

CRISPR-Mediated Epigenome Editing

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Mounting evidence has called into question our understanding of the role that the central dogma of molecular biology plays in human pathology. The conventional view that elucidating the mechanisms for translating genes into proteins can account for a panoply of diseases has proven incomplete. Landmark studies point to epigenetics as a missing piece of the puzzle. However, technological limitations have hindered the study of specific roles for histone post-translational modifications, DNA modifications, and non-coding RNAs in regulation of the epigenome and chromatin structure. This feature highlights CRISPR systems, including CRISPR-Cas9, as novel tools for targeted epigenome editing. It summarizes recent developments in the field, including integration of optogenetic and functional genomic approaches to explore new therapeutic opportunities, and underscores the importance of mitigating current limitations in the field. This comprehensive, analytical assessment identifies current research gaps, forecasts future research opportunities, and argues that as epigenome editing technologies mature, overcoming critical challenges in delivery, specificity, and fidelity should clear the path to bring these technologies into the clinic.

INTRODUCTION

The realization that phenotypic inheritance is not merely a consequence of genetic processes, but implicates responses to environmental stimuli is one of the great discoveries of the twentieth century. Conrad Waddington's seminal *Drosophila melanogaster* experiments establishing phenotypic plasticity induced by chemical or temperature stimuli [1] cemented the notion of "epigenetic landscapes" [2] as the supreme phenomenon driving cellular differentiation, and the molecular basis bridging the gap between genotype and phenotype. Today, epigenetics is a dynamic and prolific field broadly aimed at studying fundamental processes related to mitotic and meiotic stable and heritable changes—in gene

expression or cellular phenotype—that occur without alteration of DNA sequences [3].

An explosion of research spanning the last two decades has revealed remarkable insights into epigenetic mechanisms, including the discovery of myriad phenotypic outcomes associated with histone post-translational modifications (PTM[†]), DNA modifications, and non-coding RNAs (ncRNAs). Together, these concomitant processes orchestrate a highly complex system that mediates organization of chromatin structure and the epigenetic regulation of two meters of genomic DNA tightly packed into a 5 μ m [4] three-dimensional region. Despite the progress made, the precise functional roles for these epigenetic processes in development, cellular program-

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†Abbreviations: PTMs, post-translational modifications; ncRNAs, non-coding RNAs; CRISPR, clustered, regularly interspaced, short palindromic repeats; Cas9, CRISPR-associated protein 9; ZFNs, zinc finger nucleases; TALENs, transcription activator-like effector nucleases; TALE, transcription activator-like effector; HAT, histone acetyltransferase; KRAB, Krüpel-associated box; KAP1, KRAB-associated Protein 1; LSD1, lysine-specific demethylase 1; dCas9, nuclease-null, 'dead,' or 'deactivated' Cas9; VP16, virion protein 16; VP64, tetrameric VP16; sgRNA, single guide RNA; PCR, polymerase chain reaction; CHIP-qPCR, chromatin immunoprecipitation coupled with quantitative PCR; SUMO, small ubiquitin-like modifier; RING, really interesting new gene; E3, ubiquitin ligase; E2, ubiquitin-conjugating enzyme; PHD, plant homeodomain; HP1, heterochromatin protein 1; SETDB1, SET domain bifurcated 1; NuRD, nucleosome remodeling and deacetylase complex; ATP, adenosine triphosphate; NCOR1, nuclear receptor corepressor 1; TAF3, TBP-associated factor 3; DNMT, DNA methyltransferase; TET, ten-eleven translocation; TET1, TET dioxygenase 1; m⁵dC, 5-methyldeoxycytosine; hm⁵C, 5-hydroxymethylcytosine; f⁵C, 5-formylcytosine; ca⁵C, 5-carboxylcytosine; m⁶dA, N⁶-methyldeoxyadenosine; H3K14ac, histone H3 lysine 14 acetylation; H3K27me3, histone H3 lysine 27 trimethylation; CRY2, cryptochrome circadian clock 2; CIB1, cryptochrome-interaction basic helix–loop–helix 1; HIV, human immunodeficiency virus; HSV1, herpes simplex virus 1; PAM, protospacer adjacent motif; Cpf1, CRISPR from *Prevotella* and *Francisella* 1.

Keywords: Epigenome editing, epigenome engineering, CRISPR, CRISPR-Cas9, optogenetics, epigenetics, histone and DNA epigenetic modifications, transcription activation, transcription repression, CRISPR systems for epigenome editing

