Sapozhnikov and Szyf 2021). The latter authors studied several proximal promoters, including the hypermethylated *IL33* gene. After transient transfection experiments, dCas9-TET or a catalytically inactive mutated version (dCas9-dead-TET) caused hypomethylation and induction of *IL33* gene expression, suggesting a mechanism independent of TET oxygenase activity. The authors demonstrated that hypomethylation was related to DNMTs blockage, which is consistent with previous reports that showed mild hypomethylation induced by binding of dCas9-TET catalytically inactive mutants (Maeder et al. 2013; Xu et al. 2016; Morita et al. 2016), as is also known to occur upon binding of some transcription factors (Suzuki et al. 2017). Similarly, for engineered ZFs, hypomethylation was observed for targeted CpGs (Chen et al. 2014; Huisman et al. 2016). Sapozhnikov and Szyf also highlighted some important aspects with respect to promoter methylation and gene activation: demethylation of CGG repeats in the *IL33* promoter region resulted in gene re-expression, while demethylation in the proximal promoter region of other genes was not enough to induce their expression (e.g., *SERPINB5*, *TNF*). These genes required demethylation also of other regions (*cis* or *trans*) to induce gene expression. Such data illustrate the importance of studying demethylation of specific sites to better understand their relative contribution to gene expression and cause-effect dynamics. Moreover, despite effective demethylation and re-expression, the cellular functional effects might not be as expected, as was the case for dCas-TET1 induced re-expression of *FoxP3*: despite an effective increment in *FoxP3* gene expression, no increase in the functional regulatory T cell population was observed (Kressler et al. 2020).

18.3 Sustained Transcriptional States upon DNA Methylation Editing

18.3.1 Long-Lasting Transcriptional Repression

Given the maintenance of DNA methylation during cell division (and the for a long time presumed absence of active DNA demethylases), CpG methylation was initially considered a stable epigenetic mark associated with persistent silencing (Petryk et al. 2021). Currently, it is generally accepted that also this epigenetic signal is highly dynamic.

To evaluate the long-term effect of dCas9-DNMT3A without interference from the endogenous DNMT enzymes, Galonska and coworkers made use of DNMT3A/B double knockout (DKO) embryonic stem (ES) cells and DNMT1 transient repression (Galonska et al. 2018). Transient induction of dCas9-DNMT3A increased global methylation in DKO cells with a preference for hypermethylated elements or H3K27ac-enriched regions in wild-type ES cells, such as exons and repetitive elements. In contrast, unmethylated sites, such as CpG islands associated with transcription start sites, remained generally hypomethylated (Galonska et al. 2018). In these maintenance competent cells, methylation was only retained at a subset of lowly transcribed genes after 7 days post-transfection at regions devoid of histone 3 K4me3 (Galonska et al. 2018). Also in wild-type HEK293 cells, where DNA methylation was written at thousands of CGIs upon 3 days of doxycycline-induced ZN-DNMT3A expression, the introduced methylation was rapidly lost at most of them (90%) (Broche et al. 2021). The partially stable methylated CGIs (~1000) were enriched in H3K27me3, reduced in H3K4me3 and H3K27ac, and without differences in K9me3, confirming a role for the native chromatin contexts determining permissiveness for stable editing (see Fig. 18.4).

The first pioneering studies already indicated the context-dependency of maintenance of DNA methylation (Stolzenburg et al. 2015; Kungulovski et al. 2015; Vojta et al. 2016). Stolzenburg and coworkers reported a persistent tumor repression linked to sustained DNA methylation on the *SOX2* oncogene promoter using ZF-DNMT3A effector in breast tumor cells, which was not observed for the ZF-KRAB fusion. Comparing different epigenetic effector domains (EED, DNMT3B, HDAC4) with the transcriptional repressor KRAB, also Bintu and coworkers demonstrated differential dynamics of repression, with epigenetic modulators being relatively ineffective also long-term, except for DNMT3B that induced sustained silencing up to 30 days (Bintu et al. 2016). Vizoso and van Rheenen provided evidence that targeted methylation of DNA, introduced by CRISPR-dCas9-DNMT3ACD, can be inherited by daughter cells for over 48 cell divisions. The authors used methyl-specific PCR (MS-PCR) to follow up sorted single clones, and bisulfite sequencing to confirm, and indicated long-term DNA methylation for 14 out of 18 clones at day 22. Two of these HEK293 clones, randomly selected, were again clonally expanded and the 24 subclones mostly maintained methylation values after an additional 22 days of culture. Taking advantage of dCas9 system coupled to DNMT3ACD plus C-terminal DNMT3L effector (dCas9-3ACD-^{C}3L) Saunderson and coworkers targeted the p16 promoter in primary breast cells. Also here, up to 35 days post-transient transfection, maintenance of p16 CpG hypermethylation and transcript downregulation was demonstrated when compared to dCas9-3ACD-^{C}3LΔ mutant, with sustained effects on cell proliferation and senescence processes (Saunderson et al. 2017).

Yet, writing DNA methylation does not necessarily result in long-term effects (Kungulovski et al. 2015; McDonald et al. 2016; Broche et al. 2021). Rewriting a combination of classes of epigenetic marks might provide a synergistic

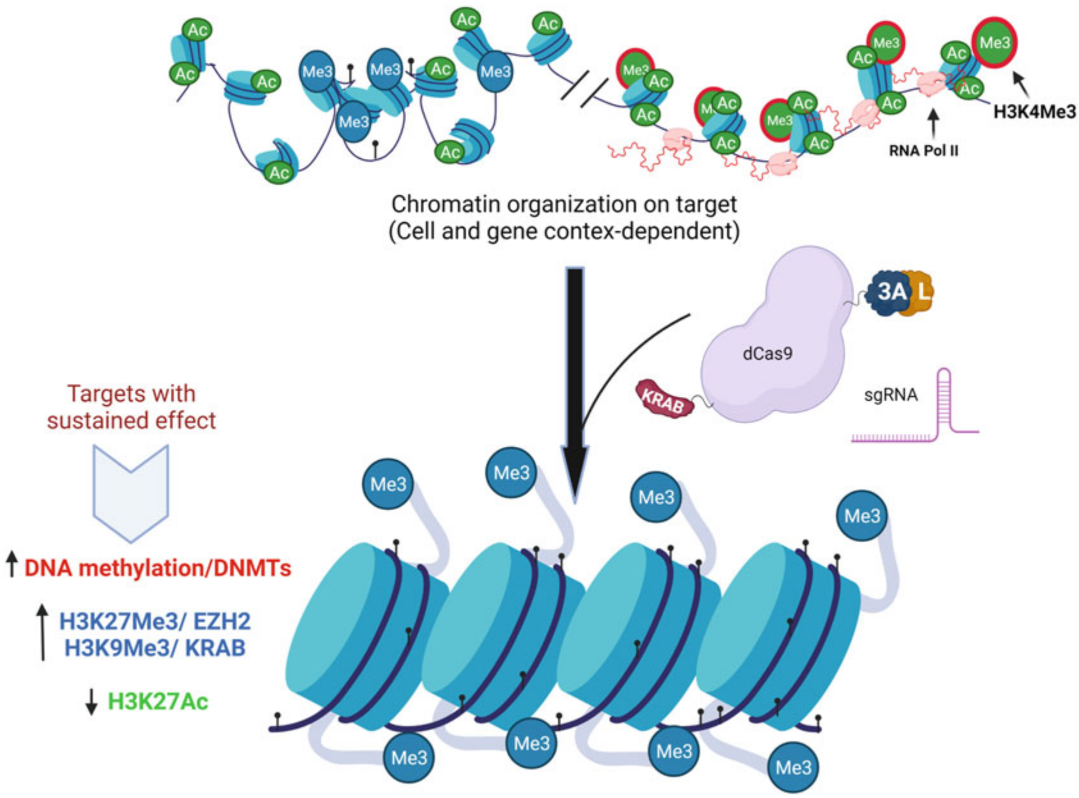

Fig. 18.4 Writing epigenetic marks to induce sustained transcriptional effects. A tripartite CRISPR-dCas9 configuration used for epigenetic long-lasting effects (KRAB-dCas9-DNMT3ACD/3L) is shown. Target genes showing mitotically sustained transcriptional reprogramming are commonly correlated with increase in DNA methylation and repressive marks on histone 3: H3K27Me3 or H3K9Me3, for EZH2 or KRAB effectors, respectively. Black hairpin decorations represent DNA methylation. Figure made in https://biorender.com

