



# Transcriptional regulation with CRISPR-Cas9: principles, advances, and applications

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CRISPR-Cas9 has recently emerged as a promising system for multiplexed genome editing as well as epigenome and transcriptome perturbation. Due to its specificity, ease of use and highly modular programmable nature, it has been widely adopted for a variety of applications such as genome editing, transcriptional inhibition and activation, genetic screening, DNA localization imaging, and many more. In this review, we will discuss non-editing applications of CRISPR-Cas9 for transcriptome perturbation, metabolic engineering, and synthetic biology.

## Addresses

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complicated design criteria and large highly repetitive TALE genes are difficult to synthesize and clone (reviewed in [2,3]). These challenges have recently been overcome using CRISPR-Cas9 based TFs. In this article we overview the biochemical properties of CRISPR-Cas9 based TFs that enable such flexibility and describe their applications to synthetic gene circuit design and multiplexed perturbation of native gene networks.

## Transcriptional regulation with CRISPR-Cas9

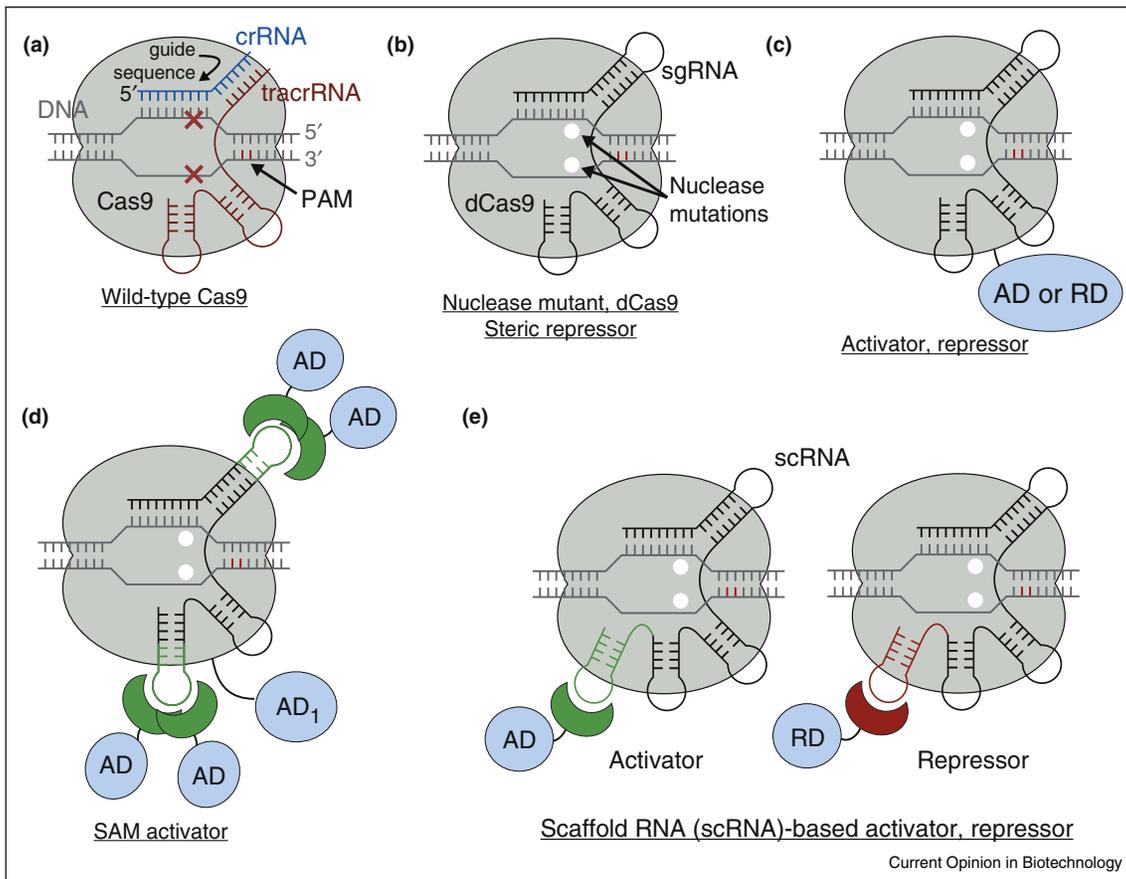
Cas9 is a key protein of bacterial Type II CRISPR adaptive immune system (reviewed in [4]). In its native context, Cas9 is an RNA-guided endonuclease that is responsible for targeted degradation of the invading foreign DNA-plasmids and phages. Cas9 is directed to its DNA targets by forming a ribonucleoprotein complex with two small non-coding RNAs: CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) (Figure 1a). By elegant engineering, crRNA and tracrRNA can be joined end-to-end and transcribed as a single guide RNA (sgRNA) that too efficiently directs Cas9 protein to DNA targets encoded within the guide sequence of sgRNA [5<sup>••</sup>]. The optimal DNA target of the complex is determined by a Watson–Crick base pairing of a short ~20-nt sequence within sgRNA (within the crRNA in wild-type), termed the guide sequence, adjacent to a few nucleotide long conserved motif recognized directly by Cas9 protein (protospacer adjacent motif, PAM) [5<sup>••</sup>,6]. Despite this, a few mismatches between guide sequence and target DNA can be tolerated [5<sup>••</sup>,7,8<sup>•</sup>,9], more so within the 5' proximal position of the guide sequence. Cas9 nuclease can be converted into deactivated Cas9 (dCas9), an RNA-programmable DNA-binding protein, by mutating two key residues within its nuclease domains (Figure 1b) [5<sup>••</sup>,6].

