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MINIREVIEW

The New State of the Art: Cas9 for Gene Activation and Repression

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CRISPR-Cas9 technology has rapidly changed the landscape for how biologists and bioengineers study and manipulate the genome. Derived from the bacterial adaptive immune system, CRISPR-Cas9 has been coopted and repurposed for a variety of new functions, including the activation or repression of gene expression (termed CRISPRa or CRISPRi, respectively). This represents an exciting alternative to previously used repression or activation technologies such as RNA interference (RNAi) or the use of gene overexpression vectors. We have only just begun exploring the possibilities that CRISPR technology offers for gene regulation and the control of cell identity and behavior. In this review, we describe the recent advances of CRISPR-Cas9 technology for gene regulation and outline advantages and disadvantages of CRISPRa and CRISPRi (CRISPRa/i) relative to alternative technologies.

The ability to regulate expression is essential to the study of biology, from basic biological research to clinical applications for the treatment of disease. Since the elucidation of the central dogma of molecular biology, we have been searching for ways to manipulate and perturb gene expression. In recent years, new technological breakthroughs have provided greater precision, ease, and throughput in the manipulation of gene regulation.

One such technology, CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats-CRISPR associated 9), has rapidly shifted the landscape for studying and manipulating the genome. Repurposed from the bacterial immune system for cleaving foreign DNA (1), this technology consists of the Cas9 endonuclease and a target-identifying CRISPR RNA (crRNA) duplex made up of two RNA components: crRNA and *trans*-activating crRNA (tracrRNA) (Fig. 1A) (2). These two RNAs can be engineered into a chimeric single guide RNA (sgRNA), simplifying its use (3). The sgRNA base pairs with the DNA target and can be easily programmed to target an 18- to 25-bp sequence of interest. The only constraint is that the sgRNA-binding site must be adjacent to a short DNA motif termed the protospacer-adjacent motif (PAM) (3, 4). In the most commonly used form of Cas9, derived from *Streptococcus pyogenes*, the PAM sequence is NGG (where N is any nucleotide and G is the base guanine), although NAG (where A is adenine) also functions sporadically, with lower efficiency than NGG (5). NGG can be found every 8 bp on average in the human genome, rendering *S. pyogenes* Cas9 an extremely versatile genetic scissor (6).

This Cas9-sgRNA complex has proven to be incredibly useful as a genome-editing tool. The simplicity of designing the 20-nucleotide (nt) DNA base pairing portion of an sgRNA and Cas9's natural RNA-directed endonuclease activity makes targeting Cas9 to new DNA sites a straightforward task. Once targeted to the DNA, Cas9 creates a blunt-ended double-stranded break (DSB) within the target sequence (3, 4). This DSB can be used to facilitate generation of indel mutations that cause a frameshift within the coding sequence of a gene due to imperfect repair by the native host DNA repair pathway (6–8). Alternatively, by supplying a repair template with homology to the cut site, Cas9 can facilitate targeted integrations of precise mutations or insertions into the genome (9, 10). CRISPR-Cas9 has been used successfully in a wide variety of organisms, from bacteria and yeast to plants and animals, both invertebrates and vertebrates (1, 8, 11–14). CRISPR's potential to expand genome engineering of previously intractable organisms cannot be overstated but is not reviewed here, as there are already several high-quality reviews on the subject (2, 15–18).

In addition to its editing potential, the CRISPR-Cas9 system offers exciting possibilities for genetic and epigenetic regulation. One strength of CRISPR technology lies in the fact that it brings together DNA, RNA, and protein in a predictable and easily programmable manner. This means that the Cas9-sgRNA complex can act as a scaffold to recruit a broad range of effectors or markers to specific DNA sequences. It is this property of CRISPR-Cas9 that has been exploited to regulate gene expression at the transcriptional level, either to activate genes (CRISPRa) or to repress genes (CRISPRi). Here, we review the characteristics and use of CRISPRa and CRISPRi (CRISPRa/i) and how these tools compare to alternative gene regulation systems.

REPURPOSING CRISPR-Cas9 FOR GENE ACTIVATION

To convert Cas9 from a DNA scissor into a gene activator, it is necessary to disrupt its nuclease activity. Cas9's two nuclease domains, the RuvC and HNH domains, are conserved among several types of nucleases, and each is responsible for cutting one strand of DNA upon binding (3, 4, 19). We and others have introduced mutations into these two domains to create a nuclease-deactivated Cas9 (dCas9) (Fig. 1A) (3, 4, 20–22). This converts the Cas9 nuclease into a generic RNA-guided DNA-binding protein. It is then possible to fuse effectors directly to dCas9, which essentially transforms the dCas9-effector fusion into an easily programmable artificial transcription factor upon being paired with a target-specific sgRNA. As the RuvC and HNH domains are conserved among Cas9s from other bacterial species, this approach provides a general strategy for repurposing orthogonal Cas9s into RNA-guided DNA-binding proteins.

