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# Epigenome Editing

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## Abstract

The regulation of chromatin structure and gene expression depends substantially on a dynamic and intricate layer of biological and chemical information that constitutes the epigenome. This epigenetic layer of information holds fundamental clues to the molecular mechanisms, not yet fully understood, by which a genotype can influence and configure a specific phenotype. A profound understanding of the molecular underpinnings of epigenetic processes is, thus, essential to wield deliberate spatiotemporal control of gene activation and repression. However, only recently has the technology required to adequately probe the functional significance of specific epigenetic mechanisms become available. This chapter provides an overview of modern epigenome editing systems, including zinc finger proteins, TAL effectors, and CRISPR–Cas systems. It highlights the use of biotechnological tools to investigate the role of DNA and histone post-translational modifications as well as regulatory RNAs to manipulate specific patterns of gene expression. This chapter further discusses the technological limitations that have limited our ability to elucidate epigenetic mechanisms in local and genome-wide contexts.

**Keywords:** epigenome editing, epigenetic manipulation, CRISPR, CRISPR–dCas9, ZFNs, TALENs, epigenome engineering, targeted gene activation and repression, epigenetics

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## 1. Introduction

Elucidating the underlying basis for the molecular links that bridge the gap between genotype and phenotype has propelled research and scientific discovery for decades. More than three-quarters of a century have passed since Conrad Waddington introduced the concept of “epigenetics.” The term signaled an attempt to describe the causal links by which genes give rise to specific phenotypes in the context of developmental changes that drive cellular

differentiation [1]. Waddington's "epigenetic landscapes" [2] laid a foundation for exploring phenotypic plasticity as a phenomenon that is not entirely dependent on genetic mechanisms, but one which can also be shaped by environmental cues that concomitantly orchestrate the process of cell differentiation from an initial totipotent state [3].

The scope of epigenetics as a scientific discipline, much like its definition, has expanded with the passage of time. Today, the field broadly refers to the study of fundamental processes related to mitotic and meiotic stable and heritable changes that emerge without alteration of DNA sequences [3–5]. Stable changes encompass durable alterations in gene expression patterns, which may be neither permanent nor heritable. In contrast, heritable changes in gene expression may constitute persistent alterations that are carried onto the progeny of cells or individual organisms [6].

In the last two decades, the emerging field of epigenetics has revealed crucial information about the regulation of chromatin states in the eukaryotic nucleus. Heterochromatic DNA is organized into compact, higher order, chromatin fibers. By contrast, euchromatic DNA comprises lightly packed chromatin that represents an active and accessible part of the genome. Both states feature an array of nucleosomes, the basic subunits of chromatin, consisting of approximately 145–147 base pairs of DNA wrapped around a core histone octamer [7]. Each histone octamer carries two copies of all of the core histone proteins—H2A, H2B, H3, H4— assembled into nucleosomes, which are subsequently stabilized into high-order chromatin structures by the presence of the linker histone H1 and linker DNA [7].

The organization and regulation of chromatin at the epigenetic level depend on dynamic and diverse combinations of covalent chemical modifications that occur on histone proteins— known as histone post-translational modifications (PTMs)—and DNA bases, as well as the expression of regulatory noncoding RNA (ncRNA) molecules [3]. Together, these epigenetic signals and ncRNAs constitute a layer of information that controls the spatiotemporal regulation of gene expression patterns by remodeling the structure of chromatin and modulating its bio-physicochemical properties.

The scientific community has made great strides in elucidating functional roles for epigenetic processes in recent years. Progress has come at the hand of biotechnologies aimed at manipulating endogenous, site-specific epigenetic targets. The technologies rely on the use of catalytically active or scaffolding epigenetic effectors fused to programmable DNA-binding proteins that target specific genetic loci. This chapter focuses on the three most important platforms for modern targeted epigenome editing: zinc finger proteins, transcription activator-like effectors (TALEs), and clustered, regularly interspaced, short palindromic repeats (CRISPR), and CRISPR-associated sequences (Cas) (CRISPR–Cas) systems.

