A CRISPR–dCas Toolbox for Genetic Engineering and Synthetic Biology

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Abstract

Programmable control of gene expression is essential to understanding gene function, engineering cellular behaviors, and developing therapeutics. Beyond the gene editing applications enabled by the nuclease CRISPR–Cas9 and CRISPR–Cas12a, the invention of the nuclease-dead Cas molecules (dCas9 and dCas12a) offers a platform for the precise control of genome function without gene editing. Diverse dCas tools have been developed, which constitute a comprehensive toolbox that allows for interrogation of gene function and modulation of the cellular behaviors. This review summarizes current applications of the dCas tools for transcription regulation, epigenetic engineering, genome imaging, genetic screens, and chromatin immunoprecipitation. We also highlight the advantages and existing challenges of the current dCas tools in genetic engineering and synthetic biology, and provide perspectives on future directions and applications.

Introduction

Tools that enable programmable control of gene expression are valuable for interrogation of genomic function and implementation of synthetic biological systems. As an interdisciplinary field, synthetic biology is becoming a universal "forward engineering" approach to study how complex cellular behaviors emerge from simple biological parts [1]. While earlier synthetic biology works focused on microbial (bacteria or yeast) engineering [2, 3], recent works aim to expand synthetic biology to understand and engineer mammalian systems. Tools that can very efficiently and precisely control gene expression in the mammalian genome are thus needed.

Proteins including zinc fingers [4, 5] and transcription activator-like effectors (TALEs) [6, 7] are the first gene editing molecules. However, these molecules function through DNA–protein interactions, requiring laborious engineering of different proteins for different genomic sequences. Nevertheless, development of zinc finger or TALE tools prepared researchers for the age of gene editing. It is thus not surprising how the CRISPR (clustered regularly interspaced short palindromic repeats) endonuclease system was rapidly developed as a powerful gene editing tool [8–14].

Elucidation of the mechanism of CRISPR–Cas systems enabled tool development. The CRISPR systems are adaptive genetic systems for counteracting foreign DNA or RNAs that are present in 40% of bacteria and almost all archaea [15]. The CRISPR–Cas systems have been categorized into two classes and six major types based on the configuration of Cas protein effectors [16, 17]. Of particular interest to gene editing, the Class 2 CRISPR systems employ a single endonuclease protein, Cas9 (Type II) or Cas12a (previously known as Cpf1, type V), and a single (Type V-A) or dual crRNAs (most other Class II systems), for RNA-guided cleavage of DNA. The high efficacy (needs to eliminate invading DNAs quickly), high specificity (needs to distinguish self from foreign DNAs), and the RNA-guided DNA recognition features made these systems superior

choices for gene editing applications in diverse organisms [9, 13–15, 18–20].

Both Cas9 and Cas12a endonucleases induce double-stranded breaks at the target DNA loci by engineering a base-pairing sgRNA [15]. Double-stranded breaks trigger host DNA repair pathways via non-homologous end joining (NHEJ), microhomology-mediated end joining (MMEJ), homology-directed repair (HDR), or a combination of these pathways. DNA repair is the process that leads to gene deletion, insertion or modification [21, 22]. Details of using CRISPR–Cas systems for gene editing and DNA repair pathways can be found in previous reviews [17].

Beyond gene editing, CRISPR–Cas systems have been repurposed for programmable, targeted gene regulation. We and others have developed the nuclease-dead Cas9 (dCas9) [23] molecule that enabled transcription regulation, epigenetic modifications, DNA looping, genome imaging, among other applications. The ability to use dCas9 or dCas12a [24] based molecules to control gene expression offers a broad class of "gene switches," which can be used for studying the casual relationship of genes and function, characterizing the function of noncoding genes and regulatory elements of the genome, and also offers a powerful strategy to design synthetic gene circuits. Thus, the dCas tools constitute a toolbox to study biology and engineer biology, an approach that we termed "discovery-based synthetic biology."

We aim to provide an overview on the up-to-date CRISPR–dCas platform with applications in mammalian systems. In particular, We focus on two aspects (Fig. 1): (1) bioengineering strategies to repurpose the dCas system for diverse uses including transcriptional or epigenome engineering, or genomic imaging, and (2) discovery strategies using dCas tools including genetic screening and chromatin immunoprecipitation. We also summarize the advantages, challenges, and future directions of the dCas platform at the end.

Programmable Gene Regulation with dCas Systems

The First Generation of dCas Fusion Proteins as CRISPR interference and CRISPR activation tools

The Central Dogma of molecular biology describes the universal process of genetic information flow in cells: information flows from DNA to RNA to protein. Synthetic control of the Central Dogma is a useful approach to understand the gene expression process and implement engineered biological systems. Indeed, synthetic control of transcription using engineered promoters driving exogenous genes has allowed for interrogation of gene function [25]. However, new tools for the programmable and quantitative control of endogenous gene expression are still needed.

The invention of dCas molecules meets such demands. As an RNA-guided DNA binding platform, dCas9 was first repurposed to regulate transcription. Despite Cas9 being a large, multi-domain protein, early work showed silencing the two endonuclease domains (the HNH domain that cleaves the target

