Review in Advance first posted online on April 25, 2016. (Changes may still occur before final publication online and in print.)

CRISPR/Cas9 in Genome Editing and Beyond

Haifeng Wang,[1] Marie La Russa,[1,2] and Lei S. Qi[1,3,4]

[1]Department of Bioengineering, Stanford University, Stanford, California 94305; email: stanley.qi@stanford.edu, hfgwang@stanford.edu, mlarussa@stanford.edu

[2]Biomedical Sciences Graduate Program, University of California, San Francisco, California 94158

[3]Department of Chemical and Systems Biology, Stanford University, Stanford, California 94305

[4]Chemistry, Engineering and Medicine for Human Health (ChEM–H), Stanford University, Stanford, California 94305

Annu. Rev. Biochem. 2016. 85:22.1–22.38

The *Annual Review of Biochemistry* is online at biochem.annualreviews.org

This article's doi: 10.1146/annurev-biochem-060815-014607

Keywords

dCas9, Cas9 structure, gene regulation, epigenetic regulation, genomic imaging, CRISPR applications

Abstract

The Cas9 protein (CRISPR-associated protein 9), derived from type II CRISPR (clustered regularly interspaced short palindromic repeats) bacterial immune systems, is emerging as a powerful tool for engineering the genome in diverse organisms. As an RNA-guided DNA endonuclease, Cas9 can be easily programmed to target new sites by altering its guide RNA sequence, and its development as a tool has made sequence-specific gene editing several magnitudes easier. The nuclease-deactivated form of Cas9 further provides a versatile RNA-guided DNA-targeting platform for regulating and imaging the genome, as well as for rewriting the epigenetic status, all in a sequence-specific manner. With all of these advances, we have just begun to explore the possible applications of Cas9 in biomedical research and therapeutics. In this review, we describe the current models of Cas9 function and the structural and biochemical studies that support it. We focus on the applications of Cas9 for genome editing, regulation, and imaging, discuss other possible applications and some technical considerations, and highlight the many advantages that CRISPR/Cas9 technology offers.

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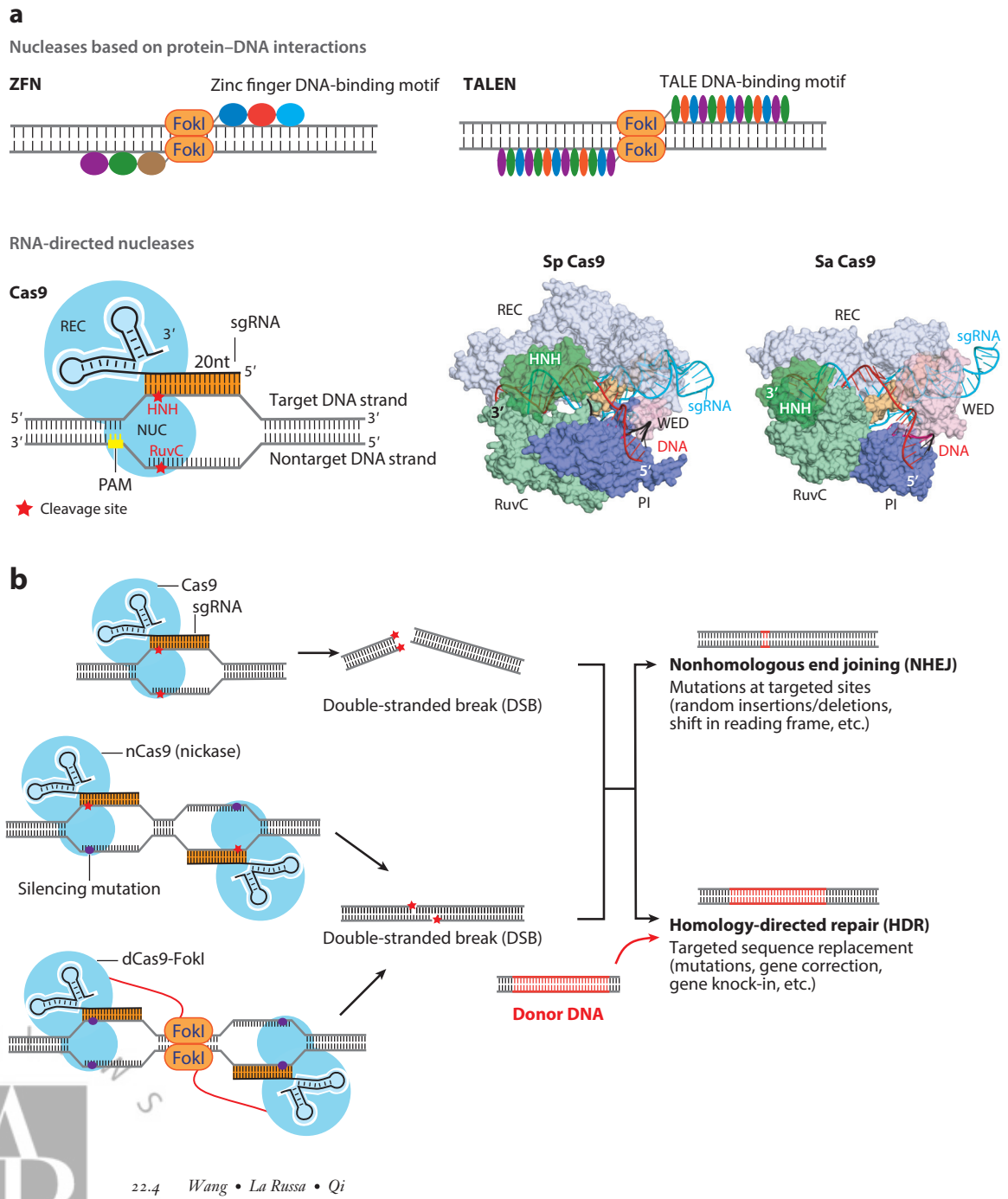
INTRODUCTION: TOOLS FOR PROGRAMMABLE GENOME EDITING, TARGETING, AND REGULATION