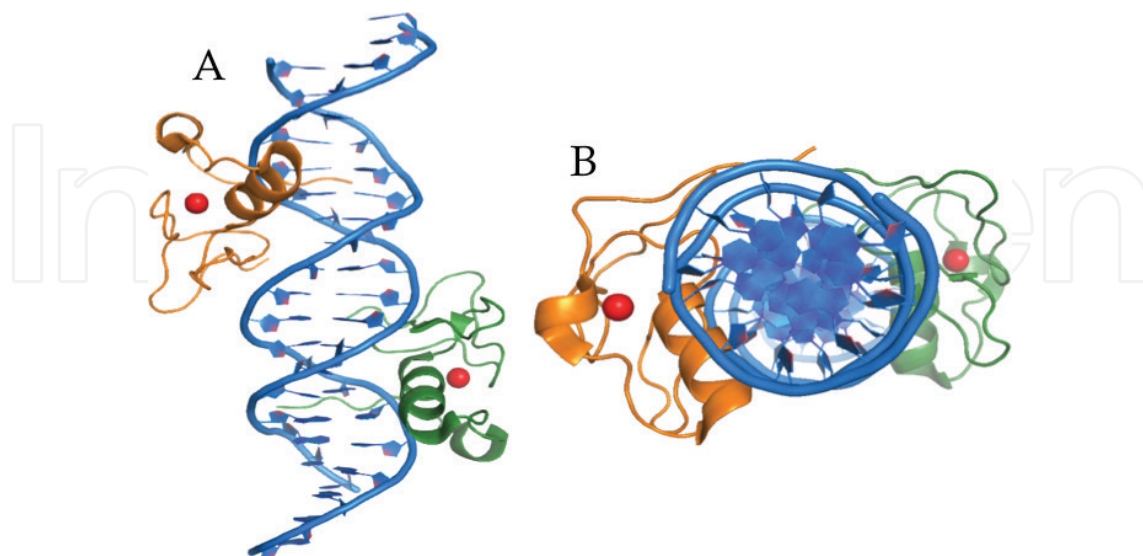
Notwithstanding the progress made in recent years, much remains to be learned about epigenetic mechanisms in development, cellular programming, disease, and personalized medicine. Epigenome editing technologies are poised to make significant contributions to the field of epigenetics. This chapter provides an overview of each epigenome editing system, highlights their use for manipulating specific patterns of gene expression, and discusses the technological limitations that have thus far limited our ability to interrogate the mechanisms of epigenetic regulation in local and genome-wide contexts.

## 2. Programmable epigenome editing systems

Regulating endogenous levels of gene expression by targeting specific epigenetic modifications is a relatively nascent field. Basic scientific research in the last few decades has provided insights that facilitated the development of technologies aimed at interrogating epigenetic processes. At their core, epigenome editing tools are based on the concept of fusing programmable DNA-binding proteins that target specific genetic loci, with catalytically active or scaffolding effector domains that exert some influence on epigenetic processes. The three most important molecular tools that have been developed for targeted epigenome editing are zinc finger proteins, TALEs, and CRISPR–Cas systems. All have been repurposed into epigenome editing platforms designed to manipulate gene expression patterns in particular contexts. An overview of each system is provided below. However, it should be noted that other, less common, tools have also been developed to probe epigenetic mechanisms and modulate gene expression in a sequence-dependent manner including synthetic polyamides [8] and triple helix-forming oligonucleotides (TFOs) [9].

### 2.1. Zinc finger-based systems

Zinc finger proteins are among the most characterized systems used for the manipulation of targeted, sequence-specific nucleic acids. Their discovery arose from research of transcriptional mechanisms in *Xenopus laevis* oocytes dating back to over three decades ago. At the time, scientists realized that a unique set of proteins, containing repetitive zinc-binding domains of roughly 30 amino acid residues, was required for transcription factor-mediated gene regulation [10]. These finger-like peptide arrangements rely on interactions between conserved pairs of cysteine and histidine residues that are anchored by a centered and tetrahedrally coordinated zinc ion (**Figure 1**). From a structural standpoint, each zinc finger



**Figure 1. Crystal structure of two zinc finger proteins in complex with DNA.** (A) Recognition of target DNA (blue) by two distinct zinc finger proteins (orange and green). A tetrahedrally coordinated zinc ion (red) stabilizes each zinc finger motif. (B) Orthogonal view of zinc finger-mediated DNA recognition. [PDB 3DFX].

comprises an antiparallel  $\beta$  sheet and  $\alpha$  helix, which are stabilized by the zinc ion and a set of hydrophobic residues [11]. The  $\alpha$  helix of each zinc finger binds directly to the major groove of B-DNA. Residues from the  $\text{NH}_2$ -terminal region of the helix mediate recognition of a three base-pair target site [11]. Thus, side chain residues in the  $\alpha$  helix that can be engineered to recognize diverse nucleic acid base-pair triplets drive the double-stranded DNA sequence specificity of each zinc finger.