and more predictable approach towards inducing sustained silencing for subsets of genes. In this respect, an elegant system based on endogenous recruitment of epigenetic players at specific loci by nanobodies (single-domain antibodies), demonstrated that co-recruitment of DNMT1 synergistically improved the sustained downregulation of a reporter gene, induced by KRAB, DNMT3A, HP1 or HDAC4 (Van et al. 2021). The first proof of the combinational enhancement via targeting KRAB and de novo DNMT3A and DNMT3L effectors was described by Amabile and coworkers who demonstrated sustained silencing of three somatic genes (Amabile et al. 2016). Tarjan and coworkers demonstrated that dCas9-KRAB, dCas9-DNMT3A or dCas9-DNMT3A3L can selectively displace the protein insulator CTCF, with dCas9-KRAB achieving 83% of CTCF binding reduction, but the effect was not sustained. When dCas9-DNMT3A or dCas9-DNMT3A3L were transiently transfected, 20–40% of DNA methylation was detected over the targeted CTCF motif, with DNMT3A3L being more effective than DNMT3A (Tarjan et al. 2019). Here, the DNA methylation on the CTCF motif persisted (~20%) upon serial passage (12 days), when the dCas9 fusions were no longer detected, congruent with ~20% reduction in CTCF binding. Again, combined treatment with single chain double effector dCas9-DNMT3A3L plus dCas9-KRAB resulted in an enhancement of CTCF displacement and in a longer sustained response (up to 27 days) (Tarjan et al. 2019).

Similarly, other reports confirmed the effectiveness of co-targeting KRAB, DNMT3A and 3L effectors to achieve sustained epigenetics changes (Mlambo et al. 2018; Nakamura et al. 2021b; Nuñez et al. 2021), however again not all genes were responsive (Mlambo et al. 2018; Nuñez et al. 2021). Using a genome-wide screen and growth as read-out, Nuñez and coworkers indicated the general applicability of transient CRISPR-Off treatment (DNMT3A, DNMT3L and KRAB fused to one dCas9 protein) to induce effective and persistent gene silencing. Interestingly, although the long-lasting silencing was not obtained for all genes, CRISPR-Off was even effective for genes lacking canonical CpG islands or with a low CpG density (Nuñez et al. 2021).

To investigate the mechanisms of maintenance in more detail, Nakamura and coworkers generated a stable cell line (HEK293T) with GFP expression under the SV40 promoter regulation, and SV40-targeting guide RNAs. This reporter allowed to evaluate gene expression effects without the context-dependent restrictions of endogenous targets, which affect accessibility and activity of CRISPR-dCas9 (Nakamura et al. 2021b). Plasmids were transiently transfected, individually or combined (dCas9-KRAB, DNMT3A, and DNMT3L) to determine the best combination and the optimal positional configuration. To evaluate the long-lasting reprogramming, cells were cultured and periodically harvested up to 30 days post-transfection with Zeocin treatments for effector enrichment during these experiments. dCas9-KRAB significantly repressed GFP expression shortly after transient transfection, with subsequent recovery of expression at longer time scales. DNA methyltransferase domains individually exhibited minor ability to generate stable silencing. When cells were cotransfected using all three dCas9 effectors, a strong reduction in GFP expression was observed for weeks post-transfection. DNA methylation analysis showed a localized hypermethylation around the TSS and more extended repressive histone marks (H3K9me3) +/– 500 bp. After experimental pairwise domain analysis and testing modular swapping combinations, Nakamura and coworkers demonstrated that C-termini configuration for DNMTs, with first DNMT3L followed by 3A, was more effective for silencing. The addition of KRAB at the N-terminus showed the highest levels of stable gene repression, and KRAB swapping by SID effector (small temporary repression), or ZIM3/KRAB effector (twofold greater maximal repression) did not further improve sustained gene repression.

Exchanging KRAB for Ezh2 (Enhancer Zeste Homolog 2) did proof effective for a gene unresponsive to KRAB/3A/3L combinations: O'Geen and coworkers confirmed that combinatorial treatment with KRAB amino-terminal fused (KRAB-dCas9) and DNMT3A-dCas9 combined with ectopically overexpressed DNMT3L was able to initiate long-term repression for six out of seven targeted genes (O'Geen et al. 2019), but the combination failed to maintain persistence at *HER2* in HCT116 cells. The dCas9 treatment combinations (KRAB + DNMT3A + DNMT3L), triggered a strong burst in H3K9me3 at the target locus, but the repressive H3K9me3 mark was completely lost after 24 days. On the other hand, histone methyltransferase Ezh2 co-treatments (Ezh2-dCas9 + DNMT3A + DNMT3L) led to a long-term *HER2* repression (O'Geen et al. 2019), with both DNA and histone methylation (H3K27me3) marks maintained through approximately 57 cell divisions. Interestingly, full-length DNMT3L was essential for Ezh2-dCas9 mediated long-term repression, and the Carboxy-terminal hybrid dCas9-DNMT3L lacking the ADD domain fused to the DNMT3A catalytic domain (dCas9-DNMT3A/L) was unable to establish long-term epigenetic memory. This report again indicated that DNA or histone methylation alone are not always sufficient for long-term repression, but that the combination of epigenetic marks is important for predictable establishment and maintenance of epigenetic memory.

18.3.2 Sustained Gene Re-expression

Long-lasting effects on gene modulation via actively inducing locus-targeted DNA demethylation have also been reported. For example, Nakamura and coworkers assessed the possibility of dCas9-reprogrammed genes to be reactivated by transient expression of various dCas9-fusions, including dCas9-TET1 and TET2. Five days post-transfection, dCas-VP64, -VPR and -p300 demonstrated the strongest gene reactivation, with negligible effect for most of the tested epigenetic effectors (full length or catalytic domains; cloned at dCas9 N- or C-termini) (KDM3A, KDM4D, KDM7B, TDG). Targeting dCas9-TET1 and -TET2 did induce GFP re-expression, and more importantly, this re-expression was stably maintained for up to 60 days, while the dCasVP64, -VPR and -p300 reactivation was transient (Nakamura et al. 2021b). Also in CHO cells, Marx and coworkers demonstrated that by using dCas9-SunTag-TET1CD targeting a constitutively silenced gene (Beta-galactoside alpha-2,6-sialyltransferase 1 -*ST6GAL1*), a stable re-expression for more than 80 days was achieved (Marx et al. 2018). A stable reactivation induced by transient dCas9-TET1-CD expression was also confirmed for an enhancer involved in *FOXP3* expression in human T-cells, although this persistent demethylation status was not sufficient to induce a stable CD4+ regulatory T-cells (Tregs) phenotype (Kressler et al. 2020). In this respect, Okada and coworkers demonstrated that despite a partial lentiviral TET1-induced demethylation of this enhancer region of *Foxp3*, no stable gene expression was induced in mouse primary T-cells, while promoter-targeted dCas9-p300 did result in partially maintained *Foxp3* expression and functionality (Okada et al. 2017). Also for *Fgf21*, DNA promoter re-methylation occurred as measured 14 days after scFv-TET1CD transient transfection (Hanzawa et al. 2020). So, not all genes were equally permissive to sustained re-expression by targeting TET alone.

