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Published in:
DNA Methyltransferases

DOI:
10.1007/978-3-319-43624-1_17

Publication date:
2016

Rewriting DNA Methylation Signatures at Will: The *Curable Genome* Within Reach?

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Abstract

Epigenetic regulation of gene expression is vital for the maintenance of genome integrity and cell phenotype. In addition, many different diseases have underlying epigenetic mutations, and understanding their role and function may unravel new insights for diagnosis, treatment, and even prevention of diseases. It was an important breakthrough when epigenetic alterations could be gene-specifically manipulated using epigenetic regulatory proteins in an approach termed epigenetic editing. Epigenetic editors can be designed for virtually any gene by targeting effector domains to a preferred sequence, where they write or erase the desired epigenetic modification. This chapter describes the tools for editing DNA methylation signatures and their applications. In addition, we explain how to achieve targeted DNA (de)methylation and discuss the advantages and disadvantages of this approach. Silencing genes directly at the DNA methylation level instead of targeting the protein and/or RNA is a major improvement, as repression is achieved at the source of expression, potentially eliminating the need for continuous administration. Re-expression of silenced genes by targeted demethylation might closely represent the natural situation, in which all transcript variants might be expressed in a sustainable manner. Altogether epigenetic editing, for example, by rewriting DNA methylation, will assist in realizing the *curable genome* concept.

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A. Jeltsch, R.Z. Jurkowska (eds.), *DNA Methyltransferases - Role and Function*, Advances in Experimental Medicine and Biology 945,
DOI 10.1007/978-3-319-43624-1_17

Abbreviations

ATF	Artificial transcription factor
ChIP	Chromatin immunoprecipitation
CpG	Cytosine–phosphate–guanine
CRISPRs	Clustered regulatory interspaced palindromic repeats
DNMT	DNA methyltransferase
ncRNA	Nonprotein-coding RNA
sgRNA	Single-guide RNA
TALEs	Transcription activator-like effectors
TDG	Thymidine–DNA glycosylase
TET	Ten–eleven translocation
ZF	Zinc finger

1 Introduction

The term epigenetics was coined by Conrad Waddington back in 1942, who defined epigenetics as the branch of biology that studies the causal interactions between genes and their products which bring the phenotype into being (Waddington 2012). This definition has evolved over time to the current understanding of epigenetics referring to the study of heritable changes in gene expression that occur independent of changes in the primary DNA sequence (Sharma et al. 2010). The basic unit of chromatin comprises the nucleosome, which consists of approximately 146 base pairs (bps) of DNA wrapped around an octamer containing two copies of each of the core histones H2A, H2B, H3, and H4. Biochemical modifications on DNA and histones, as well as the nuclear context, influence the three-dimensional structure of chromatin. The main covalent chemical modification on DNA itself is the methylation of cytosines at sites where it is followed by a guanine base (CpGs). Additionally, posttranslational histone modification (PTMs), nucleosome positioning, and the expression of nonprotein-coding RNAs (ncRNAs) are important epigenetic modifications.

A huge number of data has been generated on how epigenetics regulate gene expression; however, the majority of these data are only correlative in nature. In order to study the causative role of a particular epigenetic modification at a given genomic site, epigenetic editing approaches have been exploited in the recent years (de Groote et al. 2012; Jurkowski et al. 2015). Epigenetic editing refers to the technology of actively rewriting epigenetic signatures at a genomic locus of interest. Toward this end, molecular tools – mostly developed and used in genome engineering (Gaj et al. 2013) – have been employed that allow DNA binding at a predefined genomic locus (Kungulovski and Jeltsch 2015). The most frequently used devices for gene targeting are self-engineered zinc finger (ZF) proteins, transcription activator-like effectors (TALEs), or the recently introduced clustered regulatory interspaced palindromic repeats (CRISPRs) system, which is based on DNA targeting by RNA molecules, the so-called single-guide RNAs (sgRNAs) (Fig. 1).

