Epigenetic editing: Dissecting chromatin function in context

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Abstract
How epigenetic mechanisms regulate genome output and response to stimuli is a fundamental question in development and disease. Past decades have made tremendous progress in deciphering the regulatory relationships involved by correlating aggregated (epi)genomics profiles with global perturbations. However, the recent development of epigenetic editing technologies now enables researchers to move beyond inferred conclusions, towards explicit causal reasoning, through ‘programming’ precise chromatin perturbations in single cells. Here, we first discuss the major unresolved questions in the epigenetics field that can be addressed by programmable epigenome editing, including the context-dependent function and memory of chromatin states. We then describe the epigenetic editing toolkit focusing on CRISPR-based technologies, and highlight its achievements, drawbacks and promise. Finally, we consider the potential future application of epigenetic editing to the study and treatment of specific disease conditions.

KEYWORDS
causal, CRISPR/Cas9, dCas9, DNA methylation, epigenetic inheritance, epigenome therapy, genome regulation, histone modification, polycomb

INTRODUCTION
A fundamental goal of biology is to understand the complex regulatory systems that govern genome output. Such systems drive the distinct gene expression programs that define cell identity, and ultimately underpin phenotype. Epigenetic mechanisms represent one key layer of genome regulation, and include histone modifications, DNA methylation, chromatin topology and nuclear architecture, which may or may not be heritable. An appropriately regulated epigenome is essential for development and genome management, while a dysregulated epigenome is intimately associated with human disease, underscoring its central regulatory role. Recent decades have seen pioneering studies map the genome-wide distribution of many chromatin modifications across numerous developmental and disease contexts, and correlate them with transcription, nucleosome occupancy, genome architecture and genetic variation, among others. These comparative approaches have revealed significant associations between epigenomic features and genome regulation. Nevertheless, they do not discern to what extent epigenetic states cause gene activity states rather than merely correlate with them.

To address this, perturbation strategies have been widely employed to interrogate the functional relevance of epigenetic states. These include disruption of the upstream chromatin-modifying enzymes, typically by genetic deletion or mutation of the histone residues that bear specific epigenetic marks in species where this is technically possible. While these approaches generally trigger widespread changes in genome regulation, it remains challenging to distinguish between the direct and indirect consequences of such global perturbations. Indeed, chromatin-modifying enzymes have a broad range of non-histone substrates and non-catalytic roles, further...
FIGURE 1  Key outstanding questions towards understanding context-dependent epigenetic mechanisms. The CRISPR-Cas9 toolbox for site-specific epigenome editing enables investigation of the direct causal involvement of epigenetic marks in genome regulation and cellular phenotype. Cas9-based technologies can be harnessed to understand how individual or combinatorial chromatin modifications influence gene expression, define the non-coding genome function and shape chromatin structure or nuclear architecture. Novel experimental approaches can be designed to establish how and to what extent transcription factor binding and genomic context affect the function of epigenetic marks. Whether chromatin marks constitute an epigenetic memory and how the DNA sequence affect the potential for epigenetic inheritance constitute two important avenues for investigation. Several unaddressed questions in the epigenetic field are highlighted complicating interpretations. Therefore, molecular responses and developmental phenotypes in knockout studies cannot necessarily be attributed to disruption of epigenetic marks per se. The challenge of attributing causality is further compounded by context-dependent effects; for example, perturbation of DNA methylation leads to dramatically different consequences depending on its genomic context (CpG-density) and the cell type.

These examples not only highlight the caveats and technical limitations of the aforementioned genomics approaches, but also suggest that epigenetic marks do not have unequivocal functions. Rather, their activity stems from complex interactions with multiple contextual inputs including transcription factor binding, pre-existing chromatin architecture, genomic environment, and cell identity. As such, while epigenomic profiling, genetic studies, and data-integration have led to great progress in annotating chromatin marks with an inferred function, correlation, pleiotropy and context-dependency have made it difficult to establish the causal contribution of epigenetic states to transcriptional activity and/or cellular phenotypes.

This complexity has fueled the development of “programmable” epigenome editing technologies that target or perturb epigenetic states at specific genomic loci, and can consequently dissect their context-dependent function. As a result, the epigenetic editing toolkit is heralding an era of causal interrogation and represents a framework for precision manipulations of the epigenome that will empower both fundamental research and biomedical applications. In this review, we first highlight some of the key unresolved questions relating to epigenetic function in genome regulation (Figure 1). We then discuss the emerging epigenetic editing technologies and how they have begun to address these outstanding challenges. Finally, we consider the potential for targeted manipulations of aberrant epigenetic states in disease as a promising route towards therapeutic applications.

EPIGENOME FUNCTION: UNRESOLVED QUESTIONS

Do epigenetic marks causally drive transcriptional regulation?