Since the advent of the central dogma of molecular biology, scientists have endeavored to develop new technologies to modify or manipulate the genome. Precise editing and regulation of genomic information is essential to understanding the function of a given gene. During the past decade, technological breakthroughs have made genome editing and regulation significantly easier. One recent technology has adapted the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated protein) bacterial immune system as a simple, RNA-guided platform for highly efficient and specific genome editing and regulation in diverse organisms, thus creating revolutionary tools for biomedical research and new possibilities for treating genetic disorders (1–14).

In general, the precise editing or regulation of genomic information at the DNA level requires the action of a molecular machine composed of two major parts: a DNA-binding domain that mediates sequence-specific DNA recognition and binding, and an effector domain that enables DNA cleavage or regulates transcription near the binding site. Creating a double-stranded break (DSB) by using a sequence-specific endonuclease can stimulate the DNA repair pathway and greatly increase the rate of gene modification at the desired sequence (15–20). Thus, nuclease-mediated approaches have been extensively explored for site-specific gene editing. Meganucleases, also called homing nucleases, are among the first classes of nucleases that were engineered to target specific genomic sites for gene editing purposes (15, 16, 21). Meganucleases are a group of nucleases that recognize long nucleotide sequences and induce a DSB at their targeted site. The long recognition sequence of meganucleases may occur only once within a genome, thereby facilitating its use for site-specific genome editing. Meganucleases can be reengineered to target novel sequences through strategies such as protein engineering, structure-based design, and molecular evolution, although the procedure is usually labor intensive (20–22).

Other examples of programmable genome editing machines include zinc-finger nucleases (ZFNs) (23–25) and transcription activator-like effector nucleases (TALENs) (26–28), in which the DNA-binding domains of transcription factors have been fused with the nuclease domain of the restriction enzyme FokI, an obligate dimer (**Figure 1*a***). When targeted to paired adjacent sequences, the FokI domains of these programmable, site-specific nucleases form a dimer that activates the nuclease activity, thus creating a DSB near their binding sites (**Figure 1*a***). Researchers can exploit the cell's endogenous DNA repair pathways to create mutations at the desired DSB sites. However, because these tools function through protein–DNA interactions, targeting to a new site requires engineering and cloning a new protein, which precludes ZFNs and TALENs from being used for high-throughput applications.

In contrast to most known DNA-binding proteins, Cas9 is an RNA-guided nuclease whose sequence specificity largely arises from Watson–Crick base pairing between its guide RNA and the target DNA site, in addition to a direct interaction between Cas9 and a short protospacer-adjacent motif (PAM) of DNA (3, 4, 13, 29, 30). Thus, Cas9 can be programmed to target new sites simply by changing its guide RNA sequence, making it an ideal platform for high-throughput



sequence-specific gene editing, as well as other applications. Its natural endonuclease activity has been co-opted for sequence-specific editing of the genome in a wide range of organisms, including bacteria (31), fungi (32), plants (33, 34), and animals (5–7, 9, 10, 35, 36). To enable sequence-specific genomic regulation, nuclease-deactivated Cas9 (dCas9) has been engineered, and can be fused to a variety of effectors, such as transcriptional activators, repressors, and epigenetic modifiers (37–41).

In addition to its applications in genome editing and regulation, DNA-binding proteins, such as ZFs, TALEs, and dCas9, have been fused to fluorescent proteins (FPs) to allow direct imaging of genomic loci in living cells (42–47). Additionally, dCas9 has also been used for studying proteins that interact with specific loci (48, 49), and it may potentially be used to target RNA (50, 51). In this review, we describe the working mechanism of Cas9 based on the findings of structural and biochemical studies. We focus on the applications of CRISPR/Cas9 in genomic editing, regulation, and imaging in mammalian cells, highlighting the power of this novel system in biological research.