The intrinsic modularity of DNA recognition by zinc finger proteins led to the swift development of a range of biotechnological applications. For example, manipulation of gene expression via site-specific, DNA-binding zinc finger proteins was first established by targeting a 9 base-pair region of a *BCR-ABL* fusion oncogene [12]. Using a three-zinc finger peptide, scientists demonstrated that transcriptional repression of a leukemic oncogene in a chromosomal DNA context was possible [12].

Similarly, the development of zinc finger nucleases (ZFNs) marked an important milestone for modern genome editing biotechnologies. ZFNs are engineered chimeric fusions composed of a set of tandem zinc finger DNA-binding proteins and a nuclease domain—such as the catalytic domain of the restriction endonuclease *FokI*—that cleaves DNA [13]. Synthetic zinc finger-*FokI* fusions coupled the DNA sequence specificity of zinc finger proteins with the non-specific cleavage activity of *FokI* to trigger double-stranded breaks at desired genomic loci. Importantly, repurposing zinc finger proteins into ZFNs facilitated the path for epigenome editing biotechnologies aimed at controlling transcriptional activation and repression.

Zinc finger-related epigenome editing tools are based on the concept of fusing programmable, DNA binding, zinc finger proteins designed to target diverse sequences [14, 15] with catalytically active or scaffolding effector domains. The chimeric proteins are designed to alter gene expression patterns and act as artificial transcription factors (ATFs) [15]. Pioneer studies on transcriptional repression by directing DNA cytosine methylation [16] and local histone H3K9 methylation [17] deposition at specific promoter sequences established the feasibility of the approach in the late 1990s and early 2000s. The use of zinc finger-based epigenome editing tools has contributed valuable insights into epigenetic mechanisms, as will be discussed in the next section. However, the high cost and technical expertise required to engineer and validate context-dependent specificity in zinc finger proteins [18, 19] has greatly limited their widespread adoption.

## 2.2. TALE-based systems

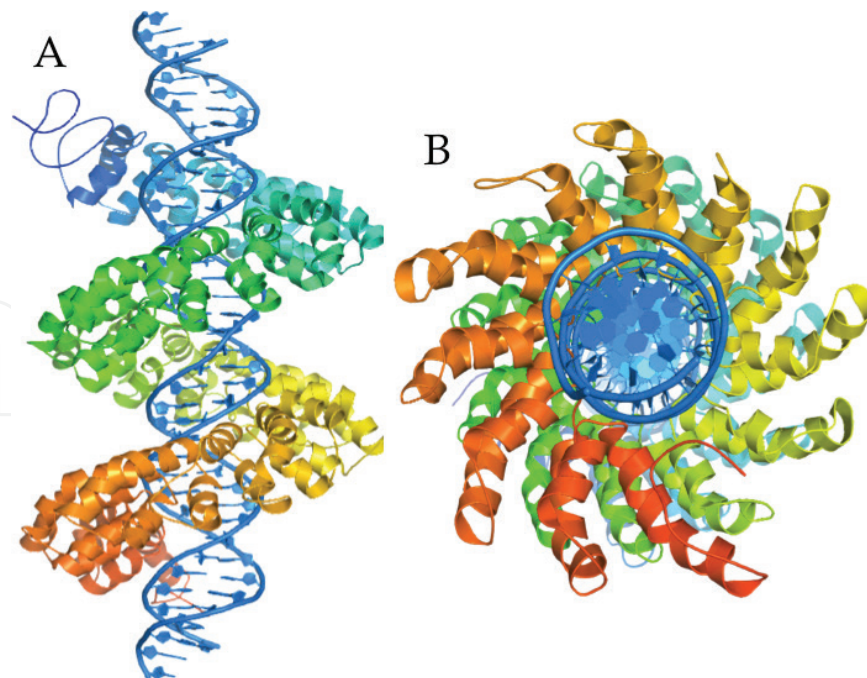
Transcription activator-like effectors (TALEs) are proteins of bacterial origin. They were first reported in the literature in 2007, after two independent research groups discovered that certain bacterial proteins can bind specific promoter sequences in eukaryotic cells [20, 21]. Upon binding to DNA, the pathogenic effector proteins induce the expression of genes that promote the spread of bacterial infection in host cells. TALEs are secreted by gram-negative bacteria of the *Xanthomonas* genus and injected into eukaryotic cells via the type III secretion system [22]. Once inside the eukaryotic cell, they translocate directly to the nucleus where they act as transcription factors to regulate the expression of genes that support bacterial infection [20, 21].