## Introduction

Since the early days of genetic engineering there has been a need for control of gene expression. Naturally occurring transcription factors (TFs) have traditionally been used to achieve this goal (reviewed in [1]). However, their limited DNA binding sequence space required installing specific sequences within the transcription regulatory elements of the target genes. This can be technically difficult and may have unintended consequences on gene expression. Zinc fingers (ZFs) and transcription activator-like effectors (TALEs) were developed to overcome the fixed binding sequence requirements of native TFs. However, both ZFs and TALEs have significant limitations. ZFs have

In the simplest case, dCas9 can repress transcription by sterically interfering with transcription initiation or elongation by being targeted to the gene of interest with a properly chosen sgRNA [5<sup>••</sup>,6,7,8<sup>•</sup>,10<sup>•</sup>,11<sup>•</sup>,12,13,14<sup>•</sup>]. The repression strength is strongly dependent on the position with respect to the target promoter as well as the nature of promoter itself [7,8<sup>•</sup>,10<sup>•</sup>,11<sup>•</sup>]. In prokaryotes, repression of up to 1000-fold was achieved when targeting dCas9 to either DNA strand within a promoter or to the non-template DNA strand downstream [7,8<sup>•</sup>,10<sup>•</sup>,15–18]. However, in eukaryotic cells such steric repression is weaker: only up to 2-fold and 20-fold repression was

Figure 1



Overview of Cas9 nuclease and dCas9-based transcription factors. **(a)** Wild-type Cas9 endonuclease guided by crRNA:tracrRNA to a specific site in DNA creates a double-stranded DNA break. **(b)** dCas9, nuclease deactivated mutant of Cas9, is an RNA programmable DNA binding protein. It can act as a steric repressor of transcription in prokaryotes and eukaryotes. sgRNA is an artificial chimeric molecule consisting of crRNA and tracrRNA molecules connected with a short loop. **(c)** dCas9 fusion with various transcription effectors can be used to repress or activate transcription. **(d)** Effector domains can be recruited by sgRNA in addition to dCas9 for enhanced activity. **(e)** sgRNA can be modified with specific protein binding hairpins to concurrently recruit repressor or activator domains in the same cell.