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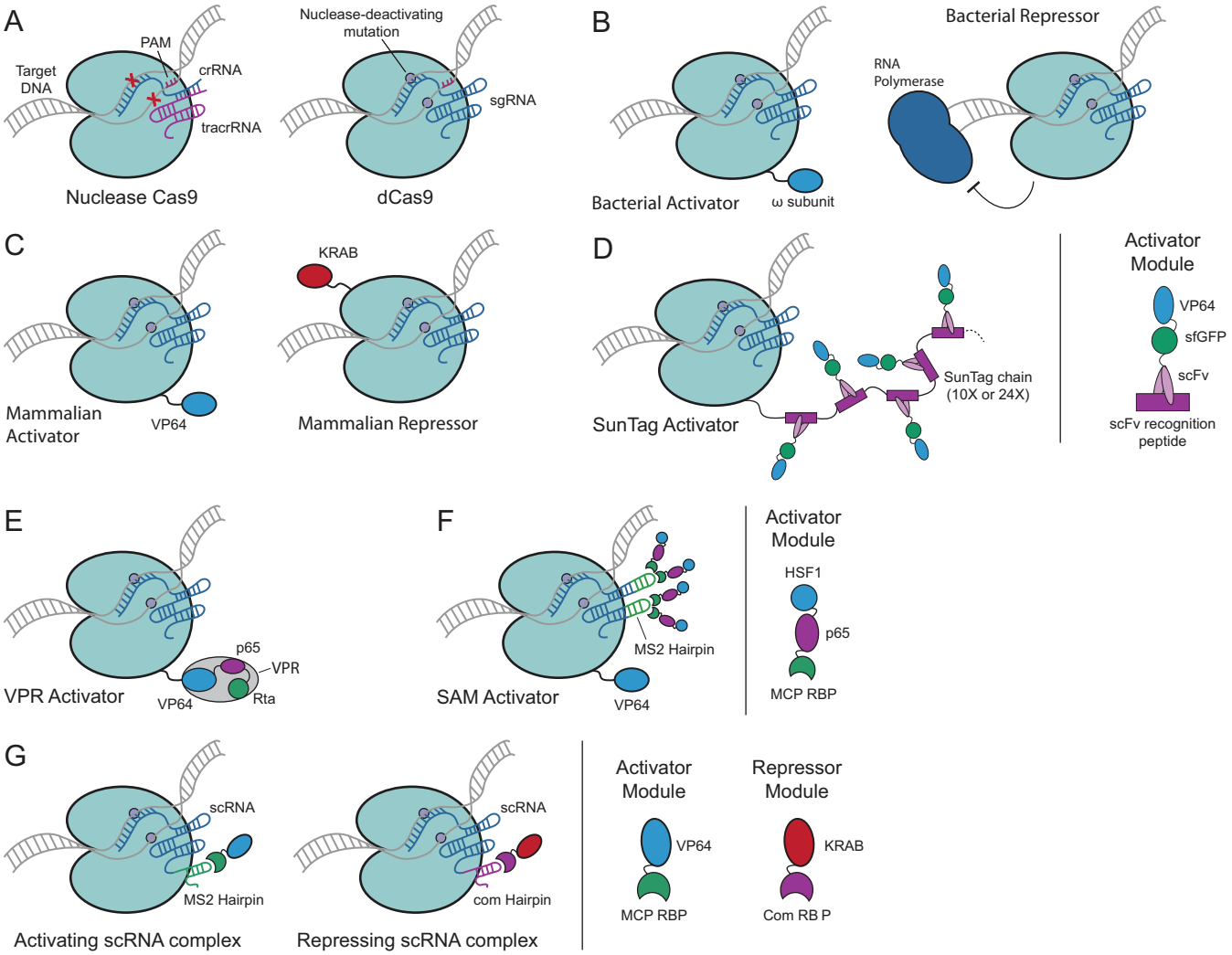

FIG 1 Engineered CRISPRa/i systems. (A) In endogenous type II-B CRISPR systems like that of *S. pyogenes* (left), there are three essential components for nuclease activity. These components are the Cas9 protein, crRNA, and *trans*-activating crRNA (tracrRNA), which base pair with each other. This Cas9-RNA complex is able to cleave the DNA targeted by the crRNA and which is adjacent to a PAM site (red). Cleavage sites are indicated by Xs. To simplify and adapt CRISPR for gene regulation (right), mutations in the nuclease domains have been introduced into Cas9, rendering it a dCas9. Additionally, the crRNA and tracrRNA have been combined into an sgRNA. (B) To control gene expression in bacterial cells, dCas9 can be fused with the ω subunit of RNA polymerase for activation (left) or can repress transcription by sterically blocking RNA polymerase (right). (C) To turn dCas9 into an artificial transcription factor in mammalian cells, it can be fused with a VP64 activator (left) or a KRAB repressor (right). (D) The SunTag activation system (left) consists of dCas9 fused to several tandem repeats of a short peptide sequence separated by linkers. The SunTag activator module (right) is an scFv, which specifically binds the SunTag peptide. The scFv is fused to sfGFP and VP64. (E) The VPR activation system is dCas9 fused to VP64, p65, and Rta linked in tandem. (F) In the SAM activation system (left), dCas9 is fused to VP64. In addition, the sgRNA has been modified such that it contains two MS2 hairpins (green). An additional activator module (right) binds to an MS2 hairpin via the RNA-binding protein MCP. The MCP is fused to the activators p65 and HSF1. (G) The scRNA system can be adapted such that it can act as an activator or repressor (left). The activator and repressor modules (right) consist of an RNA-binding protein fused to VP64 and KRAB, respectively. The activating and repressing systems can be used orthogonally when different scRNAs that recruit different modules are used. Here, the MS2 scRNA recruits the MCP activator module and the com scRNA recruits the Com repressor module.