In fact, sustained re-expression was obtained only after simultaneous targeting of TET1-dCas9 and VPR-dCas9, inducing a persistent upregulation up to 30 days which was not achieved for either dCas9-fusion construct alone (Josipovic et al. 2019). Also Nuñez et al. demonstrated that combinations of TET1-dCas9 recruiting p65-AD (activation domain of NFkB subunit) and/or Rta (transcriptional activation domain of Epstein-Barr virus) via the MS2 system increased effectivity of targeting TET1 in re-expressing genes earlier silenced by KRAB-3A3L CRISPR-off (Nuñez et al. 2021). This study again elegantly showed that repressive epigenetic states can readily be reverted using epigenetic editing in a sustained manner.

18.4 In Vivo Transcriptional Modulation via DNA Methylation Epigenetic Editing

The technology of genome editing is rapidly advancing into the clinic with over 40 ZNF, TALEN and CRISPR-Cas9 studies ongoing (https://clinicaltrial.gov). Although mainly ex vivo, the first in vivo studies have been initiated making use of lentiviral vectors or AAVs (Adenoviral Associated Vectors). Since inducing mutations in the human genome, however, is subject of societal debate, epigenetic editing, which maintains integrity on the genome sequence without introducing mutations, is explored as a more versatile and less invasive approach, with potentially equal efficiency. Despite similar limitations, including off-targets and delivery effectivity, in vivo preclinical transcriptional modulation studies have shown therapeutic effectiveness. Indeed, artificial transcriptional factors (targeting KRAB, VP64, e.g., in CRISPRi/a (Geel et al. 2018; Nakamura et al. 2021a) have induced gene expression modulation in vivo, such as gene silencing in mouse brains (Zheng et al. 2018), or activation (Bustos et al. 2017), also in mouse models of muscular dystrophy/diabetes (Liao et al. 2017), cancer (Kretzmann et al. 2019) or obesity (Matharu et al. 2019). Unless stably expressed, such agents are thought to act transiently. Using gene targeting platforms to induce epigenetic modifications of DNA and histones bears the promise for gene expression modulation to be maintained for a long time. However, only few studies actually examined the in vivo effects of epigenetic writer or eraser effector domains (Gomez et al. 2019).

As discussed already in this review, aberrant DNA methylation is associated with disease development. Despite large and ongoing efforts of the scientific world to demonstrate that modulating DNA methylation interferes with dysregulated gene expression profiles, clinical applications of interfering with DNA methylation are limited to two inhibitors of DNMTs (azacitidine (Vidaza) and decitabine (Dacogen)), which are FDA approved to treat hematological malignancies. However, these hypomethylating agents have some limitations, including a low response rate, short duration of action, and lack of specificity (Berdasco and Esteller 2019). Gene-specific DNA (de)methylation tools are thus important in assessing the causal correlation between DNA methylation status, biological function and disease development. Additionally, DNA methylation editing tools open interesting avenues to, e.g., compensate for genetic mutations, prevent therapy resistance or otherwise interfere with pathophysiology. Eventually, investing in effective DNA methylation editing techniques gives therapeutic possibilities for the numerous diseases related with aberrant up- and downregulated gene expression levels.

The few in vivo DNA methylation epigenetic editing studies available to date, described below, show promising effects, demonstrating its exciting application to create innovative disease models as well as its potential therapeutic role in the clinic. The first published mouse studies made use of injecting stable, ex vivo transduced, inducible ZF-DNMT3a expressing tumor cells. These xenograft models clearly demonstrated the correlation between tumor growth and methylation state of either the *p16* (Cui et al. 2015) or the *SOX2* (Stolzenburg et al. 2015) promoters. Similarly, the role of *Crmp4* in inducing metastases was demonstrated in prostate cancer with all control mice developing metastases, whereas 8 out of 9 animals injected with prostate cancer cells expressing a TALE-TET1 fusion designed to target the gene did not (Li et al. 2015). Using the CRISPR system, a putative tumor suppressor gene was functionally validated in a colon cancer mouse model. Targeting TET1CD to the *SARI* promoter resulted in specific demethylation and substantial gene activation of *SARI*, which is frequently downregulated in several cancers (Wang et al. 2019). Injection of transfected cancer cells into the flank of nude mice resulted in smaller tumors compared to the controls, and less angiogenesis was observed as well. Although delivery issues hamper clinical translation of such methylation editing approaches in oncology, these tools offer unique opportunities to create disease models to better understand cancer biology (Weichenhan et al. 2020).

Before the adoption of epigenetic editing, no tools were available to directly demonstrate the correlation between epigenetic changes and disease. In recent years, the DNA methylation editing approach has gained attention to create epigenome-modified animals to explore epimutations in (epigenetic) diseases. For example, to understand the role of aberrant expression of the *H19-Igf2* genes, regulated by allele-specific DNA methylation in Silver-Russell syndrome (SRS), an imprinting mouse model was created by demethylating the paternally imprinted allele (Horii et al. 2020). In this study, three different methods were compared for efficiency: reprogramming ESCs, transient transfection or stable integration of the editor-expression cassette in fertilized ovules.

The first method involved transient transfection of ESCs with dCas9-SunTag/scFv-GFP-TET1CD implanted in the uterus after 4 weeks. Even though the extent of demethylation in almost all the animals obtained was higher compared to the other two methods (75% of target sequences were demethylated), the epigenetic changes of the genomic imprinting induced by the editing were not stably inherited. The second method generated animals by transient transfection of epigenetic editor mRNA into fertilized eggs. Compared to the previous one, this approach is applicable to most animal species. However, the modification observed at the blastocyst stage was low in frequency as well as in degree of demethylation, reflecting the instability of the reprogrammed epigenetic signature in vivo.

The third approach was based on continuous modification of the epigenome of animals by stable expression of epigenetic editors by transgenes introduced at the Rosa26 locus in fertilized ovules. Although a lower percentage (50–67%) of newborn mice as compared to the first method showed transgene integration, the integration was associated with significant demethylation at seven CpG sites in the H19-DMR promoter region. Importantly, these epigenetic changes were inherited by the next generation, creating an SRS mouse model. Comparison of the three mouse models generated demonstrated that stable integration upon dCas9-ED-sgRNA delivery is a realistic approach with a high percentage of vector-integrated animals, which showed a constant expression of the epi-editor over time. However, off-target effects are a serious problem. In fact, the stable expression of epigenome-modifying factors induced DNA demethylation in two predicted off-target regions for gRNA of H19DMR_10 (2 mismatches) and H19DMR_11 (2 mismatches). This indicates that this approach could increase the risk of off-target epigenome modification.