observed with natural promoters in mammalian and yeast cells correspondingly [7,12,13]. As a notable exception, synthetic promoters specifically constructed for direct repression by dCas9 can be repressed up to 100-fold in mammalian cells [14]. Potentially stronger downregulation of natural promoters in eukaryotic cells can be achieved by fusing dCas9 with transcriptional repressor domains (RD in Figure 1c) [12,19<sup>••</sup>,20,21]. Up to 50-fold repression was achieved using a fusion with a transcriptional repressor Mxi1 in yeast [12]. In mammalian cells, dCas9 fusion with histone demethylase LSD1 can also be used to repress transcription by distal enhancers [22]. The most widely used dCas9-KRAB fusion is strong and highly specific in both yeast and mammalian cells [23,24<sup>••</sup>,25,26].

Transcriptional activation can also be achieved by engineering dCas9, sgRNA, or both to recruit transcription

effectors to the DNA (Figure 1c–e). In *E. coli*, an activator has been constructed by fusing the  $\omega$  subunit of native RNA polymerase to dCas9 in a  $\omega$  subunit deficient host [8<sup>•</sup>] (Figure 1c). This activator was moderately active with weak promoters (up to 23-fold activation), but had progressively less effect with the increasing basal strength of the target promoter. The simplest eukaryotic activators can be engineered by fusing dCas9 with VP64 (and its variations), EDLL, TAD, p65, and p300<sup>core</sup> effector domains [12,21,27–31,32<sup>••</sup>]. Activation levels using a single sgRNA per target promoter are generally weak. However, activation can be further increased by appropriately tiling the target promoter with multiple sgRNAs [29–31]. This observation led to the construction of activators that contain multiple effector domains per dCas9 molecule. First, three different activation domains (VP64, p65, and Rta) were fused to form a so-called VPR activator [33]. This activator was 1–2 orders of magnitude

more efficient than a single VP64 fusion. Second, dCas9 was fused with SunTag, a peptide containing an array of 10 or more tandem repeats [19\*\*,34]. Each repeat then recruited a VP64 transcription activator fused to an engineered antibody fragment scFv. One potential drawback of this system was an aggregation prone nature of scFv. Nevertheless, about 50-fold transcription activation was achieved using this activator guided by a single sgRNA in human K562 cells [34]. In addition to dCas9 fusions, sgRNA can also be engineered to recruit effectors [35,36]. Konermann *et al.* created so-called Synergistic Activation Mediator (SAM) by inserting two bacteriophage MS2 RNA hairpins into non-essential regions of sgRNA (Figure 1d). Each MS2 hairpin binds two molecules of MS2 coat protein MCP, each fused with a pair of transcriptional activators: p65 and human heat shock factor HSF1. Guiding dCas9-VP64 fusion protein by this chimeric sgRNA results in activation within 2-fold to  $>10^4$ -fold range and is generally better than activation of the same promoter with dCas9-VP64 guided by 8 tiled standard sgRNAs. An elegant corollary of sgRNA scaffolding is that it enables concurrent independent use of repressor, activator, or other effector domains in the same cell using a single dCas9 ortholog. Zalatan *et al.* used three mutually orthogonal RNA hairpin:RNA binding protein pairs (MS2:MCP, com:Com, PP7:PCP) to simultaneously recruit VP64 and KRAB effectors (Figure 1e) [24\*\*]. In principle, a repertoire of independent TFs can be further expanded using engineered dCas9 variants with altered specificity, natural Cas9 orthologs, as well as other CRISPR systems [37–41]. Recently, the efficiency of most modern eukaryotic dCas9-based activators and their combinations was compared directly by Chavez *et al.* within human, mouse, and fruit fly cells [42\*\*]. SAM, VPR, and SunTag activators were the most efficient, with SAM typically having up to 5-fold advantage over the other activators in human cell lines.