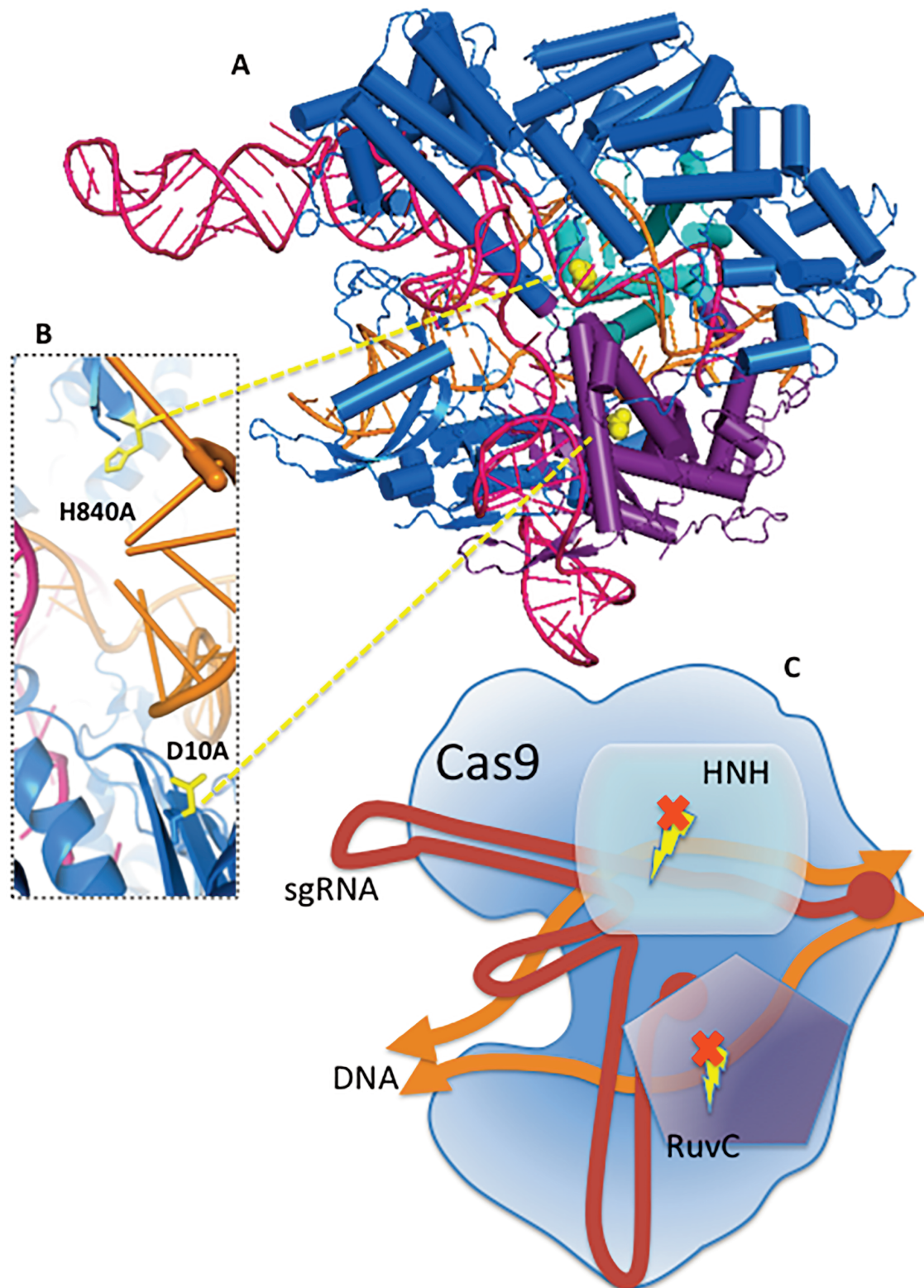


Figure 1. Structural representation of the nuclease-null Cas9 (dCas9) from *S. pyogenes*. **A.** Crystal structure of Cas9 in complex with a single guide RNA (red) and its target DNA (orange). The HNH (cyan) and RuvC (purple) catalytic domains are shown. The non-catalytic regions of Cas9 are colored in blue. Mutation of the catalytic residues (D10A and H840A) that render Cas9 inactive (dCas9) are colored in yellow. **B.** Close-up view of the active site and position of the catalytic residues shown in A. **C.** Schematic representation of nuclease-null Cas9 in complex with sgRNA and target DNA; colors as shown in A. [PDB 40O8].

ming, biology, disease, and medicine still remain poorly understood. A pressing need exists for widely accessible methods to overcome current technological limitations, which have hindered our ability to study epigenetic regulation and gene expression at local and genome-wide levels.

In recent years, the CRISPR-Cas9 (clustered, regularly interspaced, short palindromic repeats (CRISPR)-CRISPR-associated protein 9) system has been adopted as a robust and versatile genome editing tool [5] for bacterial [6] and eukaryotic [7] organisms. The CRISPR-Cas9 complex is an adaptive immunity, type II CRISPR-Cas system in bacteria and archaea that uses antisense RNAs to recognize and cleave foreign DNA [8]. CRISPR-based technologies have become popular for scientific research and the CRISPR-Cas9 system has recently been repurposed for epigenome editing by engineering a nuclease-null or “dead” Cas9 (dCas9) (Figure 1), which permits targeting specific DNA loci without cleavage [9]. CRISPR-based epigenome editing technologies are poised to become powerful tools to further our understanding of the roles that epigenetic marks and effectors play in cancer and other human diseases [10,11]. More importantly, this new technology holds great promise for the future of medicine.

This feature highlights CRISPR systems, including CRISPR-Cas9, as novel tools for targeted epigenome editing. The history behind the concept of epigenome editing, which emerged with the advent of Zinc Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs), has been the subject of two recent reviews [12,13]. As a result, I will not rehash what has already been discussed in great detail. Instead, this paper identifies current research gaps and critically examines shortcomings in the literature that have not been previously analyzed. Furthermore, it emphasizes recent discoveries that expand the catalog of DNA modifications and ncRNAs that can be interrogated using epigenome editing technologies. A summary of recent developments in the field, including integration of chemical, optogenetic, and functional genomic approaches to explore new therapeutic opportunities is provided briefly. Overall, this piece underscores the importance of mitigating current limitations in the field, forecasts future research opportunities beyond CRISPR-Cas9 systems, and argues that as epigenome editing tools mature, overcoming critical challenges in delivery, specificity, and fidelity should clear the path to bring these technologies into the clinic.

TARGETING HISTONE EPIGENETIC MODIFICATIONS

Epigenome editing technologies have demonstrated the feasibility of targeting histone epigenetic modifications. Two seminal papers published in 2015 provide important insights into the capabilities of repurposing the

CRISPR-Cas9 system as an epigenome editing platform to trigger changes in transcriptional activation or repression by targeting histone PTMs known to play important roles in chromatin structure and accessibility. One involved the activation of gene expression using a nuclease-null dCas9 tethered to an acetyltransferase (HAT) enzyme that catalyzes direct covalent modification of ϵ -lysine residues on histone tails [14]. In contrast, the other demonstrated gene silencing using a nuclease-null dCas9 fused to the Krüppel-associated box (KRAB), a naturally-occurring transcriptional repression domain involved in recruiting a heterochromatin-forming complex that mediates histone methylation and deacetylation [15].

Both approaches fuse a catalytically inactive dCas9 to an epigenetic effector. However, activation is achieved by direct catalysis to transfer an acetyl moiety onto histone lysine residues, while repression is mediated by indirect recruitment of enzymes that catalyze the transfer of methyl or removal of acetyl groups from histone residues. I discuss these epigenome editing approaches below to highlight the progress and shortcomings of the concept of targeting histone marks to study epigenetic regulation.

Transcriptional Activation

Manipulation of transcription and gene expression without directly modifying DNA sequences has been a long-standing goal in chromatin biology. Previous efforts to control transcriptional activation led to the creation of programmable, viral-based, transactivation effector domain—e.g., VP16 and its VP64 tetrameric form—fusions to TALEs [16] and Zinc Finger [17] proteins. These tools were limited by their physical and biological properties, namely, the fact that VP16 and VP64 are not chromatin modifying enzymes, but rather recruit chromatin remodeling factors that facilitate access to specific chromatin loci [18]. In the last few years, however, the first reports of TALE fusions to chromatin modifiers were published as proof-of-concept for site-specific epigenome editing [18-20], thereby opening the path for adoption of these principles to CRISPR-Cas systems.

In 2015, a study reported the use of a dCas9 fusion to the catalytic histone acetyltransferase (HAT) core of p300 [14] (Figure 2). dCas9-p300 Core fusions were shown to be more potent at achieving transactivation of genes from proximal and distal enhancers than engineered transcription factors made with activation domains [14]. Moreover, the fusion established robust gene activation by a single guide RNA (sgRNA) at promoters and characterized enhancers [14]. The study provides an important contribution to the scientific literature and marks an important step toward programmable, facile, epigenome editing of histone PTMs. However, a few significant shortcomings not discussed in the article or subsequent literature can be identified following closer scrutiny of the findings and conclusions averred by the authors. I highlight some of the shortcomings later in this section.