Alternatively, zygote microinjection of CRISPR-dCas9 tools has been used to create animal models of imprinting (Lei et al. 2017) and neurological (Lu et al. 2020) disorders. In the first, in vivo locus-specific DNA methylation was inherited for up to 3 weeks from mouse birth. Targeting CpGs of the imprinted locus of *Igf2/H19* in mice, dCas-MQ1^{Q147L} stably increased DNA methylation demonstrating the possibility to modify the methylation status of a specific gene in the early stage of embryonic development, which was maintained during cellular differentiation processes (Lei et al. 2017). This is a clear demonstration of the potency to use dCas9-MQ1^{Q147L} to introduce site-specific DNA methylation with high activity and specificity. It suggests its broad applications for the study of gene dysregulation in various disease contexts.

Zygote microinjection was also used to create a disease model for autism spectrum disorders (ASD): targeting *Mecp2* by microinjecting dCas9-DNMT3A/3L decreased the expression of *Mecp2* resulting in ASD behavior as measured up to 8 weeks after birth. These data demonstrated that DNA methylation at the *Mecp2* promoter contributes to ASD pathology and suggest that changing *Mecp2* gene expression improves treatment outcomes in individuals with ASD. The authors also applied AAV infection to express dCas9-DNMT3A/3L in the hippocampus, thereby highlighting epigenetic editing opportunities for therapeutic intervention (Lu et al. 2020).

Effective interference using epigenetic editing was also demonstrated at a later developmental stage (in utero). dCas9-SunTag-TET1CD was successfully introduced in isolated neural precursor cells (NPCs) from mouse embryos by electroporation to reactivate the expression of *Gfap* in order to induce the differentiation of NPCs into astrocytes (Morita et al. 2016). As one cytosine in the *Gfap* gene promoter is methylated in most cell types, except for astrocytes, targeted demethylation of this site was hypothesized to play a critical role in the differentiation of NPCs into astrocytes. Implantation of transfected NPCs into the ventricular zone of mouse fetal brain in utero resulted in increased expression of *Gfap*. With this article, the authors demonstrated the feasibility of implanting functionally reprogrammed cells in vivo early in development.

Using a lentiviral delivery approach, Liu and coworkers confirmed the possibility to effectively alter the methylation status and regulate the expression of a neurological gene in adult mice. Microinjection of dCas9-TET1 in the brains of GFP-transgenic mice to induce demethylation of the *Snrpn* promoter driving GFP resulted in 70% activation of GFP (Liu et al. 2016). This study set the stage to address Fragile X syndrome (FXS), the most common form of mental disability, associated with methylation-induced silencing of the *Fmr1* gene. To date, there is no effective cure for this disease. FX52 neuronal precursor cells (NPCs) were infected to express dCas9-TET1 targeting *Fmr1*, and then implanted in newborn mice brains, to study the effect of DNA methylation on *Fmr1* gene expression in vivo (Liu et al. 2018). In mice lacking *Fmr1* expression, dCas9-TET1 opened the heterochromatin state of the

Fmr1 promoter region, inducing its expression up to 1–3 months after NPCs transplantation. The increase in gene expression restored the normal condition of FXS neurons, reversing the abnormal electrophysiological phenotype, which is close to a possible therapeutic application (Liu et al. 2018). These results, retained in adult mice upon implantation in newborns, open new possibilities in this field, not only to better understand the physiology of the disease, but also to investigate its use as a potential therapeutic approach.

DNA methylation editing findings further demonstrate that epigenetic mechanisms drive pathology in neurodevelopmental disorders and confirm various neuroepigenetic editing studies using other epigenetic effector domains (Xu and Heller 2019), even in inducing differential splicing (Xu et al. 2021), which point out the use of epigenetic editing as a promising therapeutic approach for neurodevelopmental disorders. Other pathophysiologies addressed in in vivo DNA methylation editing studies concern metabolic disorders (Ou et al. 2019; Hanzawa et al. 2020) and fibrosis (Xu et al. 2018). To further understand the role of DNA demethylation on the obesity-related fibroblast growth factor 21 (*Fgf21*) gene expression in the liver, dCas9-SunTag and scFv-TET1CD were introduced into the liver of PPARα-KO mice by hydrodynamic injection into the tail vein (HTVi) (Hanzawa et al. 2020). PPARα, a nuclear receptor regulating the transcription of major genes related to hepatic metabolism, is thought to induce *Fgf21* expression via DNA demethylation, but the exact mechanism is unclear. The use of non-specific DNA methyltransferase inhibitors that demethylate the genome globally only indirectly helps to understand such specific gene regulation. Epigenetic editing, uniquely suited to address a single gene, allowed to unravel the role of epigenetic regulation mechanisms. The *Fgf21* PPARα-KO model validated that altered DNA methylation of *Fgf21* is indeed causally related to the biological activation.

Another in vivo study addressing metabolic diseases exploited the TALE platform to target TET1 to the methylated promoter of *ICR2* gene, which upon re-expression repressed p57, inducing growth of β cells, which are dysfunctional in diabetes (Ou et al. 2019). Transplantation of the TALE-TET1 expressing β cells was shown to increase proliferation, and this ex vivo approach comes very close to a possible therapeutic application for diabetic patients.

Although the above DNA demethylation in vivo studies exploited TET1 as effector domain, TET3CD was also successfully used to induce the reactivation of *Rasal1* and *Klotho* in interstitial fibroblasts and in renal tubular epithelial cells, respectively, in the unilateral ureter obstruction mouse model of nephropathy. Both genes are highly hypermethylated in these cells and their downregulation is associated with fibrosis. Using lentiviral delivery (intraparenchymal for *Rasal1*, ureter retrograde for *Klotho*), a high-fidelity dCas9 fusion (dHFCas9-TET3CD) decreased off-targets by 85% compared to conventional dCas9. Targeting the two fibrotic genes led to a reduction of 50% and 25% in the production of fibroblasts, respectively and subsequently reduced renal fibrosis (Xu et al. 2018). Combined with the ongoing efforts to improve maintenance, specificity and delivery, more in vivo preclinical studies are expected to further spark the interest for epigenetic editing, not only in providing potent disease models, but to be considered as a versatile therapeutic approach in the fight against currently uncurable diseases.

18.5 Further Considerations

Application of epigenetic editing technology in human health is desirable, as it opens novel avenues for diseases where currently no treatment or cure options are within sight. Clinical translation, however, is still challenging, although ongoing developments in applying CRISPR-Cas gene editing will certainly pave the way in overcoming delivery and off-target issues. Viruses are frequently used for efficient delivery. To circumvent the potentially harmful host genome integrations by lentiviruses, AAVs have been shown to effectively deliver dCas constructs (Thakore et al. 2018; Kemaladewi et al. 2019; Lu et al. 2020;

Matharu et al. 2019) and to exhibit low immunogenicity (Levy et al. 2020; Wu et al. 2021). Despite this, AAVs come with some limitations specific to epigenetic editing. The size of AAV restricts its application for in vivo epigenetic editing due to the inability to carry large transgenes needed to encode the fusions of the epigenetic effector domains (Colella et al. 2018). Based on the hit-and-run promise of epigenetic editing (Amabile et al. 2016; Saunderson et al. 2017), episomally maintained AAVs might not be needed for effective therapeutic effects and transient administration of proteins directly (Bailus et al. 2016) or by, e.g., lipid nanoparticles containing protein/RNA/DNA could thus be useful for future applications with effectivity shown in the first in vivo CRISPR-Cas9 trial (Gillmore et al. 2021). Indeed, advances were obtained in delivery technologies, with physical (electroporation, microinjection), chemical (lipids, polymers, nanomaterials) and biological alternatives, besides (viral) vectors. As alternative to using DNA as cargo, direct delivery of the sgRNA and dCas fusion mRNA (or protein as ribonucleoprotein (RNP)) is a very interesting and promising approach for in vivo application delivery (Wei et al. 2020; Qiu et al. 2021) as lower controllable cellular levels might reduce the off-target effects. Delivery systems based on extracellular vesicles (EVs) have shown to be an interesting approach for therapeutic genome editing (Chen et al. 2021). Also for CRISPRa delivery, applicability of EVs as vehicles has been demonstrated in mice by incorporating sgRNA and dCas9 proteins (Lainscek et al. 2018). More recently, further preclinical proof of EV-mediated delivery of CRISPR-dCas9-VP64 was reported for liver fibrosis treatment (Luo et al. 2021).