The molecular basis for DNA recognition of each TALE comes from a central tandem amino acid repeat domain of approximately 33–35 residues in length [23, 24]. This tandem repeat is flanked by an N-terminal region required for type III secretion and a C-terminal region involved in nuclear localization and transcriptional activation. Each repeat folds into two left-handed  $\alpha$  helices linked by a short loop that contains two hypervariable residues, known as the repeat variable diresidues (RVDs). The RVDs occupy positions 12 and 13 of each repeat, where the 12th residue stabilizes the RVD loop by mediating contacts with the protein backbone, while the 13th residue interacts directly with a specific nucleotide nitrogenous base (**Figure 2**). Thus, the DNA specificity of TALEs is modular and encoded in the tandem repeat sequence [25, 26].

Unlike zinc fingers, which require triplet sequence recognition sites, TALEs are able to target a single nucleotide at a time through its RVDs. This characteristic makes TALEs simpler to engineer and has enabled the rational design of artificial TALEs for biotechnological applications [26]. Indeed, borrowing from its ZFN predecessors, TALE proteins fused to the *FokI* nuclease domain gave rise to TALE nucleases (TALENs), which have been utilized for genome editing applications [27].

Similarly, fusions of TALEs to epigenetic effectors have been repurposed for epigenome editing to manipulate gene expression. For example, TALE fusions to activation domains have shown the ability to target specific genomic loci and induce robust transcriptional activation [28]. Inactivation of enhancer regulatory elements has also been demonstrated using TALE fusions to chromatin effectors targeting specific histone modifications [29], thereby establishing a platform to interrogate the function of specific regulatory elements in diverse chromatin landscapes.

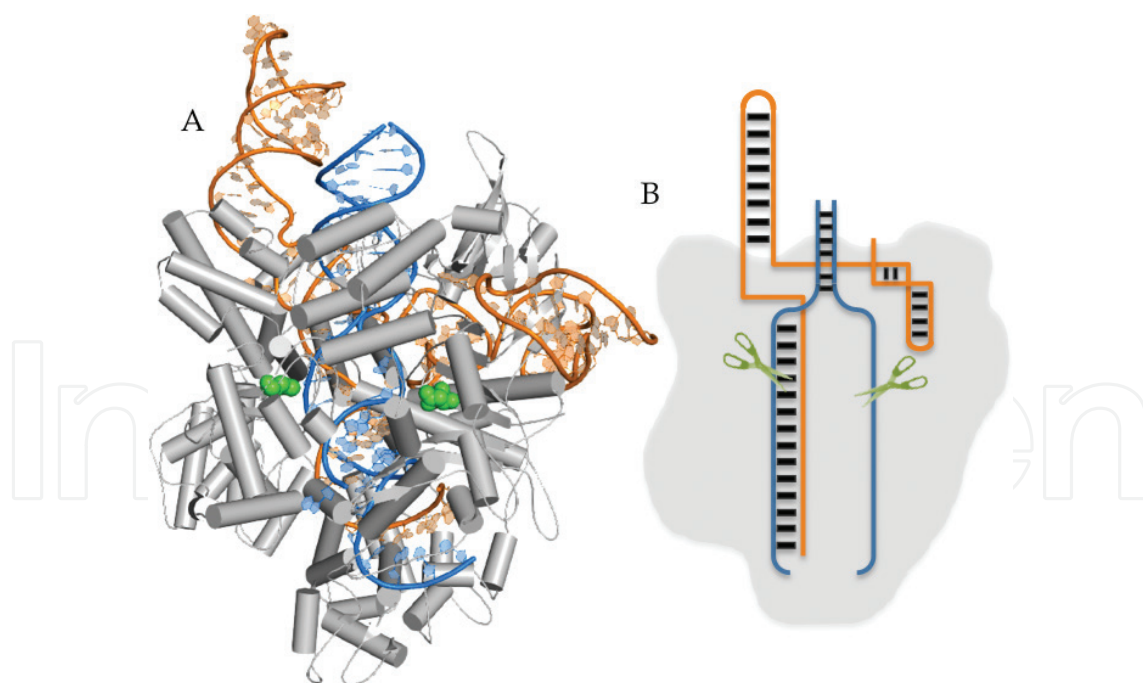


**Figure 2. Structure of a TAL effector bound to its target DNA.** (A) The TALE tandem amino acid repeats (multi-colored helices) associate into a right-handed superhelix that wraps around the major groove of the target DNA (blue). (B) Orthogonal view of target DNA recognition by a TALE [PDB 3UGM].