Beneath the apparent simplicity of dCas9-mediated transcriptional regulation, the rules that determine kinetics and specificity of DNA binding as well as the strength of transcriptional regulation are nuanced depending on context. The main categories of context are the degree of similarity between the target DNA site and the guide sequence of sgRNA, the nucleotide composition of the sgRNA guide sequence, the chromatin state of the target DNA, the host organism, and the specific ortholog of dCas9 used. In terms of DNA targeting specificity, complementarity between DNA and sgRNA is generally less important at the 5' end of the guide sequence, even tolerating DNA or RNA bulges in the bound conformation [43,44]. Both Cas9 and dCas9 appear to tolerate similar numbers of mismatches between the sgRNA guide and the target DNA [8\*,19\*\*] and have similar binding kinetics [45]. However, DNA cleavage by Cas9 has an effectively higher sensitivity to mismatches, presumably since it requires an additional conformational

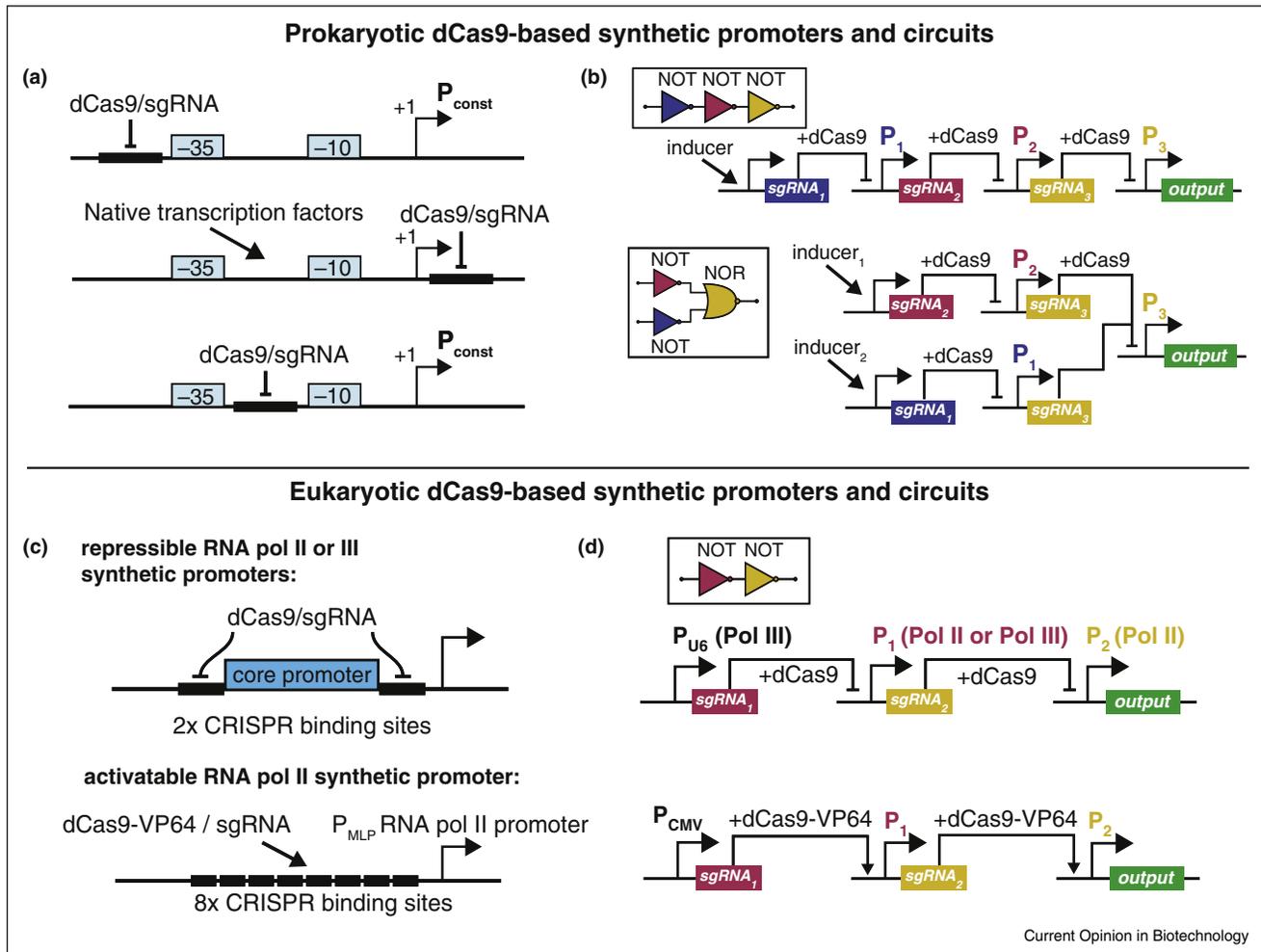
change to induce cleavage [46,47]. This property can be leveraged to achieve transcriptional regulation while avoiding DNA cleavage using nucleolytically active Cas9 guided by sgRNA with mismatches or truncations within the guide sequence [8\*,48]. Mismatch sensitivity is further affected by the concentration of dCas9/sgRNA complex relative to the target DNA [49], which itself depends on the nature and relative amounts of sgRNAs expressed [50]. In eukaryotes, efficiency of regulation is diminished by guanine enrichment and adenine depletion of the guide sequence since it decreases sgRNA stability [51]. Chromatin state also affects dCas9 DNA binding: it is much weaker within condensed chromatin regions, therefore very good match between sgRNA and target DNA sequence is required [9,46,52]. Further studies of dCas9 and specificity-improved Cas9 proteins [53,54] in the aforementioned contexts will inform further development of computational algorithms [11\*,43,51,52,55] that incorporate these effects to precisely select the types and amounts of sgRNAs that are most appropriate to specific applications.