CRISPR/CAS9: A GIFT FROM MOTHER NATURE

CRISPR: An Adaptive Immune Mechanism in Bacteria and Archaea

The CRISPR system is an adaptive immune mechanism present in many bacteria and the majority of characterized Archaea. CRISPR-containing organisms acquire DNA fragments from invading bacteriophages and plasmids before transcribing them into CRISPR RNAs (crRNAs) to guide cleavage of invading RNA or DNA (1, 13, 29, 30, 52–56). This CRISPR immune system works through the cooperation of many diverse Cas proteins. Based on differences in their components and mechanisms of action, CRISPR systems have been divided into two major classes (57). RNA-guided target cleavage in class 1 systems (types I, III, and IV) requires a large complex of several effector proteins, but in the class 2 systems [type II, putative types V (58) and VI (59)], only one RNA-guided endonuclease [e.g., Cas9 in type II and Cpf1 (CRISPR from Prevotella and

Figure 1

CRISPR-associated protein 9 (Cas9)-mediated sequence-specific genomic editing. (*a*) Comparison of programmable sequence-specific genome editing nucleases. Zinc-finger nucleases (ZFNs) and transcriptional activator-like effector nucleases (TALENs) are engineered by fusing ZF or TALE DNA-binding domains to the FokI nuclease domains (23–28). They recognize their targeted sites by sequence-specific protein–DNA interactions, and a pair of ZFNs or TALENs cleaves adjacent sequences of the DNA to create a pair of nicks on complementary strands, leading to a double-stranded break (DSB). Cas9 is a naturally evolved, RNA-guided nuclease that recognizes its target DNA through approximately 20 nucleotide (nt) base-pairing interaction between a single guide RNA (sgRNA) and its targeted DNA strand. Cas9 also interacts with the protospacer-adjacent motif (PAM) of its DNA target through its PAM-interacting (PI) domain at its C terminus. Cas9 uses its two nuclease domains (HNH and RuvC) to cleave the double-stranded DNA, creating DSBs. The HNH, RuvC, and PI domains, as well as an evolutionarily divergent wedge domain (WED), all reside in the Cas9 nuclease (NUC) lobe. The recognition (REC) lobe of Cas9 contains other regions that interact with the sgRNA–DNA duplex. (*Bottom right*) Crystal structures of Sp Cas9 and Sa Cas9. Crystal structures of *Streptococcus pyogenes* Cas9 (Sp Cas9; Protein Data Bank number 4UN3, 1368 AA) (84) and *Staphylococcus aureus* Cas9 (Sa Cas9 Protein Data Bank number 5CZZ, 1053 AA) (70) were obtained from RCSB Protein Data Bank (**http://www.rcsb.org/pdb/**), compared using PyMOL (PyMOL Molecular Graphics System, Version 1.3, Schrödinger LLC, **https://www.pymol.org/**), and domains are annotated according to References 70, 81, 83, 84. The orientation of the target DNA strand is also shown. (*b*) Cas9 in genomic editing. The DSB generated by Cas9 activates the nonhomologous end joining (NHEJ) or homology-directed (HDR) DNA repair pathways. NHEJ causes random insertions or deletions (indels) at its targeted site, and HDR can create desired mutations or indels through homologous recombination guided by donor DNA. A mutation in one nuclease domain of Cas9 creates a Cas9-based nickase (nCas9) that cleaves only one strand of DNA. The specificity of Cas9-mediated genome editing can be greatly enhanced by using a pair of nCas9s that target each strand of DNA at adjacent sites because both nCas9–sgRNA complexes must be present at the target site for DSB creation (5, 76–78). A similar strategy has been achieved by using paired nuclease-deactivated Cas9 (dCas9)-FokI–sgRNA complexes (153, 154).

Francisella-1) in type V] is required to mediate cleavage of invading genetic material (57–59). Detailed descriptions of CRISPR system classification can be found in References 53, 54, 57, 59, and 60.

In general, a CRISPR system works in three stages to carry out a full immune response to invading foreign DNA (9, 13, 14, 53–56, 61, 62). In the first stage, or acquisition stage, DNA fragments of invading plasmids or phages (termed protospacers) are incorporated into the host CRISPR locus as spacers between crRNA repeats. In the second stage, Cas proteins are expressed, the CRISPR array containing acquired spacers is transcribed into pre-crRNA, and the pre-crRNA is cleaved and processed into mature crRNAs by Cas proteins and host factors (14). The fully processed crRNA is a guide that contains a spacer sequence responsible for targeting it to the invading genome, as well as all or part of the crRNA repeat sequence, which allows for recognition of the crRNA by Cas proteins and other RNA components. In type II CRISPR systems, the presence of a noncoding *trans*-activating CRISPR RNA (tracrRNA) that hybridizes with the crRNA repeat sequence is critical for crRNA processing, Cas9 binding, and Cas9-mediated target cleavage (3, 14). In the third stage, Cas proteins recognize the appropriate target with the guidance of the crRNA and mediate the cleavage of the invading genome, thus protecting the host cells from infection. The action of many CRISPR systems depends on the presence of a sequence-specific PAM that is adjacent to the crRNA target site in the invading genome (30, 63–65). The absence of this PAM sequence at the CRISPR locus in the host genome protects it from self-cleavage in type I and type II CRISPR systems (9).