One type of effector that can be fused to dCas9 is a transcriptional activator. There are different forms of these dCas9-activator fusions. For example, Bikard et al. fused the ω subunit of RNA polymerase to dCas9 for use in *Escherichia coli* (Fig. 1B) (5, 23). This fusion was able to activate reporter gene expression up to 3-fold. Currently, the report by Bikard et al. is the only one to have been published on the use of CRISPRa in bacteria, and further development and optimization are likely needed before its use can be broadly applied to endogenous genes.

In eukaryotic cells, the first generation of dCas9 activators consisted of dCas9 fused to the activation domain of p65 or a VP64 activator, an engineered tetramer of the herpes simplex VP16 transcriptional activator domain (Fig. 1C) (24). The dCas9-VP64 fusion proved more effective than the p65 fusion and has been used more ubiquitously. A number of studies have demonstrated that dCas9-VP64 is able to activate silent endogenous genes and reporters or to upregulate already active genes (22, 24–27). This CRISPRa complex functions in eukaryotic organisms such as budding yeast or mammalian cells. However, the activation seen in mammalian cells is usually moderate, about 2-fold to 5-fold, on

average, using a single sgRNA. This activation can be enhanced by using multiple sgRNAs tiled across the promoter (25, 27), suggesting that recruiting additional activators to the target gene enhances activation. Therefore, the second generation of CRISPRa made use of strategies to corecruit multiple activators.

There have been several attempts to improve the direct fusion design for the second generation of CRISPRa. One strategy, demonstrated by Gilbert et al. and Tanenbaum et al., is to amplify activation by transforming dCas9 into a scaffold capable of recruiting many copies of an activator (28, 29). This is done by fusing dCas9 to a tandem array of peptides, called a SunTag array, which recruits many copies of the VP64 effector (Fig. 1D). The recruitment strategy involves fusing VP64 to an scFv (single-chain variable fragment), an engineered portion of an antibody that binds to the peptide repeats in the SunTag array. Compared to the ~2-fold increase observed with dCas9-VP64 alone (30), we observed a 50-fold increase at the protein level with dCas9-SunTag for endogenous genes such as the CXCR4 chemokine receptor gene in human erythroleukemia K562 cells. Activating endogenous CXCR4 using dCas9-SunTag was sufficient to produce significant increases in cell migration. This system represents a major improvement in activation efficiency, as one dCas9 can now recruit up to 24 copies of the scFv-VP64 fusion protein, rather than delivering just 1 VP64 via a dCas9-VP64 fusion. This is especially important given that simply increasing the number of copies of VP16 in a direct protein fusion (i.e., using VP160) has limited effectiveness (27).

Another strategy for CRISPR-dependent gene activation, reported by Chavez et al., employs multiple different activators to synergistically amplify activation (31). The authors created a tripartite effector fused to dCas9, composed of activators VP64, p65, and Rta (VPR) linked in tandem (Fig. 1E). These three activators were joined in a defined order to strongly activate genes. The dCas9-VPR system was successfully employed in human, mouse, *Drosophila melanogaster*, and *Saccharomyces cerevisiae* cells. Additionally, it can upregulate endogenous gene expression from 5- to 300-fold at the mRNA level compared to a single dCas9-VP64 fusion. It should be noted that this was achieved using pools of 3 to 4 different sgRNAs per endogenous gene, which has been shown to greatly increase activation for the first-generation dCas9-VP64 fusion (25, 27). In the future, it will be useful to test additional activators and see if an even greater effect can be achieved and also to look at endogenous gene activation using a single sgRNA.

A third approach, described by Konermann et al., is termed the synergistic activation mediator (SAM) system (32). Like the VPR activator, the SAM system employs multiple transcriptional activators to create a synergistic effect. This tool makes use of the first-generation version of dCas9-VP64, but the authors engineered additional features into the sgRNA to enhance activator recruitment. This new sgRNA contains two copies of an RNA hairpin from the MS2 bacteriophage, which interacts with the RNA-binding protein (RBP) MCP (MS2 coat protein) (Fig. 1F). An additional activation module was created by fusing MCP to the p65 transcriptional activator as well as to the activating domain of human heat shock factor 1 (HSF1). MCP binds to MS2 as a dimer, so up to four additional copies of the activation module can be recruited per dCas9-VP64. The SAM system can produce a wide (two- to multiple-thousand-fold) range of enhanced activation of endogenous genes at the mRNA level compared to dCas9-VP64, depending on baseline expression. This includes both protein coding genes and long noncoding RNAs (lncRNAs). In the future, it will be informative to try a similar approach to repress gene expression (see below).