### CRISPR-Cas9 regulatable synthetic promoters and gene circuits

The ability of dCas9 to regulate practically arbitrary transcriptional regulatory elements enables their interconnection into *de novo* networks, also known as synthetic gene circuits. These collections of interacting genes can be programmed with sensing, storage, or actuation functions combined to perform desired tasks [1,56]. Synthetic circuit design is facilitated by having a large panel of modular promoters with defined characteristics that can be predictably interfaced with each other. Such synthetic dCas9-regulatable promoters have been created by strategically installing dCas9/sgRNA binding sites within a parent promoter. By appropriately choosing these binding sites and their corresponding sgRNA guide sequences a panel of distinct promoter/transcription factor pairs can be produced.

In prokaryotes, custom promoters have been constructed by introducing dCas9/sgRNA binding sites in the direct vicinity of the RNA polymerase binding site of parent natural promoters (Figure 2a). Four orthogonal sgRNA/promoter pairs were computationally designed and experimentally validated within two synthetic circuits, double and triple repression cascades (Figure 2a,b, top). These dCas9-based repressors were then interfaced with native inducible promoters  $P_{BAD}$ ,  $P_{LacO-1}$ , and  $P_{LuxI}$ , resulting in predictable regulation with both synthetic and native TFs (Figure 2a, middle). Nielsen *et al.* constructed and validated five orthogonal promoter/repressor pairs (Figure 2a, bottom) [10\*]. These promoters were used to construct logical NOT and NOR gates and their combinations (Figure 2b, bottom); the output of a synthetic NOT[NOR(A,B)] logic circuit was connected to native *E. coli* network via an sgRNA directing repression

Figure 2



dCas9-based synthetic promoters and circuits. (a) Orthogonal modular dCas9-repressible promoters have been designed for building synthetic gene circuits in *Escherichia coli*. (b) Proof of principle logic circuits were built using the promoters shown in (a) [10\*,11\*]. (c) dCas9-repressible (top) and dCas9-activatable (bottom) eukaryotic modular promoters have been designed for building synthetic circuits in eukaryotes. (d) Proof of principle regulatory circuits were constructed using the promoters shown in (c) [14\*,57\*].

of an endogenous transcription factor gene. In a separate study, a panel of orthogonal T7 RNA polymerase driven dCas9-regulatable hybrid promoters was designed, tested, and used to control a reconstituted metabolic pathway [49].