Repurposing Cas9 for Sequence-Specific Genomic Targeting

Many characterized Cas proteins bind to nucleic acids; thus, the CRISPR system can form the basis of a flexible genomic engineering toolkit. Cas9, the RNA-guided endonuclease that cleaves target DNA in the class 2 type II CRISPR system, is the most widely used for genomic editing and regulation among the Cas proteins.

Cas9 target cleavage is guided by a duplex of two RNAs: the crRNA that recognizes the invading DNA through an approximately 20 base pair (bp) Watson-Crick base-pairing region and the tracrRNA that hybridizes with the crRNA and is unique to the type II CRISPR system (3, 4, 12–14, 66). Cas9, in conjunction with the crRNA–tracrRNA duplex, can be repurposed for efficient genomic editing (3, 5, 31). To simplify the system, a seminal work showed that the crRNA–tracrRNA duplex can be fused into a chimeric single guide RNA (sgRNA) (3). This single-protein, single-RNA, Cas9–sgRNA system is the most widely used for gene editing and other Cas9-based applications.

The Cas9–sgRNA complex can bind DNA that base pairs with the sgRNA and is adjacent to a PAM sequence (**Figure 1*a***). Binding of the Cas9–sgRNA complex induces cleavage within the base-pairing region. Thus, simply by customizing an approximately 20 nucleotide (nt) region of the sgRNA to pair with the DNA sequence of interest, Cas9 can be retargeted to essentially any genomic locus containing a PAM sequence, making it an easily programmable platform for specific genomic targeting.

Diversity of Cas9 Orthologs

A large variety of Cas9 proteins exist in different bacterial type II CRISPR systems. These Cas9 nucleases range from about 900 to 1,600 amino acids (AA) in three subclasses: type II-A, type II-B, and type II-C (53, 54, 67). The most commonly used Cas9 for genome engineering has been adapted from the type II-A CRISPR system from *Streptococcus pyogenes* (Sp). The Sp Cas9 has a

simple PAM (NGG, or a weaker NAG, where N is any nucleotide) and has been optimized for use in editing, as well as other contexts, using dCas9 across a wide variety of organisms (68).

Other Cas9 proteins have been studied and developed as tools. Of note is the Cas9 derived from *Staphylococcus aureus* (Sa), which is 1,053 AA in length, with NNGRRT (where R is an A or G) as its PAM (69). The relatively small size of Sa Cas9 allows it to circumvent some of the delivery issues caused by the larger Cas9s, such as Sp Cas9 (1,368 AA; see the section Use and Delivery of Cas9 for further discussion). Sa Cas9 has been developed for genome editing (69) and gene regulation (70) in mammalian cells, and it shows gene editing efficiency comparable to that of Sp Cas9 (69). In addition to Sp Cas9 and Sa Cas9, other notable orthologs include Cas9 from *Neisseria meningitidis* (Nm; PAM = NNAGAAW, where W is an A or T) and *S. thermophilus* 1 (St1; PAM = NNNNGATT), which have been used in both bacteria and mammalian cells (71–74). Both Nm Cas9 and St1 Cas9 have been engineered into dCas9 versions that have been used for gene regulation (38, 39, 71).

In addition to their distinct PAMs, these different Cas9s also have distinct crRNAs and tracrRNAs, which allow for the possibility of orthogonal genome editing, regulation, and imaging. Cas9–tracrRNA binding is sensitive to minor perturbations in the tracrRNA sequence and structure (75), which reinforces the orthogonality of these different Cas9s. Although there has been some work using multiple Cas9s simultaneously to achieve distinct targeting and function (71, 72), this area remains relatively underexplored.

Engineered Variants of the Cas9 Nuclease Domains: Nickase Cas9 (nCas9) and Nuclease-Deactivated Cas9 (dCas9)

Cas9 contains two nuclease domains: an HNH nuclease domain that cleaves the target strand of DNA (complementary to the guide RNA) and a RuvC-like nuclease domain that cleaves the nontarget strand (**Figure 1*a***) (3, 4). Mutating one of the two nuclease domains creates a nickase Cas9 (nCas9) that cleaves only one strand of DNA (**Figure 1*b***) (3, 4, 76–78). Two nCas9s can be targeted to adjacent DNA sites to cause a DSB in a manner similar to that described for TALENs above (77, 79). Pairs of nCas9s have been used to increase the specificity of Cas9-based genomic editing, as only two adjacent nicking events will generate a DSB (76, 77, 80).