Despite the advantages of possessing modular properties compared to zinc finger proteins and the improvements made in design and assembly of custom TALE-based systems [30, 31], significant technological shortcomings remain to be addressed. The highly repetitive sequences associated with TALE tandem repeats make them susceptible to rearrangements when expressed in target cells due to recombination events that trigger deletions [32]. Moreover, the sheer size of TALE repeats creates obstacles to cellular delivery using some standard viral vectors—e.g., lentiviruses [32]. Such limitations have precluded wider adaptability of TALE-based tools for epigenome editing.

### 2.3. CRISPR–Cas–based systems

Clustered, regularly interspaced, short palindromic repeats (CRISPR) and CRISPR-associated sequences (Cas) constitute bacterial and archaeal endogenous adaptive immunity systems. They were first reported over three decades ago upon publication of findings of a cryptic cluster of unknown function in the bacterium *Escherichia coli* [33]. Advances in bioinformatics—alongside greater access to bacterial genomes in public databases around the turn of the century—led to the realization that such clusters exist in a wide range of bacteria and archaea [34]. Interest about the functional significance of the clusters in the scientific community grew steadily, which eventually led researchers to empirical evidence that CRISPR–Cas systems provide adaptive immunity against attack by viruses and plasmids [35] **Figure 3**.



**Figure 3.** Structural representation of CRISPR–Cas9 in complex with target double-stranded DNA and sgRNA. (A) Crystal structure of the CRISPR–Cas9 nuclease (gray) bound to a sgRNA (orange) and double-stranded target DNA (blue) primed for cleavage. Mutation of two Cas9 residues within the RuvC and HNH nuclease domains—D10A and H840A (green spheres)—abolishes the catalytic activity of the enzyme and transforms it into dCas9, which is able to bind its target sequence without triggering DNA cleavage. (B) Schematic cartoon representation of the CRISPR–Cas9–sgRNA–DNA complex (colors as shown in A). [PDB 5F9R].

CRISPR–Cas systems are currently classified into class 1 and class 2 groups, which are further divided into six different types and multiple subtypes [36]. Adaptive immunity to foreign nucleic acids is encoded in CRISPR arrays, which contain DNA sequences derived from the integration of invasive DNA as a new CRISPR spacer. CRISPR arrays are transcribed into precursor CRISPR RNAs (pre-crRNAs) and subsequently processed into mature CRISPR RNAs (crRNAs). After processing takes place, crRNAs mediate interference by acting as guides that recruit a variety of Cas effector proteins to complementary nucleic acids from invasive genetic elements [36]. Unlike zinc finger- and TALE-based systems, which rely on protein-DNA interactions to target specific genetic loci, CRISPR–Cas systems are DNA-encoded and RNA-guided. Thus, CRISPR–Cas systems harness nature's principles of Watson-Crick base pairing of nucleic acids to identify the target genetic loci. This property confers significant advantages to CRISPR–Cas systems because they bypass the need for complex protein engineering to mediate DNA recognition.

The type II CRISPR–Cas9 system is the most characterized CRISPR–Cas system to date. This complex requires dual RNA molecules—a crRNA and a trans-activating crRNA (tracrRNA)—that hybridize together and direct Cas9, a DNA endonuclease, to a target DNA sequence. Upon target recognition, Cas9 triggers double-stranded breaks a few nucleotides away from a protospacer adjacent motif (PAM) site [37]. The RNA-guided Cas9 endonuclease cleaves double-stranded DNA via conserved RuvC and HNH nuclease domains, which form a compact catalytic core [38].

In recent years, the CRISPR–Cas9 system has been repurposed into an efficient genome editing tool by engineering a chimeric single guide RNA (sgRNA) that fuses the crRNA and tracrRNA into a programmable RNA molecule capable of sequence-specific DNA targeting [37]. Genome editing biotechnological applications take advantage of Cas9-induced double-stranded DNA breaks to mediate DNA repair via one of two pathways: nonhomologous end joining (NHEJ), which introduces insertion or deletion mutations, or homology-directed repair (HDR), which introduces specific mutations or DNA sequences by recombination with a donor template. Importantly, adapting the CRISPR–Cas9 system for gene editing has also paved the path for repurposing the system for epigenome editing applications.

CRISPR-Cas9-mediated epigenome editing is based on the use of an engineered Cas9 protein, which has been purposefully stripped of its catalytic activity into a nuclease-null or “dead” Cas9 (dCas9). Mutating two residues—D10A and H840A—located in the RuvC and HNH nuclease domains is necessary and sufficient to deactivate the nuclease activity of the enzyme [38]. The mutations allow CRISPR–Cas9 to target specific DNA sequences and bind to them without cleaving DNA. Epigenetic effector proteins can then be fused to dCas9 to trigger desired local epigenetic changes, in much the same way mentioned earlier using zinc finger and TALE fusions to epigenetic effectors.