Eukaryotic synthetic gene circuits require additional considerations for efficient linking between input and output of their components. Both RNA polymerase II and III promoters have been used to transcribe active sgRNAs. RNA polymerase III promoters can directly produce active nuclear localized sgRNA, but are significantly limited in their scope of regulation. RNA polymerase II promoters do not suffer from this drawback, but their transcripts are typically exported out of the nucleus so special methods need to be used to generate active

sgRNA within the nucleus. This can be done by (a) using non-standard RNA polymerase III terminators (U1 3' Box and MASC [35]), (b) releasing active sgRNA using site-specific RNA nucleases or self-cleaving ribozymes [57\*,58], or (c) releasing it from an intron [14\*,57\*]. Sterically repressible promoters with high fold repression (~100-fold) have been constructed by flanking a minimal RNA polymerase II or RNA polymerase III promoter with multiple dCas9 binding sites [14\*,48] (Figure 2c, top). A modular dCas9-VP64 activatable synthetic promoter has been engineered by inserting multiple dCas9 binding sites upstream of a weak RNA polymerase II promoter [57\*] (Figure 2c, bottom). Combining these custom promoters and the approaches for production of active sgRNAs enabled construction of two-stage repressor and activator circuits [14\*,48,57\*] (Figure 2d), as well

as a programmable kill switch, by combining transcription regulation and nucleolytic activity of wild-type Cas9 [48]. To demonstrate a medically relevant application, a dCas9-based logic circuit was wired to native gene network to sense and kill cancer cells [59].

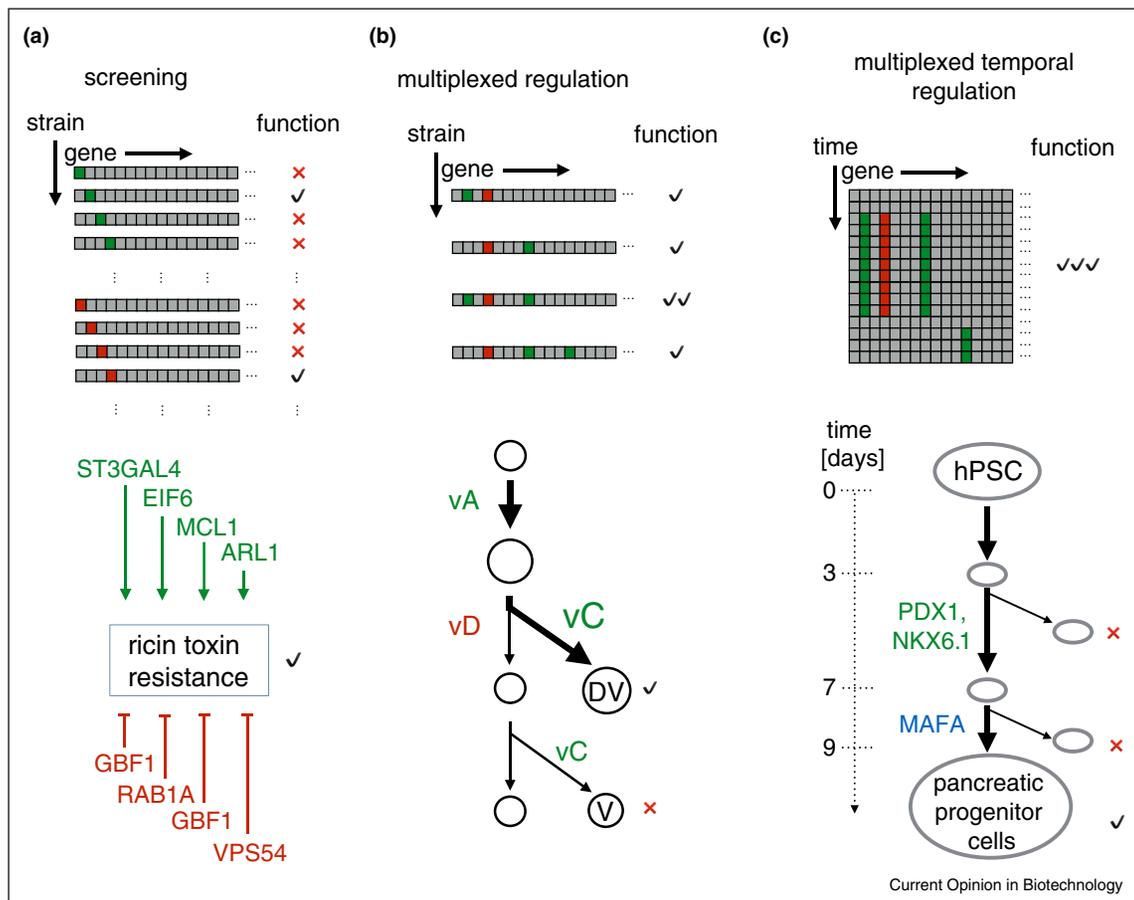
Common to both prokaryotic and eukaryotic synthetic circuit design are two fundamental considerations: (a) the crosstalk within a circuit, as well as between the circuit and the host, and (b) the dynamics of interactions between the circuit components. First, naïve choice of sgRNA guide sequences can result in undesired crosstalk that leads to interference with the circuit or host function [8<sup>\*</sup>]. This problem can be addressed by computationally designing sgRNA guide sequences and the corresponding target DNA sites in order to minimize undesired interactions [11<sup>\*</sup>]. Second, while dCas9/sgRNA complex binds its cognate target DNA very tightly, the dissociation kinetics is slow (half-life of ~6 hours or more *in vitro*