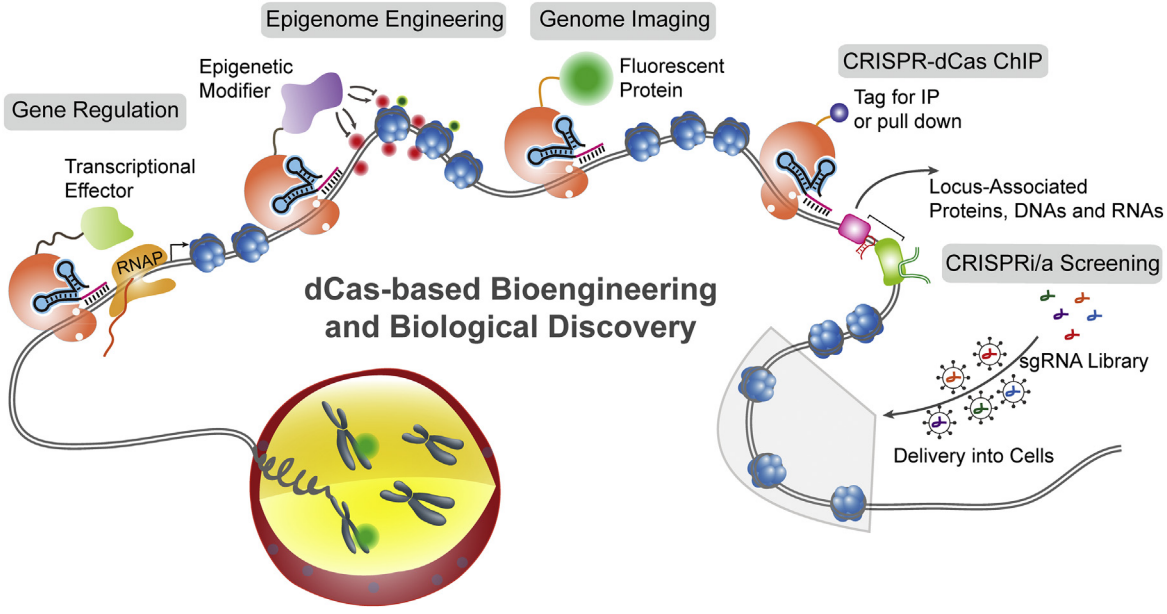

Fig. 1. CRISPR–dCas9-based bioengineering and discoveries. The applications of CRISPR–dCas9 technologies in developing bioengineering toolkits and discovering genetic elements and interactions.

DNA strand, and the RuvC domain that cleaves the non-target DNA strand) in Cas9 via point mutations resulted in a nuclease-dead dCas9 that could still bind to DNA [13, 23] (Fig. 2a). Our early discoveries demonstrated that dCas9 could repress transcription of the target genes by sterically hindering the RNA polymerase activity on the promoter or RNA polymerase processivity along the coding sequence [23] (Fig. 2b). This use of dCas9 to repress gene expression was termed CRISPR interference (CRISPRi) (Fig. 2b) [23].

As most prokaryotic organisms lack the RNA interference pathway, CRISPRi offers an easy-to-use approach for targeted gene silencing in prokaryotes [26, 27]. In addition, our later work showed partial, quantitative repression of target genes by CRISPRi further allowed interrogation of essential genes for cell survival, morphology, and antibiotic