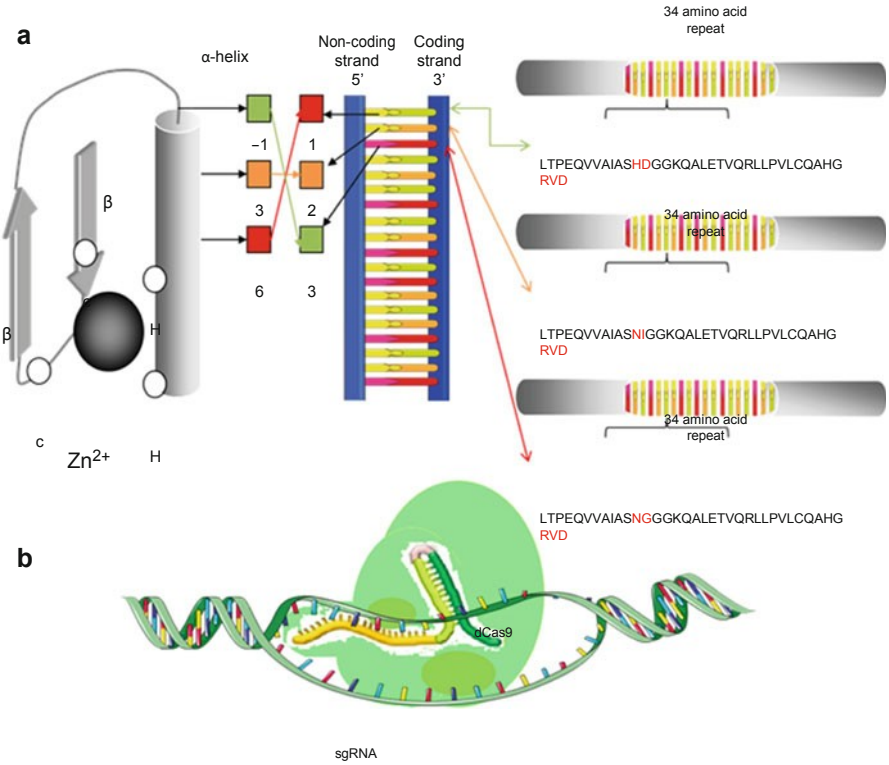

Fig. 1 Schematic representation of the DNA-binding domains of the most commonly used molecular tools in epigenetic editing. (**a**) The ZF protein on the left consists of approximately 30 amino acids, in which AAs at positions -1, 3, and 6 in the alpha-helix of the ZF protein recognize the third, second, and first base pair of the 5'−3' target sequence. Specificity can be increased by linking several ZFs together. The TALEs on the right consist of different monomers of approximately 34 AAs that are variable at positions 12 and 13 (=RVD), which are responsible for targeting a specific base pair within the DNA sequence. Notice that in comparison to the ZF, three times as much AAs are responsible for targeting the same amount of base pairs. (**b**) sgRNAs guide the CRISPR–dCas9 system to a particular sequence of approximately 20 base pairs. *ZF* zinc finger, *AA* amino acid, *TALE* transcription activator-like effector, *RVD* repeat variable di-residue, *sgRNA* single-guide RNA, *CRISPR* clustered regulatory interspaced palindromic repeat

ZF proteins are naturally occurring transcription factors forming the largest group of all transcription factors in the human genome (Vaquerizas et al. 2009). They consist of approximately 30 amino acids, wherein a stretch of seven amino acids is responsible for the recognition of 3–4 bps in the major groove of double- stranded DNA. During ZF binding, the amino acids at positions -1, 3, and 6 in the alpha-helix of the ZF protein recognize the third, second, and first base pair of the 5'−3' target sequence (Fig. 1a, left side). In 1996, Kim and Berg published the crys- tal structure of a designed ZF protein, which led to a refinement of this code, because it revealed an additional bond between a certain amino acid at position 2 in the ZF alpha-helix and the 4th base in the antisense strand of the DNA, which is at the same time the complement nucleotide of the 2 triplicate, recognized by amino acid 6 of the second ZF protein (Kim and Berg 1996).

Based on this knowledge, different DNA sequences can be targeted by engineering ZF proteins via exchanging amino acids of the α-helix to bind three base pairs of choice (Vandevenne et al. 2013). The specificity of ZF proteins is subsequently increased by linking several ZF domains together, so that, for example, a six-finger ZF protein will recognize 18 base pairs of target DNA (Fig. 1a, left side). When generating these ZF arrays, the selection procedure strongly determines the potency, and target site overlap or cross talk may complicate the array generation (Mussolino et al. 2011).