Whether individual or combinatorial chromatin marks directly affect the transcriptional state of genes or simply correlate with them remains one of the most debated points among epigenetic researchers. Different mutually non-exclusive scenarios are being considered whereby epigenetic marks could (i) actively drive changes in gene expression as upstream mediators; (ii) fine-tune precise activity states; (iii) maintain ongoing transcriptional programs to provide robustness; (iv) act as placeholders to prime future gene expression patterns in response to cues; or (v) simply represent a byproduct
of transcriptional status. Indeed, transcription per se can predict active histone modification landscapes with high accuracy,\textsuperscript{21} calling into question the directionality of relationships. So far, the role of chromatin modifications seems strongly dependent on model organism, cell type, genomic location and developmental time point, but a deeper understanding is required to dissect these inter-relationships (Figure 1).\textsuperscript{22-25}

It is noteworthy that histones are typically modified at multiple sites,\textsuperscript{26} which also raises the question: what is the contribution of combinatorial epigenetic marks in facilitating endogenous transcriptional states. Indeed, whether various chromatin marks interact to produce synergistic, antagonistic or neutral outcomes is incompletely understood. Even within well-characterized multiples such as H3K4me3/H3K27me3 bivalency, the output is nuanced and depends on multiple factors including their relative ratio and genomic context.\textsuperscript{27,28} It is also important to establish the hierarchy of chromatin features to grasp which epigenetic components are necessary and/or sufficient to elicit a given gene expression response. Finally, evidence of cell-to-cell epigenetic heterogeneity is growing.\textsuperscript{29,30} To what extent these differences are biologically meaningful in how they affect gene expression changes in response to intrinsic or extrinsic cues is an exciting area to be explored.

**What is the function of epigenetic marks at non-coding loci?**

The noncoding genome is substantially larger than its gene-coding counterpart and contains structural, regulatory, and transcribed information that is still far from being completely understood. Epigenetic marks are enriched at intergenic regions in complex patterns that are thought to demarcate gene regulatory elements and distinct classes of transposable elements (TE). For instance, co-occurrence of H3K27ac and H3K4me1 has been widely used to identify and annotate putative enhancers.\textsuperscript{31} Particularly, H3K27ac is recognized as an indicator of enhancer activity.\textsuperscript{32} However, recent studies revealed that depletion of H3K4me1 or H3K27ac from enhancer regions in murine ESC does not overtly interfere with their function,\textsuperscript{16,33} implying we have an incomplete understanding of the functional significance of these marks, and how chromatin states more generally contribute to enhancer function (Figure 1).

DNA methylation and H3K9me3 are well-established to coat TE, and play an important role in antagonizing their ability to mobilize around the genome.\textsuperscript{34-36} However, many other chromatin modifications, such as O-GlcNAc, 5hmC or H3K4me3, are also enriched on subsets of TE, suggesting that the epigenetic regulation of mobile elements may be more complex than previously anticipated and warrants further investigation.\textsuperscript{37-40} Notably, a significant proportion of mammalian noncoding sites enriched for chromatin modifications do not coincide with either enhancers or TE. Epigenetic aberrations at these unannotated regions have already been linked to human syndromes,\textsuperscript{41} implying that the functional interrogation of chromatin marks at non-genic regions will be instrumental to understand the mechanisms that govern genome regulation in both physiological and pathological circumstances.

**How do contextual interactions influence epigenetic mark function?**

Pioneering studies have demonstrated that programed de novo deposition of chromatin marks can be sufficient to induce transcriptional changes.\textsuperscript{25,42,43} However, their effect on transcription was detected at only a subset of targeted loci. This could be explained by differences in how the underlying DNA sequence, such as specific transcription factor (TF) motifs or CG-density, modulates epigenetic mark functionality. Here, while certain epigenetic features could function directly through recruitment of chromatin remodeling complexes, of the core transcription machinery or by directly affecting chromatin structure, many others could first require TF binding and/or transcriptional priming to unmask a contextual function (Figure 1).\textsuperscript{44} These possibilities betray potentially complex inter-relationships and feedback loops between chromatin, TF, and cellular context that are ripe for further exploration.

Such reciprocal relationships are evident during cellular reprogramming, where Oct4, Sox2 and Klf4 (OSK) binding promotes the deposition of H3K4me1 and H3K4me3 to remodel chromatin at regulatory elements.\textsuperscript{45} In parallel, broad domains of H3K9me3 are refractory to OSK recruitment, highlighting the importance of context in understanding TF binding and chromatin modifications.\textsuperscript{46} In addition, the binding of numerous human TF is affected by DNA methylation: approximately half of them have a lower affinity for methylated regions, while the other half have a higher affinity.\textsuperscript{47} This implies that aberrant DNA methylation patterns could influence cell identity via shaping TF occupancy. Consistently, mutations in Tet2 and Dnmt3a enzymes dramatically skew hematopoietic differentiation by modulating TAL1 and GATA1 binding.\textsuperscript{48} In mouse ESC, NRF1 binds unmethylated DNA autonomously, but is blocked by DNA methylation and consequently requires co-binding of a methylation-insensitive TF to ensure motif hypomethylation.\textsuperscript{49} These examples illustrate how dissecting the principles that govern the convoluted relationship between genetics (especially TF motifs) and epigenetics (chromatin) remains a key challenge.\textsuperscript{50,51}