Mutating both nuclease domains generates dCas9, which lacks nuclease activity but retains its RNA-guided DNA-binding activity. This allows dCas9 to be fused to other effectors to mediate site-specific genetic and epigenetic regulation without cleaving the target DNA, as well as specific DNA binding for several other applications (37–39, 41) (see discussion in sections Cas9 for Gene Regulation, Cas9 for Epigenome Editing, and Cas9 for Genomic Imaging).

Other RNA-Guided Endonucleases in the CRISPR Systems

Recently, Zetsche et al. (58) discovered that, in the class 2 type V system, Cpf1 also mediates RNA-guided target cleavage and can be adapted for gene editing in cultured human cells. In contrast to Cas9, Cpf1-mediated DNA cleavage is guided by only a crRNA, and does not require a tracrRNA. Additionally, Cpf1 uses different PAMs than those for characterized Cas9s, and it creates a staggered DSB (58). Sequence analysis has revealed that Cpf1 contains only a RuvC-like domain and lacks the HNH nuclease domain found in Cas9 (58). Based on the predicted structure of effector proteins, Shmakov et al. (59) further classified three class 2 CRISPR systems, including C2c1 and C2c3 (subtypes of putative type V) and C2c2 (a subtype of putative type VI). They showed that C2c1 is a DNA endonuclease guided by both a crRNA and a tracrRNA. The discovery of Cpf1 and other effector proteins in the diverse class 2 CRISPR systems further

expands the toolkit of programmable RNA-guided endonucleases for genome editing (57–59). These newly characterized proteins will no doubt be the source of many tool-building efforts in the future.

STRUCTURE OF Cas9 AND PROPOSED WORKING MODEL FOR GUIDE RNA BINDING AND TARGET DNA CLEAVAGE

The crystal structures of Cas9s have been reported, including Sp Cas9, Sa Cas9, and Cas9 from *Actinomyces naeslundii* (Ana Cas9). The structure of Sp Cas9 has been characterized extensively: in unbound (apo) form (81), sgRNA-bound form (82), and sgRNA–DNA-bound form (83, 84). The Ana Cas9 structure has been resolved in its apo form (81), whereas the structure of Sa Cas9 has been reported in complex with sgRNA and DNA (70). These studies have revealed the structural details of interactions among Cas9, the sgRNA, and its DNA target, and also give insight into the structural diversity of Cas9–sgRNA interactions.

Bilobed Structure of Cas9

Cas9 adopts a bilobed architecture composed of a nuclease (NUC) lobe and an α-helical recognition (REC) lobe (70, 81–83) (**Figure 1*a***). The NUC lobe contains the HNH nuclease domain, the RuvC-like nuclease domain, a PAM-interacting (PI) domain, and an evolutionarily divergent wedge domain (WED) (70, 81, 83). The RuvC and HNH nuclease domains use, respectively, a two-metal mechanism and a single-metal mechanism to cleave each of the DNA double strands (70, 81, 83). The PI domain interacts with the PAM region of DNA through base-specific interaction and contributes to the DNA target specificity of Cas9 (70, 81, 83, 84). The WED domain is important for orthogonal recognition of sgRNA scaffolds, and it also interacts with the backbone of the PAM region (70). The helical REC lobe is also diverse among different Cas9s, and it contains regions that contribute to the recognition of guide RNA–target DNA heteroduplexes, as well as specific recognition of cognate sgRNA scaffolds (70, 81, 83).

Both biochemical and structural studies have revealed that Sp Cas9 undergoes a series of conformational changes to activate its DNA cleavage activity (70, 81–86); a working model is detailed in the section Proposed Working Model for Guide RNA Binding and Target DNA Cleavage.

DNA Targeting Specificity of Cas9–Protospacer-Adjacent Motif (PAM) Interactions

The Cas9–sgRNA complex recognizes its DNA target through Watson–Crick base-pairing interactions between the sgRNA and target DNA and through Cas9's interactions with the PAM adjacent to the sgRNA targeting site. The PI domain of Cas9 is composed of two domains: a C-terminal domain and a topoisomerase-homology domain, adjacent to the C-terminal domain (70, 84). Sp Cas9 recognizes a 5′-NGG-3′ PAM, and its PI domain contains two arginine residues (Arg^{1333} and Arg^{1335}) that interact with the GG dinucleotides within the nontarget strand PAM through base-specific hydrogen bonding (84). Sa Cas9 recognizes a 5′-NNGRRT-3′ PAM, and its PI domain contains several residues [e.g., asparagine $(Asn)^{985}$, Asn^{986}, Arg^{991}, and Arg^{1015}] that form base-specific hydrogen bonds with the GRRT bases (70). These base-dependent interactions determine the specificity of PAM recognition by Cas9 orthologs.