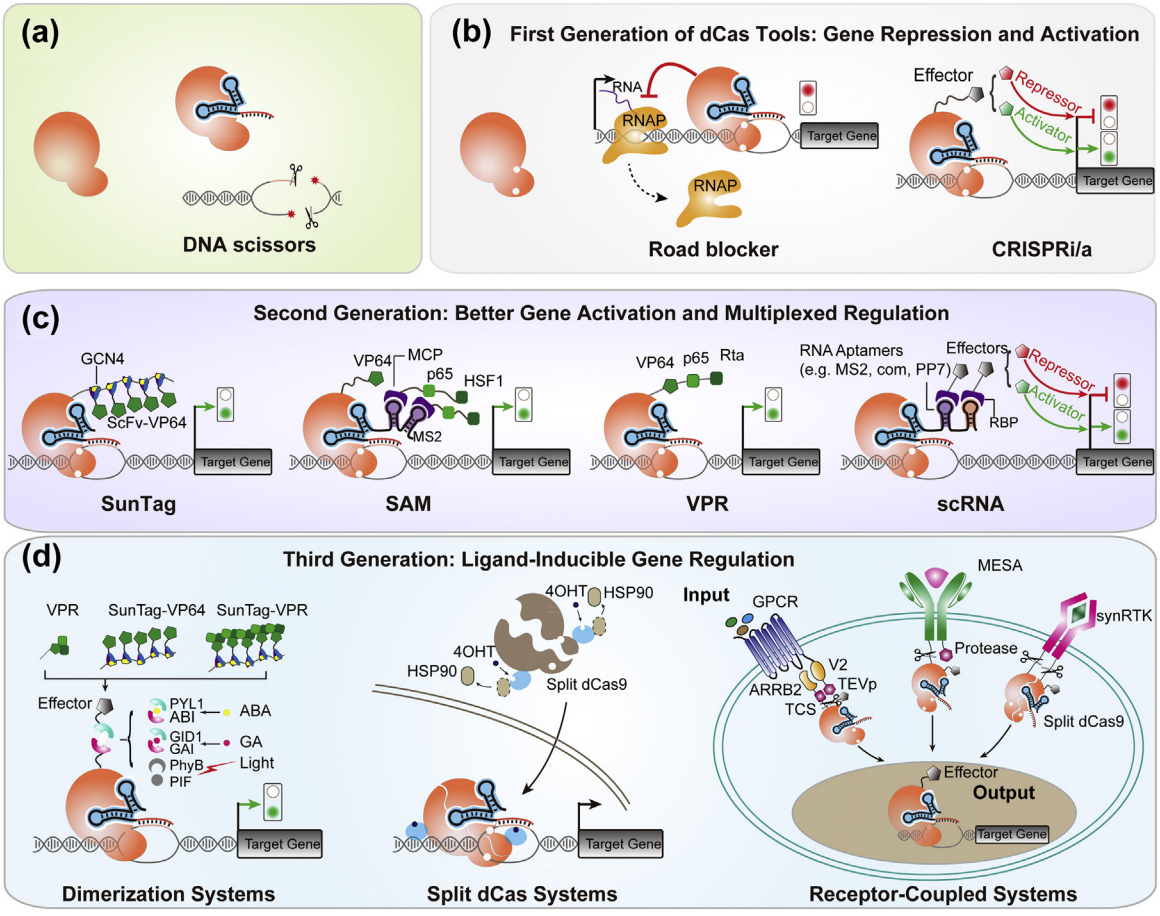

Fig. 2. Engineered CRISPR systems for gene manipulation. (a) CRISPR–Cas9-based gene editing via double strand breaking. (b) The first generation of dCas tools. RNAP: RNA polymerase. Road Blocker: dCas9 can sterically block the transcriptional elongation of RNAP. CRISPRi/a: a transcriptional activator or repressor can be coupled with dCas9 to achieve gene activation or repression. (c) The second generation of dCas tools. SunTag: activating gene expression. The tandem repeats of a small peptide GCN4 are utilized to recruit multiple copies of scFv in fusion with the transcriptional activator VP64. scFv: single-chain variable fragment. SAM: a strategy for gene activation. The dCas9 is fused to VP64 and the sgRNA has been modified to contain two MS2 RNA aptamers to recruit the MS2 bacteriophage coat protein (MCP), which was fused to the transcriptional activators p65 and heat shock factor 1 (HSF1). VPR: a strategy for gene activation. The dCas9 has been fused to the combinatory transcriptional activator VP64–p65–Rta (VPR) to amplify the activation effects. scRNA: a strategy for simultaneous gene activation and repression. A hybrid RNA scaffold coupling an sgRNA and an RNA aptamer (e.g., MS2, com, PP7) can recruit RBPs (e.g., MCP, COM, PCP) tethered to either a transcriptional activator or repressor. (d) The third generation of of dCas tools. Dimerization systems: chemical and light controlled CRISPR–dCas9 systems for inducible gene regulation. Chemical or light induced dimerizers (e.g., PYL1::ABI, GID::GAI and PhyB::PIF) have been fused to dCas9 and transcriptional effectors, respectively. The addition of corresponding chemical (e.g., abscisic acid [ABA], or gibberellin [GA]) or light can induce the gene regulations. Split dCas systems: an alternative way of controlling strategy split dCas9 for gene regulation. Receptor-Coupled systems: I/O molecular devices for gene regulations. GPCR, MESA, and synRTK systems have been introduced for dCas-based gene regulations.

resistance in bacteria [27]. Unlike traditional gene knockout strategies, CRISPRi approaches theoretically offer a route to study how quantitative gene expression (genotype) affects cellular function (phenotype). While being very efficient in bacteria, gene repression using dCas9 alone in mammalian cells is often not very efficient, likely because the eukaryotic RNA polymerase II is harder to sterically hinder [23].

Significant efforts have been devoted to improving the efficiency of targeted gene regulation in eukaryotic cells. Various transcriptional effector domains were fused to dCas9 to test if any of the effects could lead to better repression or activation in mammalian cells [28–30]. Repressive effector domains, including the KRAB (Krüppel associated box) domain of Kox1, the WRPW domain of Hes1, the CS (Chromo Shadow) domain of HP1α, and the SID4X domain, were fused to dCas9 [28, 29, 31]. Among these domains, the dCas9–KRAB fusion exhibited relatively consistent and robust effects in targeted gene repression. For activation, activating effector domains including the transactivator domain of the Herpes simplex viral protein 16 (VP16), multiple copies of VP16 (VP64 or VP160), or the transactivator domain of nuclear factor kappa B (p65) were fused to dCas9 [29, 32, 33]. Early work demonstrated decent but not great activation effects using VP64, which was termed CRISPR activation (CRISPRa) (Fig. 2b). These simple fusion molecular tools together constituted the first generation of programmable dCas gene regulation toolbox.

The Second Generation of More Effective dCas Tools via Complex Protein and sgRNA Engineering

Many strategies have been devised to improve the efficiency of dCas, with a particular focus on CRISPRa. It was observed that tiling multiple sgRNAs along the promoter allowed for better gene activation [32, 34]. This observation inspired a method that fused multiple copies of the transactivator domains to dCas9 to amplify the activation signal. Tanenbaum *et al*. [35] developed a dCas9-SunTag system (Fig. 2c). Wherein the dCas9 is fused to a multimeric peptide array (SunTag) that each peptide binds to one single-chain variable fragment (scFv) that is fused to VP64. In contrast to the SunTag system that recruits multiple copies of the same transactivator, the synergistic activation mediator (SAM) system combines dCas9–VP64 with a modified sgRNA containing two MS2 hairpins: each MS2 hairpin interacts with one MS2 binding protein (MCP) that is fused to the transactivator domain of p65 and the human heat shock factor 1 (HSF1) [30] (Fig. 2c). In the third system named dCas9–VPR, dCas9 is fused to a screened synergistic tripartite activator that consists of VP64, p65AD and Epstein–Barr virus R transactivator (Rta) [36] (Fig. 2c). More variants were designed later, including combining the SAM with SunTag, or using the SunTag system to recruit p65AD-HSF1 instead of VP64 (SPH) [37]. It remains a question how these systems perform compared with SunTag, SAM, or VPR, which needs side-by-side comparison and evaluation for different genes and in diverse cell types.

In addition to improving efficiency, dCas9 was also used for multi-gene regulation [38]. One design used scaffold RNA (scRNA), which was created by fusing the sgRNA to orthogonally acting protein-binding RNA aptamers that recruit cognate RNA-binding proteins (RBPs) to form RNA aptamer–RBP pairs (e.g., MS2–MCP, com–Com, PP7–PCP) (Fig. 2c). Although the scRNA and SAM systems share a similar concept that RNA aptamers were added to the sgRNA to link functional effector domains to the targeting genome site, the scRNA strategy expanded the scope of gene regulation by expressing RNA scaffolds containing different aptamers to permit simultaneous activation and repression of genes in the same cells.

These second-generation systems usually can more efficiently activate endogenous genes using a single sgRNA compared to dCas9–VP64 [37, 39]. Table 1 summarizes these examples of CRISPRi and CRISPRa systems. A direct comparative analysis demonstrates that SunTag, SAM, VPR, or SPH can activate a range of genes in a variety of cellular contexts, including human, mouse, and *Drosophila* cells [39].

We note that the performance of each system varies from gene to gene and from cell type to cell type. Attempts to combine SunTag, SAM, and VPR to further improve the efficiency were not very successful [39]: these combinatorial systems showed either similar or weaker gene activation. This likely suggests that recruiting many copies of the same or synergistic transactivators is only part of the mechanisms to achieve efficient gene activation. In natural systems, recruitment of transactivator domains often coordinates with Mediators, epigenetic modifying complexes, and local promoter-enhancer structure [e.g., DNA looping or three-dimensional (3D) genome organization]. Compared to natural systems, all current systems are based on the simple concept of recruiting multiple domains. Future work should explore other strategies for transcriptional regulation. This should not only elucidate novel gene regulatory mechanisms but also lead to further improvement of repression or activation efficiency.