CRISPR–dCas9 has recently been used to demonstrate the feasibility of targeting specific epigenetic modifications to trigger transcriptional activation or repression. For instance, tethering dCas9 to an epigenetic effector that acetylates histone tail residues led to transcriptional activation in one study [39], and a dCas9 fusion to a repression domain showed the CRISPR–dCas9 system's ability to recruit a heterochromatin-forming complex to silence gene expression [40]. The next section discusses these and other epigenome editing studies in more detail.



### 3. Epigenome editing to regulate gene expression

Manipulation of endogenous gene expression at target loci has thus far been achieved by coupling DNA-binding proteins with (1) transcriptional activators and repressors, which modulate gene expression by acting as site-specific ATFs, or (2) epigenetic effectors, which catalyze the deposition or removal of specific epigenetic modifications at target loci. In addition, the CRISPR–dCas9 system has recently enabled engineering of modular sgRNA molecules that carry RNA aptamers as scaffolding platforms to recruit molecules for site-specific regulation.

#### 3.1. Transcriptional activation

Transcriptional activator effector domains were first used to upregulate levels of gene expression by fusing the herpes simplex virus protein VP16—involved in recruitment of chromatin remodeling factors that increase chromatin accessibility—and its VP64 tetrameric form to zinc finger proteins [41, 42]. For instance, an early study demonstrated that zinc finger proteins targeted to 18 base-pair sequences of the 5' untranslated region of the proto-oncogene *erbB-2* could upregulate transcription in an endogenous context [41]. Upregulation was also shown to occur even under control of an exogenous chemical inducer molecule [41]. Similarly, zinc finger fusions to the VP64 activation domain have been used to reactivate dormant genes. In one study, a dormant mammary serine protease inhibitor (*maspin*) tumor suppressor gene was reactivated by targeting the *maspin* promoter region, which is often epigenetically silenced in aggressive epithelial tumors [43]. Other transcriptional activator domains, such as the p65 subunit of the NF- $\kappa$ B complex, have also been coupled to zinc finger proteins for targeted transcriptional activation [44].

The proof-of-concept studies using zinc fingers fused to transcriptional activation domains to modulate gene expression patterns served as blueprints for the subsequent use of activator domain fusions to TALEs and CRISPR–dCas9 systems. TALE activators targeted to promoter sites of the endogenous human *VEGF-A*, *NTF3*, and the microRNA *miR-302/367* cluster have been reported to induce increased target gene expression by fivefold or more [28]. Endogenous levels of gene expression of select human pluripotency factors have also been shown to increase by twofold to fivefold using TALE fusions to VP64 [45].

Furthermore, although single TALE fusions to transcriptional activators have demonstrated the ability to upregulate gene expression, combinations of TALE-VP64 fusions targeting the promoter regions of genes implicated in inflammation, immunomodulation, and cancer pathways have established the occurrence of synergistic activation effects at target sites, which presents opportunities to develop tunable transcriptional networks [46]. Interestingly, it appears that different types of transcriptional activators induce varying levels of gene expression. In one study, the mean-fold activation induced with TALE-p65 activators was lower than that with TALE-VP64 activators [28]. The combination of VP64 and p65 activators exhibits synergistic effects on endogenous gene expression, which can provide a platform not only to design tools for targeted gene expression, but also to fine-tune the fold activation induced by different combinations of activator domains fused to DNA-binding proteins [28].

Recently, researchers have built upon work on zinc finger and TALE fusions to develop technologies for targeted gene expression utilizing the CRISPR–dCas9 system. RNA-guided transcriptional upregulation using CRISPR–dCas9-VP64 and CRISPR–dCas9-p65 fusions has been reported by several research teams in the last 5 years [47–50]. Introduction of individual sgRNAs for dCas9 targeting can induce transcriptional activity at desired loci, but the use of multiple sgRNAs to target a single locus can act synergistically to induce multifold gene expression [48–50]. The CRISPR–dCas9 system offers several advantages over zinc fingers and TALEs for targeted gene activation. Because CRISPR–dCas9 is an RNA-guided, DNA-targeting system, it does not require complex protein engineering and could open additional research paths for spatiotemporal control of gene expression alongside complex chemical and optogenetic inducible systems. However, some studies have reported that dCas9-based activation tools induce weaker levels of transcription compared to TALE-based activators [50, 51].