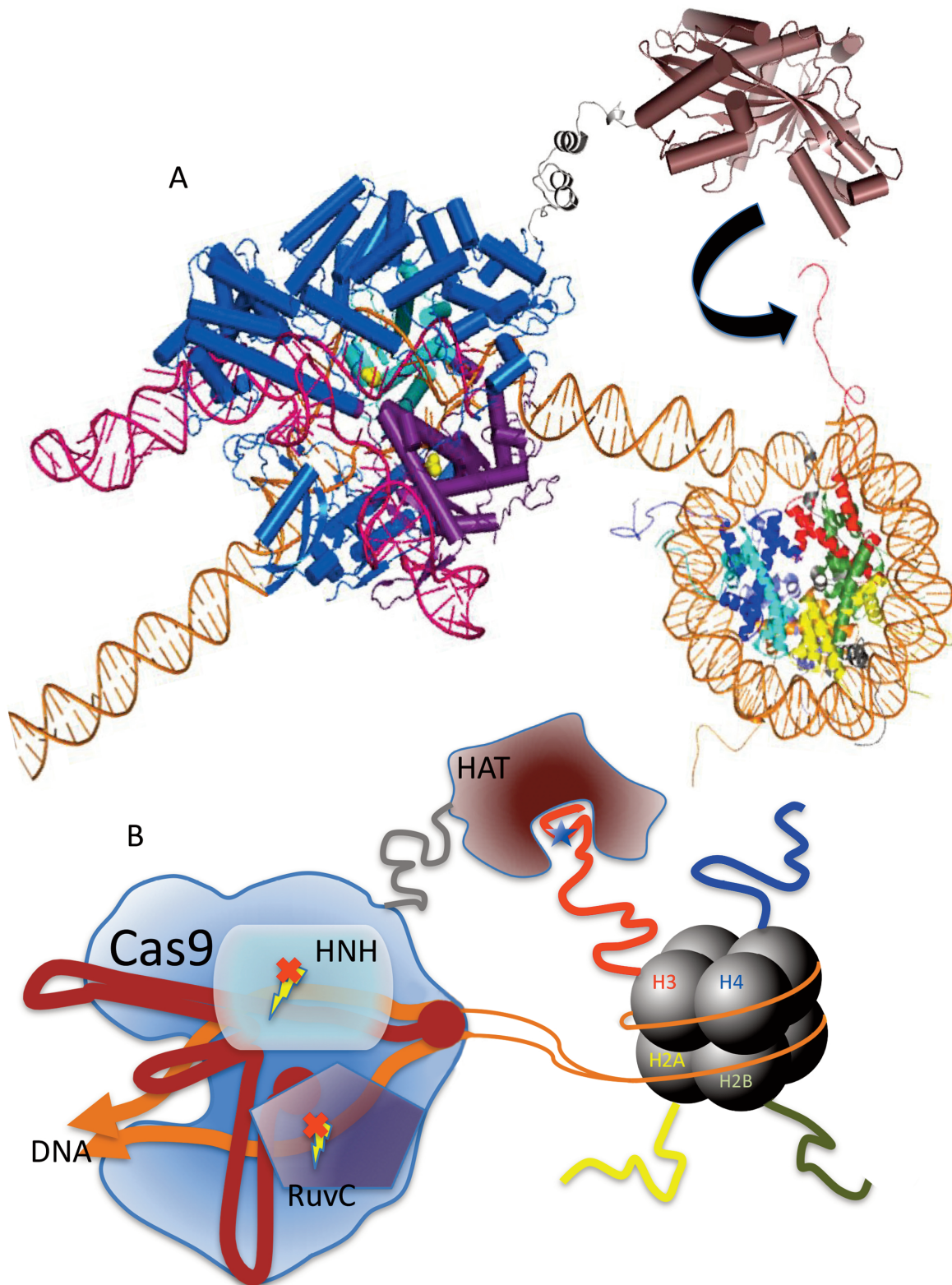


Figure 2. Schematic representation of epigenome editing using dCas9 fused to an epigenetic effector. A. dCas9 (colors are the same as in Figure 1) binds to target DNA without triggering double-stranded DNA cleavage. An epigenetic effector—p300 histone acetyltransferase (HAT) in this drawing (brown)—is fused to dCas9 by a linker region (gray). Once positioned near the target epigenetic loci, the effector catalyzes covalent modification of the target histone tail on nearby nucleosomes (the reaction is represented by the black arrow). **B.** Cartoon illustration of A. Covalent modification illustrated by a blue star on the substrate histone tail. [PDB 4008, 3BIY, 1KX5].

Transcriptional Repression

Targeting of promoter regions or other cis-regulatory elements using the DNA binding domains of Zinc Finger proteins [21,22] and TALEs [19,23] has been reported in the past as capable of achieving effective gene silencing. More recently, evidence of effector-mediated gene repression using CRISPR-dCas9 fusions has surfaced to demonstrate proof-of-concept for CRISPR-mediated epigenome editing to silence proximal and distal regulatory elements [15,24].

Two studies published in 2015 offer differing CRISPR-based approaches to repress transcription. The first utilized a nuclease-null dCas9 fused to a KRAB repressor domain [15], a motif commonly found in eukaryotic Zinc Finger proteins [25]. This type of silencing is achieved by KRAB-mediated recruitment of complexes that trigger formation of heterochromatin at specified loci, primarily through protein-protein interactions between the KRAB-associated Protein 1 (KAP1) corepressor and other factors [26-28]. The study sought to interrogate the genome-wide specificity and chromatin remodeling activity catalyzed by dCas9-KRAB at the HS2 enhancer, a 400 bp distal regulatory element comprising several enhancer regions that orchestrate expression of hemoglobin subunit genes in erythroid cells throughout development [15]. Targeting of the HS2 enhancer by dCas9-KRAB fusions was shown to silence the expression of globin genes with relatively little off-target effects in expression of non-targeted genes [15]. Additionally, the study revealed decreased levels of chromatin accessibility at targeted promoters and enhancers, as well as increased deposition of H3K9me3 repressive epigenetic marks within the selected HS2 region [15].

The second study sought to compare the repressive properties of dCas9-KRAB and a fusion of dCas9 to the Lysine-specific demethylase 1 (LSD1) histone demethylase, which is responsible for catalyzing the elimination of methyl marks on H3K4 [29] and H3K9 [30] residues, and has been associated with repression of enhancer regions [24]. The study demonstrated that dCas9-LSD1 triggers repression through decommissioning of target enhancers in an effector-dependent manner without significant disturbance of local chromatin architecture, whereas dCas9-KRAB repression is likely the result of promoter silencing or heterochromatin spreading [24]. Interestingly, these results stand in contradiction to findings reported in the dCas9-KRAB HS2 enhancer study [15], which concluded that the dCas9-KRAB system can trigger deposition of H3K9me3 marks at endogenous enhancers, suggesting that epigenome editing via dCas9-KRAB-mediated histone methylation may be context and cell-type dependent.

Current Limitations

Epigenome editing directed by CRISPR-dCas9-p300 Core, dCas9-KRAB, and dCas9-LSD1 fusions detailed in

the studies above provides tangible evidence of the feasibility of developing CRISPR-based technologies for targeted manipulation of histone epigenetic modifications. However, this proof-of-concept underscores the fact that epigenome editing has yet to come full-term. Whereas genome editing research is currently in its infancy, at this point in time, epigenome editing can be considered to be at an embryonic stage. Therefore, to propel epigenome editing forward into the clinical stage, CRISPR researchers must heed research gaps and deficiencies hidden in these foundational studies. Awareness of early technological shortcomings will permit protocol optimization and lead to data reproducibility that will prove essential in building the next generation of epigenome editing technologies.

With respect to the claim that highly specific epigenome editing was achieved across promoters and enhancers genome-wide, the data presented by the CRISPR-dCas9-p300 Core study merely corroborates targeting of desired DNA loci, not the deposition of unique epigenetic marks on histone tails. Although the authors correctly point out that acetylation of histone H3 at lysine 27 (H3K27ac) is catalyzed by p300 [14], they did not mention or consider the inherent promiscuity of the enzyme. The HAT domain of the p300 transcriptional coactivator is all but highly specific and has been shown to catalyze the acetylation of a laundry list of residues on all four—H2A, H2B, H3, and H4—core histones [31-33], transcription factors [34], and other proteins [35] to regulate a number of key biological processes. Thus, the allegation that unique targeted acetylation of H3K27 occurred at enhancers and promoters is only as good as the concession that H3K27ac was the one and only PTM the authors sought to identify. Given the poor specificity of the p300 HAT toward histone substrates, it is likely that other putative acetylation events would have been detected had chromatin immunoprecipitation followed by quantitative PCR (ChIP-qPCR) been performed using other histone PTM antibodies.

Despite evidence for robust transactivation at target genes using dCas9-p300 Core [14], it can hardly be said that highly specific epigenome editing took place in the transfected cell lines. The touchstone principle of epigenome editing concerns uncovering direct functional roles for unique PTMs in specific contexts without encumbrance from correlative approaches or the potential secondary effects driven by unintended factors. Besides transactivation of the target genes, non-H3K27 acetylation could likely have unspecified functional roles including recruitment of specific readers of other acetylation marks, structural effects on higher order chromatin structure, or PTM-dependent signaling cascades. This highlights a key obstacle for current CRISPR-directed epigenome editing technologies: contrary to the prevailing wisdom in genome editing, mitigation of off-target effects in epigenome editing is not exclusively related to the DNA-binding activity of dCas9 at specific loci, but also

off-target effects related to deposition of PTM marks at specified nucleosomes with spatio-temporal precision.

