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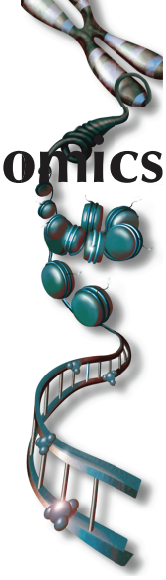
Epigenomics



CRISPR-based epigenome editing: mechanisms and applications

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Epigenomic anomalies contribute significantly to the development of numerous human disorders. The development of epigenetic research tools is essential for understanding how epigenetic marks contribute to gene expression. A gene-editing technique known as CRISPR (clustered regularly interspaced short palindromic repeats) typically targets a particular DNA sequence using a guide RNA (gRNA). CRISPR/Cas9 technology has been remodeled for epigenome editing by generating a 'dead' Cas9 protein (dCas9) that lacks nuclease activity and juxtaposing it with an epigenetic effector domain. Based on fusion partners of dCas9, a specific epigenetic state can be achieved. CRISPR-based epigenome editing has widespread application in drug screening, cancer treatment and regenerative medicine. This paper discusses the tools developed for CRISPR-based epigenome editing and their applications.

Tweetable abstract: CRISPR/Cas9 system has been remodeled for epigenome editing by juxtaposing 'dead' dCas9 with an epigenetic effector domain. This tool has applications in cancer treatment and regenerative medicine.

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"*The complex developmental processes that connect between the genotype and the phenotype*" is typically recognized as the first definition of epigenetics established by Conrad Waddington [1]. Although the term has been refined since then, it remains an accurate description of epigenetics. It encompasses a plethora of mechanisms that influence the phenotype beyond just the DNA sequence. Such mechanisms that regulate genomic functions are mediated by epigenetic factors. These factors effectuate various modifications that include DNA methylation, histone modifications [2–4], chromatin accessibility [5], 3D genome organization [6], and interactions of various RNA and protein elements with the genome (Figure 1) [7–10].

These factors orchestrate to modify the epigenetic landscape of the cell and determine which genes are turned 'on' and 'off' to control gene expression and ultimately cell fate. Genome regulation by the epigenomic mechanisms ensures that each cell is only able to produce molecules necessary for its cell or tissue function [11–13]. Epigenetic factors not only play a role in cell fate determination but also in the propagation and maintenance of the epigenetic state of the cells. Maintaining cellular memory across cell divisions is essential to maintain normal cell and organ function [14,15].

Owing to the significant role of epigenetics in normal physiology, it is not surprising that the epigenetic landscape is altered in a number of human disorders [16] including cancer [17–19], heart disease [20–22], obesity [23,24], neurological disorders [25,26], and so on. Understanding how epigenetic factors individually and collectively contribute to modulation of the epigenetic landscape, and how aberrant epigenetic profiles promote disease progression, requires the development and expansion of epigenetics research tools. The utilization of such tools will be valuable in both basic and applied research [16]. Identifying aberrant epigenetic patterns may serve as biomarkers in diagnostics [27,28], and in the development of epigenetic drugs called 'epidrugs' that target epigenetic mechanisms in cancer and other human disorders [27,29]. Despite their potential use in a number of conditions, the nonspecificity of epidrugs poses a serious challenge. Recently, several biochemical and bioinformatic tools have been developed to study and alter epigenetic marks, mostly focusing on methylation and histone modifications. However, these techniques have some limitations in terms of their versatility and precision [30,31]. Therefore, it is critical to develop novel technologies

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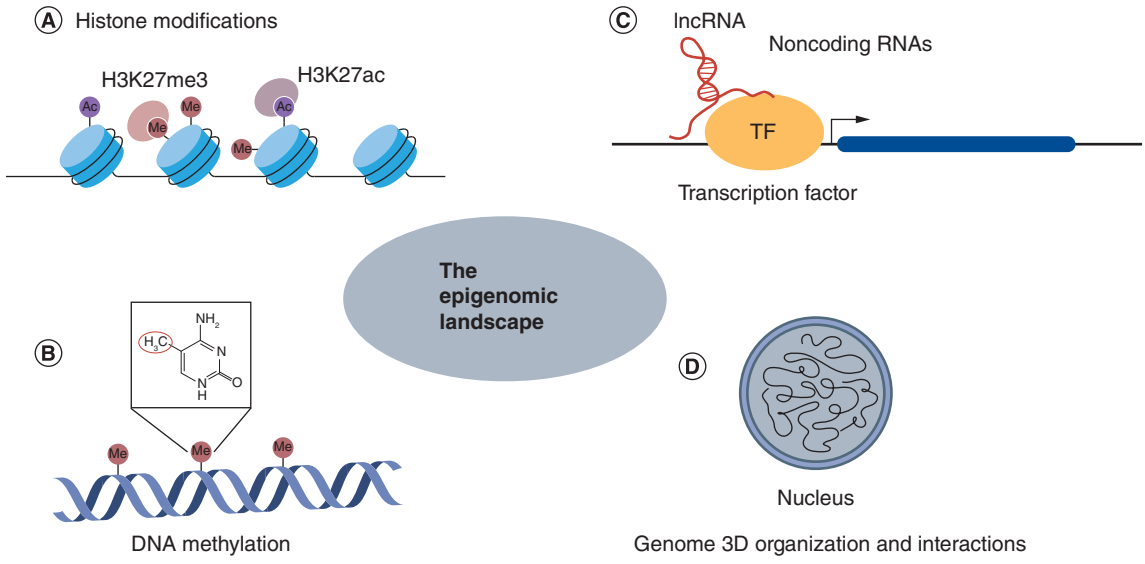

Figure 1. Molecular mechanisms modulating the epigenome. The epigenome encompasses a variety of dynamic molecular processes crucial for cell function and development. **(A)** Nucleosomes, composed of DNA (black line) wrapped around histones (blue) are the basic units of chromatin packaging in eukaryotes. Histones tails can be modified by the covalent additions of chemical groups catalyzed by enzymes (colored circles), ultimately effecting chromatin accessibility. **(B)** Methyltransferases may add a methyl group to the C-5 position of cytosine, which may affect transcription status. **(C)** Noncoding RNA elements such as lncRNAs (red line) can modulate gene expression by binding TFs (yellow circle). **(D)** Chromatin is intricately folded into a 3D configuration in the nucleus, constituting chromatin domains and interactions of regulatory elements that affect gene expression.
lncRNA: Long noncoding RNA; TF: Transcription factor.

that are precise in inducing and interfering with epigenetic marks at the specific genomic regions. One such tools is a modified version of CRISPR-Cas system.