In addition to the use of transcriptional activators for targeted gene expression, research teams have demonstrated the efficacy of using epigenetic effectors fused to DNA targeting proteins to manipulate epigenetic landscapes. In 2015, a study reported the use of CRISPR–dCas9, TALE, and zinc finger fusions to the p300 histone acetyltransferase (HAT) catalytic domain, which deposits H3K27 and other acetylation marks on histone tails, to trigger transactivation of genes [39]. Notably, the study showed that CRISPR–dCas9-p300 Core fusions led to more efficient and robust activation from proximal and distal enhancer regions compared to synthetic transcription factors engineered to carry activation domains that target promoters. Other epigenetic effectors that catalyze covalent modifications on DNA have also been fused to DNA-binding proteins to induce gene expression. For example, ten-eleven translocation (TET) demethylase enzymes that catalyze the sequential oxidation of 5-methyldeoxycytosine ( $m^5dC$ ) to form 5-hydroxymethylcytosine ( $hm^5C$ ), 5-formylcytosine ( $f^5C$ ), and 5-carboxylcytosine ( $ca^5C$ ) have been used with zinc fingers [52–54], TALEs [55], and CRISPR–dCas9 [56, 57] to direct promoter-specific DNA demethylation and, thus, upregulate gene expression.

As mentioned earlier, CRISPR–dCas9 systems represent promising tools for the next generation of complex inducible systems. More recently, a novel system comprising CRISPR–dCas9 fused to a tripartite activator featuring the constituent activation domains VP64, p65, and Rta (VPR) was shown to activate endogenous coding and noncoding regions while targeting several genes simultaneously [58]. Another platform built with CRISPR–dCas9 and a protein scaffold—namely, a repeating peptide array called the SuperNova tagging system (SunTag)—capable of recruiting multiple copies of an antibody fusion protein showed robust levels of endogenous gene activation at target sites [59]. Lastly, it has been demonstrated that CRISPR–Cas sgRNAs can be engineered to carry protein-binding cassettes, artificial aptamers, and other ncRNAs directly into the sgRNA stem-loop structures, thereby creating modular CRISPR–dCas9 complexes that enable locus targeting, multiplexing, and highly inducible regulatory action to upregulate patterns of gene expression [60–62].

### 3.2. Transcriptional repression

Silencing endogenous gene expression at target loci has been achieved in similar ways to gene activation by fusing repressor domains or epigenetic effectors to DNA-binding proteins.

The domain most commonly used to trigger gene silencing is the Krüppel-associated box (KRAB). KRAB repression is mediated by the recruitment of complexes that elicit formation of heterochromatin via interactions between the KRAB-associated protein 1 (KAP1) corepressor and other factors that catalyze histone methylation and deacetylation [17, 63]. KRAB fusions to zinc finger proteins [41, 64], TALEs [65], and CRISPR–dCas9 [40, 47] have demonstrated robust inactivation from promoter as well as proximal and distal regulatory elements.

Although the KRAB repressor is the most common domain used for recruitment of heterochromatin-forming factors, researchers have also reported efficient gene silencing with other repressor domains such as the mSin interaction domain (SID) [65] and Mxi1 [47]. One study comparing dCas9-KRAB and dCas9-Mxi1 showed that the Mxi1 fusion could induce more repression by nearly threefold levels [47]. A separate study found that TALE-SID repressors could lead to 26% more transcriptional repression than its TALE-KRAB counterparts [65]. Moreover, concatenating four SID (SID4X) domains—much like combining VP16 units into VP64 activator proteins—and fusing them to DNA-binding proteins can induce greater repression than single SID domain fusions [66].

In contrast to transcriptional repressors, which aptly recruit heterochromatin-forming complexes to desired loci, epigenetic effectors can directly catalyze specific modifications on histones and DNA that trigger targeted epigenetic repression. Several studies have thus far tested and confirmed robust repression efficiency rates of histone methyltransferases [17], histone demethylases [29, 67], and DNA methyltransferases [68–70] with zinc finger, TALE, and CRISPR–dCas9 proteins at target promoter and enhancer regions.

Notably, a study featuring a CRISPR–dCas9 fusion to the catalytic domain of DNMT3A, a *de novo* DNA methyltransferase, found that multiplexing with several sgRNAs exhibits synergistic effects on site-specific CpG methylation [70]. Thus, CRISPR–dCas9-DNMT3A multiplexing can lead to a greater increase of methylation levels at target regions. Despite the synergistic effects seen with DNMT3A repressor fusions, it appears that dCas9-DNMT3A epigenetic editing of target CpG methylation sites is not wholly stable and does not persist beyond the loss of expression of the editing construct [70]. To overcome this type of transient epigenetic editing, a study recently reported a system for inheritable silencing of endogenous genes by transiently expressing combinations of DNA-binding proteins fused to transcriptional repressors, including KRAB and DNMT3 domains, to prompt long-term memory of repressive epigenetic states [71]. Lastly, the modular nature of sgRNAs has enabled the creation of scaffolding systems to achieve multiplexing and multimerization of epigenetic effectors, which will be useful to study endogenous and synthetic gene repression [62, 72].