**Can epigenetic marks directly alter local and global genome architecture?**

Epigenetic marks have the potential to directly influence nucleosome dynamics and stability, as illustrated by histone acetylation that affects local chromatin structure by changing the affinity of histone tails for DNA, or histone methylation, that provides docking sites for the recruitment of chromatin remodeling enzymes.\textsuperscript{52,53} The recent development of biochemical approaches has enabled the synthetic incorporation of several histone modifications into nucleosomes both in vitro and in vivo.\textsuperscript{54} These approaches have shed light onto the mechanisms by which chromatin remodelers discriminate between different
epigenetically-modified nucleosome substrates,[55] and further studies will help understanding the interplay between epigenetic marks and nucleosomal structure in living cells.

At a global scale, a strong correlation has been observed between the 3D structure of the genome and chromatin states,[56] with specific histone modifications occupying distinct nuclear domains.[57] The extent to which chromatin marks actively take part in the formation of genomic compartments or topological associated domains (TAD) is still under debate.[58] Notably, DNA methylation and H3K9me3 have both been shown to disrupt chromatin binding of the CTCF insulator, thereby blocking DNA looping and inducing mis-expression of neighboring genes.[42,59,60] However, genome-wide loss of either H3K9me2, H3K27me3 or H3K4me1 in mESCs does not affect TAD structure.[61,62] Thus, understanding the full relationship between epigenetic marks and chromatin architecture, at both local and genome-wide levels, and the directionality of relationships are key outstanding questions.

Are epigenetic marks necessary and/or sufficient for cellular memory?

In multicellular organisms, cell identity is maintained by specific gene expression programs that are inherited through genome replication and cell divisions. The mechanisms that allow cells to retain “memory” of the state of their predecessors post-mitotically are the subject of intense investigation. Both transcription factors and epigenetic marks provide attractive candidates for transmitting cellular memory.[63] Sequence-specific transcription factors can form self-maintaining gene regulatory networks that are stable over long periods of time. However, the cellular pool of transcription factors alone is not sufficient to define its spectrum of gene activity,[64] neither are they able to explain phenomena such as genomic imprinting or X-chromosome inactivation.[65]

It is still unclear whether chromatin states per se can propagate in the absence of continuous inducing inputs and/or independently from the underlying DNA sequence. DNA methylation can be inherited through cell division owing to the activity of the maintenance DNA methyltransferase DNMT1.[66] A recent study even suggested that DNA methylation memory can be propagated across timescales of million years in yeast,[67] but its utility as a carrier of cellular memory seems to be context-dependent in mammals.[44] Likewise, H3K9me3 is considered amongst the classical self-reinforcing epigenetic systems, but in yeast transmission of ectopic H3K9me3 requires additional disruption of the putative H3K9 demethylase Epe1, indicating H3K9me3 may not be sufficient for epigenetic memory.[68] In Drosophila, cellular memory is enabled via regulatory elements called PREs, that are the substrate of the Pcg/TxrG proteins that deposit H3K27me3, H3K4me3, H3K36me3 and H3K27ac to maintain silent or active gene expression states.[69] In mammals, PREs have proved challenging to identify, and testing whether H3K27me3 alone can induce long-lasting gene repression led to contradictory results.[70,71] Thus, the interplay of molecular mechanisms that mediate cellular memory remains unresolved but likely involves the cooperative activity of several regulatory systems including transcription regulators and epigenetic marks. The influence of DNA context on the potential for epigenetic inheritance is an important avenue for investigation, especially towards understanding the potential functional significance of epialleles.[72]

Overall, while tremendous progress has been made in understanding the role of distinct epigenetic states, the ability to tease apart the directionality of complex functional relationships with precision approaches has been lacking. We anticipate that the emergence of epigenetic editing technologies will contribute to resolving this and will therefore address many of the outstanding issues in the field, including those discussed above.

THE EPIGENETIC EDITING TOOLKIT

The comprehensive cataloguing of genomes, epigenomes, and transcriptomes across model organisms and cell types has created a demand for innovative strategies that functionally interrogate the relationships between chromatin state, genome regulation and cellular phenotype. The development of artificial DNA binding domains (DBD) coupled with transcriptional modulators or epigenetic modifiers has effectively met this demand by enabling locus-specific modulation of gene expression and/or epigenome editing.[73] To date, three main types of customizable DBDs have been employed: zinc finger proteins,[74] transcription activator–like effectors,[75] and the CRISPR/dCas9 system.[76] The latter has emerged as the most widely used platform due to the reduced time and costs needed to adapt and implement the technology, and for its high efficiency and modularity (Figure 2).