Cas9 also contains a phosphate lock loop with residues [e.g., glutamate $(Glu)^{1108}$ and serine $(Ser)^{1109}$ in Sp Cas9, and aspartate $(Asp)^{786}$ and threonine $(Thr)^{787}$ in Sa Cas9] that interact with the

target-strand DNA backbone directly adjacent to the PAM (70, 84). These interactions appear to kink the DNA and facilitate the local, adenosine triphosphate–independent DNA strand separation required to initiate the formation of the sgRNA–DNA duplex (70, 84).

DNA-Targeting Specificity of Cas9: Guide RNA–Target DNA Base-Pairing Interactions

Cas9–sgRNA targeting specificity is largely ensured by base-pairing interactions between the sgRNA and its complementary target DNA strand. Mechanically, the activation of Cas9 nuclease activity requires an HNH domain conformational change that depends on proper base-pairing interactions between the guide RNA and its DNA target, providing another mechanism to ensure the specificity of Cas9 in addition to PAM recognition and guide RNA–target DNA complementarity (86). The extent of this HNH domain conformational change, monitored by intramolecular Förster resonance energy transfer (FRET) assays, is sensitive to mismatches within the guide RNA–target DNA base-pairing region (86).

Base pairing in the PAM-proximal region, referred to as the seed region, is where DNA double-strand separation and sgRNA–DNA heteroduplex formation start. This has a crucial role in determining the binding and cleavage specificity of Cas9 (3, 68, 69, 87–90). The PAM-distal regions are more tolerant of mismatches as assayed by Cas9 binding and cleavage (3, 68, 69, 87). This is consistent with the model in which the Cas9–sgRNA complex first surveys the genome for the PAM site before unwinding DNA, starting with the PAM-proximal portion of the target DNA sequence (87).

Structurally, the PAM-proximal RNA seed region bound to Cas9 maintains an A-form conformation to facilitate sgRNA–DNA heteroduplex formation (70, 82). Biochemical work has revealed that binding to at least 10 nt of the guide RNA seed region is required to trigger a conformational change within Sp Cas9 for DNA recognition (82), and FRET assays have shown that shorter guide RNAs induce lower levels of conformational changes than a full-length guide RNA (86).

Interactions between the Single Guide RNA–DNA Duplex and Cas9

Structural studies also have demonstrated that the sgRNA–DNA duplex interacts with Cas9 through sequence-specific and nonspecific interactions, providing more insights into its functional mechanism (70, 82–85). The sgRNA consists of three key regions from 5′ to 3′: a guide RNA–target DNA heteroduplex region where the sgRNA base pairs with the target DNA, a repeat–antirepeat duplex that represents a hybridization region between crRNA and tracrRNA, and additional stem-loops that are found in the tracrRNA in the endogenous CRISPR locus (3, 14, 83). In general, the guide RNA–target DNA heteroduplex and the repeat–antirepeat duplex are both located in a positively charged groove between the two lobes of Cas9. The additional sgRNA stem-loops also interact with charged residues on the surface of Cas9 to enforce the interaction between Cas9 and its cognate sgRNA.

Cas9 recognizes the guide RNA–target DNA heteroduplex region in a sequence-independent manner, primarily through interactions with its phosphate backbone, but interactions between many other regions of the sgRNA and Cas9 depend on the sgRNA sequence and folding (70, 75, 83, 85). These aspects of Cas9 structure allow it to flexibly target any PAM-adjacent DNA sequences with paired sgRNAs while at the same time precisely recognizing guide RNAs containing specific sequences and structures (70, 83, 85).

Orthogonality of Cas9–Single Guide RNA Interactions

The comparison of different Cas9 structures provides insights into the orthogonal DNA-targeting mechanism of Cas9s (70, 81, 83, 84). In addition to binding to their specific PAM motifs, Cas9 orthologs recognize their cognate sgRNA scaffolds through sequence-specific and structure-specific interactions (70, 83, 84). For example, the repeat–antirepeat duplexes are significantly different between Sp sgRNA and Sa sgRNA. Their distinct structural features are recognized by the WED domains and REC lobes of their respective Cas9s in a highly specific manner (70, 83, 84). Moreover, three stem-loops of the Sp sgRNA are required for efficient Cas9-mediated DNA cleavage in vivo (68, 83), but sequence predictions suggest that the Sa sgRNA may contain only two stem-loops (69, 70). The phosphate backbones and some residues in these stem-loops also interact with different regions of their cognate Cas9s in a structure-specific or base-specific manner (70, 83, 84). The structural dependence of Cas9–sgRNA interactions forms a basis for orthogonal recognition of sgRNA and, thus, orthogonal DNA targeting, and it underscores the importance of maintaining interaction-relevant nucleotides when optimizing the sgRNA scaffold.