To further improve selectivity, light-inducible approaches seem versatile, responsive, precise and reversible (Wu et al. 2021); however, short wave excitation limits its application at in vivo level. To get over this hurdle, near-infrared optical control has been proposed (Chen et al. 2020). On the other hand, concerns regarding off-target effects might turn out to be less significant for epigenetic editing versus genetic engineering: Cas9-mediated double-strand breaks can be induced by the (unspecific) binding of one Cas9 molecule, while various events are thought to be required in epigenetic editing to achieve gene expression modulation. Indeed, various combinations of effector domains are required for sustained expression modulation (Amabile et al. 2016; Josipovic et al. 2019; O'Geen et al. 2019; Halmai et al. 2020; Nuñez et al. 2021; Nakamura et al. 2021b), offering options to further reduce the off-target toxic effects. Importantly, the (off-target) stable reprogramming can be reversed by targeting counteracting enzymes (Amabile et al. 2016; Nuñez et al. 2021), allowing possibilities to reset the intervention. So, although the goal to reach to a system that allows straightforward and very efficient sustained gene expression modulation, with low off-target and immunological effects, seems far, companies are founded and developments are promising with exciting results obtained.

18.6 Conclusions

The study of DNA methylation in vivo is rapidly developing, and helps to understand epigenetic dysregulations at the single gene level and its association with disease. By direct interference at the level of DNA methylation, restoration of cellular function can be induced. As discussed in this review, some stumbling blocks have slowed the development of epigenetic editing, but ongoing technological improvements (especially sustained reprogramming) and the increasing list of preclinical therapeutic successes spark a wide interest to develop methylation-based epigenetic editing strategies for a wide variety of diseases. As any genomic locus can be targeted, epigenetic editing might open avenues for diseases without any current treatment options.

Acknowledgements The authors thank Sabine Stolzenburg for her assistance in writing the chapter on targeted methylation in the earlier book version (Stolzenburg et al. 2016). FCM acknowledges his Scholarship funding from the Colombian Ministry of Science, Technology and Innovation (Minciencias -COLCIENCIAS/COLFUTURO-Doctorados en el exterior 2017 N°783) and Instituto Tecnológico Metropolitano

(ITM). FS is supported by VALERE program, Vanvitelli per la Ricerca. H2020 European Cooperation in Science and Technology (COST) Training Actions (www.INC-COST.eu and www.EpiChemBio.eu) are acknowledged for facilitating network activities.

References

Amabile A, Migliara A, Capasso P, Biffi M, Cittaro D, Naldini L, Lombardo A (2016) Inheritable silencing of endogenous genes by hit-and-run targeted epigenetic editing. Cell 167:219–232 e14

Bailus BJ, Pyles B, Mcalister MM, O'Geen H, Lockwood SH, Adams AN, Nguyen JT, Yu A, Berman RF, Segal DJ (2016) Protein delivery of an artificial transcription factor restores widespread Ube3a expression in an Angelman syndrome mouse brain. Mol Ther 24:548–555

Becker S, Boch J (2021) TALE and TALEN genome editing technologies. Gene Genome Editing 2:100007

Berdasco M, Esteller M (2019) Clinical epigenetics: seizing opportunities for translation. Nat Rev Genet 20:109–127

Bernstein DL, Le Lay JE, Ruano EG, Kaestner KH (2015) TALE-mediated epigenetic suppression of CDKN2A increases replication in human fibroblasts. J Clin Invest 125:1998–2006

Bintu L, Yong J, Antebi YE, Mccue K, Kazuki Y, Uno N, Oshimura M, Elowitz MB (2016) Dynamics of epigenetic regulation at the single-cell level. Science 351:720–724

Broche J, Kungulovski G, Bashtrykov P, Rathert P, Jeltsch A (2021) Genome-wide investigation of the dynamic changes of epigenome modifications after global DNA methylation editing. Nucleic Acids Res 49:158–176

Bustos FJ, Ampuero E, Jury N, Aguilar R, Falahi F, Toledo J, Ahumada J, Lata J, Cubillos P, Henríquez B, Guerra MV, Stehberg J, Neve RL, Inestrosa NC, Wyneken U, Fuenzalida M, Härtel S, Sena-Esteves M, Varela-Nallar L, Rots MG, Montecino M, van Zundert B (2017) Epigenetic editing of the Dlg4/PSD95 gene improves cognition in aged and Alzheimer's disease mice. Brain 140:3252–3268

Cano-Rodriguez D, Gjaltema R, Jilderda R, Jellema P, Dokter-Fokkens J, Ruiters M, Rots MG (2016) Writing of H3K4Me3 overcomes epigenetic silencing in a sustained but context-dependent manner. Nat Commun 7:1–11

Chen H, Kazemier HG, De Groote ML, Ruiters MH, Xu GL, Rots MG (2014) Induced DNA demethylation by targeting ten-eleven translocation 2 to the human ICAM-1 promoter. Nucleic Acids Res 42:1563–1574

Chen Y, Yan X, Ping Y (2020) Optical manipulation of CRISPR/Cas9 functions: from ultraviolet to near-infrared light. ACS Mater Lett 2(6):644–653

Chen H, Wang L, Zeng X, Schwarz H, Nanda HS, Peng X, Zhou Y (2021) Exosomes, a new star for targeted delivery. Front Cell Dev Biol 9:751079

Choudhury SR, Cui Y, Lubecka K, Stefanska B, Irudayaraj J (2016) CRISPR-dCas9 mediated TET1 targeting for selective DNA demethylation at BRCA1 promoter. Oncotarget 7:46545–46556

Colella P, Ronzitti G, Mingozzi F (2018) Emerging issues in AAV-mediated in vivo gene therapy. Mol Ther Methods Clin Dev 8:87–104

Cui C, Gan Y, Gu L, Wilson J, Liu Z, Zhang B, Deng D (2015) P16-specific DNA methylation by engineered zinc finger methyltransferase inactivates gene transcription and promotes cancer metastasis. Genome Biol 16:252

de Groote ML, Verschure PJ, Rots MG (2012) Epigenetic Editing: targeted rewriting of epigenetic marks to modulate expression of selected target genes. Nucleic Acids Res 40:10596–10613

Devesa-Guerra I, Morales-Ruiz T, Perez-Roldan J, Parrilla-Doblas JT, Dorado-Leon M, Garcia-Ortiz MV, Ariza RR, Roldan-Arjona T (2020) DNA methylation editing by CRISPR-guided excision of 5-methylcytosine. J Mol Biol 432:2204–2216

Ehrlich M (2019) DNA hypermethylation in disease: mechanisms and clinical relevance. Epigenetics 14:1141–1163

Felsenfeld G (2014) A brief history of epigenetics. Cold Spring Harb Perspect Biol 6