The CRISPR/dCas9 system typically comprises a catalytically inactive (dead) variant of Streptococcus pyogenes Cas9 (dCas9) and a synthetic guide RNA (gRNA). The gRNA recruits dCas9 to complementary target DNA sequences, located upstream of a PAM, the trinucleotide NGG. By changing the sequence of the gRNA, the CRISPR/dCas9 system can be used to target virtually any genomic region of interest.[77] Additionally, multiple loci can be targeted in parallel, allowing efficient multiplexing.[78] To elicit specific perturbations, a wide range of effectors domains have been complexed with dCas9 (Table 1). These can be grouped into two categories: (i) transcriptional regulators, which in turn recruit other proteins including chromatin remodeling complexes, chromatin modifying enzymes, and transcriptional cofactors, and (ii) chromatin modifying enzymes per se, which directly catalyze the addition or removal of epigenetic marks. For example, potent transcriptional regulators such as the herpes simplex VP16 and the KRAB domain, or their variants, have been successfully employed to activate and suppress gene expression, respectively.[79] A range of chromatin modifiers have also been used to add or remove chromatin marks from histones or DNA (Table 1), providing direct evidence that, at least in some cases, the alteration of established epigenetic states can be sufficient to induce transcriptional changes.[42,43,80,81] Importantly, the catalytic domains of modifiers can often edit the epigenome as efficiently
FIGURE 2  Schematic overview of CRISPR-dCas9 technologies for epigenome editing. Shown are various Cas9-based systems to programably edit chromatin states. The specialised function of each is indicated in brackets. (A) Catalytically dead Cas9 (dCas9) from Streptococcus pyogenes (Sp) is used in combination with different effector domains, including transcriptional regulators and chromatin modifiers, to achieve targeted epigenome editing. The dCas9-fusion protein is recruited to genomic locations of interest via a guide RNA (gRNA) that recognises a complementary DNA sequence located upstream of the protospacer adjacent motif (PAM) “NGG”, where N stands for any nucleotide. (B) The SpCas9-SunTag technology consists of a SpCas9 enzyme fused to several (5-20) tandem repeats of the GCN4 binding motif (SunTag), which recruits any proteins coupled with a small-chain variable fragment (scFv) domain. This system allows recruitment of multiple copies of the same effector domain (or of different ones) to a specific genomic site, thus promoting high ON-target editing efficiency and increased spreading of the induced epigenetic state, while minimizing OFF-target effects. (C) The SpCas9-SAM system is made up of a SpCas9 enzyme fused to the transcriptional activator VP64. The accompanying gRNA is modified to include two RNA aptamers for binding with MS2 coat proteins, which, in turn, recruit two additional transcriptional activators, p65 and HSP1. (D) The Staphylococcus aureus (Sa) dCas9 is 1053 amino acids smaller than its SpCas9 counterpart. This makes the SaCas9 ortholog suitable for delivery within adeno-associated virus (AAV) vectors that can be used in vivo, while also allowing space for effector domains and gRNA. The enzyme recognizes the “NNGRRT” PAM. (E) Orthogonal dCas9 moieties (e.g., SpCas9 and SaCas9) are linked to proteins that form heterodimers in the presence of light or chemical compounds. By exploiting the features of different gRNAs this system can be successfully used to bring two different regions of the genome into close proximity and to drive de novo DNA loops. (F) RNA editing has recently been enabled by the development of the programmable, catalytically dead, ribonuclease dCas13 (Figure 2) and its ortholog dCasRx. Such a technology has already been used to image RNA in living cells\cite{90} to edit ribonucleotides\cite{91,92} and to manipulate alternative splicing\cite{92,94} and could also be applied to the study of non-coding RNAs, which constitute another important layer of epigenetic regulation.

Achievements and promises of epigenome editing

The exploitation of the CRISPR/dCas9 technology has facilitated the completion of a growing but still limited number of studies aimed at deciphering the mechanisms that govern genome regulation. Epigenetic editing tools have been used to understand if and how chromatin modifications affect transcription, and imply that at least some epigenetic marks can directly instigate changes. As such, dCas9 associated with KRAB drives gene silencing at target genomic loci, potentially through recruiting TRIM28, SETDB1 and H3K9me3\cite{79,95,96}. Indeed, a direct involvement for H3K9me3 in transcriptional repression was observed.
### Table 1: Applications of epigenome editing tools