Finally, Hilton et al. were able to fuse a histone acetyltransferase to dCas9, creating a dCas9-p300^{core} fusion activator capable of acting as an epigenome editing platform (33). This fusion was able to enable activation at both proximal and distal enhancers of genes. This is in contrast to the dCas9-VP64 activator, which must be targeted to a promoter to achieve significant gene activation. Furthermore, the dCas9-p300^{core} fusion achieved higher activation than dCas9-VP64 alone. In the future, it will be interesting to fuse other epigenome modifiers to dCas9. Such tools could be used to specifically probe the effects that epigenetic changes have on gene expression levels.

Together, these transcriptional activation systems function across a range of cell types and species and provide many options for transcriptional and epigenetic manipulation. Each strategy comes with its own advantages and disadvantages. For example, while the VPR activator relies on fewer components, it has not yet been validated for larger-scale screens like the SunTag and SAM activators. The high activation levels of the VPR system depended on using a pool of 3 to 4 sgRNAs, making it more difficult to use effectively in genome-wide screens. In addition, there may be cell type-specific efficiency or toxicity issues with each of these technologies. All of these tools are relatively new, and so it will be interesting to compare their efficiencies and specificities directly in a range of cell types and for a variety of genes.

TRANSFORMING dCas9 INTO A TRANSCRIPTIONAL REPRESSOR

In addition to being fused to transcriptional activators, dCas9 can also function as a repressor. This was first demonstrated in bacterial cells, where dCas9 alone was able to act as a transcriptional repressor by sterically hindering the transcriptional activity of RNA polymerase (Fig. 1B) (20, 23). This provides a very efficient way to silence transcription in bacteria, usually in the range of 1,000-fold. Repression is tunable, as the choice of sgRNA site determines the strength of its repressive effect. It is also rapidly reversible using inducible promoters to control expression of dCas9. This system is advantageous because genes can be efficiently repressed without the addition of specific effectors, making the repression system simpler and more transferable across genes, species, and cell types than the activation system.

This steric hindrance strategy for repression has been employed in yeast and mammalian cells (20, 24). While the simple dCas9 transcriptional blockade has been found to work in these cells, the efficiency of repression is much lower. This is likely because the binding of dCas9 to DNA is not sufficient to disrupt the action of eukaryotic RNA polymerases. One strategy to improve the efficiency of repression in mammalian cells has been to fuse transcriptional repressors to dCas9 (Fig. 1C) (21, 24). These repressors include the KRAB (Krüppel-associated box) domain of Kox1, the CS (chromoshadow) domain of HP1α, the WPRW domain of Hes1, and four concatenated copies of the mSin3 interaction domain (SID4X) (21, 24). Of these, the KRAB-dCas9 fusion has proven to be the most effective. The most active sgRNAs can achieve repression levels in the range of 90% to 99%, although it may be necessary to screen through 5 to 10 sgRNAs to find 1 or 2 of the most highly active guides (29). The efficacy of sgRNAs for CRISPRa/i may be further improved by bioinformatically model-

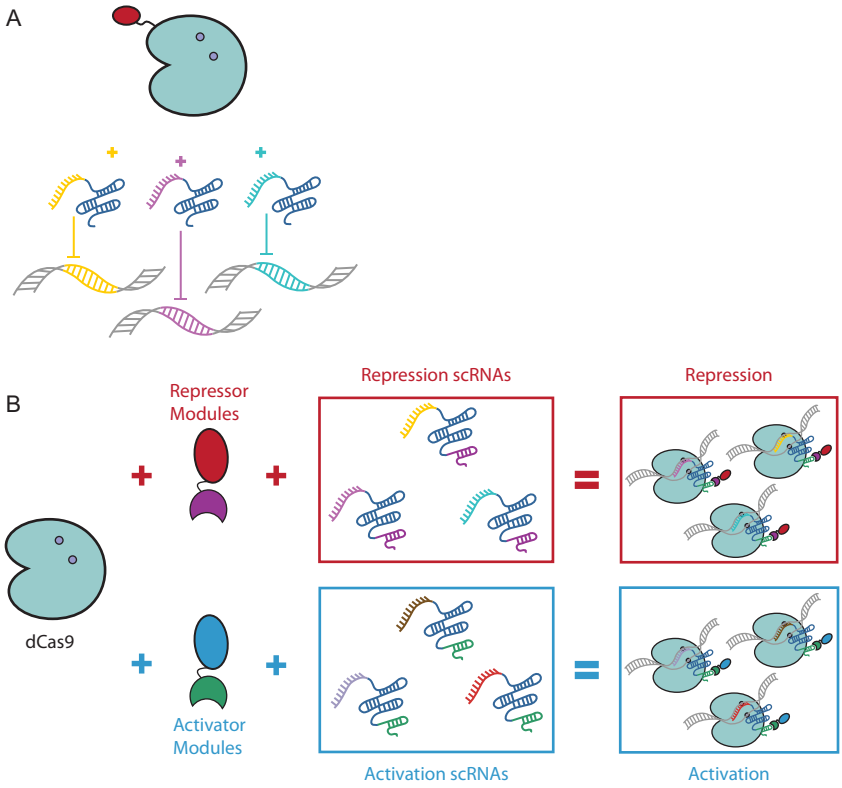

FIG 2 Multiplexed gene regulation with CRISPRa/i. (A) A dCas9 effector fusion is capable of perturbing expression of multiple genes simultaneously. In the example here, multiple genes are repressed simultaneously. (B) With the scRNA system, it is possible to use dCas9 to activate and repress multiple distinct genes simultaneously. The diversity of function arises from the different RNA hairpins attached to the sgRNA, which recruit orthogonal effectors. Here, the com RNA hairpin (purple) recruits a repressive effector module and the MS2 RNA hairpin (green) recruits an activating effector module.