The potential for non-specific hyperacetylation of nucleosomal substrates by dCas9-p300 Core also raises concerns about antibody specificity during epigenetic experiments. It has been reported recently that some commercially available histone PTM antibodies cannot frequently detect their intended targets [36]. Although the issue of histone antibody specificity arguably applies to many epigenetic experiments, the problem is of particular importance in dCas9 fusions to catalytic domains. For instance, evidence shows that many site-specific H4-acetyl antibodies preferentially bind to poly-acetylated histone peptides [36]. Based on these observations, hyperacetylation of proximal nucleosomes by a tethered dCas9-p300 Core HAT might confound ChIP-qPCR experiments using anti-H3K27ac antibodies. Thus, potential non-H3K27 acetylation events may have played a role in detecting highly enriched regions of H3K27ac at the enhancers and promoters surveyed.

The underlying principles applicable to transcriptional activation also apply to epigenome editing involving transcriptional repression and regulation of gene expression. Similar to HATs, other enzymes including histone deacetylases, methyltransferases, demethylases, kinases, phosphatases, ubiquitin and SUMO ligases, etc. have varying degrees of substrate specificity, and involve the use of commercial antibodies to detect PTM marks deposited on histones.

Although the effectiveness of transcriptional repression directed by epigenome editing via the dCas9-KRAB system has been aptly demonstrated, the use of KRAB domains as scaffolding platforms for recruitment of heterochromatin-forming complexes raises numerous questions, particularly concerning the dCas9-KRAB system's future applicability in clinical settings. Compared to the relatively straight forward characterization of functional roles for individual epigenetic effectors—e.g. LSD1—that catalyze either deposition or removal of specific histone epigenetic marks responsible for transcriptional suppression, the manipulation and control of functional outcomes associated with KRAB-recruited protein complexes working in tandem to trigger suppression is inherently more intricate.

Consider the KAP1 corepressor, a multi-domain protein that features a putative catalytic RING finger domain—a common E3 ubiquitin ligase motif that mediates the transfer of ubiquitin from an E2 ubiquitin-conjugating enzyme to substrate [37]—and two epigenetic reader domains [38]—a PHD domain and a bromodomain—capable of reading methyl- [39] and acetyl- lysine [40] residues. Recently, the KAP1 RING domain was characterized as a specific small ubiquitin-like modifier (SUMO) E3 ligase involved in the regulation of innate immunity [41].

Although SUMOylation has been linked to transcriptional repression [42,43] and, thus, fits within the overall purpose of the dCas9-KRAS system, new SUMOylation functional roles beyond repression have been reported, such as activation of DNA damage signaling cascades [44]. Like ubiquitination, it is likely that SUMOylation is involved in a widespread range of context-dependent, substrate functional roles including proteasome-dependent proteolysis, signaling, assembly, cellular localization, and others [37].

Consequently, dCas9-KRAB epigenome editing aimed at repression could be confounded by physical and biochemical events that obfuscate the specific contributions of each component of the system's repressing machinery. This could have serious repercussions for clinical therapies, in which CRISPR-dCas9-KRAB-mediated epigenome editing is capable of eliciting a desired silencing event, but triggers a deleterious secondary effect caused by unknown or unforeseen processes. Indeed, on this point, KRAB's corepressor, KAP1, is known to serve as a scaffold to recruit a myriad of factors and complexes associated with repressive epigenetic states including heterochromatin protein 1 (HP1) [45], SETDB1—a methyltransferase that catalyzes deposition of methyl histone marks on H3K9 [46] and other substrates [47], NuRD—a corepressor complex that catalyzes histone deacetylation and ATP-dependent chromatin remodeling [48], and N-COR1—a histone deacetylase complex [49]. KAP1 has also been linked to functional roles in DNA double-stranded repair [50], restriction of retrovirus replication [51], and regulation of self-renewal in embryonic stem cells [52].

Given the vast range of KRAB- and KAP1-mediated interactions, it is not far-fetched to foresee hurdles associated with epigenome editing, particularly when repression is orchestrated by multiple enzymes of varying degrees of substrate specificity, and proteins involved in long-range interactions. This is a significant concern—even if recruitment of repressive complexes is only targeted to a specific locus—mainly because of potential effects on chromatin architecture, and looping of discrete distant regulatory regions. Thus, individual characterization of functional roles for repressive epigenetic effectors using CRISPR-based methods will be an important first step in the path toward targeting of concomitant repressive processes.

Overcoming Current Limitations

As pointed out above, major limitations currently exist in the development and future translational applications of epigenome editing technologies. Nevertheless, the scientific community is working hard to try to tackle these obstacles head-on. As new research and technologies improve, it is likely that current impediments to efficient epigenome editing will gradually be surmounted.

One promising and appealing technique to ameliorate the problem of antibody specificity and reproducibility that often plagues epigenetic experiments was recently published and highlights the feasibility of using recombinant epigenetic readers as reliable substitutes for histone PTM antibodies [53]. The study validated the use of recombinant TAF3 PHD domain, a motif that specifically reads H3K4me3 histone marks [54], as a robust anti-H3K4me3 affinity reagent in techniques ranging from western blotting to ChIP experiments coupled with qPCR and deep sequencing [53]. This study illustrates a key methodology to circumvent current problems associated with commercial antibodies. In the last decade and a half, the epigenetics research community has characterized numerous epigenetic readers—many of which exhibit high substrate specificity and can be easily produced recombinantly—that could be repurposed as anti-histone PTM reagents to abolish the need for substandard commercial antibodies.

Other existing limitations concerning inadequate antibody specificity for epigenome editing experiments are likely to become less prominent with increased collaborations and partnerships between industry and academia that give rise to common interests for developing highly specific antibodies. The research community ought to speak in unison to exercise its commercial power and demand improved standardized protocols and internal quality controls to minimize batch-to-batch variations commonly seen among commercial suppliers of antibodies, especially polyclonal ones. Given the great significance of using reliable and adequate antibodies that facilitate reproducibility of results by independent researchers, it is also imperative that the research community avail itself of open source resources aimed at characterizing and validating antibody performance in epigenetic experiments such as the Antibody Validation Database and the Antibody Specificity Database [36,55].

The limitations surrounding substrate specificity of HAT and other epigenetic effector enzymes present a greater obstacle to the development of efficient and programmable CRISPR-mediated epigenome editing. Detailed structural information and cumbersome protein engineering is likely necessary to boost efforts to reduce substrate promiscuity and increase cognate target selectivity among certain epigenetic effectors. Structural characterization of readers, writers, and erasers of the epigenome—in their isolated states as well as within the complexes they form—is also critical to guide structure-based drug design efforts aimed at developing chemical probes to modulate epigenetic regulatory proteins. Unfortunately, many of these endeavors are time-consuming, labor-intensive, and require expenditure of considerable resources, which may not always be available to researchers. As CRISPR-mediated epigenome editing technologies mature, it will also be important to incorporate functional genomic, pharmacogenomic, and high-through-

put approaches to ensure studies properly identify predictable events, as well as non-specific epigenome editing of target loci.

INTERROGATION OF DNA EPIGENETIC MODIFICATIONS

Following propitious reports describing the viability of CRISPR-mediated epigenome editing of histone PTMs to trigger transcriptional activation or repression in the last year, CRISPR researchers have swiftly begun to probe DNA epigenetic modifications at select regulatory elements. In 2016, several reports from independent research teams established—almost simultaneously—proof-of-concept for up- or down-regulation of target genes elicited by dCas9 fusions to catalytic domains, directing either methylation or demethylation of CpG islands that span promoter regions [58,59,66,67].