The Third Generation of Synthetic dCas Input/Output Molecular Devices

Ligand-inducible control of gene expression is a powerful approach to understand temporal and spatial expression patterns, and engineer designer

Table 1. Examples of CRISPR–dCas9-based gene regulation in mammalian cells

Rep/Act	Cell type	Promoter–reporter	sgRNA	Fold change	Effector domains	dCas9 fusion	Imput (inducer)	Refs.
Rep[a]	HEK293	SV40–EGFP	1/locus	2-fold	–	–	–	[23]
Rep[a]	HEK293	SV40–EGFP	1/locus	15-fold	KRAB	BFP–KRAB	–	[29]
Act[a]	HEK293	GAL4 UAS–CMV–GFP	1/locus 3 targetable sites	25-fold	VP64	VP64–BFP	–	[29]
Act[a]	HEK293	GAL4 UAS–CMV–GFP	1/locus 3 targetable sites	12-fold	P65AD	P65AD–BFP	–	[29]
Act[a]	HEK293T	Endogenous IL1RN	4/locus	1632-fold	VP64	VP64	–	[33]
Act[b]	K562	Endogenous CXCR4	1/locus	50-fold	Multi-VP64	SunTag10x_v4	–	[35]
Act[b]	HEK293FT	Endogenous IL1B	Modified sgRNA 1/locus	(10,000–15,000)-fold	VP64&MS2–p65–HSF1	VP64	–	[30]
Act[b]	HEK293T	Endogenous TTN	3–4/locus	20,000-fold	VPR	VPR	–	[36]
Act[b]	HEK293T	Endogenous CXCR4	Modified sgRNA 1/locus	5-fold	2 × MS2–MCP–VP64	None	–	[38]
Act[b]	HEK293T	tetO–EGFP	Modified sgRNA 1/locus 7 targetable sites	(100–150)-fold	2 × MS2–MCP–VP64	None	–	[38]
Act[c]	HEK293T	pTRE3G–EGFP	1/locus	165-fold	VPR–PYL1	ABI	ABA	[40]
Act[c]	HEK293T	pTRE3G–EGFP	1/locus	94-fold	VPR–GIDI	GAI	GA	[40]
Act[c]	HEK293T	Endogenous CXCR4	3/locus	34-fold	VPR–PYL1	ABI (SadCas9)	ABA	[40]
Act[c]	HEK293FT	Endogenous ASCL1	4/locus	57-fold	VP64	dCas9(N)–FRB–2 × NES; dcas9(C)–FKBP–2 × NLS–VP64	Rapamycin	[41]
Act[c]	HEK293T	Endogenous ASCL1	1/locus	48.3-fold	SAM–VP64	VEGFR2: TEV(N)–NES–TCS–dCas9(N); VEGFR1: TEV(C)–NLS–TCS–dCas9(C)VP64	VEGFA121	[42]
Act[c]	HEK293T	Endogenous IFNG	1/locus	20-fold	VPR	ARRB2–TCS–dCas9–VPR–mCherry	Neuromedin B	[43]
Act[c]	HEK293FT	Endogenous IL-2	1/locus	5.2	TF	VEGF–MESA–dCas9–TF	VEGF	[44]
Act[c]	Jurkat	Endogenous IL-2	1/locus	4.7	TF	VEGF–MESA–dCas9–TF	VEGF	[44]

Some examples of the three generations of CRISPRi/a were shown.
The fold change in the third generation of CRISPRi/a indicates the change after and before the addition of inducer.
Act, Activation; Rep, repression.
[a] The first generation of dCas9-based gene regulation.
[b] The second generation of dCas9-based gene regulation.
[c] The third generation of dCas9-based gene regulation.

cells that can sense input signals and generate functional outputs. Coupling the dCas activity via external perturbation (human added external ligands) or autonomous methods (cells detecting native cellular or microenvironmental signals) has been a goal for developing more powerful CRISPRi/a tools to enable spatial and temporal control of gene function.

Two main strategies to create input/output (I/O) dCas9 molecular devices have been reported: (1) coupling dCas9 or split-dCas9 to chemical or optogenetic sensing domains, and (2) coupling dCas9 to ligand-sensing receptor domains. Both strategies aim to transduce input signals into programmable transcription responses via dCas9.

To sense synthetic inputs, chemical-induced dimerizing domains (CIDs) or optogenetically inducible dimerizing domains (OIDs) have been fused to dCas9 and its respective regulator domain (the repressive or activating domain), which are expressed as two separate proteins. The presence of ligands (chemical or light with a specific wavelength) can induce dimerization of CIDs or OIDs that recruit the effector domain to the dCas9 binding site on the genome. Various CIDs or OIDs have been combined with the dCas9 platform, including abscisic acid (ABA)-inducible ABI–PYL1 [40, 45, 46], gibberellin (GA)-inducible GID1–GAI24 [40], rapamycin-inducible FKBP–FRB [41, 45], blue light-inducible CRY2-CIB1, Magnet pMag–nMag [47–50], and phytochrome-based red light-inducible PhyB–PIF [51] (Fig. 2d). In addition to dCas9, dCas12a was also fused to the rapamycin analog A/C herterodimerizer domains [52].

In addition, an alternative way to accomplish ligand-inducible control of dCas9 is via engineering split dCas9 molecules. For example, a split dCas9

was fused with the ligand-binding domain estrogen receptor (ERT), which interacts with the cytosolic chaperone Hsp90 [53] (Fig. 2d). While the split dCas9 protein is sequestered in the cytoplasm, addition of the ligand 4-hydroxytamoxifen disrupts the interaction between Hsp90 and ERT, which promotes nuclear translocation of dCas9 for gene regulation. The split dCas9 system can provide an AND gate for the dimerization systems to control gene expression. These systems often allow for spatial and temporal control of gene expression, as well as ligand dose-dependent gene regulation [54].

To sense native cellular or microenvironmental inputs, cellular receptors have been coupled to the dCas system. Mammalian cells have evolved a diverse class of transmembrane receptors that can sense and respond to physiologically important ligands, including small molecules, metabolites, hormones, antigens, and other. These ligands interact with their cognate receptor molecules on the cell surface and binding of ligands to cognate receptors induces conformational changes in the receptors which triggers signaling in cells. To engineer synthetic devices that can sense these ligands as input to control gene expression as output, chimeric molecular devices were designed. These chimeric devices are often composed of a ligand-sensing extracellular receptor domain (input) and an intracellular gene regulatory domain (output). Ideally, the two domains are modular, allowing researchers to flexibly alter the specificity of the input and the targets of the output.