ing the efficacy of large pools of sgRNAs. While this has been done for both CRISPR knockouts (KO) and CRISPRa/i, iteratively testing and analyzing sgRNA efficiency can enormously improve the system.

While these strategies have proven quite effective at repressing transcription, there are improvements that can be made. For example, we have found that using an N-terminal KRAB fusion is more effective at repression than using a C-terminal fusion (compare the repression reported in reference 29 to that reported in reference 24) (additional data not published). In addition, the repression system might also be improved by combining several synergistic repressors in a manner similar to the activation systems described above (31, 32).

ENGINEERING COMPLEX REGULATORY PATTERNS USING CRISPRa/i

These CRISPRa/i systems are remarkably versatile. The activating or repressing dCas9 fusions can regulate a single target or be multiplexed to regulate multiple targets at once (Fig. 2A) (24, 27, 32). In mammalian cells, multiple sgRNAs can be used in the same cell while still efficiently regulating any one target (24, 32). This ability can be used to upregulate or downregulate multiple genes within the same pathway.

One exciting use of CRISPRa/i is to regulate multiple genes in multiple ways (i.e., using activation and repression) within a single cell. One disadvantage of the direct fusion of effectors to dCas9 as described above is that only one type of perturbation can occur within a given cell: dCas9 either activates or represses genes but does not do both. To work around this, Zalatan et al. turned the sgRNA into a scaffold to recruit different types of effectors (30) (Fig. 1G). This is made possible by fusing the effectors to RNA-binding proteins (RBPs) from bacteriophages, which recognize specific RNA hairpin structures. These RNA hairpins can be fused to the sgRNA, creating a scaffold RNA (scRNA). By fusing different RNA hairpins to the sgRNA, different RBP-effector combinations can be recruited. Thus, the scRNA encodes both the target gene location (through the DNA-base-pairing region) and the type of gene regulation (through the additional RBP-recruiting RNA hairpin). This strategy has been used in both yeast and mammalian cells to regulate genes in orthogonal directions simultaneously (30) (Fig. 2B). With three distinct sets of RBP-scRNA pairs, there are many possibilities for synthetic biology using this technology.

The varied dCas9 and Cas9-mediated regulatory strategies can be combined in diverse ways to create unprecedented levels of control. This is particularly relevant to synthetic biology and cellular engineering studies. This has been illustrated by various groups that have used dCas9 or Cas9 to create logic gates that influence cellular outcomes. Zalatan et al. reprogrammed a branched metabolic pathway in yeast to control the production of various product metabolites (30). Liu et al. used a nuclease Cas9 to create a promoter-based "AND" logic gate to identify and control a specific type of cancer cell (34). Promoter-based logic gates (e.g., AND, OR, and NOT) could also be combined with the scRNA

components to create complex regulatory patterns that are induced only under certain conditions. Tuning defined sets of genes with such precision will allow extraordinary control over cell behavior and identity.

LARGE-SCALE SCREENS WITH CRISPRa/i

In addition to its use for multiplexed regulation, the CRISPR-Cas9 system can be adapted for use in pooled genetic screens to interrogate the functions of many genes at once. This was first shown by several groups using nuclease-active Cas9 for genome-wide knockout (KO) screens in mammalian cells (35–37). These groups were able to pool sets of tens of thousands of sgRNAs, with a coverage of approximately 3 to 10 sgRNAs per gene, to investigate a range of phenotypes from cell growth to drug resistance to host factors influencing viral susceptibility. While these have been powerful demonstrations of CRISPR-Cas9 technology, we have been able to broaden our ability to perform genome-wide screens by adapting CRISPRa/i to screening technology as well.

Recently, both CRISPRa and CRISPRi have been employed in pooled genetic screens in mammalian cells (29, 32). This is a particularly revolutionary technique for gene activation, as CRISPRa overcomes limitations of previous gene overexpression methods (discussed further below). The CRISPR KO and CRISPRa/i screening systems can be complementary to each other, as each can enrich for different sets of genes responsible for a certain phenotype. For example, the genes that are most highly enriched with CRISPRa are likely those that "drop out" of a CRISPRi or KO screen; likewise, the genes that are most highly enriched with CRISPRi or KO are likely those that drop out of a CRISPRa screen. Since there is less sensitivity and more noise in the sgRNAs that drop out of a screen, upregulation and downregulation may offer complementary sensitivities (see examples in references 29 and 36 versus reference 32), although this is not necessarily always true.

