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 (sgRNA) 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 from 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 act as a scaffold to recruit a broad range of effectors or markers necessary to disrupt its nuclease activity. Cas9’s two nuclease domains, the RuvC and HNH domains, are conserved among several bacterial species, this approach provides 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.

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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One type of effector that can be fused to dCas9 is a transcriptional activator. 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 so 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.
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 βt (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-p300core 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-p300core 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 PWRP 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-
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 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 a small library as possible, which is particularly important in working with systems in which 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...
CRISPRI VERSUS PREVIOUS REPRESSION METHODS

| Method | Cas9 | dCas9 | TALEs | ZF | sgRNA cloning
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<tr>
<td><strong>Activation</strong></td>
<td>Medium</td>
<td>Low</td>
<td>Minimal</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Ease of production</strong></td>
<td>Moderate</td>
<td>Low</td>
<td>High</td>
<td>NA</td>
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| **Activation** | Easy (simplesgRNA) | Low | Minimal | No | Can target specific
| **Off-target effects** | Low | Low | Low | Low | Low |
| **Expression of mutant alleles or specific splice variants** | Limitations in use | Low | Low | Low | Low |
| **Gene repression** | Requires an “NGG” PAM | Low | Low | Low | NA |

**Comparison of CRISPRa to other activation methods**

CRISPR activators (CRISPRa) can target specific sequences, but they require more complicated cloning from the cDNA, one may be missing physiologically relevant smaller and easier-to-amplify genes. Additionally, when a small interfering RNA (siRNA) is used, it is possible to overexpress a certain splice variant or a mutant allele of an ORF. However, overexpression is not the best choice for studying miRNA pathways for processing and function (75). Additionally, when a specific splice variant is needed, it may be advantageous to use CRISPRi over CRISPRa, as CRISPRi can target specific sequences and CRISPRa can target large regions of DNA. However, CRISPRi can also be used for precise gene editing, such as gene knockouts or knock-ins. Another advantage of CRISPRi is that it can be used for multiplexing, allowing the targeting of multiple genes simultaneously. Additionally, CRISPRi can be used for genome-wide screens, making it a powerful tool for identifying genes involved in specific pathways or processes. On the other hand, CRISPRa has limitations, such as the need for precise sgRNA design and the potential for off-target effects. However, CRISPRa can be used for gene activation, making it a useful tool for studying transcriptional regulation. Overall, the choice of CRISPR method depends on the specific experimental needs and goals.
Minireview

Requires an “NGG” optional repression module; these may be collected on one vector in some cases

RNAi The target mRNA is sequestered or degraded

Yes Minimal Yes Only if different

Ability to target specific splice variants

Limitations in targeting PAM adjacent to a target sequence

No Only if different TSSs

TABLE 2 Comparison of CRISPRi to other repression methods

| Repression method | Used in pooled genome-wide screens | Off-target effects | 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

| CRISPRi | Yes | NA |
| CRISPR | Yes | Yes |
| TALE or ZF | No | Yes |
| RNAi | No | Yes |
| miRNA sponges or antagomirs | Yes | Yes |

Both miRNA sponges and antagomirs act as dominant negatives by binding to miRNAs and preventing them from acting on their target mRNAs.

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| CRISPRi | Yes | NA |
| CRISPR | Yes | Yes |
| TALE or ZF | No | Yes |
| RNAi | No | Yes |
| miRNA sponges or antagomirs | Yes | Yes |

Both miRNA sponges and antagomirs act as dominant negatives by binding to miRNAs and preventing them from acting on their target mRNAs.

TABLE 2 Comparison of CRISPRi to other repression methods

| Repression method | Used in pooled genome-wide screens | Off-target effects | 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 IncRNAs 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 antagonism of 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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