[45,60]). The DNA dissociation rate of dCas9/sgRNA complex can likely be increased while maintaining sufficiently tight DNA binding by introducing a small number of strategically designed mismatches or truncations within the sgRNA guide sequence [61]. Alternatively, by tagging dCas9 for degradation [32<sup>\*\*</sup>,62] the effective half-life of DNA-bound dCas9/sgRNA may be decreased. Further advances in addressing these considerations will enable construction of complex circuits with precise dynamics expanding the scope of applications.

### Applications

dCas9-based transcriptional regulation is a useful tool for basic science and biotechnology applications, especially those employing screening and network engineering (Figure 3). Forward genomic screens enable identification of genes involved in cellular functions of interest by creating several strain variants with single gene expression perturbations and observing which variants produce

Figure 3



Applications of transcriptional regulation with dCas9. **(a)** Gain of function (green) and loss of function (red) monogenic screening. Below is an example of ricin toxin resistance screen [19<sup>\*\*</sup>]. **(b)** Combinatorial screening combining multiple transcriptional perturbations. Below is an example of this approach to maximize deoxyviolacein (DV) production relative to violacein (V) by upregulating vA and vC (green) and downregulating vD genes (red) [24<sup>\*\*</sup>]. **(c)** Reversible perturbations to multiple genes. Below is an example of using a conditionally degradable dCas9-based activator to differentiate human pluripotent stem cells to pancreatic progenitor cells. Upregulation of PDX1 and NKX6.1 (green) during days 3–7 followed by activation of MAFA (blue) during days 7–9 leads to differentiation [32<sup>\*\*</sup>].