It will be important to carefully consider all of these aspects when designing genome-wide functional screens. Investigators who wish to thoroughly probe as many of the genes involved in a given process as possible may need to perform 2 to 3 different types of CRISPR screens.

SPECIFICITY OF CRISPRa/i

While there have been several studies examining the off-target effects for the nuclease version of Cas9, investigation into the specificity of the CRISPRa/i system is still in its nascent stages. One study used RNA sequencing in cells expressing an sgRNA targeting an exogenously added green fluorescent protein (GFP) compared to a nontargeting sgRNA control (24). While the GFP gene was the only gene which was significantly repressed genome wide, only a single sgRNA was investigated. Other studies, using both the first-generation version of dCas9-VP64 and the second-generation SAM system, have used a similar technique to show the specificity of gene activation using CRISPRa (32, 33, 39).

It is difficult to directly compare nuclease Cas9 and dCas9 effectors, since the activity readouts of the two are different. However, there are some indications that the dCas9 effector function may be more sensitive to mismatches (and thus less prone to off-target effects) than Cas9. This possibility is supported by work from Gilbert et al., where dCas9-KRAB and Cas9 nuclease were tested for their ability to function with sgRNAs containing mismatches to a given target site (29). In this study, sgRNAs containing 1- to 5-bp mismatches were systematically tested for activity and normalized to fully on-target sgRNAs for both nuclease Cas9 and dCas9-KRAB. Across the entire panel of sgRNAs tested, dCas9-KRAB repression was more affected by mismatches than nuclease Cas9 cleavage. In the future, it will be important to continue comparing dCas9 effectors with Cas9 nuclease so that we can better assess which technology to use for a given application.

In one genome-wide chromatin immunoprecipitation sequencing (ChIP-seq) study by Polstein et al., a hemagglutinin (HA)-tagged dCas9 was found to bind quite specifically (between 4 and 31 off-target sites) (39). The authors also correlated this with dCas9-VP64 activation as assayed by RNA-seq and found that the dCas9-VP64 activation was also quite specific. This is in contrast to the results of two genome-wide ChIP-seq studies performed by Kuscu et al. and Wu et al. using an HA-tagged dCas9, where dCas9 was found to bind promiscuously (up to 100s to 1,000s of off-target sites) to sequences matching the "seed" region in the sgRNA portion adjacent to the PAM (40, 41). Interestingly, nuclease Cas9 using the same sgRNAs was quite specific, cleaving only the few off-target sites with extensive base pairing between the sgRNA and off-target DNA.

One possible explanation for the apparent promiscuity of dCas9 reported by Kuscu et al. and Wu et al. could be that, while dCas9 and Cas9 interrogate many sites transiently, prolonged interactions occur only with extensive matching between the sgRNA and target DNA (42). Transient interrogations would be captured in the assays described above, since formaldehyde cross-linking was used to fix samples. If the three studies had used different fixation protocols and peak-calling or thresholding methods to process the ChIP-seq data, they could conceivably have generated quite different results. The studies described above are consistent with the model that Cas9 is fully functional as a nuclease after only extensive base pairing between the sgRNA and target DNA (43). The specificity of nuclease Cas9 function due to the necessity of prolonged binding may also apply to dCas9 effectors, especially those that need to recruit other proteins to be active. More work must be performed to continue probing dCas9 off-target binding and effector function.

Altogether, while research on dCas9 effector specificity is still in its early stages, the initial studies have been quite promising with respect to the future use of CRISPRa/i. In the future, it will be important to continue to probe for off-target effects for a wide variety of sgRNAs and for all of the new types of dCas9 effector combinations, as different mechanisms of action for each effector may result in different levels of functional promiscuity. With greater knowledge about off-target effects from the use of CRISPRa/i, we can better judge the rate of false positives in using these technologies for screens. Furthermore, such knowledge can be used to inform us what level of sgRNA coverage per gene is needed to extract the maximum amount of information from as small a library as possible, which is particularly important in working with systems where it is difficult to scale up the number of cells used.

CRISPRa VERSUS PREVIOUS ACTIVATION METHODS

CRISPRa offers many advantages over alternative gene overexpression or activation methods (Table 1). One technique to overexpress genes is to clone the open reading frame (ORF) or cDNA of the gene of interest (reviewed in reference 45). For longer or GC-rich genes, this alone can be technically difficult. In cloning many genes at once using this method, there would be a bias to-

ward smaller and easier-to-amplify genes. Additionally, when cloning from the cDNA, one may be missing physiologically relevant splice variants. However, there are some applications where the use of ORF overexpression constructs is more suitable. For example, it is possible to overexpress a certain splice variant or a mutant version of an allele (45). This is particularly useful when studying disease-associated mutations or variants. Additionally, ORF overexpression is useful for introducing fluorophore- or peptide-tagged versions of proteins, which can be invaluable for tracking the location or binding partners of the given protein when no good antibody is available (57).