Proposed Working Model for Guide RNA Binding and Target DNA Cleavage

A working mechanism of Cas9 has been proposed by combining structural studies (70, 81–85) and in vitro assays (86–88, 91). In this model, the Cas9 protein maintains an autoinhibited conformation when not bound by sgRNA in which the active sites in the HNH domain are blocked by the RuvC domain (81). The binding of an sgRNA induces a conformational change to create a central channel between the two lobes for DNA binding (70, 81–86), thus entering into a DNA recognition–competent state (82). The resulting Cas9–sgRNA pretargeting complex can survey DNA for PAMs by three-dimensional diffusion (87, 92). The Cas9–sgRNA complex binds to a PAM through its PI domain, which initiates local DNA strand separation in the PAM-proximal region to facilitate sgRNA–DNA heteroduplex formation (84). The Cas9–sgRNA complex will continue to unwind the DNA only if there is a significant match between the guide RNA segment and the target DNA (82, 86). The strong guide RNA–target DNA base-pairing interactions further promote DNA double strand separation and RNA–DNA heteroduplex formation, which proceeds from the PAM-proximal region and forms a complete R loop (86–88). Finally, the complete R loop causes another conformational change in the HNH domain, activating the nuclease activity of both the RuvC and HNH domains to induce DNA cleavage (81–88). Sp Cas9 creates a DSB 3 bp upstream of PAM in the target DNA.

Cas9 FOR GENOME EDITING

Mechanism of Genome Editing: DNA Cleavage Followed by DNA Repair

Since its discovery, Cas9 has been extensively used for genome editing in multiple organisms. Cas9, like engineered ZFNs and TALENs, is a programmable, sequence-specific endonuclease. Similar to other nucleases, Cas9-mediated genome editing is achieved by a two-step process: DNA cleavage followed by DNA repair (**Figure 1*b***). The sgRNA directs Cas9 to a specific genomic locus where Cas9 creates a DSB (3, 4), which triggers DNA repair through intrinsic cellular mechanisms, such as nonhomologous end joining (NHEJ) or homology-directed repair (HDR) (15–19).

NHEJ causes nearly random insertion and deletion mutations (i.e., indels) at the DSB site and, thus, may lead to gene knockout (e.g., by causing a shift in the target gene's reading frame

or mutating a critical region of the encoded protein) (**Figure 1*b***) (93). HDR can be exploited to generate the desired sequence replacement at the DSB site through homologous recombination guided by a donor DNA template, causing targeted gene deletion, mutagenesis, insertion, or gene correction (**Figure 1*b***) (17, 19). Thus, the CRISPR/Cas9 system provides a powerful platform for sequence-specific genome editing, including gene knockout, gene knockin, and site-specific sequence mutagenesis and corrections (9, 10, 35).

Applications of Cas9-Mediated Genome Editing for Studying Gene Function, Disease Modeling, and Gene Therapy

The Cas9-mediated gene-editing system has been broadly used in reverse genetics studies to understand the role of specific genes, for disease modeling, and for demonstrating new therapeutic schemes in a number of models of genetic and infectious diseases (9, 10, 94).

Retargeting Cas9 to a new DNA site is easy to achieve by simply creating a new sgRNA that pairs with the desired DNA targeting site adjacent to PAM. In the case of Sp Cas9, the NGG PAM motif occurs, on average, once every 8 bp within the genome, thus allowing almost any gene of interest to be targeted (9, 10, 35). Cas9s from other species have different PAMs, of different sizes and comprising a variety of sequences, which further expands the range of Cas9-targetable genomic sequence [e.g., Sa Cas9 (69), St1 Cas9, and Nm Cas9 (71)]. The molecular evolution of existing Cas9s has also led to the creation of new versions of Cas9 with altered PAM sequences (95, 96), thus expanding the targetable space within the mammalian genome.

The use of the Cas9 platform has greatly increased the efficiency of generating transgenic organisms, from fungi (32) and plants (33, 34, 97, 98) to a variety of animals (5–7, 36, 99–102) (reviewed in 9, 10, 35). This technology also makes it much easier to generate disease models for genetic disorders and diseases such as cancer, which aids our understanding of the molecular mechanisms of these pathological processes (reviewed in 9, 10, 103).

Cas9 can be easily programmed to edit multiple genomic loci at the same time by introducing several sgRNAs simultaneously. This can be applied to generate large-scale chromosomal rearrangements. For example, creating a pair of DSBs at nearby regions within the same chromosome may produce targeted deletions or inversions of the intermediate segment of DNA (104–110), and creating two DSBs in different chromosomes may lead to a targeted chromosomal translocation (107, 111). These Cas9-mediated, targeted rearrangements may be useful for creating disease models by mimicking rearrangements that occur in human disease states (e.g., cancers and heritable genetic disorders) (107, 110, 111).

The Cas9 system also has the potential to cure or treat many maladies, including HIV, genetic diseases, and cancer (94, 112). For example, when Cas9 is introduced into infected cells together with sgRNAs targeting crucial viral genomic elements, it helps to inactivate or clear the viral genome and, thereby, defends the cells or organism from infections with HIV (113, 114), hepatitis B virus (115–117), human papillomavirus (118), and Epstein–Barr virus (119). Moreover, it has been shown by using CRISPR/Cas9 (120, 121) or ZFNs (122–124) that editing the genes of HIV coreceptors (e.g., *CCR5*) in the host genome, which encodes a coreceptor of HIV, creates cellular resistance to the HIV-1 virus and, thereby, may help to combat infection.