| DNA targeting module | Effector protein | Applications | References |
|----------------------|------------------|--------------|------------|
| **Transcriptional regulators** | | | |
| SpdCas9 | KRAB | Transcriptional repression | [79, 95, 96, 106] |
| SpdCas9 | KRAB | In vivo transcriptional repression | [143] |
| dCpf1 | KRAB | Transcriptional repression | [171] |
| SpdCas9 | VPR | Transcriptional activation | [116, 157] |
| dCpf1 | VPR or p65 | Transcriptional activation | [172] |
| SpdCas9 | VP64 (VP16 tetramer) | Transcriptional activation | [79, 95, 126] |
| SpdCas9-SunTag | VP64 (VP16 tetramer) | Transcriptional activation and cellular reprogramming | [83, 117] |
| SpdCas9-SAM | VP64 + p65 + HSF1 | Transcriptional activation | [173] |
| dCpf1 | p300 catalytic domain | Transcriptional activation | [174] |
| **Chromatin marks** | | | |
| SpdCas9 | DNMT3A full length | Targeted DNA methylation | [42] |
| SpdCas9 | DNMT3A catalytic domain | Targeted DNA methylation | [42, 82, 106, 175] |
| SpdCas9-SunTag | DNMT3A full length or catalytic domain | Targeted DNA methylation | [86, 89] |
| SpdCas99SodCas9 | DNMT3A catalytic domain | Targeted DNA methylation | [176] |
| SpdCas9 | TET1 catalytic domain | Targeted DNA demethylation | [42, 102, 106] |
| SpdCas9-SunTag | TET1 catalytic domain | Targeted DNA demethylation | [88] |
| SpdCas99SodCas9 | TET1 catalytic domain | Targeted DNA demethylation | [176] |
| SpdCas9 | p300 catalytic domain | Targeted H3K27ac | [43, 106] |
| SpdCas9-SunTag | p300 catalytic domain | Targeted H3K27ac | [117] |
| SpdCas9 | EZH2 full length and catalytic domain | Targeted H3K27me3 | [70, 100] |
| SpdCas9 | G9a catalytic domain | Targeted H3K9me3 | [70] |
| SpdCas9 | SUV39H1 catalytic domain | Targeted H3K9me3 | [70] |
| SpdCas9 | HDAC3 full length | Targeted histone deacetylase | [81] |
| SpdCas9 | LSD1 | Targeted histone demethylation | [80] |
| SpdCas9 | PRDM9 catalytic domain | Targeted H3K4me3 | [25] |
| SpdCas9 | DOT1L catalytic domain | Targeted H3K79me2 | [25] |
| **Chromatin remodelling** | | | |
| SpdCas9 | FOG1 catalytic domain | Chromatin condensation and indirect targeting of H3K27me3 | [70] |
| SpdCas9 | BAF | Targeted chromatin remodelling | [107] |
| SpdCas9 (with modified gRNA containing two MS2 RNA elements) | | | |
| **Nuclear architecture** | | | |
| SpdCas9 | Leucin zipper proteins | Artificial DNA looping in bacteria | [177] |
| SpdCas9 | PYL1 and ABI1dimerization proteins | Artificial DNA looping | [109] |
| SpdCas9 | CIBN (dimerization induced by CRY2) | Light inducible DNA looping | [178] |

(Continues)
suggested when specific H3K9 methyltransferases, including SUV39H1 and G9a, were used as the epigenome editing effector domain of different DNA-binding proteins. Importantly, negative controls in the form of catalytically inactive enzymes were used to confirm that the histone mark per se was responsible for the observed phenotypes.

Available data on the function of H3K27me3 are harder to interpret. O’Geen and colleagues directed either the full-length or a truncated version of the H3K27 methyltransferase EZH2 to selected genomic loci via dCas9. Although both versions of the enzyme induced significant transcriptional repression, only the full-length EZH2 was able to deposit H3K27me3. Moreover, long-term maintenance of repression was achieved exclusively when the full-length EZH2 was used in combination with the DNA methyltransferase DNMT3A.

By contrast, a role for DNA methylation in instructing gene silencing has been more convincingly demonstrated, at least at some CpG-dense promoters, by targeting either DNMT3A or the DNA demethylase TET1 to endogenous genes in different cell types. Similarly, a dCas9-p300 histone acetyltransferase fusion has been used to successfully deposit the H3K27ac mark and activate transcription from MyoD and Oct4 promoters and distal enhancers in HEK293T cells. Finally, sustained gene expression was achieved by local induction of H3K4me3 when targeting the PRDM9 catalytic subunit to selected gene promoters. Interestingly, persistence of the induced transcriptional state was observed only at a subset of targeted loci and was facilitated by co-deposition of the H3K79me2 mark. These data demonstrate the potential utility of epigenetic editing for disentangling causal relationships between chromatin modifications and genome regulation via precise perturbations. However, these strategies require further optimization and eradication of confounding influences to achieve their full potential.

Whether engineered epigenetic changes can persist and be mitotically inherited has also been the subject of intense investigation and can be directly addressed with epigenome editing. Bintu et al. analyzed the dynamics of silencing and reactivation of an extra-chromosomal reporter after de novo deposition/removal of four different epigenetic modifications: DNA methylation, histone acetylation, H3K9me3 and H3K27me3. While histone deacetylation was able to repress the reporter in a very potent and fast manner, silencing was not durable. H3K9me3 and H3K27me3 on the other hand promoted slower silencing, which was maintained in a fraction of the cell population, implying a stochastic penetrance effect. Finally, DNA methylation gave rise to slow-building gene silencing which stably persisted in most cells. These results were supported by another study in which DNA methylation was transmitted across several cell divisions to give rise to heritable gene silencing. Nonetheless, emerging evidence suggest that the concomitancy of different chromatin marks is more likely to reinforce long-lasting epigenetic memory. Particularly, DNA methylation in combination with either H3K9me3 or H3K27me3 was shown to instruct robust and self-sustaining repressive epigenetic states.