In 1994, Klug and colleagues engineered the first ZF protein successfully targeting and repressing the *BCR–ABL* fusion oncogene (Choo et al. 1994). Since this pioneering work was conducted, engineered ZF proteins have been used in fusion with nucleases (*molecular scissors*) or transcriptional activators and repressors (*artificial transcription factors*, ATFs) to target a multitude of endogenous genes (de Groote et al. 2012). The relatively small size and low immunogenicity of ZF proteins are a major advantage compared to other DNA-targeting proteins (Falahi et al. 2015; Mussolino et al. 2011). Importantly, the potential of ZF proteins as molecular scissors for therapeutic applications is explored in clinical trials (Ledford 2011).

TALEs are derived from plant pathogenic bacteria where they are used to modulate host gene expression (Boch and Bonas 2010). Upon injection into the plant cells, TALEs are imported into the nucleus where they bind specific sequences of the host cell genome and activate transcription. Like ZFs, TALEs also consist of individual modules (Jurkowski et al. 2015): each monomer (of 33 or 34 amino acids) differs at amino acid positions 12 and 13, a region called repeat variable di- residue (RVD) (Fig. 1a, right side). These hypervariable residues mediate binding to the target DNA site. Each RVD recognizes one nucleotide within the DNA-binding site (HD=C, NI=A, NG=T, NN=G), allowing for a straightforward design. Subsequently, transcriptional activators, repressors, or nucleases can be fused to the TALE DNA-binding domain (DBD) for targeted gene expression modification. Targeting efficiencies of the TALE DBD range from 25 to 95 % (Miller et al.2011; Maeder et al. 2013b), and new assembly methods are now available to improve the generation of more efficient TALEs (Reyon et al. 2012; Briggs et al. 2012). Considerable progress has been made in the design, development, and characterization of TALEs (Cermak et al. 2011).

Another breakthrough technology was introduced early in 2013 the flexible CRISPRs–CRISPR-associated proteins (CRISPR–Cas) system, which revolutionized biomedical research because of its ease, low cost, and flexibility (Fig. 1b). This system is derived from the bacterial defense system where the CRISPR–Cas system recognizes foreign DNA and the nuclease activity of Cas9, which is guided to a particular sequence by sgRNAs, cleaves the invading DNA. However, in order to modulate gene expression without altering the DNA sequence, the endonuclease activity of Cas9 is inactivated and instead linked to transcriptional or epigenetic modulators (CRISPR–dCas9) (Sander and Joung 2014).

To summarize, all three systems have in common a programmable DNA-binding platform, designed to recognize a specific genomic DNA sequence. Subsequently, for epigenetic editing, an epigenetic modulator (or a catalytic domain thereof) is recruited to the locus of interest by tethering the effector domain to the DNA-binding platform,

Fig. 2 Epigenetic editing is used to actively rewrite epigenetic signatures at a genomic location of interest. The molecular tools used for this purpose consist of (**a**) a DNA-binding platform to recognize the target sequence (see Fig. 1) and (**b**) an epigenetic modulator (or a catalytic domain thereof) which exerts its activating or repressive function by rewriting the epigenetic signature at a desired location

either directly (to ZF proteins or TALEs) or to the catalytically inactive Cas9 protein (which is recruited by sgRNAs). Upon delivery into target cells, the DNA-binding platform finds its DNA sequence, so the epigenetic modulator can expose its enzymatic activity at the desired genomic site (see Figs. 1 and 2). The epigenetic editing approach faced much disbelief in its early days, as the epigenetic marks were not generally considered to be instructive for gene expression, and if so, it was expected that their effect would be overruled by the native chromatin environment on a longer term. Moreover, the generally accepted inaccessibility of heterochromatic genes was thought to hamper successful editing of silenced genes. Pioneering studies by us and others and the introduction of straightforward DNA-targeting approaches set the stage for the recent boom in epigenetic editing (Jurkowski et al. 2015).