Galonska C, Charlton J, Mattei AL, Donaghey J, Clement K, Gu H, Mohammad AW, Stamenova EK, Cacchiarelli D, Klages S, Timmermann B, Cantz T, Scholer HR, Gnirke A, Ziller MJ, Meissner A (2018) Genome-wide tracking of dCas9-methyltransferase footprints. Nat Commun 9:597

Geel TM, Ruiters MHJ, Cool RH, Halby L, Voshart DC, Andrade Ruiz L, Niezen-Koning KE, Arimondo PB, Rots MG (2018) The past and presence of gene targeting: from chemicals and DNA via proteins to RNA. Philos Trans R Soc Lond B Biol Sci 373

Gillmore JD, Gane E, Taubel J, Kao J, Fontana M, Maitland ML, Seitzer J, O'Connell D, Walsh KR, Wood K, Phillips J, Xu Y, Amaral A, Boyd AP, Cehelsky JE, Mckee MD, Schiermeier A, Harari O, Murphy A, Kyratsous CA, Zambrowicz B, Soltys R, Gutstein DE, Leonard J, Sepp-Lorenzino L, Lebwohl D (2021) CRISPR-Cas9 in vivo gene editing for transthyretin amyloidosis. N Engl J Med 385:493–502

Gomez JA, Beitnere U, Segal DJ (2019) Live-animal epigenome editing: convergence of novel techniques. Trends Genet 35:527–541

Greenberg MVC, Bourc'his D (2019) The diverse roles of DNA methylation in mammalian development and disease. Nat Rev Mol Cell Biol 20:590–607

Gregory DJ, Zhang Y, Kobzik L, Fedulov AV (2013) Specific transcriptional enhancement of inducible nitric oxide synthase by targeted promoter demethylation. Epigenetics 8:1205–1212

Halmai J, Deng P, Gonzalez CE, Coggins NB, Cameron D, Carter JL, Buchanan FKB, Waldo JJ, Lock SR, Anderson JD, O'Geen H, Segal DJ, Nolta J, Fink KD (2020) Artificial escape from XCI by DNA methylation editing of the CDKL5 gene. Nucleic Acids Res 48:2372–2387

Hanzawa N, Hashimoto K, Yuan X, Kawahori K, Tsujimoto K, Hamaguchi M, Tanaka T, Nagaoka Y, Nishina H, Morita S, Hatada I, Yamada T, Ogawa Y (2020) Targeted DNA demethylation of the Fgf21 promoter by CRISPR/dCas9-mediated epigenome editing. Sci Rep 10:5181

Hofacker D, Broche J, Laistner L, Adam S, Bashtrykov P, Jeltsch A (2020) Engineering of effector domains for targeted DNA methylation with reduced off-target effects. Int J Mol Sci 21

Horii T, Morita S, Hino S, Kimura M, Hino Y, Kogo H, Nakao M, Hatada I (2020) Successful generation of epigenetic disease model mice by targeted demethylation of the epigenome. Genome Biol 21:77

Huang YH, Su J, Lei Y, Brunetti L, Gundry MC, Zhang X, Jeong M, Li W, Goodell MA (2017) DNA epigenome editing using CRISPR-Cas SunTag-directed DNMT3A. Genome Biol 18:176

Huisman C, Van Der Wijst MG, Schokker M, Blancafort P, Terpstra MM, Kok K, Van Der Zee AG, Schuuring E, Wisman GB, Rots MG (2016) Re-expression of selected epigenetically silenced candidate tumor suppressor genes in cervical cancer by TET2-directed demethylation. Mol Ther 24:536–547

Huo M, Zhang J, Huang W, Wang Y (2021) Interplay among metabolism, epigenetic modifications, and gene expression in cancer. Front Cell Dev Biol 9: 793428

Jain S, Shukla S, Yang C, Zhang M, Fatma Z, Lingamaneni M, Abesteh S, Lane ST, Xiong X, Wang Y, Schroeder CM, Selvin PR, Zhao H (2021) TALEN outperforms Cas9 in editing heterochromatin target sites. Nat Commun 12:606

Jeziorska DM, Murray RJS, De Gobbi M, Gaentzsch R, Garrick D, Ayyub H, Chen T, Li E, Telenius J, Lynch M, Graham B, Smith AJH, Lund JN, Hughes JR, Higgs DR, Tufarelli C (2017) DNA methylation of intragenic CpG islands depends on their transcriptional activity during differentiation and disease. Proc Natl Acad Sci U S A 114:E7526–E7535

Josipovic G, Tadic V, Klasic M, Zanki V, Beceheli I, Chung F, Ghantous A, Keser T, Madunic J, Boskovic M, Lauc G, Herceg Z, Vojta A, Zoldos V (2019) Antagonistic and synergistic epigenetic modulation using orthologous CRISPR/dCas9-based modular system. Nucleic Acids Res 47:9637–9657

Jurkowski TP, Ravichandran M, Stepper P (2015) Synthetic epigenetics-towards intelligent control of epigenetic states and cell identity. Clin Epigenetics 7:18

Kemaladewi DU, Bassi PS, Erwood S, Al-Basha D, Gawlik KI, Lindsay K, Hyatt E, Kember R, Place KM, Marks RM, Durbeej M, Prescott SA, Ivakine EA, Cohn RD (2019) A mutation-independent approach for muscular dystrophy via upregulation of a modifier gene. Nature 572:125–130

Kressler C, Gasparoni G, Nordstrom K, Hamo D, Salhab A, Dimitropoulos C, Tierling S, Reinke P, Volk HD, Walter J, Hamann A, Polansky JK (2020) Targeted de-methylation of the FOXP3-TSDR is sufficient to induce physiological FOXP3 expression but not a functional treg phenotype. Front Immunol 11: 609891

Kretzmann JA, Evans CW, Moses C, Sorolla A, Kretzmann AL, Wang E, Ho D, Hackett MJ, Dessauvagie BF, Smith NM, Redfern AD, Waryah C, Norret M, Iyer KS, Blancafort P (2019) Tumour suppression by targeted intravenous non-viral CRISPRa using dendritic polymers. Chem Sci 10:7718–7727

Kungulovski G, Nunna S, Thomas M, Zanger UM, Reinhardt R, Jeltsch A (2015) Targeted epigenome editing of an endogenous locus with chromatin modifiers is not stably maintained. Epigenetics Chromatin 8:12

Lainscek D, Kadunc L, Keber MM, Bratkovic IH, Romih R, Jerala R (2018) Delivery of an artificial transcription regulator dCas9-VPR by extracellular vesicles for therapeutic gene activation. ACS Synth Biol 7:2715–2725

Ledhord H (2018) First test of in-body gene editing shows promise. Nature. https://doi.org/10.1038/d41586-018-06195-6

Lei Y, Zhang X, Su J, Jeong M, Gundry MC, Huang YH, Zhou Y, Li W, Goodell MA (2017) Targeted DNA methylation in vivo using an engineered dCas9-MQ1 fusion protein. Nat Commun 8:16026

Levy JM, Yeh WH, Pendse N, Davis JR, Hennessey E, Butcher R, Koblan LW, Comander J, Liu Q, Liu DR (2020) Cytosine and adenine base editing of the brain, liver, retina, heart and skeletal muscle of mice via adeno-associated viruses. Nat Biomed Eng 4:97–110

Li K, Pang J, Cheng H, Liu WP, Di JM, Xiao HJ, Luo Y, Zhang H, Huang WT, Chen MK, Li LY, Shao CK, Feng YH, Gao X (2015) Manipulation of prostate cancer metastasis by locus-specific modification of the CRMP4 promoter region using chimeric TALE DNA methyltransferase and demethylase. Oncotarget 6:10030–10044