The application of modular epigenetic editing tools has also been extended to the study of chromatin structure and spatial genome organization. Different dCas9-based strategies have been employed to recruit the BAF chromatin remodeling complex to target genomic loci and to reposition portions of the genome to specific nuclear compartments, including nuclear periphery, Cajal bodies and promyelocytic leukemia bodies. In all cases, alteration of either local or global chromatin architecture led to changes in gene expression. Similarly, the induction of an artificial chromatin loop between a silent gene and an enhancer region was shown to be sufficient to initiate transcription. Looping was indeed followed by RNAPII recruitment and acquisition of H3K4me3 at the silent gene promoter. Interestingly, the aforementioned studies make use of dCas9 variants fused to proteins that can form heterodimers in the presence of light or chemicals (Figure 2), further highlighting the versatility and the possibilities for exploitation of the CRISPR/dCas9 system.

One of the most impactful applications of the CRISPR-based technology has been the high-throughput functional interrogation of the genome by means of gRNA libraries. Initial screens made use of the nuclease-active version of Cas9 to systematically knockout genes genome-wide and dCas9 has been used more recently in combination with epigenetic modifying domains such as p300 and

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### Table 1 (Continued)

| DNA targeting module | Effector protein | Applications | References |
|----------------------|-----------------|--------------|------------|
| SpdCas9              | CIBN (dimerization induced by CRY2) | Light inducible DNA looping | [178] |
| RNA editing          |                 |              |            |
| dCas13               | ADAR2           | Conversion of adenosine to inosine | [91] |
| dCas13               | Modified ADAR2  | Conversion of cytosine to uracil | [179] |
| dCas13               | METTL3, METTL14 | RNA methylation | [92] |
| dCas9                | METTL3, METTL14, ALKBHS, FTO | RNA methylation | [180] |
| dCasRx               | Splicing factors | Manipulation of alternative splicing | [93,94] |
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Targeted genomic loci. RNA-seq can also be employed to verify both dCas9 binding and the level of induced epigenome editing at non-methodologies, such as Cut & Run-seq, should be used to directly assess and lasting manner, as successfully done in both uncommitted and differentiated cell lines. Finally, epigenome editing has the potential to correct disease phenotypes that do not arise from genetic mutations but rather from the misregulation of epigenetic and/or transcriptional states (discussed below).

Beyond the basic understanding of the epigenome functionality, epigenetic editing tools can be exploited to reprogram cells in a specific and controlled manner. This can be circumvented using Cas9 variants or orthologs that utilize alternative PAM sequences (Figure 2).

A third pitfall of dCas9-based systems is represented by data mis-interpretation. It is indeed important to make sure that the observed effects observed after epigenome editing result from the catalytic activity of the effector domain rather than from the recruitment of other chromatin remodelers and transcriptional cofactors. Thus, it is preferable to use only the catalytic domain rather than full-length effectors that may promote further protein-protein interactions. Negative controls in the form of effector mutants that lack catalytic activity should be included in each experiment. In addition, dCas9 coupled to “fake” effectors, such as GFP, should also be used to control for steric hindrance effects. Finally, it is important to remember that the efficacy and the stability of the editing depend on a plethora of variables including (i) inherent features of the effector domain and of the selected genomic location, (ii) gRNA position within the targeted site, (iii) presence of existing modifications, (iv) cell type and cell culture conditions, and (v) the chosen method for the delivery of the editing tools, particularly when working in vivo.

### THERAPEUTIC PROMISE OF EPIGENETIC EDITING

Beyond its potential for unravelling biological mechanisms, epigenetic editing is also an exciting prospect for future therapeutic development. Epigenetic editing represents a potentially precise and non-invasive strategy for combating specific disease states, and the variety of possible applications make it both complementary and, in cases, preferred over gene therapy or wild-type Cas9 genetic approaches. In principle, epigenetic editing has a better safety profile than genetic editing because it does not alter host DNA sequence and is reversible by nature. Moreover, it relies on endogenous cis-elements to regulate target gene expression, making it a more physiological approach than gene (cDNA) delivery. For example, it facilitates switching endogenous genes ON while also retaining the co- and post-transcriptional signals for appropriate expression regulation. Moreover, because several genes can be targeted at the same time, multiplex epigenetic editing could be used (i) to upregulate the expression of several genes simultaneously, or (ii) to switch some genes on and others off in parallel by taking advantage of the orthogonality between CRISPR-Cas9 systems. Given that the first clinical trial for CRISPR-Cas9 started in 2016, CRISPR-based epigenetic therapy holds great promise for subsets of human diseases.

### Potential pitfalls of the epigenome editing tools

Despite the promise that dCas9-based systems hold, several pitfalls should be carefully considered before designing an epigenome editing experiment. First, dCas9 genomic targeting is limited by its PAM requirement, which can pose difficulties when targeting GC-poor regions. This can be circumvented using Cas9 variants or orthologs that utilize alternative PAM sequences (Figure 2).