The by far most studied epigenetic mark is DNA methylation. It predominately occurs on cytosine followed by a guanine (CpG) sites; however, also non-CpG methylation has been detected in stem cells (Lister et al. 2009) and in the brain (Lister et al. 2013). 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 (Ehrlich et al. 1982; Saxonov et al. 2006). These CpG-rich promoters are usually unmethylated, with a few exceptions, including tissue-specific methylation during development (Bird et al. 1985; Song et al. 2005). Gene promoters found with high levels of DNA methylation are generally transcriptionally inactive (Boyes and Bird 1992; Siegfried et al. 1999; Jones and Takai 2001). Epigenetic editors will be very helpful to investigate whether methylation precedes gene inactivation or whether it is rather a consequence of inactivation, since (de)methylation can now be induced at will at specific genomic sites. This will also shed light on the order of events during the process of DNA methylation at, e.g., promoters, gene bodies, or enhancers.

2 Targeted DNA Methylation

Pioneering work in the field of targeted DNA methylation has been performed by Xu and Bestor who were the first to use a fusion protein consisting of an engineered ZF protein and a DNA methyltransferase to target DNA methylation to a predefined

DNA sequence (Xu and Bestor 1997). Several other studies of targeted DNA methylation using ZF proteins fused to human or bacterial DNA methyltransferases have been published, showing that the induction of DNA methylation indeed results in transcriptional repression (Smith and Ford 2007; Li et al. 2006, 2007; Minczuk et al. 2006; Smith et al. 2008; Carvin et al. 2003; McNamara et al. 2002; van der Gun et al. 2010). However, these early studies have only been performed on exog- enous or nonmammalian sites. Endogenous gene repression by targeted DNA meth- ylation was shown for the first time, in 2012 for the human gene promoters of *VEGF-A* (Siddique et al. 2012) *SOX2* and *MASPIN* (Rivenbark et al. 2012). Both studies used designed ZF proteins, engineered to bind a stretch of 18 bps within the promoter of the intended target gene. The ZF proteins were fused to the catalytic domain of the murine and human DNA methyltransferase 3A (DNMT3A) and a fusion of murine Dnmt3a or human DNMT3L, respectively.

The mouse Dnmt3a fusion resulted in a mean yield of 14.4 % DNA methylation over all CpG sites at the interrogated region of the *VEGF-A* promoter leading to a downregulation of mRNA expression by 36 % (Siddique et al. 2012). For certain CpGs, the induced methylation even reached efficiencies of 100 %. This average effect was further improved – up to a mean of 28.6 % DNA methylation and 56 % *VEGF-A* mRNA downregulation – when the effector domain consisted of the C-terminal domain of Dnmt3a fused to DNMT3L. This finding further proved that DNMT3L stimulates *de novo* methylation through Dnmt3a, as DNMT3L has no catalytic activity itself (Gowher et al. 2005).

Targeted DNA methylation of the tumor suppressor gene *MASPIN* using the catalytic domain of the human DNMT3A (598–908 amino acids) increased DNA methylation up to 60 % at single CpGs within the *MASPIN* promoter (Rivenbark et al. 2012). Increase of DNA methylation was detectable up to 500 bps downstream of the ZF target site and translated into a 50 % downregulation of mRNA and protein expression compared to an empty vector control. As expected, the downregulation of the tumor suppressor gene resulted in an increased proliferation rate and a more aggressive phenotype of breast cancer cells in vitro. In addition, the transcription factor *SOX2* was targeted using an inducible ZF–DNMT3A fusion (Rivenbark et al. 2012). Cell lines were stably transduced with the ZF–DNMT3A fusion, and as a control the same ZF protein fused to the transient repressor SKD (Kruppel-associated box domain) was used. This system is induced upon addition of doxycycline (Dox) to the culture medium, which causes expression of the fusion proteins. In turn, discontinuation of the Dox treatment led to depletion of ZF–DNMT3A and ZF–SKD expression, respectively. The expression of the *SOX2*-targeted ZF–DNMT3A construct translated into a 60–80 % downregulation of mRNA and protein expression, respectively. In a subsequent cell proliferation assay, the ZF–SKD construct was included as a control and both, ZF–SKD and ZF–DNMT3A, were initially able to decrease cell growth. However, when Dox was removed from the culture media 48 h after induction, only the ZF–DNMT3A fusion was able to attenuate cell proliferation over the time course of the experiment, suggesting stable gene repression mediated by DNA methylation, although DNA methylation at the *SOX2* promoter was not directly shown (Rivenbark et al. 2012). In a follow-up study, the