In this paper we will discuss the tools developed for CRISPR-based epigenome editing and their applications. We will begin by discussing the basic principles of CRISPR and how it can be utilized to target the epigenetic landscape. We then discuss the different tools that have been developed for epigenome modulation, and lastly review the current state of knowledge on the applications of CRISPR systems in this field.

Harnessing CRISPR for epigenome editing

the CRISPR system, originally identified in bacteria and archea, is a revolutionary gene-editing tool that typically uses guide RNA (gRNA) to target a specific DNA sequence. Once the gRNA binds to the target DNA sequence, Cas proteins are recruited to the site and cut the DNA to allow insertions, deletions and modifications on the target site using indigenous cell DNA-repair mechanisms [32]. The CRISPR-Cas system exhibit high functional and structural diversity. CRISPR-Cas can be categorized into six different types (I–VI), each type consisting of a distinct Cas nuclease protein. The six different types have been further regrouped into two classes depending on the composition of Cas effectors and CRISPR RNA ribonucleoprotein (crRNP) complexes.

Class 1 systems include (Types III, IV, and I) and consist of multiple Cas proteins in their crRNPs that can include nuclease components. While Class 2 systems (types II, V, and VI) only consist of a single Cas effector Protein in their crRNP complexes [33,34]. This diversity also includes the nucleic acid substrates that can be recognized and targeted by CRISPR-Cas effector Complexes. Types I, II, V and likely IV target DNA, while type III systems can target both DNA and RNA [35]. Type VI systems exclusively target RNA [36,37]. CRISPR system research tools were originally developed utilizing class 2 type II Cas9 and type V Cas12 single effector complexes that originally target and cleave DNA sequences. The CRISPR-based toolbox expanded to include nuclease 'null' Cas proteins dCas9 and dCas12 [38]. A concerted effort is now underway to include CRISPR systems that bind and cleave RNA molecules based on type III and VI crRNP effector complexes [35,39–42].

Recently, CRISPR/Cas9 technology has been remolded for epigenome editing by generating a 'dead' Cas9 protein (dCas9) or 'nuclease-null' that allows us to take advantage of its DNA-binding abilities to target specific loci without

Table 1. Types of CRISPR systems that have been repurposed for epigenome editing.

Type	I	II	III	IV	V	VI	Ref.
Class	1 (multisubunit)	2 (single unit)	1 (multisubunit)	1 (multisubunit)	2 (single unit)	2 (single unit)	
Endonuclease protein	Cas3	Cas9	Cas10	Scf1	Cas12	Cas13	
Target molecule	DNA	DNA	DNA/RNA	–	DNA	RNA	
Epigenome editing platforms	–	dCas9-DNMT3A dCas9-SunTag-DNMT3A dCas9-DNMT3A-DNMT3L dCas9-DNMT3A-DNMT3L-KRAB dCas9-TET1CD dCas9-Tet1-CD and MS2-Tet1-CD dual system dCas9-TET1 TET1-dSaCas9 and VPR-dSpCas9 dCas9-SunTag-TET1 dCas9-p300 dCas9-HDAC3 dCas9-KRAB dCas9-LSD1 dCas9-Ezh2 dCas9-VPR (CRISPRa)	DiCas7-11	–	–	CRISPR-Cas13d dCas13b-ADAR2 CRISPR-Cas13a	[36,46,47,49,53–76]

cleavage [43]. Epigenetic effectors can be tethered to the catalytically inactive Cas9 complex, allowing it to localize the effectors to a specific locus to induce epigenetic alterations. The same dCas9 protein can be reused to target other loci just by changing the sgRNA sequence. CRISPR technology offers great advantages as an engineering tool from targeting DNA sequences of any length, to specific and multiplex modifications [44–46]. The developments of dCas9 fusion constructs provided us with a diverse epigenome control toolkit, to control transcriptional activation and repression of genes at the level of histone modifications [47,48], DNA methylation [49,50], and even of genes coding for noncoding RNAs (ncRNAs) [51,52]. Table 1 summarizes the tools developed from various classes of CRISPR systems. In the following section, we will discuss the mechanisms that have been employed to design tools for CRISPR-based epigenome editing and their applications (summarized in Table 2).

Mechanisms of epigenome targeting by CRISPR systems

DNA methylation

DNA methylation is a major epigenetic repressive chemical modification that influences gene expression. This modification is established by the addition of a methyl group to the fifth carbon position of cytosine to form 5-methylcytosine (5mC). 'Writer' proteins, namely DNA methyltransferases (Dnmts), catalyze this process [4], while TET1–3 enzymes control active removal of the methylation modification by adding a hydroxyl group to the methyl group of 5mC to produce a 5hmC modification that can be converted back to bare cytosine by the oxidase activity of TET enzymes [90]. Many epigenome-editing technologies developed for DNA methylation control make use of these 'writer' and 'eraser' proteins to add or remove chemical modifications to specific target genome loci using dCas9 systems when fused with Dnmts or TET domains. This is demonstrated in previous studies [49,53–61] where Dnmt3a, Dnmt3L and TET1 catalytic domains were fused to CRISPR/dCas9 to create targeted DNA methylation control tools. This has been used along with engineered sgRNA molecules to target and bind genomic loci, recruit enzymatic DNA modification activity, and eventually modulate upregulation or downregulation of gene expression of the target genes. These systems has given us the means to investigate transcriptional changes in response to alterations in localized epigenomic marks. Other research by Pflueger *et al.* aimed to optimize the specificity of such systems that relies on fusion proteins for DNA methylation modulation [62]. As the understanding of the off-target effects of such systems is lacking, and due to the importance of specificity in the implementation of epigenome-editing tools, a modular dCas9-SunTag DNMT3A dual system was designed. The system consists of a dCas9 protein fused to a SunTag peptide array and a counterpart antibody to a DNMT3A catalytic domain. This research revealed that the dual transfection of dCas9-SunTag and DNMT3A single-chain antibody fusion (αGCN4-D3A) construct leads to more specific and potent methylation of target sites. This is attributed to the epitope activity of the SunTag peptide that recruits multiple (αGCN4-D3A) molecules to the target site. The dual nature of this system also allows

Table 2. dCas9-mediated epigenome editing involves multiple epigenetic mechanisms.