There are several types of chimeric receptors that have been reported before coupling to dCas. For example, chimeric antigen receptors (CARs) are a type of receptors whose customized input domains (antigen-binding scFvs) can trigger T cell activation [55]. Notably, the use of CAR-T cells as an immunotherapy has recently been clinically approved for B-cell acute lymphoblastic leukemia [56]. The extracellular domain of Notch can be replaced with a scFv, and the intracellular domain can be replaced with a synthetic transcriptional factor (TF), which resulted in synthetic Notch (synNotch) receptors. synNotch allows cells to detect a spectrum of customized signals and in response to control on custom genes [57]. G protein-coupled receptors (GPCRs) were also engineered. Termed the Tango system, the intracellular domain of a GPCR molecule was replaced with a proteolytically cleavable synthetic TF (e.g., tTA), and the adaptor protein Beta Arrestin (ARRB2) was fused to a specific protease (e.g., tobacco etch virus, or TEV) [58]. ARRB2 conditionally interacts with GPCR upon binding to ligand, which releases the synthetic TF into the nucleus for activation of gene expression. This system has been used to assay GPCR activity by conditionally driving reporter gene expression in response to certain ligands.

Coupling dCas9 to these receptors theoretically allows for control of endogenous genes by diverse cellular inputs [42–44]. A system termed modular extracellular sensor architecture (MESA) was designed by fusing dCas9 to the dimerizing target chain via a cleavable linker and fusing TEV protease to the other protease chain [44, 59] (Fig. 2d). Another system fused a receptor tyrosine kinase (RTK) to the split dCas9 architecture to detect VEGF, which displayed high output induction and no significant ligand-independent activity [42]. The split dCas9-based transcription factors were also coupled to GPCRs using the Tango design. Besides dCas9, dCas12a from *Acidaminococcus* sp. BV3L6 was used to construct the I/O device following the Tango design [24].

The reported ChaCha system expanded the previous Tango GPCR system (Fig. 2d) [43]. In this system, the Tango design is reversed, replacing the intracellular domain of the GPCR with the TEV protease and fusing the dCas9 transcriptional regulators to the adaptor protein Beta Arrestin (ARRB2) via a proteolytically cleavable linker. Theoretically, the system preserves the GPCR molecule after each adaptor recruiting and cleavage event, thus potentially amplifies the signal from a single ligand-receptor binding events. The ChaCha system is dose dependent and reversible, and can sense a broad category of signals, including chemokines, mitogens, synthetic compounds, fatty acids, and hormones [43].

Altogether, these I/O systems offer a promising toolkit to control endogenous genes with programmable inputs. Notably, most systems still displayed significant ligand-independent background with a moderate dynamic range of gene regulation. Further improvements of the efficiency, signal sensitivity, and reduction of the size of the devices are critical for broader applications.

dCas-Mediated Epigenome Engineering

The mammalian genome contains a broad spectrum of epigenetic chemical modifications on DNA or histone proteins [60], which have profound influence on chromatin organization and gene expression [61–63]. It has been challenging to understand the causal relationship between epigenetic modifications and gene expression at specific genomic loci due to a lack of tools. The dCas9 fused to epigenetic enzymatic domains has enabled locus-specific epigenetic modifications.

DNA methylation regulates many biological processes in mammalian cells [64] and is an important modification to target using epigenome engineering. In mammalian cells, the methyltransferases DNMT3A and DNMT3B catalyze methylation of unmethylated CpGs [65], with the co-factor

DNMT3L as an important stimulatory factor for DNMT3A [66]. The dCas9 molecule has been fused to the catalytic domain of DNA methyltransferase (DNMT3A), which allowed site-specific methylation of the CpG islands around the dCas9 target site and repression of nearby genes [67–69] (Fig. 3). A combination of dCas9 fusion proteins, in which KRAB, DNMT3A, and DNM3L domains were individually fused to dCas9, further allowed for long-term epigenetic silencing of the target endogenous genes [67]. To activate genes, the catalytic domain of the demethylase TET1 was fused to dCas9 to induce site-specific DNA demethylation, leading to upregulation of related genes [70–72] (Fig. 3). The dCas9–SunTag strategy was adapted for targeted DNA demethylation. Fusing TET1 to the scFv domain of the dCas9–SunTag system showed demethylation of CpGs and upregulation of targeted genes in embryonic stem cells, cancer cell lines, primary neural precursor cells, and *in vivo* in mouse fetuses [71].

Posttranslational modifications of histones encode important epigenomic features that are associated with the gene expression program. There are at least eight types of characterized histone modifications that are involved in transcription regulation, including acetylation, methylation (lysine and arginine), phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deamination, and proline isomerization [73]. dCas9 has been fused to different histone-modifying enzymes. For example, the catalytic core of the human acetyltransferase p300 was fused to dCas9, allowing for acetylation of histone H3 lysine27 (H3K27) and activation of target genes [74] (Fig. 3). The histone demethylase LSD1 was coupled to dCas9 (Fig. 3), which repressed the genes by decreasing the epigenetic modification level of H3K4Me2 and H3K27Ac around the targeted Tbx3