DNA Methylation

Programmable DNA methylation at specific CpG islands has been reported via fusion of dCas9 to the catalytic domain of the *de novo* DNA methyltransferase 3A (DNMT3A), a cytosine-5 methyltransferase [56,57], at the human *CDKN2A* and *ARF* promoters, and the mouse *Cdkn1a* promoter [58]. This dCas9-DNMT3A study showed that the induction of DNA methylation was confined within a region spanning approximately 50 bp of the sgRNA target site, and is strongest between adjacent and inwardly directed sgRNA binding sites [58]. The induced methylation at the promoter sites was sufficient to lower expression of all three genes targeted and generated little off-target activity [58].

Similarly, a separate published study using a dCas9-DNMT3A fusion, featuring a Gly₄Ser flexible linker, showed efficient and specific CpG island methylation of the *BACH2* and *IL6ST* promoters [59]. Notably, the study provides evidence that targeting multiple promoter sites by co-expression of diverse sgRNAs produces synergistic effects that lead to increased methylation of larger regions of the promoter [59]. Despite previous attempts to alter methylation patterns using older technologies such as Zinc Finger proteins and TALEs [60-62], which suffered from low induction levels of methylation and off-targeting effects, CRISPR-mediated approaches appear to be more robust and reliable at inducing methylation across promoters. Moreover, CRISPR-based methods can help overcome the DNA binding domain sensitivity to CpG methylation that have been shown to occur with TALEs [63].

DNA Demethylation

Promoter-specific demethylation of CpG islands has been shown to increase the expression of endogenous genes using TALE-demethylase fusions [20,64]. However, due to technological shortcomings of TALEs—e.g.,

the need for intricate protein engineering, high-throughput epigenome editing to regulate demethylation of promoter regions has been an unreachable goal. Two reports recently described alternative approaches using CRISPR-dCas9 fusions to the catalytic domain of Ten-Eleven translocation dioxygenase 1 (TET1), a maintenance DNA demethylase that prevents abnormal hypermethylation of CpG islands [65].

The first study involved using dCas9-TET1 alongside various sgRNAs targeting the promoter region of *BRCA1*, a tumor suppressor gene [66]. Experiments showed that demethylase-directed epigenome editing selectively targeted the *BRCA1* promoter and induced robust gene expression [66]. The second study used dCas9-TET1 to direct promoter-specific DNA demethylation as well [67]. However, this report also disclosed a method to exploit sgRNA plasticity by inserting bacteriophage MS2 RNA elements—which bind the MS2 coat proteins—into the scaffolding of the sgRNAs [67]. Guided by these engineered sgRNAs, dual and simultaneous recruitment of dCas9-TET1 and MS2-TET1 fusions to targeted loci was shown to occur [67]. This unusual CRISPR-based demethylase system was able to significantly upregulate *RANKL*, *MAGEB2*, and *MMP2* gene expression through demethylation of their respective promoters with little off-target activity [67]. Engineering modular elements into sgRNAs could offer many advantages for high-throughput screening of genome-wide targets for epigenome editing.

Using CRISPR-mediated Approaches to Probe New DNA Epigenetic Modifications

As the above studies demonstrate, it is evident that epigenome editing can also be used as a tool to tease out the function and mechanisms of epigenetic marks deposited directly on DNA [58,59,66,67]. Early efforts have currently focused on studying epigenetic effectors responsible for the regulation of patterns commonly associated with the well-established methylation of cytosine deoxynucleotides at the C-5 position to create 5-methyldeoxycytosine (m^5dC) [68]. However, CRISPR-based approaches could also be used to study functional roles for other marks. To this effect, recent findings have expanded our knowledge of the range of DNA modifications, which had previously been confined to m^5dC . Catalytically inert Cas9 fusions and future editing technologies will likely facilitate ripe opportunities to further our understanding of underlying mechanisms inherent in a diverse repertoire of newly discovered DNA epigenetic marks.

Oxidation of m^5dC Derivatives

The m^5dC epigenetic mark is covalently attached to DNA at CpG and non-CpG sites by a class of enzymes known as DNA Methyltransferases (DNMTs) [69], and has been shown to mediate heritable transcriptional repression in the cell [70]. For nearly six decades, m^5dC was

thought to be the only DNA modification in higher eukaryotes until the recent discovery that Ten-Eleven Translocation (TET) “writer” proteins are capable of catalyzing the sequential oxidation of m^5dC to form 5-hydroxymethylcytosine (hm^5C) [71], 5-formylcytosine (f^5C) [72], and 5-carboxylcytosine (ca^5C) [72,73].

Although very little is known about the function of these m^5dC oxidation derivatives, studies report the accumulation of such epigenetic marks at major satellite repeats [74], within exons [75], and near transcriptional start sites enriched with dual histone H3 lysine 27 trimethylation (H3K27me3) and histone H3 lysine 4 trimethylation (H3K4me3) marks [75]. Following the identification and characterization of the m^5dC derivatives and their “writers,” scientists have discovered sets of “reader” proteins that exhibit preferential and specific binding to hm^5C , f^5C , or ca^5C [76,77]. Moreover, the marks appear to be involved in recruitment of distinct transcription regulators and DNA repair proteins in mouse embryonic stem cells [76]. These findings suggest that m^5dC -oxidized intermediates play important roles in regulation of transcription, control of gene expression, and chromatin dynamics.

Given the recent reported findings using CRISPR-mediated epigenome editing in DNA methylation, it is quite likely that future studies probing functional roles for m^5dC derivatives will shed insights into the molecular and mechanistic underpinnings of these DNA epigenetic modifications.

Discovery of N^6 -methyldeoxyadenosine

Within the last year, a trio of papers marked the debut of methylation of adenine deoxynucleotides at the N-6 position of the purine ring as the latest epigenetic mark decorating the DNA of higher eukaryotes [78-80]. N^6 -methyladenosine had previously been known as a ubiquitous RNA modification found in viruses [81] and eukaryotes [82,83], including mammals [84,85]. However, although N^6 -methyldeoxyadenosine (m^6dA) had been detected as an epigenetic feature of bacteria, it had not been identified in higher eukaryotes. Thanks in part to technological advances in methods of detection to overcome low sensitivity thresholds, m^6dA has now been identified in the genomes of *Caenorhabditis elegans* [78], *Drosophila* [79], and *Chlamydomonas* [80]. Interestingly, unlike m^5dC , which is associated with gene repression, m^6dA appears to localize at ApT di-nucleotides near transcription start sites and is linked to gene activation [80]. The identification of m^6dA in *C. elegans* also dispels the prevailing view that the nematode's genome lacks any DNA methylation and suggests possible mechanisms of m^6dA -mediated epigenetic inheritance in eukaryotes [78].

During the first half of 2016, two additional studies established that the m^6dA mark is widely distributed across the genomes of frogs, mice, mouse embryonic stem cells, and humans [86,87]. Remarkably, the published reports on *Drosophila* [79] and *Chlamydomonas* [80]

squarely contradict findings in vertebrates and mammals [86,87]. Whereas m⁶dA appears to be enriched near transcription start sites and is associated with gene activation in simpler eukaryotes and invertebrates, m⁶dA seems to play opposite roles in vertebrates and mammals in which the mark triggers transcriptional suppression [87]. Future work will likely reveal a rationale for this peculiar contradiction. A hypothesis proposes that m⁶dA acquired new functions during evolution [87]. But there is also the possibility that m⁶dA activation or repression could depend on the type of tissue or species assayed.