Modification	Effector domains	Target	Mechanism	Ref.
DNA methylation	dCas9-DNMT3A dCas9-SunTag-DNMT3A dCas9-DNMT3A-DNMT3L dCas9-DNMT3A-DNMT3L-KRAB	*BDNF* promoter *MyoD* enhancer CTCF loops *CDKN2A* promoter, *ARF* promoter, *Cdkn1a* promoter *IL6ST*, *BACH2* *HOXA5* *EpCAM*, *CXCR4* and *TFRC* promoters *UNC5C* promoter GAPDH-Snrpn reporter system	Targeting of dCas9-DNMT3a fusion proteins to unmethylated promoter sequences Targeting of the dCas9-DNMT3a fusion domain along with repetitive peptide epitopes (SunTag) that can recruit and amplify the local concentration of DNMT3a domains for robust methylation of target loci Targeting of a dCas9-DNMT3A-DNMT3L methyltransferase chimeric construct to induce higher methylation efficiency compared with a system that utilizes a single methyltransferase catalytic domain Development of 'CRISPRoff' composed of dCas9-DNMT3A-DNMT3L-KRAB fusion domains to target and induce gene silencing memory	[46,49,56–59,62,77]
DNA demethylation	dCas9-TET1CD dCas9-Tet1-CD and MS2-Tet1-CD dual system dCas9-TET1 TET1-dSaCas9 and VPR-dSpCas9 dCas9-SunTag-TET1 enzyme free steric interference with DNA methyltransferases dCas9-TETv4 with sgRNA modified with MCP (VP64, p65-AD and Rta)	STAT3 binding site upstream of GFAP *BRCA1* gene promoter *RANKL*, *MAGEB2*, *MMP2* neighboring CpGs *BDNF* promoter *MyoD* enhancer Dazl-Snrpn-GFP reporter *HNF1A*, *MGAT3* *FMR1*, *SerpinB5*, *Tnf* *CLTA*	Targeted DNA demethylation using a dCas9 fusion construct with a TET1CD catalytic domain Simultaneous transfection of dCas9-TET1-CD and a MS2 coat protein fusion construct guided with a sgRNA2.0 transcription system to demethylate target genomic regions and promote gene expression Induction of *de novo* demethylation of target gene regions with the dCas9-TET1 construct Demethylation of regulatory regions via dual TET1-dSaCas9 and VPR-dSpCas9 fusion constructs for synergistic modulation of gene expression Targeting of the dCas9-TET1 fusion domain along with repetitive peptide epitopes (SunTag) that can recruit and amplify the local concentration of TET1 proteins for robust demethylation of target loci Targeting of the enzyme-free dCas9 system to CpGs adjacent to transcription start sites to interfere with the binding of DNA methyltransferases leading to induced site-specific gene expression Development of 'CRISPRon' composed of a dCas9-TETv4 fusion domain with modified sgRNAs with MCP and various combinations of transactivation domains to reverse 'CRISPRoff'-mediated gene silencing	[46,53–57,60,61,77]
H3K27 acetylation	dCas9-p300 dCas9-p300 fused with PYL or ABI proteins dLbCpf1-P300/dCpf1-SunTag dCas9-p300 and MCP-VP64 dual system	I*L1RN (MYOD)*, *POU5F1*/*OCT4* *IL1RN* GRM2 *MYOD*, *HS2* enhancer, *TAL1* *IL1RN, GRM2, HBA, MYOD* *IL1RN, RHOXF2, TTN*	Targeting of dCas9-p300 acetyltransferase domain to promoters and distal enhancers leading to gene activation Utilizing CIP for temporal control of histone modifications, where p300 and dCas9 proteins can be fused to ABI and PYL that can dimerize when the small chemical inducer ABA is introduced to reversibly recruit p300 to target sites System based on a Cpf1 and p300 fusion domain for targeted histone acetylation as an alternative to dCas9 to activate multiple genes with a single sgRNA enhancer targeting by 'enCRISPRa' system for activation	[47,63–65,78–82]
H3K27 deacetylation	dCas9-HDAC3 dCas9-KRAB	*Smn1, Mecp2, Isl1* *OCT4, Tbx3, SOX2*	Targeting of the dCas9-HDAC3 deacetylase domain to promoters and enhancers leading to a repressive state Targeting of the dCas9-KRAB deacetylase domain to promoters and enhancers leading to a repressive state	[66,67]
H3K4 demethylation	dCas9-LSD1	*OCT4, Tbx3, SOX2*	Targeting of the dCas9-LSD1 demethylase domain to promoters and enhancers leading to activation	[67]
H3K4 methylation	dCas9-SET	*ICAM1, RASSF1a, EpCAM* *IL1RN, GRM2*	Targeting of the dCas9-SET histone methyltransferase domain to promoters leading to gene reactivation	[63,83]

CIP: Chemically induced proximity; LADL: Light-activated dynamic looping; MCP: MS2 coat protein; ssRNA: Single-stranded RNA; TF: Transcription factor; vlincRNA: Very long intergenic noncoding RNA.

Table 2. dCas9-mediated epigenome editing involves multiple epigenetic mechanisms (cont.).

Modification	Effector domains	Target	Mechanism	Ref.
H3K9 methylation	dCas9-G9A[SET]/dCas9-SUV[SET] dCas9-KRAB	HER2, MYC, EPCAM HS2 enhancer	Repression of target genes by histone methyltransferases fusion domains with dCas9 Targeting of dCas9-KRAB to enhancer regions and recruitment of repressive chromatin methylation modifiers	[68,84]
H3K27 methylation	dCas9-olEzh2 dCas9-Ezh2	*Arhgap35, Pfkfb4a, Nanos3, Dcx, Kita, Tbx16, Slc41a2a* *HER2, MYC, EPCAM* *C/ebpa* promoter	Repression of target genes by histone methyltransferases fusion domains with dCas9	[68,69,85]
RNA	DECKO (double-excision CRISPR knockout) CRISPR-Cas13d dCas9-VPR (CRISPRa) dCas13b-ADAR2 DiCas7-11 CRISPR-Cas13a	*UCA1, MALAT1, TFRC* *mCherry, B4GALNT1, ANXA4, HOTTIP, MALAT1, EGFR, EZH2, HRAS, KRAS, NF2, NFKB1, NRAS, PPARG, RAF1, SMARCA4, STAT3* vlincRNAs (ID30, ID332, ID-372, ID-604) RP11-326A19.4 Cluc W85X Gluc/Cluc luciferases PPIB, KRAS, MALAT1 CXCR4 RFP Trans-ssRNA	Knock out of long noncoding RNA genes by the deletion of promoters Programmable RNA targeting and cleavage Targeted knockdown of vlincRNAs Activation of long noncoding RNA promoter region inducing genome wide transcriptional changes ADAR2 domain fusion with dCas13 for targeted RNA editing through the direct adenosine to inosine deaminase activity of ADAR2 Engineering of Cas7-11 for RNA targeting and knockdown Guided cleavage of ssRNAs by CRISPR-Cas13a effector	[36,70–76,86]
TF hinderance	CRISPR-dCas9	*OCT4, Nanog, Pax6*	By Steric hinderance of transcription factors by dCas9 targeted binding leading to disturbed TF-DNA interaction	[87]
Genome organization	dCas9-DNMT3A dCas9 fused with PYL or ABI proteins (CLOuD9) dCas9-CIBN	*miR290, Pou5f1* *β-globin* promoter, *LCR HS2, OCT4* *Klf4, Zfp462* promoter	Targeted methylation of CTCF anchor sites blocking CTCF binding and altering DNA looping and expression of genes located in neighboring loops Utilizing CIP for selective formation of *de novo* chromatin loops dCas9 proteins can be fused to ABI and PYL that can dimerize when the small chemical inducer ABA is introduced to reversibly bring two genomic regions into juxtaposition LADL system that drives *de novo* chromatin looping by the heterodimerization of dCas9-CIBN fusion proteins when blue light is applied using cryptochrome 2 inducible bridging factor	[56,88,89]