Liao HK, Hatanaka F, Araoka T, Reddy P, Wu MZ, Sui Y, Yamauchi T, Sakurai M, O'Keefe DD, Nunez-Delicado E, Guillen P, Campistol JM, Wu CJ, Lu LF, Esteban CR, Izpisua Belmonte JC (2017) In vivo target gene activation via CRISPR/Cas9-mediated trans-epigenetic modulation. Cell 171:1495–1507 e15

Lin L, Liu Y, Xu F, Huang J, Daugaard TF, Petersen TS, Hansen B, Ye L, Zhou Q, Fang F, Yang L, Li S, Floe L, Jensen KT, Shrock E, Chen F, Yang H, Wang J, Liu X, Xu X, Bolund L, Nielsen AL, Luo Y (2018) Genome-wide determination of on-target and off-target characteristics for RNA-guided DNA methylation by dCas9 methyltransferases. Gigascience 7:1–19

Liu XS, Wu H, Ji X, Stelzer Y, Wu X, Czauderna S, Shu J, Dadon D, Young RA, Jaenisch R (2016) Editing DNA methylation in the mammalian genome. Cell 167:233–247 e17

Liu XS, Wu H, Krzisch M, Wu X, Graef J, Muffat J, Hnisz D, Li CH, Yuan B, Xu C, Li Y, Vershkov D, Cacace A, Young RA, Jaenisch R (2018) Rescue of fragile X syndrome neurons by DNA methylation editing of the FMR1 gene. Cell 172:979–992 e6

Lo CL, Choudhury SR, Irudayaraj J, Zhou FC (2017) Epigenetic editing of Ascl1 gene in neural stem cells by optogenetics. Sci Rep 7:42047

Lu Z, Liu Z, Mao W, Wang X, Zheng X, Chen S, Cao B, Huang S, Zhang X, Zhou T, Zhang Y, Huang X, Sun Q, Li JD (2020) Locus-specific DNA methylation of Mecp2 promoter leads to autism-like phenotypes in mice. Cell Death Dis 11:85

Luo N, Li J, Chen Y, Xu Y, Wei Y, Lu J, Dong R (2021) Hepatic stellate cell reprogramming via exosome-mediated CRISPR/dCas9-VP64 delivery. Drug Deliv 28:10–18

Maeder ML, Angstman JF, Richardson ME, Linder SJ, Cascio VM, Tsai SQ, Ho QH, Sander JD, Reyon D, Bernstein BE, Costello JF, Wilkinson MF, Joung JK (2013) Targeted DNA demethylation and activation of endogenous genes using programmable TALE-TET1 fusion proteins. Nat Biotechnol 31:1137–1142

Marx N, Grunwald-Gruber C, Bydlinski N, Dhiman H, Ngoc Nguyen L, Klanert G, Borth N (2018) CRISPR-based targeted epigenetic editing enables gene expression modulation of the silenced beta-galactoside alpha-2,6-sialyltransferase 1 in CHO cells. Biotechnol J 13: e1700217

Matharu N, Rattanasopha S, Tamura S, Maliskova L, Wang Y, Bernard A, Hardin A, Eckalbar WL, Vaisse C, Ahituv N (2019) CRISPR-mediated activation of a promoter or enhancer rescues obesity caused by haploinsufficiency. Science 363

McDonald JI, Celik H, Rois LE, Fishberger G, Fowler T, Rees R, Kramer A, Martens A, Edwards JR, Challen GA (2016) Reprogrammable CRISPR/Cas9-based system for inducing site-specific DNA methylation. Biol Open 5:866–874

Mlambo T, Nitsch S, Hildenbeutel M, Romito M, Muller M, Bossen C, Diederichs S, Cornu TI, Cathomen T, Mussolino C (2018) Designer epigenome modifiers enable robust and sustained gene silencing in clinically relevant human cells. Nucleic Acids Res 46: 4456–4468

Morita S, Noguchi H, Horii T, Nakabayashi K, Kimura M, Okamura K, Sakai A, Nakashima H, Hata K, Nakashima K, Hatada I (2016) Targeted DNA demethylation in vivo using dCas9-peptide repeat and scFv-TET1 catalytic domain fusions. Nat Biotechnol 34: 1060–1065

Nakamura M, Ivec AE, Gao Y, Qi LS (2021a) Durable CRISPR-based epigenetic silencing. BioDesign Res 8

Nakamura M, Gao Y, Dominguez AA, Qi LS (2021b) CRISPR technologies for precise epigenome editing. Nat Cell Biol 23:11–22

Nicoglou A, Merlin F (2017) Epigenetics: A way to bridge the gap between biological fields. Stud Hist Philos Biol Biomed Sci 66:73–82

Nuñez JK, Chen J, Pommier GC, Cogan JZ, Replogle JM, Adriaens C, Ramadoss GN, Shi Q, Hung KL, Samelson AJ, Pogson AN, Kim JYS, Chung A, Leonetti MD, Chang HY, Kampmann M, Bernstein BE, Hovestadt V, Gilbert LA, Weissman JS (2021) Genome-wide programmable transcriptional memory by CRISPR-based epigenome editing. Cell 184:2503–2519 e17

O'Geen H, Bates SL, Carter SS, Nisson KA, Halmai J, Fink KD, Rhie SK, Farnham PJ, Segal DJ (2019) Ezh2-dCas9 and KRAB-dCas9 enable engineering of epigenetic memory in a context-dependent manner. Epigenetics Chromatin 12:26

Okada M, Kanamori M, Someya K, Nakatsukasa H, Yoshimura A (2017) Stabilization of Foxp3 expression by CRISPR-dCas9-based epigenome editing in mouse primary T cells. Epigenetics Chromatin 10:24

Onodera A, Gonzalez-Avalos E, Lio CJ, Georges RO, Bellacosa A, Nakayama T, Rao A (2021) Roles of TET and TDG in DNA demethylation in proliferating and non-proliferating immune cells. Genome Biol 22: 186

Ou K, Yu M, Moss NG, Wang YJ, Wang AW, Nguyen SC, Jiang C, Feleke E, Kameswaran V, Joyce EF, Naji A, Glaser B, Avrahami D, Kaestner KH (2019) Targeted demethylation at the CDKN1C/p57 locus induces human beta cell replication. J Clin Invest 129:209–214

Petryk N, Bultmann S, Bartke T, Defossez PA (2021) Staying true to yourself: mechanisms of DNA methylation maintenance in mammals. Nucleic Acids Res 49: 3020–3032

Pflueger C, Tan D, Swain T, Nguyen T, Pflueger J, Nefzger C, Polo JM, Ford E, Lister R (2018) A modular dCas9-SunTag DNMT3A epigenome editing system overcomes pervasive off-target activity of direct fusion dCas9-DNMT3A constructs. Genome Res 28: 1193–1206

Policarpi C, Dabin J, Hackett JA (2021) Epigenetic editing: Dissecting chromatin function in context. Bioessays 43:e2000316

Qiu M, Glass Z, Chen J, Haas M, Jin X, Zhao X, Rui X, Ye Z, Li Y, Zhang F, Xu Q (2021) Lipid nanoparticle-mediated codelivery of Cas9 mRNA and single-guide RNA achieves liver-specific in vivo genome editing of Angptl3. Proc Natl Acad Sci U S A 118