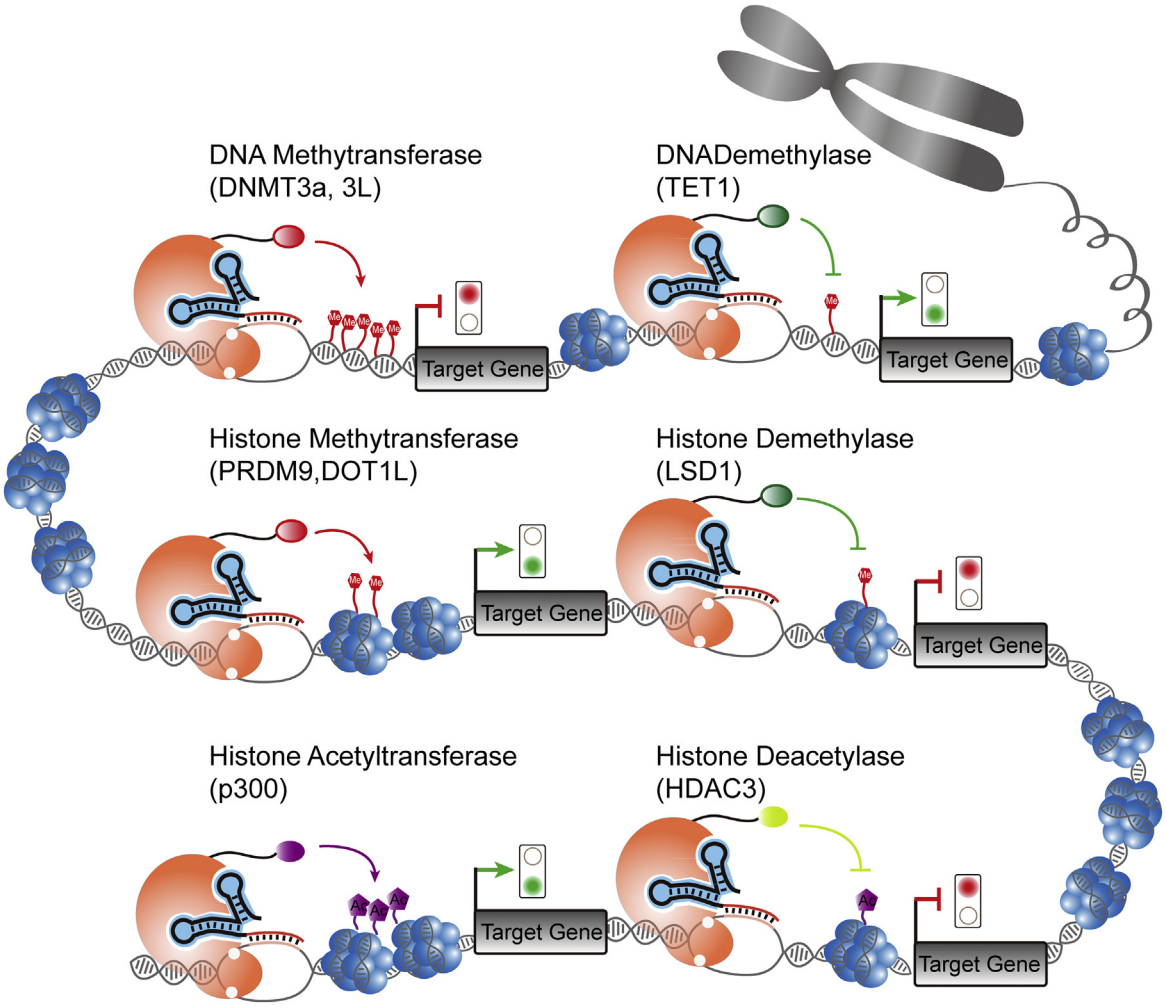

Fig. 3. CRISPR–dCas9-based epigenome engineering. The epigenetic landscape of DNA and histone can be programmed in a site-specific manner to control gene expression by coupling dCas9 with epigenetic modifying enzymes. The reported examples are displayed in the figure.

enhancer region [75]. Other histone modification dCas9 tools were also developed, including histone methyltransferases (DOT1L, SMYD3, and PRDM9) [76, 77], and histone deacetylases (HDACs) [78] (Fig. 3). For instance, dCas9–PRDM9 and dCas9–DOT1L can upregulate 2-oxoglutarate 5-dioxygenase 2 (*PLOD2*) expression moderately by inducing their intended histone mark around the TSS region (H3K4Me3 by dCas-PRDM9 and H3K79Me3 by dCas-DOT1L) [76]. In addition, strategies used for signal amplification or the I/O devices in the second and third generations of dCas9 gene regulators have also been applied in dCas9-based epigenome engineering [46, 79].

These studies opened a door for site-specific epigenetic engineering in mammalian cells, which not only alter the local epigenetic marks but also regulate the expression of related genes. Orthogonal dCas9s can be used to introduce different marks at different loci and to study the genetic interactions between these epigenetic modifications and relevant cellular phenotypes. However, the causal link between epigenetic modifications and the local and global gene regulation still remain to be elucidated using the tools. Furthermore, off-target epigenetic modifications caused by some epigenetic modifying domains need caution and further investigation [80].

dCas-Mediated Genome Imaging

The dCas9 system has been developed as a tool to visualize the genome in living cells. The 3D organization of chromosomes in the nucleus affects gene expression, recombination, and function [81, 82]. Various imaging tools have been developed to interrogate how the chromatin organization affects biological function. Most tools are based on nucleotide base-pairing interactions or protein–DNA interactions, or a combination of the two, including fluorescence in situ hybridization (FISH), a method using fluorescently labeled oligos to detect DNA sequence in “dead” fixed cells [83]. Imaging in living cells is more challenging. Methods using DNA binding domains (DBDs) fused with fluorescent proteins allow for labeling of specific genomic loci. Some genomic loci have naturally associated specific DBDs, such as centromeres and telomeres; thus, it is convenient to visualize them by fusing FPs to the native DBDs. However, most genomic sequences lack a specific binding protein. Therefore, programmable DBDs-based dCas9 enable genomic imaging at specific loci in cells. For a detailed comparison of available genomic imaging methods, the readers can refer to a previous review [17]. As dCas9s can be reprogrammed to target specific DNA sequences by changing the guide RNAs, this tool is promising to study the temporal and spatial behavior of the genome in living cells.

The rationale of using dCas9 for genome imaging is to target a fluorescently labeled dCas9 to a genomic locus of interest using sgRNAs. The dCas9 protein was fused to fluorescent proteins to visualize repetitive sequences using a single sgRNA, or to visualize non-repetitive genomic loci using multiple sgRNAs [84], which successfully detected centromeres, telomeres, and pericentric regions in living cells [85].

There have been several approaches to improve or expand dCas9-based imaging. To enhance the imaging sensitivity and reduce the number of required guide RNAs, several labs have tried to amplify dCas9 fluorescent signals. For example, the SunTag system can be used to amplify the dCas9 fluorescent signal [35]. In addition, the sgRNA scaffold was engineered to integrate with up to 16 MS2 binding domains for signal amplification to detect low- and non-repetitive genomic loci using relatively few sgRNAs [86]. To detect more loci, multiple repetitive genomic loci can be imaged simultaneously in living cells using dCas9 orthologs fused to different fluorescent proteins [87]. In addition, studies used scRNAs containing RNA aptamers to recruit different fluorescent protein-tagged effectors, enabling multicolor tracking of genomic loci [88–91]. A method that combines CRISPR imaging and DNA FISH was developed to track genomic loci dynamics using CRISPR and subsequently assign each loci to respective chromosomes in cells using FISH [92].