CIP: Chemically induced proximity; LADL: Light-activated dynamic looping; MCP: MS2 coat protein; ssRNA: Single-stranded RNA; TF: Transcription factor; vlincRNA: Very long intergenic noncoding RNA.

for independent variation in the expression of the DNA-targeting module (dC9Sun) and the effector module (GCN4-D3A) [62]. A more recent innovative approach uses a dCas9-based systems with no epigenetic modifying enzymes to control DNA methylation in a less confounding way *via* steric hindrance. This method of epigenome control is based on the fact that dCas9-gRNA binding to a specific site will block binding and subsequently the activity of DNA methyltransferases in dividing cells. Blocking access of the enzymes to the target site will lead to passive demethylation of the site by dilution as the cells divide. Considering that steric hindrance requires strong, tight binding between the dCas9-gRNA and the target site, off-target effects will be averted [77]. In a seminal paper by Nuñez *et al.*, novel CRISPR-based epigenome engineering tools, 'CRISPRon' and 'CRISPRoff', were reported. The development of these systems used the dCas9 and catalytic protein fusion approach, where CRISPRoff is composed of ZNF10 KRAB, Dnmt3A (D3A) and Dnmt3L (D3L) protein domains fused to catalytically null dCas9. This system showed the potential to silence the vast majority of human genes robustly and specifically. In addition, it can target and disturb enhancers as well as genes lacking canonical CpG islands. The silencing of genes by CRISPRoff expression is stably maintained throughout cell divisions and stem cell differentiation, which provides a powerful tool to study the mechanisms and roles of epigenetic modifications and inheritance. CRISPRoff also provides a platform for genome-wide screens, multiplexed cell engineering and, in general, a tool to rewrite the human epigenome. Effects of CRISPRoff can be reversed by CRISPRon and can even optimize the gene expression of target genes. CRISPRon is composed of a TETv4 and MCP-transactivator domain (VP64, p65-AD and Rta) fusion proteins that recruit the transcriptional machinery leading to a higher level of expression in target genes [46].

Histone modifications

Histone proteins are the basic components of chromatin that are post-translationally modified in a number of ways such as acetylation, methylation, phosphorylation, ubiquitination, and so on [91]. These modifications can regulate and remodel the chromatin structure to up- or down-regulate the transcription of genes by the recruitment of regulatory proteins and complexes [91,92]. Epigenome editing tools have been developed that make it possible to target and 'tweak' those chemical modifications to control cellular behaviors. Changes in chromatin state can be achieved by the dCas-based recruitment of effectors to deposit or remove histone acetylation and methylation marks [47,63–66,78–81,93]. Depositing histone acetylation patterns by this method have been a successful approach in the locus-specific transactivation of gene expression [47,63–65,78–81,93]. For instance, acetylation of the histone3 lysine 27 residue is an extensively targeted modifications by CRISPR-based editing systems. This was done by designing a nuclease-null dCas9 protein fused to the catalytic domains of acetyltransferases such as p300 [47,64,65,78–81] to modulate proximal and distal enhancer-regulated genes. These studies showed that dCas9-p300 fusion proteins are effective in transactivating endogenous genes. To analyze the functions of active enhancers based on enhancer-associated chromatin modifications, enCRISPRa and enCRISPRi dual-effector editing systems were developed. The enCRISPRa system combines acetylation writing domain p300 and transcriptional effector domain VP64 for enhancer activation, while the antagonistic enCRISPRi system is composed of the LSD1 lysine demethylase domain along with a KRAB transcriptional repressor domain for maximal perturbation of enhancer functions [80]. CRISPR-Cas9 histone deacetylases (HDACs) protein fusions for transcriptional repression have also been developed, wherein the dCas9-HDAC3 fusion system represses transcription at endogenous promoters [66]. Histone lysine methylation is another critical epigenetic modification that regulates gene expression in a context-dependent manner. Typically, methylation of H3K4, H3K36 and H3K79 residues is associated with an active state, while H3K9, H3K27 and H4K20 methylation is associated with a repressed chromatin state [82,94,95]. Histone methylation dynamics is regulated by methyltransferases, the writers, and demethylases, the erasers. Several studies reported the development of dCas9-based systems to modulate histone lysine methylation marks [63,67–69,83,85,96,97]. The dCas9 fusion proteins were constructed with different methyltransferases that target different histone lysine residues: repressive trimethylation at H3K27Me3 by EZH2 fusion systems [68,69,85,96], transcription-activating H3H4me3 modifications by SMYD3 and PRDM9 fusions [83,97], and H3K4Me3 demethylation by LSD1 domains to perturb enhancers [63,67]. This targeted recruitment of epigenetic effectors not only achieved the respective epigenetic state with corresponding gene-expression patterns but also allowed us to study the crosstalk and interplay between epigenetic modifications [63]. It is pertinent to note that persistent and potent modulation using dCas9 fusion proteins can be better achieved by multiple effector systems [46,68,80]. This approach suggests promising avenues for the optimization of existing dCas9-based epigenome editing tools.