CRISPR-mediated epigenome technologies can now be used by researchers to tease out answers to many important questions posed by these latest studies focusing on previously uncharacterized DNA modifications. Now that putative “writers,” “readers,” and “erasers” of m⁶dA marks have been reported, chromatin biologists and structural biochemists will lead efforts to further characterize the molecular and structural underpinnings of the repertoire of factors involved in this novel mode of epigenetic regulation as well as its potential unique roles in simpler and more complex eukaryotes.

Uncovering New DNA Epigenetic Modifications

The discovery of hm⁵C, f⁵C, ca⁵C, and m⁶dA marks, alongside the ensuing development of modern high-throughput technologies and highly sensitive methods to detect transient DNA modifications, brings exciting prospects for future research. It may be the case that other types of DNA epigenetic modifications await characterization in higher eukaryotic organisms. For instance, hypermodification to produce the DNA base β-d-glucosyl-hydroxymethyluracil, commonly known as base J [88], has been documented in unicellular eukaryotes as an epigenetic factor regulating Polymerase II (Pol II) transcription initiation [89]. Phosphothioration, which involves the incorporation of sulfur directly into the DNA phosphate backbone, has been shown to occur pervasively in bacterial genomes [90]. Earlier in 2016, insertion of 7-deazaguanine derivatives into DNA was shown to be a previously unrecognized bacterial and phage defense system targeting foreign DNA [91]. Although neither of these modifications have been identified in higher eukaryotes to date, it is possible that their future characterization will be facilitated by upcoming breakthroughs in detection methods. After all, hm⁵C was first discovered in phage DNA nearly sixty-five years ago [92], but only recently documented in Purkinje neurons and the human brain [93]. Similarly, m⁶dA was known to occur in bacterial DNA for decades before evidence of m⁶dA in higher eukaryotes surfaced within the last year [78-80,86,87].

Because DNA modifications likely occur in distinct contexts, probing functional roles of specific modifications in different tissues, settings, and genomic regions will be pivotal to linking sets of epigenomic marks to phenotypic outcomes. Furthermore, dynamic and reversible

DNA epigenetic modifications are likely to not only affect global processes such as replication, transcription, and gene regulation, but also chromatin dynamics and the three-dimensional structure of the DNA biopolymer. This could be significant in ways we do not yet understand.

For example, earlier in 2016, the first reported crystal structure of a DNA deoxyribozyme revealed an intricate network of tertiary interactions stabilizing the DNA catalyst [94]. It is not difficult to imagine the structural consequences triggered by DNA-modified steric interference, which may ultimately promote or inhibit unknown DNA catalytic mechanisms. Thus, the new generation of epigenome editing biotechnologies aimed at interrogating the functional roles for known and yet-unknown DNA epigenetic effectors (i.e., writers, readers, and erasers) will prove invaluable to elucidate biological processes that have remained out of our technological reach.

PROBING FUNCTIONAL EPIGENETIC ROLES FOR NON-CODING RNAs

Epigenome editing can now be used to probe functional roles for non-coding RNAs (ncRNAs) involved in gene regulation and epigenetic chromatin dynamics. Although we have long known that RNA is an integral part of chromatin, our understanding and appreciation of the role RNA plays in chromatin regulation has only begun to form within the last decade [95,96]. Few recent studies demonstrate the feasibility of using CRISPR-systems to probe ncRNA function by repurposing the sgRNAs that bind dCas9 as scaffolding molecules to trigger loci-specific regulation [97,98]. Orthogonal incorporation of engineered sgRNA molecules featuring protein-binding RNA aptamers—e.g., MS2, PP7—has been established as an efficient platform for parallel gene regulation in mammalian and yeast cells [97]. Protein-binding cassettes, RNA aptamers, and long ncRNAs at least 4.8 kb long have been used to build CRISPR-Cas9 complexes that may enable precise ectopic targeting of functional RNAs and ribonucleoprotein complexes to specific genomic loci [99]. Incorporation of protein-binding motifs and ncRNAs directly into the stem-loop structures of sgRNAs—at the 5' or 3' positions—is likely to usher in a long-awaited era of ncRNA functional characterization relevant to chromatin regulation.

CHEMICAL- AND PHOTO-INDUCIBLE EPIGENOME EDITING

Controlling gene activation or repression in a spatiotemporal fashion to mimic the natural chromatin dynamics of gene expression is the next frontier of epigenome editing-based biotechnologies. To date, several publications detail the use of CRISPR-Cas9 systems for chemical-[100] and photo-inducible [101-103] genome editing and transcriptional regulation. Chemically inducible