### 3.3. Technological limitations

Epigenome editing directed by catalytically active or scaffolding epigenetic effectors fused to programmable DNA binding proteins has contributed important insights about the causal relationships between epigenetic states and gene regulation. However, despite the progress made in recent years to uncover the molecular basis of epigenetic processes, epigenome editing

remains a nascent field that must overcome many technological challenges related to editing efficiency, delivery, cytotoxicity, and specificity.

A major current limitation concerns the spatio-temporal specificity of epigenome editing, which may involve multiple factors including, but not limited to, non-specific deposition or removal of select epigenetic marks on target DNA loci or histones, mitigation of off-target DNA binding effects, and temporal expression of epigenetic effectors in different cell types and chromatin contexts [3]. For example, enzymes that catalyze the deposition of histone PTMs—e.g., p300's HAT domain, which catalyzes acetylation of multiple residues on all four core histones—have various degrees of histone substrate specificity. Thus, the inherent promiscuity of an enzyme may pose challenges to the study of direct functional roles for unique PTMs in specific contexts [3].

Similarly, the expression levels and times at which an epigenetic effector is present in a cell type can exert effects on the efficiency of DNA binding specificity. Following target site saturation, off-target binding activity is likely to increase if the effector remains, or continues to be expressed, in the cell. This could partly explain the extensive global off-target effects reported with the use of dCas9–methyltransferase fusions in one study [73]. Given the lack of target site specificity previously reported for dCas9–sgRNA complexes [74], further research is needed to establish whether other dCas9–effector fusions exhibit similar off-target activities. The use of zinc finger and TALE fusions to epigenetic effectors offers an alternative to mitigate possible dCas9-related off-target effects. However, zinc finger and TALE epigenome editing biotechnologies are limited by the complex protein engineering required to mediate DNA recognition.

Progress in other areas associated with challenges in delivery, efficiency, and stability of epigenetic states has come at a rapid pace [75–77]. Chemical and optogenetic molecules have expanded the toolbox for reversible and inducible epigenome editing in endogenous contexts [3, 66, 78]. And emerging technologies for RNA editing [79] could soon provide new insights about the epigenetic roles of ncRNAs. As the field matures, epigenome editing will undoubtedly make significant contributions to the advancement of basic and applied research.

## 4. Conclusion

Recent technological breakthroughs in epigenome editing have expanded our understanding of the underlying mechanisms responsible for the regulation of chromatin structure and the spatiotemporal control of gene activation and repression. As detailed in this chapter, modern epigenome editing systems are revolutionizing the field of epigenetics. Programmable DNA-binding proteins fused to catalytically active or scaffolding epigenetic effectors represent invaluable tools to uncover the functional significance of site-specific epigenetic mechanisms in a myriad of contexts including development, cell differentiation, and disease. Undoubtedly, progress in this emerging field will offer great contributions to translational medicine and biotechnology in the near future.



## Conflict of interest

The author declares no conflicts of interest.

## Acronyms and abbreviations

PTM	post-translational modification
ncRNA	noncoding RNA
ZFN	zinc finger nuclease
ATF	artificial transcription factor
TALEN	transcription activator-like effector nuclease
RVD	repeat variable diresidue
CRISPR	clustered, regularly interspaced, short palindromic repeats
Cas	CRISPR-associated sequences
pre-RNA	precursor CRISPR RNA
crRNA	CRISPR RNA
tracrRNA	trans-activating crRNA
sgRNA	single-guide RNA
NHEJ	nonhomologous end joining
HDR	homology-directed repair
TFO	triple helix-forming oligonucleotide
MASPIN	mammary serine protease inhibitor
HAT	histone acetyltransferase
TET	ten-eleven translocation
m <sup>5</sup> dC	5-methyldeoxycytosine
hm <sup>5</sup> C	5-hydroxymethylcytosine
f <sup>5</sup> C	5-formylcytosine
ca <sup>5</sup> C	5-carboxylcytosine
VPR	VP64, p65, and Rta

SunTag	SUPerNOVA tagging
KRAB	Krüppel-associated box
KAP1	KRAB-associated protein 1
SID	mSin interaction domain

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