RNA engineering

Although ncRNAs do not encode proteins, they are involved in many cellular functions to regulate gene expression. Recently, many classes of regulatory ncRNAs have been discovered due to advances in transcriptome sequencing techniques [98]. One of the classes of ncRNAs is long non-coding RNAs (lncRNA), which are RNA transcripts usually with a length of more than 200 nucleotides. lncRNAs play major roles in both transcriptional and post-transcriptional regulation, genomic imprinting and chromatin remodeling [99–101]. Owing to their roles in cellular processes and human disorders, the functional studies of lncRNAs are of paramount importance to elucidate their diverse roles in gene regulation and cellular functions. While RNAi for ncRNAs has limited success, CRISPR-based systems proved to be powerful in the genetic manipulation of lncRNAs, providing versatile and effective tools for lncRNA studies. The potential of CRISPR systems as a screening tool was demonstrated in numerous studies [86,102–108]. CRISPR-based lncRNA editing approaches vary from targeted deletions or mutations [86,102,103,108], targeting splice sites [104] and CRISPRi/CRISPRa systems [105,107], where transcription is repressed or activated by dCas9 with transcription effector domain fusions such as KRAB and VP64. In addition, the lncRNA targeting toolbox also includes direct targeting of the lncRNA molecules by the RNA binding Cas13 variant to induce knockdowns of lncRNAs without genome alterations [36,70]. Although these tools show efficiency and specificity, they still have limitations and challenges including off-target effects on neighboring gene-expression levels as a result of transcription effector recruitment and unrelated promoter interactions [71].

As discussed earlier, type VI CRISPR-Cas systems contain RNA targeting Cas13 effectors that can bind and cleave single-stranded RNAs, making them attractive tools for RNA editing and transcriptome modulation. Cas13 proteins contain conserved higher eukaryote and prokaryote nucleotide-binding domains that have nuclease

activity for RNA cleavage and degradation of single-stranded RNAs [42,109]. This RNAse activity induces transcript knockdowns in mammalian cells [72–75,110], thus demonstrating transcriptome engineering without permanent alterations to the DNA sequence. Moreover, a catalytically inactive Cas13 protein was engineered that serves as an RNA-targeting platform without the induction of RNA cleavage. This allowed the fusion of RNA editing proteins such as adenosine deaminases acting on RNA (ADARs) that can catalyze the conversion of adenosines to inosines with effects on translation and splicing [111,112], creating an RNA-editing platform with a similar approach as dCas9 fusion effectors [72]. The dCas13b RNA targeting platform has a number of applications including transcript modifications, splicing modification, RNA imaging and transcripts localization within the cell. With this promising expansion of CRISPR epigenome editing tools, further research and consideration is needed to interrogate the toxic effects of targeted RNA degradation. A recent study by Özcan *et al.* explored the use of class 1 subtype III-E effector Cas7-11 derived from fusion between a Cas11 domain and multiple Cas7 subunits, as a programmable RNA cleavage system. In contrast to RNA knockdown systems based on Cas13 effectors, Cas7-11 RNA knockdown exhibited negligible collateral activity and cell toxicity [76]. This finding could pave the way for the development of new RNA-targeting platforms based on cas7-11 orthologs that are more effective and safer than currently available platforms in the pursuit of RNA-targeting therapeutics.

Regulatory regions

Traditionally known as 'junk DNA', the noncoding region of our genome plays a pivotal role in the regulation and 3D organization of our genome. These *cis*-regulatory regions modulate the epigenetic landscape *via* interactions with histones, sequence-specific DNA-binding transcription factors and other regulatory proteins, thus regulating gene expression at distal sites [113]. Alterations in noncoding regions may lead to the deregulation of cognate genes, even if the regulatory sequences are located far away from the gene itself [114]. Identification and unraveling of functions of noncoding genomic regions will help better understand interactions between gene regulatory programs. CRISPR-based regulatory region annotation screens [80,84,87,115–123] provided a high-throughput assay that enabled us to study the activities of endogenous regulatory elements and identify target genes of regulatory regions. In general, CRISPR-cas9 systems rely on introducing indels to regulatory genomic locations such as transcription factor binding sites and enhancers to examine the effects of loss of function in these regions on target genes [124]. However, such approaches fail to quantify the impact of the regulatory elements on the transcriptional output, along with the fact that these only rely on loss-of-function analysis and do not include gain-of-function studies. To mitigate these limitations, dCas9-KRAB repressor and 9Cas9-p300 activator epigenetic-modifying fusion proteins have been utilized to study both loss- and gain-of-function effects on gene promoters and both proximal and distal genomic enhancer regions [125,126]. This approach has proven to be instrumental in studying gene regulation as it allows us to precisely manipulate regulatory regions and dissect their functions. Furthermore, dCas9 fusion proteins can be combined with other screening approaches such as RNA fluorescence *in situ* hybridization labeling, high-throughput sequencing and flow cytometry to rigorously quantify target transcript levels and identify candidate regulatory elements [127].

3D genome organization is another level of regulation that determines how the genome is hierarchically packaged inside the nucleus. This entails organization of the chromatin as chromosome territories, euchromatin and heterochromatin compartments, topologically associating domains, and chromatin loops. 3D chromatin architecture is a key determinant of gene expression patterns and is altered in response to environmental stress and developmental cues [128,129]. CRISPR-based epigenome regulation has been employed to delineate the nature of chromatin looping. One system employed by Morgan *et al.* is CLOuD9, a dCas9-based approach to forcibly introduce loops in the chromatin structure, bringing together any two genomic regions to gain insight into their interactions and effects on gene expression. CLOuD9 consists of dCas9 fusion with components of the plant ABA signaling pathway. dCas9 fusion proteins are targeted to each genomic loci by sgRNAs; upon the addition of phytohormone S-(+)-abscisic acid (ABA), dimerization occurs between the chemical-induced proximity proteins. This results in interaction and juxtaposition of the two chromosomal loci [88]. Similarly, Kim *et al.* developed an optogenetic-inducible CRISPR-based tool called the light-activated dynamic looping system to direct a stretch enhancer to the Zfp462 promoter using CRISPR gRNAs and light-induced heterodimerized cryptochrome 2 and a dCas9-CIBN fusion protein [89]. Such platforms offer inducible, reversible and broadly applicable tools for 3D chromatin manipulation that likely will prove to be of significance in studying abnormal chromatin effects in human disorders. Figure 2 summarizes the tools in use for epigenome and RNA editing.