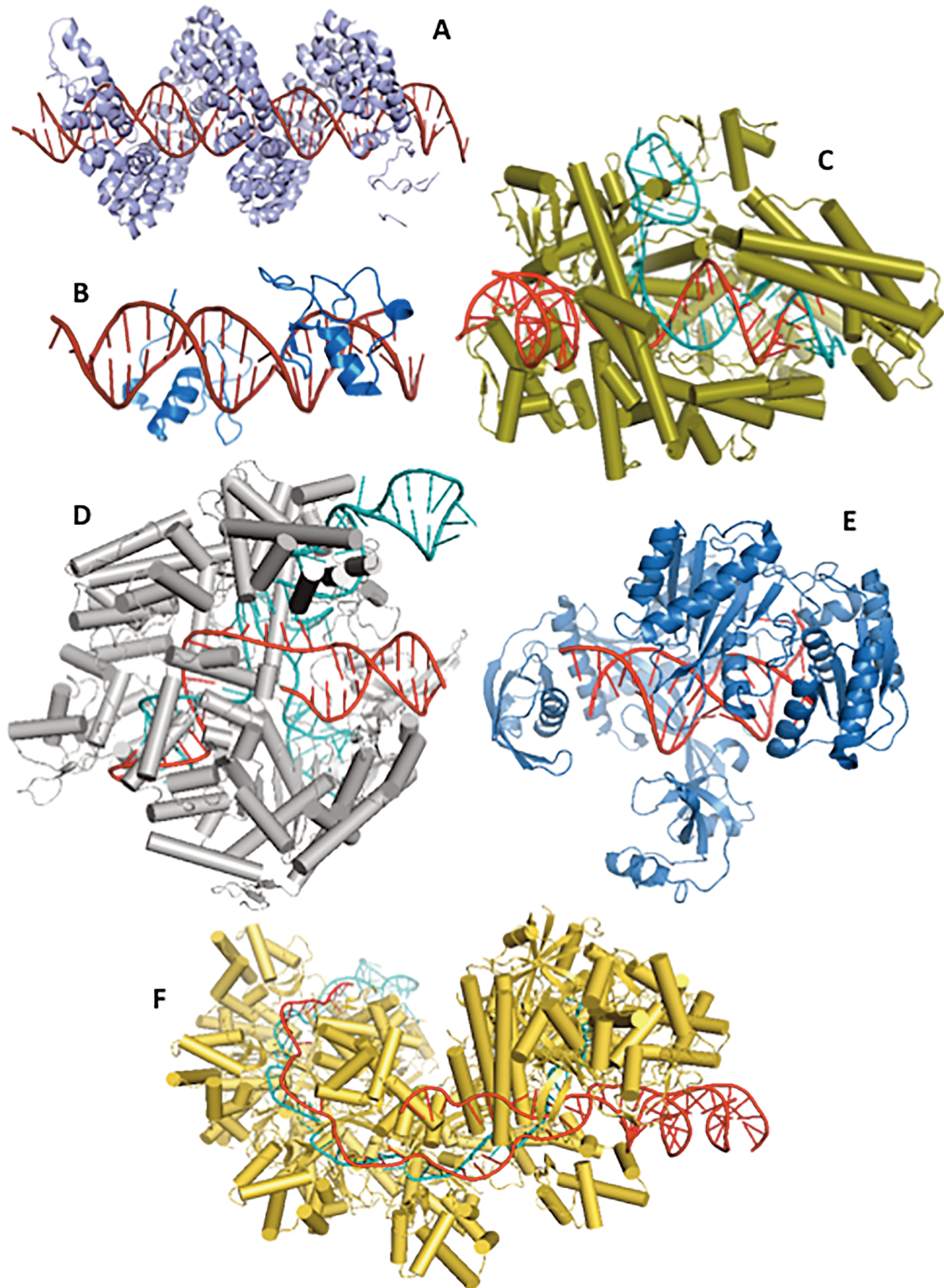


Figure 3. Repurposing CRISPR- and non-CRISPR-based tools for epigenome editing. **A.** Structure of a TALE bound to its target DNA. **B.** Zinc Finger proteins in complex with DNA. **C.** Structure of Cpf1 in complex with crRNA and target DNA. **D.** Cas9 bound to sgRNA and PAM-containing target DNA. **E.** Structure of an Argonaute protein in complex with guide and target DNA fragments. **F.** *E. coli* Cascade bound to crRNA and PAM-containing dsDNA. All structures feature DNA in red and RNA (where applicable) in teal. [PDB 3UGM, 3DFX, 5B43, 4UN3, 4NCB, 5H9F].

epigenome editing comprises the use of small molecules that act as chemical inducers. For example, rapamycin-sensitive dimerization domains have been shown to chemically induce CRISPR-Cas9-based genome editing and transcription regulation when Cas9 is split into two fragments that, upon chemical induction, reconstitute a full-length, catalytic nuclease [100]. This approach could also be applied with other chemically inducible dimerization domains, such as abscisic acid or gibberalin sensing domains [100]. A myriad of small molecules have been established as chemical inducers such as anhydrotetracycline [9], doxycycline [104], rapamycin [100], and steroid hormone receptor ligands [105], all of which could be exploited in epigenome editing platforms.

Many photo- or light-inducible “optogenetic” approaches typically rely on a light-dependent dimerization system comprising the proteins cryptochrome circadian clock 2 (CRY2) and its binding partner CIB1 [106]. Using this approach, groups have tested the feasibility of fusing a split dCas9 together with the CRY2-CIB1 system for photoactivation of genome editing [101], or demonstrated that CRY2 and CIB1 can be separately fused to dCas9 and a transcriptional activator respectively for subsequent photo-inducible transcriptional regulation [102,103]. Interestingly, a new approach using engineered protein photoswitches (“magnets”) to enhance photo-induced heterodimerization was recently reported in the literature [107]. These optogenetic “magnets” overcome some of the obstacles inherent with the CRY2-CIB1 system and have already been shown to be a more efficient and powerful tool for optogenetic manipulation of transcription regulation [101].

Chemical and photo-induced epigenome editing to interrogate histone PTMs and modulate endogenous gene expression has been demonstrated in the past using older technologies such as TALE and Zinc Finger proteins [108,109]. It will be interesting to witness the repurposing of chemistry- and photo-based platforms into CRISPR-directed epigenome editing as a means to interrogate unexplored areas of chromatin biology and dynamics. Few significant limitations such as optimization of light delivery methods, availability of effectors capable of generating endogenous light sources with spatiotemporal precision, need for expensive equipment, and the use of favorable intensities and wavelengths for the activation of select photosensitive proteins and chemicals will need to be addressed as the technologies continue to develop. But promising signs of progress offer a glimpse into the bright future ahead for these chemical- and photo-inducible systems [110].

Given that optogenetic induction systems have already been used to study neural processing, behavior, and epigenetic chromatin modifications in mammalian cells [108,111,112], there is no doubt that the use of these spatio-temporal technologies coupled to epigenome editing

will elucidate fundamental biological processes and causal relationships underlying human disease.

LINKING EPIGENOME EDITING TO PATHOLOGY AND THERAPEUTIC OPPORTUNITIES

Targeted epigenome editing can be an extremely useful tool for basic scientific research. However, for many researchers in the field, one important goal is to develop technological advances that may, in the near future, be used to combat disease and form the foundation of epigenetic therapies. We are still some time away from taking the leap into the realm of translational medicine. Until that time arrives, it is imperative that the scientific community continues to document and expand the knowledge related to the extensive catalog of pathologies associated with misregulation of the epigenome, which arise as a result of aberrant patterns of DNA and histone epigenetic modifications, as well as defects in regulation by ncRNAs.

Recent studies in animal models of human disease and human cell lines have demonstrated the promise of genome editing technologies to clinically address human pathologies brought on by viruses, such as HIV [113,114], hereditary diseases like Cystic Fibrosis and hereditary tyrosinemia type I [115,116], neurological disorders [117], and various monogenic conditions [118]. Likewise, genome editing has facilitated testing disease-associated gene disruption in large animals [119]. Despite the fact that many diseases have genetic components, we have only begun to uncover the epigenetic mechanisms underlying some of these conditions. CRISPR systems appear to be particularly well poised to make substantial contributions to shed light on epigenetic studies just as they have done in the field of genome editing. Indeed, many reports are already providing important insights into the functional roles played by epigenetic factors in a plethora of pathologies.

For example, recent publications reveal crucial roles for DNA methylation and histone PTM patterns for synaptic plasticity, learning, and memory, which have been useful to study behavioral abnormalities associated with fetal alcohol spectrum disorder [120]. Complex patterns of DNA methylation at CpG islands and other di-nucleotides within promoters have uncovered key roles for epigenetic processes in dementia and neurodegenerative diseases [121]. Studying putative functional roles for methylation patterns other than the canonical CpG methylation is important given that methyltransferases and demethylases have various degrees of specificity and have been shown to catalyze reactions at CpA and CpT di-nucleotides—both of which are poorly understood in the field [122].

Targeting the tumor suppressor *BRCA1* promoter to decrease levels of DNA methylation has been shown to cause reactivation and restoration of *BRCA1* functional activity in cervical and breast cancers [66]. Epigenome

editing to achieve epigenetic silencing or activation could be modulated to treat active or latent viruses such as HSV1 and HIV [123]. Induction of H3K9 methylation and reduced histone H3 acetylation has shown that the human epidermal growth factor receptor-2 (HER2/neu/ERBB2), which is over expressed in many types of cancer, is amenable to down regulation [124]. Diabetic retinopathy has been associated with several epigenetic markers, including DNA methylation and demethylation of H3K4 and H3K9 [125]. Epigenetic silencing of genes that over express factors linked to protein aggregation diseases could open avenues for treatment, and slowing down the progression, of devastating neurodegenerative disorders [126].

Another key area of research where epigenome editing technologies could have a lasting impact is the study of therapeutic avenues to ameliorate imprinting disorders. Genomic imprinting is a unique type of epigenetic mechanism where differential patterns of allele-specific DNA methylation result in discrete expression of “imprinted” genes, depending on whether the paternal or maternal allele is inherited [127]. For instance, differential methylation patterns of the same locus in chromosome 15q form the underlying molecular basis for the neurogenetic disorders Prader-Willi syndrome—paternally-linked—and Angelman syndrome—maternally-linked [128]. Imprinting has been associated with DNA methylation [129,130], as well as histone PTMs [131], and ncRNAs [132]. CRISPR-mediated, allele-specific, epigenome editing offers remarkable possibilities to alter specific epimutations and epigenetic modifications at precise targeted genomic regions, thereby opening a path for rational therapeutic opportunities to correct aberrations associated with genomic imprinting disorders.