Binding of multiple sgRNAs and dCas9s to the chromatin may alter the local genome organization and dynamics. While it has been reported that the dCas9 fused with a single EGFP protein does not affect the dynamics of telomeres [84], recruiting multiple copies of fluorescent proteins using a complex structure (e.g., SunTag) might increase the probability of altering chromatin dynamics. Further efforts are needed to characterize such perturbations on the chromatin. Moreover, methods to enhance the sensitivity and specificity of dCas9 fusions for detecting any genomic loci are necessary for future studies.

New Discoveries Enabled by the CRISPR–dCas Platform

Synthetic regulation of gene expression offers a powerful approach to determine causal relationships between gene expression and phenotype. In addition to regulating individual gene expression and modifying epigenetic marks, the CRISPR–dCas9 platform provides a powerful tool for high-throughput genetic screens to probe gene function and genetic interactions. Combined with chromatin immunoprecipitation, dCas9 was also used to identify new genome-associated regulatory elements (DNAs, RNAs, or proteins) and their functions.

CRISPR–dCas for interrogating novel genetic elements and interactions

Genetic screens can be used to interrogate the function of multiple genes simultaneously in a high-throughput manner. CRISPRi or CRISPRa has been employed to perform large-scale loss-of-function or gain-of-function screens [28, 30]. These screens are often based on pooled approaches (Fig. 4a), in which mixtures of oligos are used to generate libraries of sgRNA expression vectors. These libraries are packaged into viral particles and then stably integrated into the genome of mammalian cells, with each cell receiving no more than one sgRNA. As sgRNAs are barcoded (or their DNA-target sequences are used as barcodes), their identities and relative enrichment can be easily confirmed via deep sequencing after phenotype selection.

With the help of CRISPRi/a, a large number of new genes and regulatory mechanisms have been identified for cell growth and drug resistance [28, 93–98]. One early demonstration of the CRISPRi/a screening studied cell growth and sensitivity to a cholera–diphtheria toxin in human myeloid leukemia K562 cells [28]. In another study, combined CRISPRi/a chemical–genetic screening identified the target and mechanism of cancer cell killing by rigosertib, an anti-cancer drug that is in clinical trials for high-risk myelodysplastic syndrome [94]. Since there are many elegant studies using CRISPR or CRISPRi/a screens, please refer to other reviews for detailed summary [99–101].

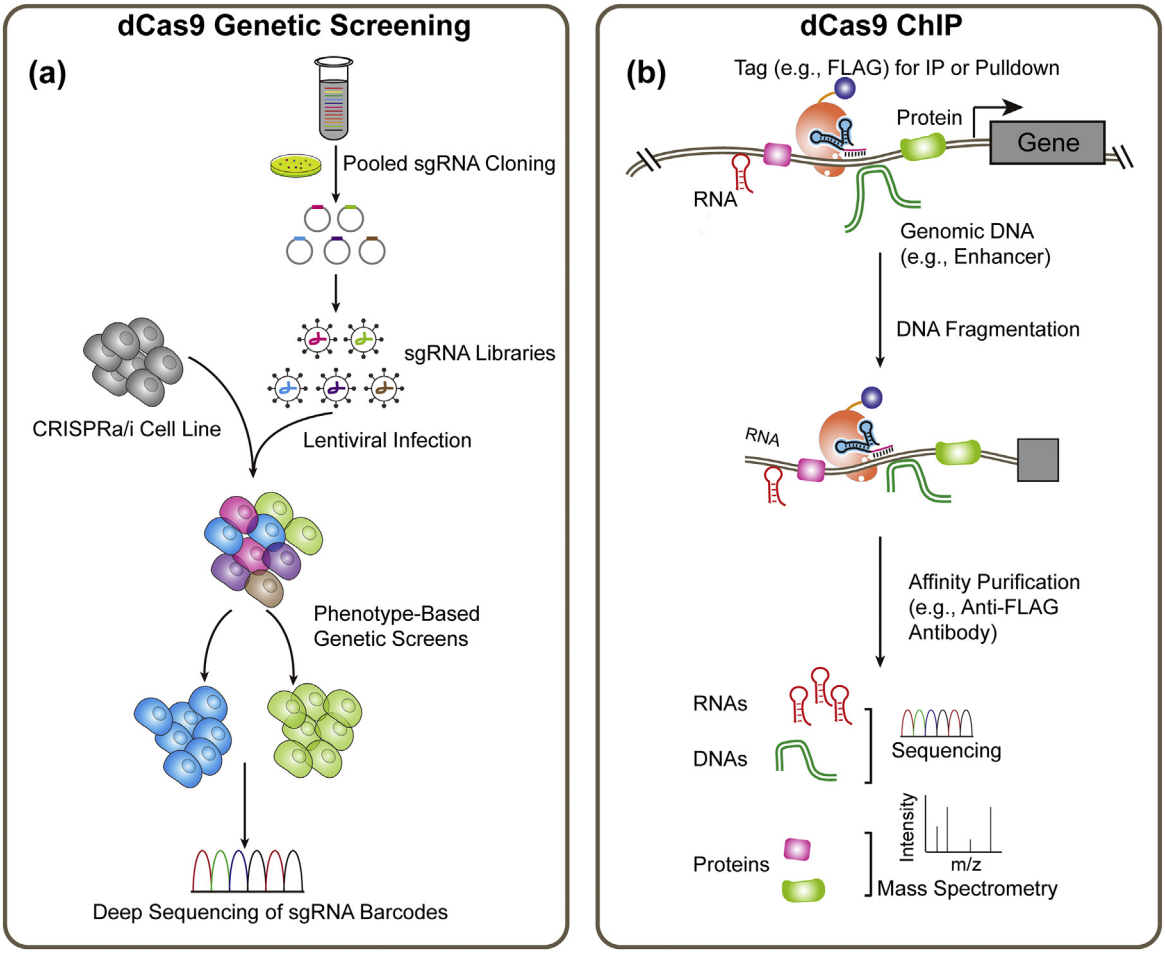

Fig. 4. New discoveries based on CRISPR–dCas9 platform. (a) CRISPR–dCas9-based screening for interrogating novel genetic elements and interactions. An overview of loss-of-function or gain-of-function screens mediated by CRISPRi or CRISRPa. The enrichment or depletion of sgRNA in the final population based on a specific phenotype can be assessed using deep sequencing. (b) CRISPR–dCas9-based chromatin immunoprecipitation (IP). The purification of a tagged dCas9 can capture site-specific complexes. The regulatory elements (DNA, RNA, and proteins) that immunoprecipitated with dCas9 can be characterized by sequencing or mass spectrometry.