Qu J, Zhu L, Zhou Z, Chen P, Liu S, Locy ML, Thannickal VJ, Zhou Y (2018) Reversing mechanoinductive DSP expression by CRISPR/dCas9-mediated epigenome editing. Am J Respir Crit Care Med 198:599–609

Rivenbark AG, Stolzenburg S, Beltran AS, Yuan X, Rots MG, Strahl BD, Blancafort P (2012) Epigenetic reprogramming of cancer cells via targeted DNA methylation. Epigenetics 7:350–360

Sapozhnikov DM, Szyf M (2021) Unraveling the functional role of DNA demethylation at specific promoters by targeted steric blockage of DNA methyltransferase with CRISPR/dCas9. Nat Commun 12:5711

Saunderson EA, Stepper P, Gomm JJ, Hoa L, Morgan A, Allen MD, Jones JL, Gribben JG, Jurkowski TP, Ficz G (2017) Hit-and-run epigenetic editing prevents senescence entry in primary breast cells from healthy donors. Nat Commun 8:1450

Sgro A, Blancafort P (2020) Epigenome engineering: new technologies for precision medicine. Nucleic Acids Res 48:12453–12482

Shayevitch R, Askayo D, Keydar I, Ast G (2018) The importance of DNA methylation of exons on alternative splicing. RNA 24:1351–1362

Siddique AN, Nunna S, Rajavelu A, Zhang Y, Jurkowska RZ, Reinhardt R, Rots MG, Ragozin S, Jurkowski TP, Jeltsch A (2013) Targeted methylation and gene silencing of VEGF-A in human cells by using a designed Dnmt3a-Dnmt3L single-chain fusion protein with increased DNA methylation activity. J Mol Biol 425: 479–491

Slaska-Kiss K, Zsibrita N, Koncz M, Albert P, Csabradi A, Szentes S, Kiss A (2021) Lowering DNA binding affinity of SssI DNA methyltransferase does not enhance the specificity of targeted DNA methylation in E. coli. Sci Rep 11:15226

Stepper P, Kungulovski G, Jurkowska RZ, Chandra T, Krueger F, Reinhardt R, Reik W, Jeltsch A, Jurkowski TP (2017) Efficient targeted DNA methylation with chimeric dCas9-Dnmt3a-Dnmt3L methyltransferase. Nucleic Acids Res 45:1703–1713

Stolzenburg S, Beltran AS, Swift-Scanlan T, Rivenbark AG, Rashwan R, Blancafort P (2015) Stable oncogenic silencing in vivo by programmable and targeted de novo DNA methylation in breast cancer. Oncogene 34:5427–5435

Stolzenburg S, Goubert D, Rots MG (2016) Rewriting DNA methylation signatures at will: the curable genome within reach? Adv Exp Med Biol 945:475–490

Suzuki T, Maeda S, Furuhata E, Shimizu Y, Nishimura H, Kishima M, Suzuki H (2017) A screening system to identify transcription factors that induce binding site-directed DNA demethylation. Epigenetics Chromatin 10:60

Tarjan DR, Flavahan WA, Bernstein BE (2019) Epigenome editing strategies for the functional annotation of CTCF insulators. Nat Commun 10:4258

Thakore PI, Kwon JB, Nelson CE, Rouse DC, Gemberling MP, Oliver ML, Gersbach CA (2018) RNA-guided transcriptional silencing in vivo with S. aureus CRISPR-Cas9 repressors. Nat Commun 9:1674

Tiane A, Schepers M, Riemens R, Rombaut B, Vandormael P, Somers V, Prickaerts J, Hellings N, Van Den Hove D, Vanmierlo T (2021) DNA methylation regulates the expression of the negative transcriptional regulators ID2 and ID4 during OPC differentiation. Cell Mol Life Sci 78:6631–6644

Van MV, Fujimori T, Bintu L (2021) Nanobody-mediated control of gene expression and epigenetic memory. Nat Commun 12:537

Vizoso M, Van Rheenen J (2021) Diverse transcriptional regulation and functional effects revealed by CRISPR/Cas9-directed epigenetic editing. Oncotarget 12:1651–1662

Vojta A, Dobrinic P, Tadic V, Bockor L, Korac P, Julg B, Klasic M, Zoldos V (2016) Repurposing the CRISPR-Cas9 system for targeted DNA methylation. Nucleic Acids Res 44:5615–5628

Wang Q, Dai L, Wang Y, Deng J, Lin Y, Wang Q, Fang C, Ma Z, Wang H, Shi G, Cheng L, Liu Y, Chen S, Li J, Dong Z, Su X, Yang L, Zhang S, Jiang M, Huang M, Yang Y, Yu D, Zhou Z, Wei Y, Deng H (2019) Targeted demethylation of the SARI promotor impairs colon tumour growth. Cancer Lett 448:132–143

Wang H, Han M, Qi LS (2021) Engineering 3D genome organization. Nat Rev Genet 22:343–360

Wei T, Cheng Q, Min YL, Olson EN, Siegwart DJ (2020) Systemic nanoparticle delivery of CRISPR-Cas9 ribonucleoproteins for effective tissue specific genome editing. Nat Commun 11:3232

Weichenhan D, Lipka DB, Lutsik P, Goyal A, Plass C (2020) Epigenomic technologies for precision oncology. Semin Cancer Biol 84:60–68

Wu X, Li G, Xie R (2018) Decoding the role of TET family dioxygenases in lineage specification. Epigenetics Chromatin 11:58

Wu H, Wang F, Jiang JH (2021) Inducible CRISPR-dCas9 transcriptional systems for sensing and genome regulation. Chembiochem 22:1894–1900

Xiong T, Meister GE, Workman RE, Kato NC, Spellberg MJ, Turker F, Timp W, Ostermeier M, Novina CD (2017) Targeted DNA methylation in human cells using engineered dCas9-methyltransferases. Sci Rep 7:6732

Xu GL, Bestor TH (1997) Cytosine methylation targetted to pre-determined sequences. Nat Genet 17:376–378

Xu SJ, Heller EA (2019) Recent advances in neuroepigenetic editing. Curr Opin Neurobiol 59:26–33

Xu X, Tao Y, Gao X, Zhang L, Li X, Zou W, Ruan K, Wang F, Xu GL, Hu R (2016) A CRISPR-based approach for targeted DNA demethylation. Cell Discov 2:16009

Xu X, Tan X, Tampe B, Wilhelmi T, Hulshoff MS, Saito S, Moser T, Kalluri R, Hasenfuss G, Zeisberg EM, Zeisberg M (2018) High-fidelity CRISPR/Cas9-based gene-specific hydroxymethylation rescues gene expression and attenuates renal fibrosis. Nat Commun 9:3509

Xu SJ, Lombroso SI, Fischer DK, Carpenter MD, Marchione DM, Hamilton PJ, Lim CJ, Neve RL, Garcia BA, Wimmer ME, Pierce RC, Heller EA (2021) Chromatin-mediated alternative splicing regulates cocaine-reward behavior. Neuron 109:2943–2966 e8

Zhang XH, Tee LY, Wang XG, Huang QS, Yang SH (2015) Off-target effects in CRISPR/Cas9-mediated genome engineering. Mol Ther Nucleic Acids 4:e264

Zheng Y, Shen W, Zhang J, Yang B, Liu YN, Qi H, Yu X, Lu SY, Chen Y, Xu YZ, Li Y, Gage FH, Mi S, Yao J (2018) Author Correction: CRISPR interference-based specific and efficient gene inactivation in the brain. Nat Neurosci 21:894