phenotypes of interest (Figure 3a, top). Gene knock-downs using dCas9 can be more effective than traditional RNAi based approaches, which are susceptible to incomplete knockdown and off-target effects (reviewed in [63,64]). This was explicitly demonstrated by comparing shRNA and dCas9-KRAB/sgRNA in screens for genes responsible for resistance to the ricin toxin in human K562 cells [19\*\*] (Figure 3a, bottom). More importantly, not only does dCas9-based screening extend the scope of RNAi regulation to cells that do not use it (e.g., prokaryotes), it can also easily be adapted for gain of function screening. Recently, Peters *et al.* screened the microbe *Bacillus subtilis* with dCas9 to identify the essential gene target of newly discovered antibiotic MAC-0170636 as well as find genetic determinants of cell morphology and growth [17]. A gain of function screen with dCas9-VP64 identified genes that modulate resistance to inhibition of BRAF, a human proto-oncogene [36]. Combining dCas9-based activation and repression screening can lead to new regulatory network information; this approach enabled characterization of complementary pathways in glycosphingolipid biosynthesis related to cholera and diphtheria toxin resistance [19\*\*]. These studies foreshadow the potential of dCas9 for next-generation functional genomic screens.

The ability to guide dCas9 with multiple sgRNAs concurrently allows straightforward extension of screening results to testing strain variants with multiple transcriptional perturbations (Figure 3b, top). This is particularly useful for adjusting groups of enzymes in metabolic pathways to optimize production of desired metabolites. While broad genetic screens using nucleolytically active Cas9 have been widely used to find genes that increase metabolite yield [65], there are many cases in which graded dCas9-based modulation of multiple enzyme concentrations is required for optimal metabolite production. This has been demonstrated in *E. coli* and *C. glutamicum*, where multiplexed promoter repression resulted in increased yields of naringenin [66], 4-hydroxybutyrate [67], as well as lysine and glutamate [16]. Such an approach has also been successfully utilized in *E. coli* [49] and *S. cerevisiae* [24\*\*] to increase the flux in the violacein metabolic pathway (Figure 3b, bottom). Here, the authors employed concurrent upregulation and downregulation of enzymes at several metabolic flux branch points enabling quick construction of subtle metabolic pathway variants. While dCas9-based metabolic engineering has thus far been limited to unicellular organisms or cells in tissue culture, first steps are being taken for engineering complex multicellular organisms used in agriculture [21]. Besides improving production of valuable compounds, we envisage that dCas9 will be used to engineer metabolic networks for bioremediation [68], biosensing [69] and endowing cells with other useful properties (e.g. stress resistance [70]).

Controlling cell fate holds great promise for regenerative medicine and oncology. Regulation of cell pluripotency

and tissue morphogenesis is achieved by transcriptional regulation of unique combinations of gene targets at precise times during development. The multiplexability and reversibility of the dCas9/sgRNA system is thus well suited to study these programs (Figure 3c, top). Both VP64 activator and KRAB repressor versions of dCas9 have been used to reprogram fibroblasts to myocytes [26], guide human pluripotent stem cell differentiation [23,32\*\*], or revert fibroblasts to induced pluripotent stem cells [32\*\*]. To quickly relieve dCas9-mediated activation, Balboa *et al.* fused an inducible destabilization domain (DHFR from *E. coli*) to dCas9. This enabled the execution of a transient overexpression of differentiation genes PDX1 and NKX6.1 that achieved a differentiation of human pluripotent stem cells into pancreatic progenitor cells [32\*\*] (Figure 3c, bottom). To detect and retard bladder cancer cell growth Liu *et al.* integrated oncogenic hTERT and bladder-specific hUP II gene upregulation with dCas9/sgRNA-induced apoptosis [59]. These studies recapitulate that dCas9-mediated interactions with transcriptional programs enable researchers to both explore the coordination of genetic networks that underlie cellular functions and to leverage this knowledge for regulation of these functions. Harnessing this ability in a diversity of environmental circumstances is germane to applications spanning all of the life sciences.

## Conclusion

In this review we have described how dCas9 has been developed to efficiently regulate transcription in a variety of contexts, including generation of novel gene regulatory circuits, screens for discovering functions of genes, and perturbations enabling efficient production of compounds and cell types. As the technology matures and new applications emerge we foresee an increasing importance of CRISPR-based tools for basic science, biotechnology, and medicine.

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