Second, the system is prone to off-target effects which can arise from multiple sources. For example, the selection of a suboptimal gRNA due to the limited availability of PAM sequences. Other causes for off-target effects are the expression level and the time span of expression of the dCas9 enzyme and/or of the effector domains. Off-target candidate genomic regions can be predicted computationally. Additionally, next-generation sequencing-based methodologies, such as Cut & Run-seq, should be used to directly assess both dCas9 binding and the level of induced epigenome editing at non-targeted genomic loci. RNA-seq can also be employed to verify whether “unintended” changes to the epigenome have led to altered gene expression states within the edited cells. To reduce off-target effects, use of inducible promoters and drug systems that precisely control the quantitative and temporal expression of editing tools represents a good strategy. Moreover, the dCas9-SunTag system allows for fine-tuning the dCas9:effector ratio and can therefore be used to optimize on-target efficiency.

A third pitfall of dCas9-based systems is represented by data mis-interpretation. It is indeed important to make sure that the observed effects observed after epigenome editing result from the catalytic activity of the effector domain rather than from the recruitment of other chromatin remodelers and transcriptional cofactors. Thus, it is preferable to use only the catalytic domain rather than full-length effectors that may promote further protein-protein interactions. Negative controls in the form of effector mutants that lack catalytic activity should be included in each experiment. In addition, dCas9 coupled to “fake” effectors, such as GFP, should also be used to control for steric hindrance effects. Finally, it is important to remember that the efficacy and the stability of the editing depend on a plethora of variables including (i) inherent features of the effector domain and of the selected genomic location, (ii) gRNA position within the targeted site, (iii) presence of existing modifications, (iv) cell type and cell culture conditions, and (v) the chosen method for the delivery of the editing tools, particularly when working in vivo.

### Human disease types responsive to epigenetic editing

Several classes of disease represent attractive targets for development of epigenetic therapy (Figure 3). For example, hundreds of human diseases are associated with haploinsufficiency and selectively enhancing gene expression could compensate for the deficient gene product. This can be achieved by targeting a dCas9 fused with a transcription activation module such as VP64 and its derivatives, or with a histone modification enzyme such as p300, PRDM9 or DOT1L. So far, several studies have reported successful target gene activation in different mouse organs in vivo, including in the brain. For example, Colasante and colleagues have stimulated Scn1a transcription in a mouse model of a severe epileptic encephalopathy called Davet syndrome and showed a significant attenuation of the symptoms. In another study, Matharu et al. took advantage of a SNP present within the promoter region of their target gene to regulate expression in an allele-specific manner, resulting in rescue of the mouse obesity phenotype. Other pathologies that could profit from the in vivo development of the CRISPR activation system are the X-linked disorders, that arise...
FIGURE 3  Potential therapeutic applications of epigenome editing. Various classes of disease could benefit from the development of distinct CRISPR/Cas9-driven epigenome editing strategies. Shown here are examples of how mutant or wild-type alleles could be manipulated in specific disease contexts. The molecular aetiology underlying each disease type, as well as the dCas9-based rescue strategy, are shown. See text for references.

from the expression of a faulty allele from the active X chromosome while the inactive X chromosome contains a functional copy. Rett syndrome, caused by a mutation in the MeCP2 gene,\[136] and Cdkl5 disorder,\[137] are good examples of such diseases. Using programable epigenetic editing to upregulate the expression of the functional gene copy from the inactive X chromosome in brain cells could complement the mutant allele. Remarkably, in the case of the Rett syndrome, induced expression of an exogenous wild-type MeCP2 is sufficient to reverse multiple aspects of disease phenotype,\[138,139] underscoring the potential of such an approach.

Epigenetic editing can potentially apply to several neurological disorders. For example, fragile X syndrome is the most prevalent form of intellectual disability in males, where abnormal DNA methylation mediates epigenetic silencing of the FMR1 gene in neurons. Interestingly, using a dCas9-Tet1 system to specifically demethylate the FMR1 promoter reversed its heterochromatic status and restored FMR1 expression in iPSC derived neurons.\[140] This strategy could be extended to imprinted disorders that arise from an aberrant DNA methylation pattern. Prader-Willi (PWS) and Angelman (AS) syndromes are good examples for the successful use of a similar approach, because some forms of PWS are due to abnormal methylation of the paternal allele, while 4% of AS results from the absence of methylation on the maternal allele.\[141] Therefore, an allele-specific therapy based on targeting of dCas9-Tet1 for the former and dCas9-Dnmt3 for the latter could hold promise. Besides, targeting the brain precludes any consideration for the persistence of epigenetic modifications through cell division as brain cells are mainly in a quiescent state. Finally, a recent study linked the haploinsufficiency of the brain-specific TF C11orf46 to callosal hypoplasia, leading to a mental retardation syndrome.\[142] Remarkably, the authors
used that same TF as a dCas9 fusion effector to rescue the neurodevelopmental disorder, showing that deciphering the molecular mechanisms of brain disorders can be crucial for therapeutic development.