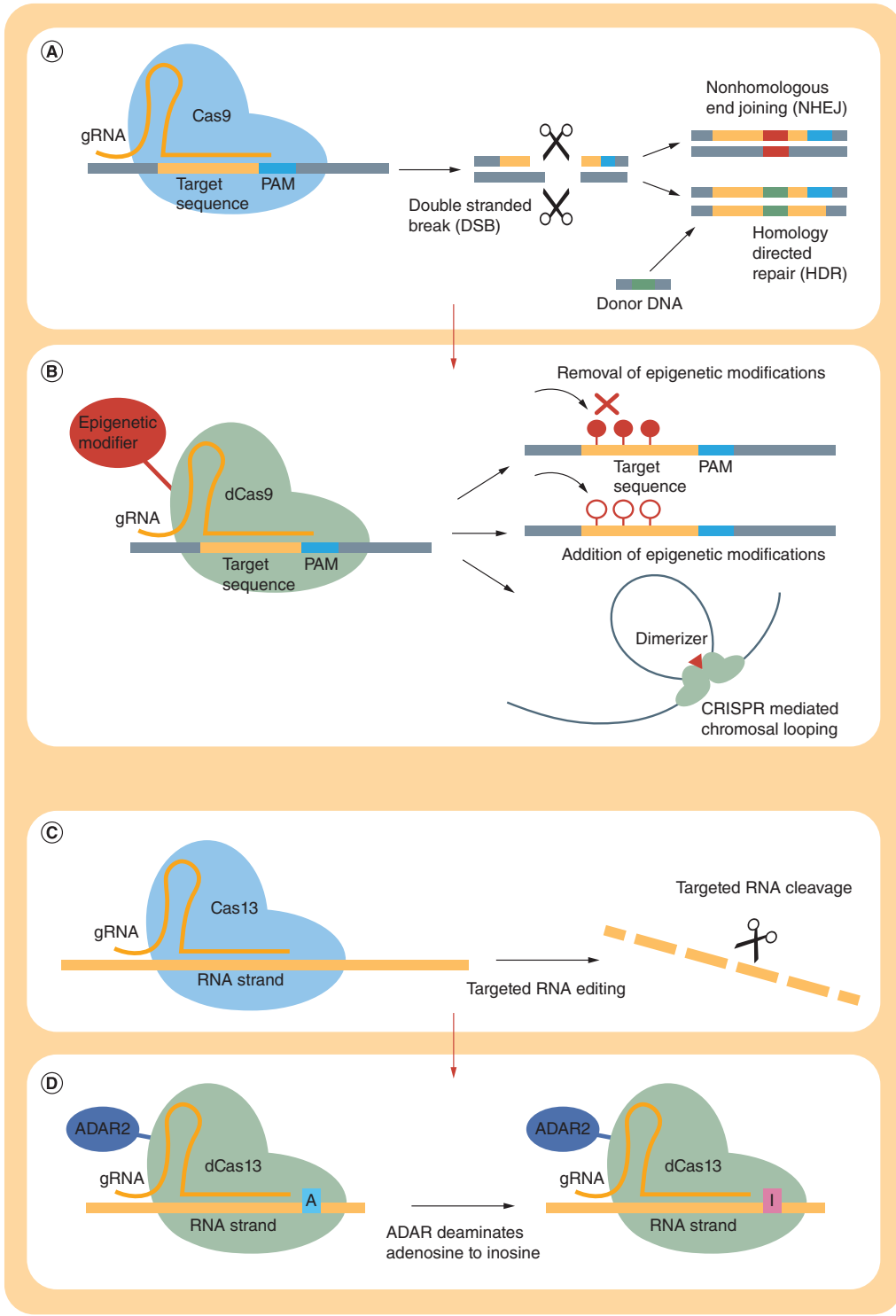

Figure 2. Epigenome and RNA editing via CRISPR. (A) Cas protein (light blue) binds DNA target sequence (orange) via guide RNA (orange line) near protospacer adjacent motif (PAM) (blue). This leads to cleavage of the target sequence due to nuclease activity of the Cas protein, producing double-stranded breaks that will be mend by the endogenous DNA repair machinery. **(B)** dCas lacks the ability to induce breaks in target sequences. However, can still bind to target sequences *via* gRNAs and add/remove specific epigenetic marks or modulate chromosome looping. **(C)** Cas13, guided by gRNA, targets the RNA (yellow) molecule for cleavage. **(D)** However, dCas13 juxtaposed with ADAR2 carries out RNA editing by converting A to I.
ADAR: Adenosine deaminase acting on RNA; dCas9: Nuclease-defective Cas9; gRNA: Guide RNA.

Table 3. Applications of CRISPR mediated epigenome editing in cancer research and regenerative medicine.

Application	Target	Mechanism	Ref.
Cancer therapeutics	GRN in Hep3B hepatoma cells SARI promoter in colon cancer tissue DKK3 promoter in PCa cells ΔNp63 in in lung and esophageal SCCs Two class II TSGs, MASPIN, REPRIMO in H157 lung cancer cells and AGS gastric cancer cells MDM2 in osteosarcoma cells KLF4 in UBC Rnd3 in MM RPMI 8226 and JJN3 cell lines	Targeting of a dCas9 fusion system with DNMT3a, EZH2 and KRAB epi-suppressors to the GRN promoter, leading to knockdown and reduction of tumor formation Targeted demethylation of the SARI promoter by a dCas9 TET1 fusion system, leading to restoring SARI antitumor function Targeted demethylation of the DKK3 promoter by a dCas9-VPR fusion system, leading to induction of DKK3 tumor suppressive roles CRISPR interference system (dCas9-KRAB) targeted to the transcription start site of ΔNp63, leading its suppression and induced apoptosis of cancer cells CRISPR/dCas9 fused with a number of effector domains (VP64, p300, VPR, SAM complex) for dormant tumor suppressor gene reactivation to inhibit cell proliferation Control of the MDM2 proto-oncogene by dCas9-KRAB in osteosarcoma cells, leading to effective inhibition of tumor growth Upregulation of KLF4 expression by dCas9-VP64 (CRISPRon), leading to the inhibition of tumorigenesis Silencing of Rnd3 in multiple myeloma cells by dCas9-KRAB	[96,135–145]
Regenerative medicine	UCP1 in adipose-derived stem cells (ADSCs) Pparγ2, Prdm16, Zfp423, Ucp1 in adipocytes Ascl1, Lmx1a, Nr4a2 (ALN) or Ascl1, Lmx1a, NeuroD1 transcription factors FMR1 CGG expansion in induced pluripotent stem cells Brn2, Ascl1, Myt1l in fibroblasts	Upregulation of UCP1 expression in engineered adipocytes using a CRISPR activation system (CRISPRa) composed of dCas9 and activation domains MCP-p65-HSF1 Upregulation of genes involved in adipocyte differentiation and function by the CRIPSRa SAM system for adipocyte engineering Reprogramming of striatal astrocytes into mature neurons by the CRISPRa system dCas9-VP64/SAM for voluntary motor behavior rescue in Parkinson's disease Targeted demethylation and reactivation of FMR1 by dCas9-TET1 Reprogramming mouse embryonic fibroblasts to induced neuronal cells utilizing dCas9-VP64 to activate endogenous neurogenic genes *Brn2*, *Ascl1*, *Myt1l*	[146–154]

GRN: Granulin; MM: Multiple myeloma; PCa: Prostate cancer; SAM: Synergistic activation mediator; SCC: Squamous cell carcinoma; UBC: Urothelial bladder cancer.