same lab showed that the silencing of *SOX2* expression was indeed mediated by targeted DNA methylation (Stolzenburg et al. 2015). Furthermore, depletion of Dox and subsequent discontinuation of the expression of the ZF–SKD and ZF–DNMT3A fusions led to the re-expression of *SOX2* only in ZF–SKD-transduced cells but not in cells that previously expressed the DNMT3A construct. Therefore, in this context, DNMT3A was a more stable mediator of expression than the SKD. Interestingly, *SOX2* mRNA and protein repression was stronger 8 days after Dox removal than the initial downregulation, suggesting that DNA methylation is reinforced by cellular mechanisms during subsequent cell divisions.

Using the mouse Dnmt3a fused to a ZF protein targeting the cell adhesion molecule *EpCAM* (van der Gun et al. 2013), an increase in DNA methylation at the endogenous *EpCAM* promoter by 20–25 % after transient transfection of an ovarian cancer cell line was observed (Nunna et al. 2014). At specific CpGs an increase of DNA methylation of more than 80 % was detected. In addition, two cell lines were generated that stably express ZF–Dnmt3a. Importantly, both cell lines showed an increase of DNA methylation at the *EpCAM* promoter of more than 40 %. The induction of promoter DNA methylation decreased the expression of *EpCAM* mRNA (60–70 %) and protein (50 %) in the examined cell line, and furthermore, the reduction of EpCAM expression translated into a decrease of the proliferative character of the examined ovarian cancer cell line.

Researchers have also employed TALEs for targeted DNA methylation studies, fused to either DNMT3A (Li et al. 2015) or DNMT3A–DNMT3L (Bernstein et al. 2015), targeting the promoters of *CRMP4* and *CDKN2A*, respectively. Both studies successfully showed induced DNA methylation at their respective TALE target sites. In both studies, the DNA methylation was associated with target gene repression and resulted in the intended physiological downstream effects. Although for the *CRMP4* promoter, DNA methylation only increased to about 5.5 % upstream to 6.4 % downstream of the TALE target site (numbers represent mean values of the interrogated region, with peaks of max 9–12 % at individual CpGs). The induced methylation, however, was sufficient to virtually completely knock down mRNA and protein expression in a nonmetastatic prostate cancer cell line (Li et al. 2015). This targeted DNA methylation was then shown to spread over 300 bps up- and downstream of the TALE–DNMT3A binding site. Importantly, the downregulation of *CRMP4*, a metastasis suppressor gene in a nonmetastatic cell line, led to the formation of metastasis in vivo. Furthermore, the crucial impact of DNA methylation at the *CRMP4* promoter for prostate cancer patients' survival was shown by DNA methylation analysis of prostate cancer specimen. The analysis revealed that 64 % of *CRMP4* methylation positive samples were indeed confirmed as metastatic.

Bernstein et al. (2015) engineered a TALE–Dnmt3a–DNMT3L (TALE–DNMT) construct to target the *CDKN2A* locus in HeLa cells, primary human fibroblasts, and coronary artery smooth muscle cells. The *CDKN2A* locus encodes the cyclin-dependent kinase inhibitor p16, a tumor suppressor, which is regulated by DNA methylation. The induced DNA methylation across the *CDKN2A* CpG island varied between 10 % (human fibroblasts) and 13.8 % (HeLa cells) after lentiviral transduction and 17 % in HeLa cells after sorting for successfully transfected cells. At

individual CpGs, the methylation levels increased even up to 66 % in the sorted population after transfection and 30–50 % after lentiviral transduction. The TALE–DNMT3A-mediated DNA methylation was associated with a 50 % decrease in *p16* mRNA expression in human fibroblasts accompanied by an increase in cell cycle progression. Recently, another group demonstrated ZF-induced methylation of the *CDKN2A* locus to promote migration and invasion of cancer cells (Cui et al. 2015).

These publications demonstrate that induction of endogenous DNA methylation at will by epigenetic editing tools at a specific target is not only possible but also effective, as treatment results in the intended physiological downstream effects. The induced DNA methylation needed to downregulate gene expression varied highly between the studies, and in one case as little as 10 % was sufficient to achieve target gene suppression. This strengthens the notion that a single CpG can be crucial for gene regulation at a given locus (Pogribny et al. 2000).