Of course, it would be impossible to list every example of the potential therapeutic benefits of targeted epigenome editing for mitigating human diseases in the context of this review. However, taken together, these findings and research paths present a promising and hopeful picture of the future of epigenetic therapies.

BEYOND CRISPR-Cas9

CRISPR-Cas9 has been widely adapted in laboratories across the globe at an exponential rate for genome editing and, more recently as detailed in this piece, a tool for programmable epigenome editing. However, the future of the field will likely witness introduction of orthogonal and other repurposed systems. The bulk of published literature presently describes use of *Streptococcus pyogenes* nuclease-null Cas9, but other orthogonal Cas9 proteins such as those from *Neisseria meningitidis* [24], *Streptococcus thermophilus* [133], *Staphylococcus aureus* [134], and *Treponema denticola* [135] have been used to demonstrate genome editing and transcriptional regulation. Researchers could avail themselves of these and other yet-

uncharacterized orthogonal Cas9 proteins to interrogate the function of epigenetic marks genome-wide or at specific loci. The untapped diversity of Cas9 proteins is likely to prove invaluable as distinct Cas9s target unique, longer, and more discriminatory PAM sites. Ultimately, the advantage of targeting PAM sites other than the NGG motif characteristic of *S. pyogenes* Cas9 (Figure 3D) may offer alternatives to ameliorate some of the off-target effects reported in this system [136].

In addition to exploiting orthogonal CRISPR-Cas9 systems for epigenome editing purposes, other CRISPR and non-CRISPR-based systems are also poised to contribute important insights to the advancement of the field. Older technologies such as Zinc Finger Nucleases (Figure 3B) and TALENs (Figure 3A) have proven valuable, and may continue to be used, as epigenome editing tools [24,137]. Newly-discovered systems, however, are likely to accelerate the rate of epigenome editing-related biotechnologies. For instance, the Cpf1 enzyme from *Francisella novicida* (Figure 3C) was shown in 2015 to mediate robust DNA interference distinguishable from Cas9 [138]. Orthogonal Cpf1 enzymes from *Acidaminococcus sp. BV3L6* and *Lachnospiraceae bacterium ND2006* are capable of mediating genome editing in human cells, suggesting that Cpf1-family proteins from diverse bacteria could also be repurposed for epigenome editing applications [138].

Moreover, the type I-E CRISPR-Cas system in *Escherichia coli* can be programmed to efficiently suppress gene expression by the DNA-binding Cascade complex (Figure 3F) [139,140] and could also be co-opted for epigenome editing uses. Surprisingly, the most recent addition to the list of genome editing systems in the literature was recently reported for the *Natronobacterium gregoryi* Argonaute endonuclease (Figure 3E—*Thermus thermophilus* Argonaute), which was shown to mediate DNA-guided genome editing in human cells and does not require a PAM for DNA targeting [141]. Collectively, these Cas- and non-Cas-effector enzymes, along with orthogonal and still-uncharacterized systems, may usher a new era of discoveries in basic research that could lay the foundation to propel epigenome editing into the clinical setting.

CONCLUSION AND FUTURE PERSPECTIVES

CRISPR systems are revolutionizing genome editing and are well-positioned to exert a similar effect in the nascent field of epigenome editing. The next generation of tools to investigate the functional and biological relevance of DNA and chromatin modifications as well as regulatory RNAs *in vivo* will provide scientists with a unique opportunity to study long awaited fundamental questions in chromatin biology. As the field continues to develop, it is imperative that researchers focus not merely on performing experiments to study particular epigenetic modi-

fications with a myopic lens, but also on identifying critical obstacles that must be overcome in order to uncover the specific contributions—in local and genome-wide contexts—of writer, reader, and eraser mono- and multivalent epigenetic effectors. Likewise, as chemical- and photo-inducible epigenome editing systems become more accessible, spatio-temporal optogenetic manipulation of chromatin remodeling and epigenetic landscapes will likely provide invaluable insights into precise mechanisms for numerous biological processes including development, cellular reprogramming, and the epigenetic basis of human pathophysiology.

It is incumbent upon the scientific community to galvanize enthusiasm for using CRISPR-based technologies for designing complementary experiments that will potentially provide answers to a myriad of important questions in the field. For example, now that CRISPR systems have been established as useful tools for multiplex genome editing [142], it will be important to study the structure-function relationships of specific histone residues directly—by epigenome editing—and indirectly—by probing the epigenome with mutations of all copies of histones at the genomic level. While some progress has been made toward interrogating the function of metazoan histone residues without the use of CRISPR systems [143], it has been a difficult challenge to overcome. Although the latter approach concerns genome editing, insights from these experiments can offer valuable complementary information to those gathered through epigenome editing experiments aimed at studying the same marks from multi-faceted approaches.

Consider acetylation of the H3K14 mark. Mutating the mark to a residue that cannot be acetylated may result in a phenotype that is distinct from either rationally manipulating the acetyltransferase that deposits the mark, or purposefully preventing the locus-specific deposition of the mark. Similarly, the resulting phenotype from mutating all histones at H3K14 loci may not necessarily correlate to H3K14 acetylation alone. This is particularly true given that the histone-modifying acetyltransferase responsible for catalyzing covalent attachment of the acetyl moiety is likely to have many non-histone targets; the H3K14 site may be a cognate target for other types of modifications—e.g. methylation, butyrylation, crotonylation, ubiquitination, etc.; and there may be various readers and erasers of the mark unable to signal downstream factors and effectors. All of these scenarios—individually—are likely to mask or confound interpretation of results from a single experiment. But the collective study of these functional relationships could be used to methodically elucidate complementary roles for epigenetic marks, effectors, and other signaling factors targeting the same epigenetic locus. In short, epigenetic regulation is far more complex than its genetic counterpart and the only way to truly understand epigenetic mechanisms is through care-

ful investigation of epigenetic processes from multiple perspectives.

Other problems that will need to be addressed in coming years relate to issues of chromatin accessibility, specificity, fidelity, and delivery of CRISPR systems repurposed for epigenome editing applications. It has been reported that nucleosomes inhibit Cas9 activity *in vitro*, suggesting that higher order chromatin structures are more inhibitory to Cas9 activity unless targeting stimulates chromatin remodeling at the targeted site [144]. The issues of specificity and fidelity to mitigate off-targeting effects of CRISPR-Cas systems are currently a critical area of study [145-147]. Similarly, research into optimizing delivery methods of CRISPR-based systems into target cells and tissues has paved the way for development of modern adeno-associated virus technologies and promising nanoparticle delivery approaches [148]. All these factors will play indispensable roles in determining whether CRISPR-based epigenome editing biotechnologies will successfully reach the clinical realm.

The development of new bioinformatics tools for Cas9 orthologs and other CRISPR systems is also an area that deserves attention. Currently, genome-wide off-target effects prediction is available for *S. pyogenes* Cas9, but other CRISPR systems are lagging behind in this regard [149]. Lastly, a combination of genome and epigenome technologies coupled to high-throughput functional genomics [150,151] to modulate transcriptional activity is likely to yield identification of much needed novel therapeutic targets for many diseases.

It will be up to the scientific community to ensure that this nascent scientific field develops into an established discipline. Already, exciting signs of progress are illustrating how epigenome editing can be used as an efficient, programmable tool to uncover biological roles of epigenomic marks and effectors in their native contexts. Undoubtedly, as detailed in this analytical assessment, epigenome editing technologies hold great promise for the future of chromatin biology and personalized medicine. But more importantly, pushing beyond the scientific frontiers of the epigenome may one day, in the near future, establish a solid foundation for the development of powerful tools to combat the suffering inherent in human disease.

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