Applications of CRISPR epigenome editing

As epigenetic reprogramming underlies a number of developmental and environmental disorders [130,131], manipulation of the epigenome offers a multitude of biological applications. CRISPR-mediated epigenome editing can be applied to address challenges in the fields of drug screening, disease modelling and cell fate engineering. Soon after conception, CRISPR epigenome editing has been used to investigate both basic biological questions and clinical applications in various fields. One exciting area of basic exploration includes identifying and exploring the functions of noncoding regulatory regions of the genome. Enhancers are one such region with characteristics epigenetic marks, H3K4me1/2 and H3K27ac [132–134]. CRISPR/dCas9-based enhancer-targeting epigenetic editing systems have been developed that provide opportunities to identify corresponding genes regulated by specific enhancers as well as interrogating enhancer function in native biological contexts [80,127]. In addition to these basic biological discoveries, the expansion in the CRISPR epigenome engineering toolbox served as a catalyst for major developments in stem cell research, biotechnology, regenerative medicine and basic research (summarized in Table 3). Here we overview their applications in the field of cancer biology and regenerative medicine.

Cancer therapeutics

Epigenetic anomalies contribute to various human diseases, imprinting disorders [135,155,156] and cancer [136,157] in particular due to their critical roles in growth related pathways. The development of CRISPR-based systems has ushered in a new era of cancer therapy, with the potential to precisely target and correct epigenetic alterations that drive tumor growth. Epimutations of tumor-suppressor genes and oncogenes can be targeted via dCas9 fusion

proteins with epigenomic 'writers' and 'erasers' of epigenetic marks such as DNA and histone methylation (Dnmts, EZH2, TET) or transcriptional effectors (VP64, p300, KRAB) to modulate the expression of genes of interest. Studies of dCas9 targeting platforms demonstrated promising results in the suppression of cell proliferation and tumor growth in different types of cancers both *in vivo* and *in vitro* following epigenetic targeting. Hypermethylation is found to contribute to carcinogenesis through the silencing of tumor suppressor genes [137], leading gaining malignancy hallmarks. For example, the inactivation of SMARCA2, a key component of the SWI/SNF complex with vital roles in chromatin remodeling gene regulation by promoter hypermethylation, leads to loss of control over cell growth and a significantly reduced survival rate in lung cancer patients. This was experimentally verified by hypermethylation-mediated downregulation of SMARCA2 by targeting the promoter CpG with the dCas9-DNMT3A fusion protein [138]. Thus, the role of epigenome in diseases can be unravelled by CRISPR-mediated modeling of epigenetic aberrations. Several studies have demonstrated the applications of dCas9 fusions with epigenetic effectors that act on DNA methylation marks to target tumor-suppressor genes and oncogenes to modulate their functions for tumor growth inhibition [96,139,140]. For example, *BRCA1*, a tumor-suppressor gene silenced by DNA methylation, was demethylated at the promoter by dCas9–TET1 fusion, leading to rescued expression of *BRCA1* and inhibition of cell proliferation in a cancer cell line [54]. Besides epigenetic effectors, dCas9 systems can be fused to transcription effector domains to target tumor-suppressor genes and oncogenes [141–144,146,147]. However, transcriptional modulators affect gene expression in a broad way, while epigenetic effectors target specific epigenetic changes. This specificity is a key feature of precision therapy, which aims to treat cancer by targeting the specific genetic changes that drive it [146]. Besides therapeutic applications, dCas9 platforms can be used to induce the expression of endogenous oncogenes to generate *in vitro* models for preclinical studies of cancer therapeutics [145,158].

Regenerative medicine

Acquiring the ability to reinstate the function of a lost/damaged organ has been the target of stem cell biologists. The seminal work of Takahashi and Yamanaka identified unique factors that can reprogram differentiated cells to a pluripotent state [159]. These cells, termed induced pluripotent stem cells (iPSCs), are valuable tools for both basic research and clinical applications [148,149]. In basic research, iPSCs can be used to model and study the development of various diseases, which can help us to better understand these processes and screen and develop new drugs. In clinical settings, iPSCs have been used to generate patient-specific cell types for transplantation [150]. Multiple versions of CRISPR have been applied for endogenous gene manipulation to achieve reprogramming [151–153,160]. A dCas9-synergistic activation mediator (SAM)-based screen not only successfully reprogram primed murine epiblast stem cells to a pluripotent embryonic stem cell state but also identified novel molecules implicated in reprogramming that were previously unknown regulators of pluripotency [161].

Cellular reprogramming, differentiation and transdifferentiation of cells into a required cell type are complex processes that involve the coordinated expression of many genes. By activating or inactivating the required genes, it is possible to direct cells for guided differentiation or transdifferentiation. The ability to activate multiple genes simultaneously is a challenging task but essential for cellular reprogramming. Robustness of CRISPR-dCas9 has been used to achieve this task. For instance, the dCas9-VP64 activator and dCas9-SAM and dCas9-SunTag systems have been successfully used to simultaneously activate several endogenous genes in human PSCs and mesenchymal stem cells with robustness and specificity [154]. Importantly, the systems were effective for single and multiplexed gene activation. Direct reprogramming of mouse fibroblasts to neuronal cells was achieved *via* activation of neurogenic factors Brn2, Ascl1 and Myt1l (BAM factors) genes by a dCas9-VP64 activation system. This study also suggests that targeting endogenous genes is a more effective approach to achieve sustained cell reprogramming compared with forced expression of transgenes [162]. DNA methylation modulation by dCas9–TET1 fusions showed potency in ameliorating repressive hypermethylation of the causative CGG expansion mutation present in the *FMR1* gene in iPSCs derived from fragile X syndrome patients. These adjustments in DNA methylation patterns were shown to be stable and restore the functionality of the iPSC-derived neurons [160]. All these studies demonstrate the power of using CRISPR-mediated epigenome editing in understanding molecular mechanisms that mediate complex cellular processes in regenerative medicine.