3 Stability of the Induced DNA Methylation Changes

It is well documented that promoter DNA methylation plays an important role in permanent gene silencing and that established DNA methylation is maintained during cell divisions to achieve stable gene repression (Riggs 1975; Holliday and Pugh 1975; Lister et al. 2009; Chen et al. 2007; Stein et al. 1982). However, nowadays it is presumed that DNA methylation by itself might not be enough to maintain stable gene repression in any given context. It is much more agreed that DNA methylation and a myriad of additional epigenetic mechanisms, such as histone modifications, nucleosome positioning, ncRNAs, and others, work together to create a stable context-dependent gene repression pattern (Raynal et al. 2012). Epigenetic editing provides unique tools to address sustainability of DNA methylation in different chromatin contexts.

The first study to address maintenance of written DNA methylation marks was performed by the Blancafort team. Toward this end, engineered ZF proteins targeting the *MASPIN* gene were fused to DNMT3A and retrovirally delivered into breast cancer cells (Rivenbark et al. 2012). The downregulation of the tumor suppressor *MASPIN* would lead to a more aggressive phenotype of the host cells. To prove this hypothesis, retrovirally transduced cells were seeded in soft agar for colony formation. After colonies were formed (several weeks later), single colonies were picked from the soft agar, disrupted, and cultured for subsequent sodium bisulfite sequencing to investigate the methylation state of *MASPIN*. The data revealed that even 50 days post-transduction DNA, methylation was maintained in the host cells (Rivenbark et al. 2012). Interestingly, knockdown of *UHRF1* (ubiquitin-like containing PHD and RING finger domains 1, a protein required for the maintenance of DNA methylation patterns (Bostick et al. 2007; Sharif et al. 2007)) led to re-expression of *MASPIN* in these cells (Rivenbark et al. 2012).

The longevity of the induced DNA methylation was further tested in vivo for the *SOX2* promoter in a xenograft mouse model (Stolzenburg et al. 2015). To do so, the advantages of the Dox-inducible system were exploited: the Dox-inducible system

allows controlled expression of the ZF–DNMT3A fusion by administration of a Dox-containing diet, whereas the switch to a Dox-free diet leads to the discontinuation of the ZF–DNMT3A expression. The results showed a strong tumor growth inhibition in the cells that expressed the ZF–DNMT3A fusion. This was associated with DNA methylation at the *SOX2* promoter together with a decrease in *SOX2* expression. Although DNA methylation at the *SOX2* promoter was largely sustained for 53 days post-Dox removal, after the removal of ZF–DNMT3A expression, tumor growth inhibition was only maintained for 10 days. Interestingly, examination of ZF–DNMT3A and *SOX2* expression at day 10 after Dox removal revealed a maintained repression of *SOX2* and no detection of ZF–DNMT3A, implying – once being induced – a long-term effect of DNA methylation on *SOX2* repression.

However, to truly verify the long-term effect of written DNA methylation signatures, DNA methylation and target gene expression should be validated at later time points. This notion is underpinned by a recent publication showing that ZF-targeted DNA methylation at the *VEGF-A* promoter by means of transient adenoviral transfer was not stably maintained (Kungulovski et al. 2015). The authors examined, after targeting a ZF–DNMT3A fusion to the *VEGF-A* promoter, the longevity of the induced DNA methylation mark at the *VEGF-A* promoter and *VEGF-A* expression over a time course of 15 days. In contrast to Stolzenburg et al., the loss of experimental ZF–DNMT3A expression was associated with a loss of DNA methylation at the target site and target gene re-expression (Kungulovski et al. 2015). Interestingly, the authors also looked into secondary effects of the induced DNA methylation on histone modifications. After induction of the targeted DNA methylation at the *VEGF-A* promoter, the authors examined whether the methylation mark at the DNA level is reinforced by a change in the silencing mark H3K9me3. However, no changes in H3K9me3 were detected using ChIP–qPCR (Kungulovski et al. 2015). As epigenetic editing is uniquely suited to address the parameters allowing or preventing maintenance of DNA methylation, ongoing research efforts are expected to yield important insights in this respect.