In addition to identifying the function of individual genes, CRISPRi/a have been used to study genetic interactions. When two genes are simultaneously perturbed, a cell may exhibit a phenotype that is different from the case when individual gene is perturbed. Studying combinatorial phenotypes thus can allow us to understand how genes interact with each other [102]. Pooled genetic screens based on CRISPRi, where pairs of sgRNAs are delivered simultaneously, allow for the discovery of genetic interactions at a large scale [103]. Using two sgRNAs may cause some problems, such as the recombination between homologous sequences on the sgRNA vectors and the competition for dCas9 protein between the distinct sgRNAs. A recent report used orthogonal Cas9 proteins from *Streptococcus pyogenes* and *Staphylococcus aureus* to perturb gene pairs in dual screen, which can solve these problems [104]. In addition, they also used two different genomic engineering strategies, in which one gene was activated and another gene was deleted in the same cell, allowing for determination of the directionality of genetic interactions.

dCas chromatin immunoprecipitation for discovering interacting elements at genomic loci

Immunoprecipitation of genomic DNA is a useful method to elucidate how DNA interacts with regulatory elements (proteins or RNAs). Previously, DNA sequences of DBDs such as LexA and LacI were inserted into a genomic region of interest [105–107]. A tagged DNA-binding molecule can target a specific genomic locus in cells, which will be pulled down in the process of affinity purification of the DNA-binding molecule with an antibody against its tag [108–110] (Fig. 4b). The captured complex may include DNA fragments, RNAs, or regulative proteins that have physical interactions with the target genomic region. These genetic elements can be analyzed by sequencing for interacting DNAs or RNAs, or mass spectrometry for proteins. These methods require a tedious DNA sequence insertion process. An immunoprecipitation technology named Engineered DNA-Binding Molecule-Mediated Chromatin Immunoprecipitation (enChIP) was developed to address the issue. For example, sequence-specific molecules, such as TALEs [108] and locked nucleic acids (LNAs) [111], were used to characterize proteins associated with specific genomic loci.

Following a similar concept, dCas9-based chromatin immunoprecipitation has been developed to identify proteins interacting with specific genomic loci. For example, one study profiled proteins interacting with the interferon regulator factor-1 promoter responding to interferon-γ stimulation [112]. In addition to proteins, DNAs and RNAs associated with specific genomic regions can be detected using dCas9 chromatin immunoprecipitation paired with next-generation sequencing and RNA sequencing [113–116]. Recently, a work reported an approach termed CAPTURE [117], where a biotinylated dCas9 was combined with proteomics, chromosome conformation capture (3C)-seq and RNA sequencing to identify locus-specific chromatin regulatory complexes and long-range DNA interactions at any genomic location.

Given the off-target Cas9-binding events that might cause non-specific interactions [118–120], it is necessary to reduce binding to off-target genomic loci. As the number of off-target binding events by dCas9 is dependent on the sgRNA [119], the on-target enrichment and specificity should be evaluated when designing the sgRNAs. Bioinformatic tools have been developed to help evaluate highly specific sgRNAs. These tools are compared in the on-target efficiency and off-target detection algorithms in a recent review, which is helpful for choosing proper tools [121]. Furthermore, the same cells lacking the binding sites of interest can be used as controls. The same analysis process conducted in control cells can indicate some non-specific binding of regulatory elements, which provides a good reference for quantitative analysis. After characterizing the genetic elements from CRISPR–dCas9 based ChIP, additional experiment should be conducted to verify their biological functions.

Concluding Perspectives

The discovery of CRISPR–Cas9 as an RNA-guided DNA binding system has revolutionized gene editing. Beyond editing, dCas9 offers a broad platform for regulating gene expression and has been exploited in discovery-based synthetic biology, which aims to make use of natural genetic elements as tools for biological and biomedical discoveries. The two main arms of synthetic biology research are (1) the development of novel tools and (2) the pursuit of new discoveries enabled by these tools. The two arms complement each other. Novel technologies utilizing genetic elements as tools enable understanding of unknown genes. In return, new discoveries could offer more genetic modules for developing new powerful tools.

Currently, dCas9 fusions can act as a gene switch tool to execute a variety of different functions. However, our understanding of the endogenous regulatory mechanisms of genome function is still limited. Therefore, in the future, it is necessary to characterize more chromatin-associated molecules and also in specific cellular contexts. This will help understand natural regulation mechanisms and inform the design of synthetic circuits. In addition to discovering novel genetic elements and interactions, it will be interesting to see how dCas9 facilitates understanding epigenetic modifications, probing 3D

architectures of the genome, and profiling the causal relationship between epigenetics, genome organization, and gene regulation. In the complicate and delicate cell environment, it will be interesting to see how inducible dCas9 tools allow for exploration of creating novel mammalian cell functions for diagnosis and therapeutics.

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Abbreviations used:
TALEs, transcription activator-like effectors; CRISPR, clustered regularly interspaced short palindromic repeats; dCas9, dead Cas9; CRISPRi, CRISPR interference; CRISPRa, CRISPR activation; scFv, single-chain variable fragment; SAM, synergistic activation mediator; MCP, MS2 binding protein; HSF1, human heat shock factor 1; scRNA, scaffold RNA; RBPs, RNA-binding proteins; I/O, input/output; CIDs, chemical-induced dimerizing domains; OIDs, optogenetically inducible dimerizing domains; TF, transcriptional factor; GPCRs, G protein-coupled receptors; TEV, tobacco etch virus; FISH, fluorescence in situ hybridization; DBDs, DNA binding domains.

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