Another, alternative approach is to use other engineered transcriptional activators to turn on gene expression. These include zinc finger (ZF) effectors (58–60) and transcriptional activator-like effectors (TALEs) (38, 50, 61) fused to a set of activation domains similar to those described above for CRISPRa. As with dCas9 activators, ZF and TALE activators target specific sequences of DNA and recruit transcriptional machinery to activate transcription. However, Cas9 finds its DNA target through simple Watson-Crick base pairing interactions between the sgRNA and target DNA, whereas ZFs and TALEs rely on protein-DNA interactions. Typical engineered zinc finger tandem arrays possess between 3 and 6 individual zinc finger motifs for binding target DNA ranging from 9 to 18 bp in length (59). An individual zinc finger motif targets 3 or 4 nucleotides, and creating a composite ZF protein to target novel DNA sites can require much engineering and testing (51). TALEs are much easier to program than ZFs. A TALE contains a series of repeat variable domains (RVDs), each targeting a single DNA nucleotide. The RVD code for all DNA nucleotides has been well characterized (62). Thus, a TALE can easily be programmed by creating an array of RVDs that bind to the nucleotides in the target DNA.

While there are limited reports directly comparing ZF, TALE, and CRISPR activators, they seem to achieve similar levels of activation (31, 33, 39). The main disadvantage of TALEs and ZF effectors compared to CRISPR-Cas9 is that they require more complicated cloning to assemble, making them less user-friendly overall and not amenable to genome-wide screens. However, the use of TALEs in particular can be advantageous when a particular target area lacks a PAM, since TALEs can be programmed to target any sequence. TALEs are able to distinguish between methylcytosine (mC) and cytosine (C), which may be advantageous or disadvantageous, depending on the situation (52, 53, 63). The methylation status of the targeted site must be taken into account when designing TALEs, which is not the case for Cas9 targeting. Finally, some of the CRISPRa systems require many components, making them potentially much more complicated to deliver than the one-component ZF or TALE activators. These characteristics must all be taken into account when deciding which activation system to use.

CRISPRi VERSUS PREVIOUS REPRESSION METHODS

CRISPRi also offers several advantages over previous forms of gene repression, although it is not necessarily the best choice for every assay (Table 2). The most widely used repression tool predating CRISPRi is RNAi technology (65). RNAi can refer to either to small interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs), which make use of the cell's endogenous siRNA and microRNA (miRNA) pathways for processing and function (75). These pathways are naturally occurring cellular processes used to

TABLE 1 Comparison of CRISPRa to other activation methods[a]

Activation method	Activated gene	Ease of production	Throughput of production	Used in pooled genome-wide screens	Off-target effects	Blocked by methylated DNA	Expression of mutant alleles or specific splice variants	Limitations in use	No. of components required	References
CRISPRa	Endogenous	Easy (simple sgRNA cloning)	High	Yes	Minimal	No	Can target specific variants only if different variants have different TSSs; not ideal for expressing mutant alleles	Requires an "NGG" PAM adjacent to a target sequence	2 or 3 (dCas9, sgRNA, optional activation module); these may be collected on one vector in some cases	3, 4, 24, 26, 29, 30, 32, 44
ORF overexpression	Exogenously added	Medium to difficult (entire ORF of gene must be cloned)	Low	Yes, but libraries are burdensome to create and can contain biases toward smaller ORFs or certain splice variants	Nonexistent	NA	Yes	Longer or GC-rich genes can be difficult to clone	1	45, 46–49
TALE or ZF	Endogenous	Medium to difficult (requires complicated cloning [TALEs] or protein engineering [ZFNs])	Low	No	Minimal	TALEs can be blocked by methylated DNA but can also recognize it specifically	Can target specific variants only if different variants have different TSSs; not ideal for expressing mutant alleles	TALEs have no sequence limitations. ZFNs may require some engineering to target a given sequence	1	39, 50, 51, 52–53, 54–56

[a] NA, not applicable; TSS, transcription start site.

TABLE 2 Comparison of CRISPRi to other repression methods

Repression method	Mechanism of action	Used in pooled genome-wide screens	Off-target effects	Ability to target small RNAs	Ability to target specific splice variants	Limitations in targeting	No. of components required	References
CRISPRi	Transcriptional elongation of mRNA is sterically blocked; the KRAB fusion recruits repressive chromatin marks	Yes	Minimal	Yes	Only if different variants have different TSSs	Requires an "NGG" PAM adjacent to a target sequence	2 or 3 (dCas9, sgRNA, optional repression module); these may be collected on one vector in some cases	3, 4, 20, 24, 26, 29, 30, 64
RNAi	The target mRNA is sequestered or degraded	Yes	Extensive	No	Yes	No	1	65–69
TALE or ZF	Transcriptional elongation of mRNA is sterically blocked; the KRAB fusion recruits repressive chromatin marks	No	Minimal	Yes	Only if different variants have different TSSs	TALEs have no sequence limitations; ZFNs may require some engineering to target a given sequence	1	39, 50, 51, 54–56
miRNA sponges or antagomirs	Both miRNA sponges and antagomirs act as dominant negatives by binding to miRNAs and preventing them from acting on their target mRNAs	No	miRNAs in a family sharing the same seed bind to sponges; antagomirs can distinguish between family members	Yes	NA	Sponges are not ideal for selectively targeting 1 miRNA in a family sharing the same seed	1; also, a single sponge can repress multiple miRNAs simultaneously	70–74

regulate mRNA translation and stability, and both employ small (approximately 18- to 24-nt) double-stranded RNAs (dsRNAs). During siRNA and miRNA processing, the precursor dsRNA is loaded into the RNA-induced silencing complex (RISC), where one strand of the RNA is degraded, while the other (the guide strand) remains. The mature RISC is then guided to its target mRNA, where it causes repression in various ways (reviewed in more detail in references 76 and 70).