4 Targeted DNA Demethylation

Epigenome-wide association studies result in increasing lists of aberrantly hypermethylated loci associated with various clinical phenotypes. Mimicking these methylation profiles by epigenetic editing will provide valuable insights into the biological function of these modifications. More importantly, the actual removal of such epimutations will open new therapeutic avenues. Indeed, in the clinical setting, inhibitors of DNA methyltransferases are used to prevent hypermethylation of tumor suppressor genes. Unfortunately, such conventional epigenetic drugs will affect methylation patterns genome-wide. In contrast, epigenetic editing approaches might exploit the reversibility of epigenetic marks in a gene-targeted manner and in this way avoid potentially dangerous side effects.

Before the identification of active DNA methylation-modifying enzymes, DNA repair mechanisms were exploited for their role in reducing local DNA methylation

profiles. Indeed, Gregory et al. reported that the targeting of thymidine–DNA glycosylase (TDG) by fusion to engineered ZF proteins did result in lowering of DNA methylation, allowing improved induction of the target gene *Nos2* (Gregory et al. 2013). The identification of ten–eleven translocation (TET) enzymes and their role in modifying methylated cytosines allowed epigenetic editing approaches to actively reduce hypermethylation states of target genes without introducing temporary changes to the DNA. Indeed, we were the first group to report on the potency of targeting the TET domains to induce active DNA demethylation (Rots and Petersen-Mahrt 2013; Chen et al. 2014). Targeting either of the three TET members to the hypermethylated *ICAM* gene demonstrated that both TET1 and TET2 are effective reducers of DNA methylations. Although DNA demethylation in this experimental setting was relatively low (minus ca 5 approx.), gene expression was increased two-fold. Obviously, when compared to targeting of VP64, a strong viral transcription activator, this gene expression modulation was modest. However, such mild increases might be physiologically more relevant, and these findings do generate opportunities to realize therapeutically relevant localized DNA demethylation. Indeed, we confirmed the robustness of the TET2-targeting approach in inducing DNA demethylation for four other genes (*EPB41L3*, *C13ORF18*, *CCNA1*, and *TFPI2*; all putative hypermethylation biomarkers for cervical cancer) (Huisman et al. 2015a; Huisman et al. 2015b). Although the observation that modest local demethylation is less effective in gene re-expression than targeting a transcriptional activator is understandable, the large size of TET domains is also partially responsible for this effect. Interestingly, when cells were co-treated with the epigenetic drug trichostatin A (TSA), which is a histone deacetylase inhibitor that might increase gene accessibility, induced expression of silenced target genes was detectable (Huisman et al. 2015a).

Despite the large size of the TET domain, also in fusion with the relatively larger TALE domains, TET1 was able to induce targeted DNA demethylation (Maeder et al. 2013a; Li et al. 2015). Maeder et al. were the first to show targeted demethylation using TALEs fused to TET1. In total, they engineered 25 TET1-containing TALEs targeting *KLF4*, *HBB*, and *RHOXF2* (Maeder et al. 2013a). Comparison of the TALE constructs – either fused to the full-length TET1 or the constructs containing only the TET1 catalytic domain (TET1c) – showed that the catalytic domain had a stronger effect on demethylating its target genes (up to 30 % at *KLF4* and even 84 % at *HBB*) than the full-length TET1 (Maeder et al. 2013a). The most effective demethylation was observed within 30 bps up- and downstream but also up to 200 bps away from the target sequence. This is in accordance with a study published by Li et al., who examined two regions located 4 bps upstream and 95 bps down- stream of the TALE–TET1c target site for their methylation status. Both regions showed demethylation with the more distant region being more efficiently demeth- ylated. In this study, DNA demethylation was associated with a re-expression of target gene mRNA expression, followed by an induction of protein expression (Li et al. 2015). As expected, the demethylation and re-expression of *CRMP4* (a metas- tasis suppressor gene) showed a decrease in migration and invasion in otherwise metastatic cell lines. Furthermore, the re-expression of *CRMP4* after active DNA

demethylation abolished the metastatic character of these cells even in an in vivo mouse model of prostate cancer (Li et al. 2015). However, demethylation of the target gene by TALE–TET1c did not always result in induced gene expression. Only four out of ten demethylating TALE–TET1c constructs targeting *HBB* indeed increased the expression of HBB mRNA. Similarly, in the case of *RHOXF2*, two out of five demethylating TALEs–TET1c induced mRNA expression (Maeder et al. 2013a). The authors suggested that the artificial demethylation at the target gene was not stably transmitted, and therefore, demethylated CpGs became remethylated, as TALE–TET1c coding constructs became cleared from the transfected cells.