Limitations of current methods

Although CRISPR systems show high potential as a therapeutic epigenome-editing approach, many challenges persist and make it challenging to implement the technology in clinical trials. First, CRISPR systems are generally

prone to off-target effects, which can be a result of using suboptimal gRNA molecules or might be caused by the expression levels of effector domains or dCas proteins, as with increasing expression levels the off-target effects also increase. This is besides the influence of their intrinsic binding specificity and exposure time (time span of expression in the cell) and can be circumvented by using tunable dCas fusions and inducible systems for better control of these parameters. Second, persistence of the therapeutic effects poses another challenge, as epigenetic marks in growing cells may fade away during successive cell divisions by dilution. Therefore, it is important to carry out epigenetic editing in a way that mimics the endurance of natural epigenomic stability. This can be potentially done by creating systems that can actively induce endogenous maintenance mechanisms, to ensure 'self-sustainability' of the process. Last, one of the major challenges that limits the application of epigenome editing systems is delivery to target cells. The ideal vehicle is a system that is safe, specific and stable, and with high loading capacity. Although lentiviral systems are most commonly used in clinical settings, the issues of immunogenicity, limited loading capacity and genomic integrations remain major concerns. Recently, delivery systems have been developed that utilize nanoparticles as carriers for CRISPR systems. Nanocarrier systems are an attractive approach as they exhibit high loading capacity and stability. However, more studies are required to characterize and improve their safety [163–165].

Conclusion

CRISPR based epigenome editing has rapidly evolved in relatively short period of time. The development of nuclease-null mutant, dCas9, has led to expansion of additional tools that have been instrumental in our understanding of epigenetic mechanisms in gene regulation. Due to these versatile tools and ease of targeting, this system has become the method of choice for epigenetic research. In addition to deciphering the role of various DNA elements, the CRISPR system has also been harnessed to modify RNA molecules. These tools have been successfully applied both in basic and clinical research. It is anticipated that the methodology will continue to evolve in future, and with the development of efficient tools for safe and targeted delivery, epigenome editing will have broader clinical applications in cancer therapeutics, regenerative medicine and other diseases.

Future perspective

It has been just 10 years after the first use of CRISPR in eukaryotic system, and the developments in the field have been astounding. Two developments have been progressing in parallel; the identification of new classes of the CRISPR system in widespread organisms, and remodeling of existing classes for diverse uses. Soon after its use in eukaryotes, CRISPR-Cas9 was repurposed for epigenome editing and a number of tools have been developed for this purpose. Epigenome editing has widespread applications ranging from basic biological research to clinical applications. One of the fundamental questions epigenome editing addressed is that it delineated the causal relationships between epigenetic marks and gene expression. The systems developed have the potential to identify novel therapeutic targets. RNA-targeting CRISPR-based platforms have been developed for use in transcriptome engineering and diagnostics.

Despite all these strides, significant work is needed to improve the specificity of the developed tools. The collateral effect of Cas13, for instance, is a major challenge that has to be addressed, which is being done with the identification of more CRISPR platforms harboring RNA-targeting activity. Another major concern is the utility of these developed tools *in vivo*. For the *in vivo* expression of dCas9 fusion proteins in transgenic mice, two conditional transgenic mouse lines were recently developed: Rosa26:LSL-dCas9-p300 for gene activation and Rosa26:LSL-dCas9-KRAB for gene repression [166]. Adeno-associated viruses are the most commonly used vector systems for the *in vivo* delivery of CRISPR-mediated editing systems [161,167,168] that have been used for disease modeling and treatment of various conditions. Cell-penetrating peptides [169], gold nanoparticles [170], lipid nanoparticles [171] and microinjections [172] are other available methods that have been used for *in vivo* disease models. However, further development of safe and effective methods to deliver epigenome-editing components still presents a major goal for *in vivo* genome-editing therapy. In addition, reducing off-targets and ensuring efficient delivery have to be addressed before making a transition from bench to clinic.

While these limitations currently exist, the efforts to identify novel and efficient genome-editing tools continue. In an effort to identify a eukaryotic counterpart of CRISPR, the Zhang laboratory recently identified the 'Fanzor' protein, which is a eukaryotic programmable RNA-guided endonuclease that makes more precise changes in human cells [173]. This not only complements the existing tools, but also points to the possibility of even more

diverse genome-editing tools in nature. The identification of new genome-editing tools and their modified versions for epigenome editing hold great promise for future research and therapeutics.

Executive summary

- The CRISPR system is a breakthrough gene-editing technology that typically employs a guide RNA to target a specific DNA region.
- Six different forms of CRISPR-Cas (I–VI) can be distinguished, and each type includes a unique Cas nuclease protein. Based on the makeup of the Cas effectors and CRISPR RNA ribonucleoprotein complexes, the six distinct types have been further classified into two classes.
- Recently, CRISPR-Cas9 technology was modified for epigenome editing by creating a 'dead' Cas9 protein (dCas9) or 'nuclease-null', which enables us to take advantage of its DNA-binding capabilities to target specific loci for epigenetic alterations without cleavage.

Mechanisms of epigenome targeting by CRISPR systems

- A significant epigenetic constrictive chemical change that affects gene expression is DNA methylation. To make methylation more effective and specific, dCas9-SunTag and DNMT3A single-chain antibody fusion (GCN4-D3A) constructs can be integrated.
- Another mechanism by which CRISPR can be used for gene editing is through histone modifications. Histone proteins can be altered post-translationally in a variety of ways, including acetylation, methylation, phosphorylation, ubiquitination, and so on.
- The development of these epigenome editing technologies has made it possible to make targeted chemical modifications to control cellular behaviors.
- Several noncoding RNAs have been investigated for their role in epigenetic modification, one important one being long noncoding RNAsn (lncRNAs). lncRNAs play a variety of roles in gene regulation and cellular function, as well as a significant role in cellular processes and diseases in humans. CRISPR-based systems have demonstrated effectiveness in lncRNA genetic manipulation, proving to be a flexible and useful tool for lncRNA investigations.
- CRISPR systems that have been developed to target RNA include the Cas13 effector systems and Cas7–11 RNA systems. These systems allow for RNA editing and transcriptome modification even without the induction of RNA cleavage.

Regulatory regions

- Using CRISPR-based regulatory area annotation screens, we were able to identify and decipher the roles of noncoding genomic regions.
- dCas9 fusion proteins can be used in conjunction with other screening techniques to study both loss- and gain-of-function effects on gene promoters.
- The nature of chromatin looping has been defined using CRISPR-based epigenome regulation. The use of 3D chromatin manipulation systems such as the CLOuD9 and light-activated dynamic looping systems is pivotal for understanding the role of aberrant chromatin looping in human illnesses.

Applications of CRISPR epigenome editing

- Oncogenes and tumor suppressor genes can undergo epimutation, which can be targeted by dCas9 fusion proteins. The normal expression of tumor suppressors may be rescued.
- Cells can be directed for pluripotency using a dCas9-synergistic activation mediator-based screen.
- The CRISPR-dCas9 system has been utilized to simultaneously activate many genes that have important implications in regenerative medicine.
- Numerous fields, including fundamental biological research and therapeutic applications, use CRISPR-based epigenome editing.
- Further work on CRISPR-based system for epigenome editing holds great significance for the future.

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