In addition to targeted activation, programed gene silencing can potentially address disease phenotypes, particularly those arising from dominant-acting genes. For example, dCas9-KRAB has been used in a mouse model of hypercholesterolemia to target the Pcsk9 gene, resulting in lower cholesterol levels in treated mice.\cite{143} Many inflammatory and pain-related diseases could benefit from this strategy by targeting (i) cytokines involved in the responsible inflammatory pathway, as envisioned to treat degenerative disc disease,\cite{144} or (ii) pain receptors located in the skin.\cite{145} Indeed, the skin constitutes an accessible and attractive organ for CRISPR-mediated gene silencing as it is associated with numerous monogenic and autosomal dominant disorders,\cite{146} such as Olmsted syndrome\cite{147} or the familial primary localized cutaneous amyloidosis.\cite{148}

Importantly, to ensure a functional gene copy remains active, allele-specific epigenetic silencing could be utilized in dominant-acting disorders. Such strategies have emerged to silence the mutant HTT allele in distinct clinical populations presenting the neurological Huntington disease.\cite{149} Similarly, a recent study shows that targeting the mutant DMD allele in a mouse model of Duchenne Muscular Dystrophy with the CRISPR/Cas9 system greatly improved the mouse muscle contractility.\cite{150} Finally, complex diseases such as cancers could potentially profit not only from the strategies discussed above but also from multiplex epigenetic editing, by activating tumor suppressor genes and inhibiting oncogene expression at the same time.\cite{151-154} Indeed, concomitant activation and repression of different genes within the same cell has been achieved in vitro by coupling dCas9 with chemical- and light-inducible effector domains\cite{155} and by engineering scaffold gRNA molecules that can recruit transcriptional regulators.\cite{156} Moreover, the proof of concept for simultaneous gene activation has been shown in vivo in mice.\cite{85,152,157} Nonetheless, in order for combinatorial gene targeting strategies to be successful in cancer therapy, more vigorous effort will be needed to design potent and precise delivery methods that enable targeting the entire population of disease-associated cells; a major challenge.

**Delivery**

A key limitation on the potential for epigenetic editing as a biomedical strategy in humans is delivery of the system into the relevant cells. Indeed, this represents a fundamental bottleneck for many genome therapy approaches. Nevertheless, several vehicles for CRISPR/Cas9 in vivo delivery have been identified.\cite{158} and each presents their own characteristics of safety, efficiency and target specificity for clinical application. Viral delivery is the leading platform for (epi)genome therapy delivery.\cite{159} Lentiviral systems have been widely used in clinical trials where they facilitate efficient gene therapy, but suffer from compound issues associated with integration into the host genome.\cite{160} As such, they are being progressively replaced by AAV, which do not integrate genomically, and are linked with an excellent safety profile. However, AAV only have limited cargo capacity, which has stimulated the development of smaller CRISPR/Cas9 systems, such as those built around staphylococcus aureus (sa)Cas9 (Figure 2).\cite{161} Notably, engineered AAV capsids can overcome immunogenicity issues\cite{160} and may enable a degree of tissue specificity. Thus, while the application of AVV to therapeutic delivery remains challenging, they potentially fit all the requirements of safety, efficiency and specific delivery.

While not yet broadly applicable, nanoparticles potentially offer a wide range of applications including efficient and site-specific delivery of a CRISPR/Cas9 cargo in vivo.\cite{162-164} They possess a high loading capacity and good stability, as well as the potential for timely control of their cargo release. The current focus is on the conjugation of different nanomaterials to improve their safety, cellular intake and specificity towards a designated cell type or tissue.\cite{165} In addition, new tools are being developed for epigenome therapy. For example, extracellular vesicles offer a promising cargo system for targeted delivery of epigenome editing molecules.\cite{166,167}

One of the major challenges of delivering an epigenome editing system in vivo is to ensure that the cargo won’t hit the germline – thus avoiding the ethical issue of transmitting the biological changes to the offspring – and to minimize the effects of epigenome therapy in non-relevant tissues. Some nanocarriers target specifically the liver and spleen and ex vivo epigenome editing can also be considered as a reliable technique with minimum off-target effects, although its application is limited to a few cell types.\cite{168} AAV can be further engineered to direct their tropism to the cell type of interest through a ligand-receptor interaction.\cite{169,170} These considerations make therapeutic delivery an important area of focus when it comes to clinical applications of epigenetic editing.

**Concluding remarks**

The development of novel technologies for epigenetic editing represents a major advance for both fundamental and applied research into genome regulation, as it allows scientists to manipulate chromatin states with a precision and ease previously unthinkable. This provides a means to deconstruct complex regulatory circuits, identify causal relationships, and ultimately shed light on (epi)genome function in health and disease. Indeed, we anticipate epigenome editing will enable a robust reassessment of the specific functional role of chromatin across contexts and scales, and therefore advance our understanding of the critical question; how is one genome regulated to give rise to many molecular-, cellular-, and organismal-phenotypes. Moreover, the ability to manipulate chromatin in a programable manner also holds great promise for biomedical applications and therapeutic strategies designed to alter gene activity states, particularly as we enter the era of personalized medicine.

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CONFLICT OF INTEREST
No conflicts of interest.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are openly available.

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