A very interesting aspect of the targeted DNA demethylation, namely, its effect on histone modifications, was examined by Li et al. for both regions that showed a decrease in TALE–TET1c-mediated DNA methylation (Li et al. 2015). While the region directly upstream to the TALE–TET1c target site showed a reduction of repressive histone modifications (H3K9me3, H3K27me3, H3K79me3), this was not seen at the region 95 bps downstream of the target site, although this region showed stronger demethylation (Li et al. 2015).

5 Concluding Remarks and Future Perspectives

Epigenetics has been receiving a lot of attention in this post-genomic era: many abnormalities in the epigenetic landscape have been identified in numerous diseases, and so-called epigenetic drugs, including inhibitors of DNA methyltransferase, have entered the clinical arena. Epigenetic editing – to mimic or reverse such epimutations – is currently gaining widespread acceptance, and many research groups join the field. Initially, the technology had to overcome some hurdles: genome specificity was not likely to be achieved, accessibility of silenced genes was thought to be impossible, and the instructive nature of epigenetic marks with respect to controlling gene expression was highly questioned. As reviewed here, these assumptions have been proven untrue. To increase the specificity of targeting, a considerable progress has been made in the field of enzyme engineering, where split enzymes allow activity to take place only when two split parts are brought close together via their fusion to two closely binding DNA-targeting modules (Kiss and Weinhold 2008). Genome-wide specificity can also be achieved using CRISPR–dCas9 technology (Hilton et al. 2015), and the progress in sgRNA design is expected to rapidly improve our understanding of the off-target effects. Taken together, this allows the prediction and prevention of unwanted side effects due to unintended endogenous binding.

Also – against common belief – heterochromatin is not hampering accessibility per se, as re-expression of silenced genes has now been shown for many heterochromatic genes (tumor suppressors) by ATFs (Beltran et al. 2007; Lara et al. 2012; van der Wijst et al. 2015; Falahi et al. 2013; Huisman et al. 2013) and epigenetic editors (e.g. Chen et al. 2014; Huisman et al. 2015a, b). Although the transcriptional activators/repressors in ATFs are relatively small in size, also larger constructs can gain gene access even though size is likely to affect effectiveness for heterochromatic genes.

On top of that, the heterochromatin landscape, which is unique for each gene and cell, needs further investigation in order to completely understand the mechanism of action of different epigenetic editors.

With respect to the cause versus consequence relationship of epigenetic marks and gene expression, strong indications that epigenetics is instructive in gene expression regulation have been obtained by targeting effector domains to artificial loci (e.g., plasmids and integrated sites) as reviewed by us in 2012 (de Groote et al. 2012). At that time, only two examples were published, which confirmed that editing of epigenetic marks at a predetermined endogenous site was effective in modulating gene expression (Snowden et al. 2002; Rivenbark et al. 2012). These days, epigenetic/epigenome editing has been declared a method to watch (Rusk 2014), and a rapid increase in publications confirms efficiency of the approach (Ledford 2015). An important open question concerns the chromatin microenvironment conditions allowing sustained re-expression, but the technology of epigenetic editing is uniquely qualified to address this question (Cano-Rodriguez et al. 2016). As also the CRISPR–dCas9 platform is currently exploited for targeted (de)methylation (Choudhury et al. 2016; McDonald et al. 2016; Vojta et al. 2016; Xu et al. 2016), we expect epigenetic editing tools to soon reprogram the genome in a sustained manner, which will provide a clinically relevant hit-and-run approach to cure currently incurable diseases, including imprinting (Bashtrykov et al. 2015) or behavioral disorders (Dekker et al. 2014).

Acknowledgments We would like to acknowledge the EU funding for D.G. (H2020-MSCA- ITN-2014-ETN 642691 EpiPredict). M.G.R. serves as vice-chair of H2020-COST CM1406, and her team is partially funded by NWO-Vidi-91786373 and EU-FP7-SNN-4D22C-T2007.

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