When the guide strand shares perfect complementarity with its mRNA target, the target is directed down the siRNA pathway and is degraded through endonucleolytic cleavage (76). When there is imperfect complementation between the guide and the target mRNA but a match in the guide strand's seed region (nucleotides 2 to 8), the mRNA is directed down the miRNA pathway (71). Repression through the miRNA pathway can cause degradation by mRNA deadenylation or suppression of translation initiation or elongation (70, 76).

Since RNAi makes use of the cell's endogenous small RNA processing machinery, only a single, small component must be added to achieve repression: the shRNA or siRNA. This may make RNAi more advantageous in some systems, as other repression mechanisms may be more cumbersome to deliver. Some caution must still be taken, as there is evidence that hijacking the RISC using RNAi may prevent endogenous miRNAs from functioning (66).

RNAi-based screens have revolutionized our ability to test the function of many genes at once through pooled, genome-wide knockdown screens. However, there are many concerns about off-target effects and reproducibility. For example, meta-analyses of RNAi screens have shown little reproducibility between different studies (67, 77). This is in large part due to the prevalence of RNAi off-target effects. siRNAs and shRNAs act through a miRNA-like mechanism when the seed region of the dsRNA binds to an mRNA (68, 78). Hundreds of transcripts with a given seed may exist, and there is not currently a high-confidence method to rank these alternative targets for the likelihood of being a true off-target (71). It is possible that a large number of RNAi screen "hits" are due to off-target effects from such a mechanism. While this can be accounted for, it requires testing with mutated versions of the siRNA or shRNA and looking for a matching phenotype, which can be burdensome (79).

One way to reduce the rates of false negatives and false positives is to have many unique shRNAs (or sgRNAs in the case of CRISPR) that target each gene in the library pool. To find the ideal shRNA or sgRNA coverage and design, multiple studies have performed small-scale pooled screens with very high coverage of shRNAs or sgRNAs per gene (29, 69, 80, 81). These studies each identified high-confidence hits and then computationally subdivided their libraries to (i) discover the number of sgRNAs required to distinguish true hits from the background and (ii) define rules that make more effective shRNAs and sgRNAs. Thus, bioinformatic modeling and iterative analysis and testing large pools of sgRNAs have resulted in significant improvements in both RNAi and CRISPRi (29, 69). It is now possible to use a library with 10 shRNAs or 10 sgRNAs per gene to produce robust results in a screen, although both RNAi and CRISPRi will no doubt benefit from further refinement in the future.

Ultimately, the choice of whether to use CRISPRi or RNAi will depend on the requirements of a given user. For small-scale use targeting only a few genes, CRISPRi has simpler design rules can

achieve very high levels of knockdown (29). However, RNAi can be advantageous in that one can target specific splice variants over others, which is not possible with CRISPRi unless the different variants have different transcription start sites. Additionally, it has been shown that off-target effects from siRNAs can result in cell toxicity in a cell type-dependent manner (82). This has not yet been seen with CRISPRi, although the possibility has not been systematically investigated. It may be that certain cell types tolerate RNAi or CRISPRi better, which will need to be determined empirically. Finally, CRISPRi is a two-component system, whereas RNAi is a one-component system. In assays where delivering two components may be an issue, it may be more desirable to use RNAi.

In the study of noncoding RNAs (ncRNAs), CRISPRi also offers many advantages. Noncoding RNAs such as microRNAs (miRNAs) and lncRNAs can be targeted by CRISPRi in the same manner as coding genes (29, 64). Since many miRNAs are redundant, one can potentially make use of the multiplexing capability of CRISPRi to hit all miRNAs in the same targeting "family" at once. One alternative approach to CRISPRi is the use of antagomir miRNA inhibitors, which are modified oligonucleotides that are antisense with respect to the target miRNA (72, 73). They bind to the miRNA with high affinity and prevent it from acting on its target mRNA. However, these miRNA inhibitors can be expensive and are specific for a single miRNA. An alternative approach is to create miRNA "sponges," an array of tandem repeats of miRNA seed sequences, which act by sequestering active miRNAs and preventing them from acting on their true targets (74). Since sponges consist of an array of repeats, they can be difficult to synthesize or clone. However, they may be the better choice if a user wants to repress all miRNAs of the same family, which share the same seed.

CONCLUSIONS

The present is an exciting time for biologists, bioengineers, and clinicians—anyone who has an interest in the effect of genes and how to control them. CRISPR technology is ushering us into an unprecedented era of biological control. Our toolkit keeps expanding, with each new addition bringing greater precision and power. While we have advanced much in such a short time, much work remains to be done to continuously refine this technology. We have only just begun to use these tools, and many basic technical, biological, and biomedical questions remain. With CRISPRa/i, we have a powerful new means of answering them.

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