In addition, many studies have reported using the Cas9-mediated genome editing system for correcting disease-related mutations in animal somatic (125) and germ line cells (126–128), as well as in human stem cells (129) and induced pluripotent stem cells (130–136). A partial list includes the *Fah* gene in hereditary tyrosinemia (125), *Dystrophin* in Duchenne's muscular dystrophy (126, 133), *Crygc* in cataracts (127, 128), *CTFR* in cystic fibrosis (129), *HBB* in β-thalassemia

(132, 134, 135), *JAK2* in polycythemia vera (136), and *SERPINA1* in α-1 antitrypsin deficiency (136) (reviewed in 94, 112, 137).

Applications of Cas9-Mediated Genome Editing for Genome-Wide Functional Screening

Significantly, the Cas9 platform has been used for large-scale genome-wide knockout screens that had been previously unfeasible (138–145). Previously, genomic loss-of-function screening relied on the RNA interference (RNAi) approach, which represses gene expression at the RNA level without affecting the DNA sequence (146–148). In RNAi, a small interfering RNA (siRNA) that base pairs with its target messenger RNA (mRNA) will lead to a decrease in the stability and translation of its target. The siRNA can be synthesized or produced from a vector encoding a short hairpin RNA (shRNA), an artificial RNA molecule containing a hairpin that is then processed into the mature siRNA form by the cell's endogenous small RNA pathway. In this way, large-scale gene knockdown screening can be achieved using a library of siRNAs or shRNAs.

Similarly, by creating a library of sgRNAs targeting gene coding regions, researchers can exploit the CRISPR/Cas9 platform to screen for genes contributing to a biological process. The Cas9–sgRNA approach generates indels at the targeted loci and may cause complete loss of gene function, whereas the RNAi method may lead to only partial gene suppression. Thus, when targeting the same gene, the CRISPR/Cas9 technique may generate a more pronounced phenotype than RNAi, which may make identification of relevant genes easier. One avenue to validate candidate genes identified with the CRISPR/Cas9 approach is to re-express the targeted gene in the knockout strain (143). Similarly, hits discovered with the RNAi approach may be validated by expression of an RNAi-resistant transcript (143).

In terms of limitations in targeting, the CRISPR/Cas9 method can target only a sequence adjacent to PAM, and not all exons contain such a targetable sequence, whereas an siRNA or shRNA library, in principle, can target any mRNA sequence. In addition, a complete gene knockout by CRISPR/Cas9 requires all alleles of the same gene to be mutated, which makes the screening more challenging for cells containing several alleles, such as cancer cells (147). Furthermore, use of the CRISPR/Cas9 knockout approach to study essential genes is challenging, because deletion of essential genes causes a lethal effect that prevents most functional assays. Both methods can form the basis of a successful screen, and the method choice will depend on the needs of the experiment.

Challenges in Cas9-Mediated Genome Editing

Despite Cas9's great potential for both research and therapeutics, improvements can still be made in its specificity, efficiency, and spatiotemporal control (149). One concern with the commonly used Sp Cas9 system is the possibility of off-target effects because the 20 bp targeting sequence in the sgRNA plus the 3 bp PAM may potentially be present elsewhere in the genome (68, 150, 151). Different strategies have been developed to improve Sp Cas9 specificity: optimizing the sgRNA design (68, 142, 152); using paired nCas9s (5, 76–78), paired dCas9-FokI nucleases (153, 154) (**Figure 1*b***), enhanced Sp Cas9 with improved specificity (155), shorter (17–18 bp) sgRNAs or sgRNAs with two unpaired Gs on the 5′ end that are more sensitive to mismatches (156, 157); or decreasing the concentration of the Cas9–sgRNA complex or its length of active time within the cell (68, 158). Although these strategies have greatly improved specificity, they sometimes come at the cost of efficiency.

Another major challenge is to improve the efficiency of precise genome editing via HDR while reducing indel generation through NHEJ. To address this, strategies have been developed to

modulate the HDR:NHEJ ratio, including altering the expression of DNA repair components, using small molecules, synchronizing the cell cycle, and optimizing delivery timing and methods (159–163). It is also imperative to develop tightly regulated platforms, as well as safe and efficient delivery methods, for precise control of Cas9 activity, especially for potential applications in gene therapy.

Responsible Use of Cas9

The rapid progress of Cas9 as a tool for genome editing has transformative potential for use in applications ranging from clinical treatments to agricultural production to population control of disease-carrying insect species. The rapid advances in Cas9 technology have introduced challenges concerning the regulatory controls governing its safe, secure, and ethical applications. Concerns within the community flared up after it was reported that one group had used Cas9 to edit a gene in human embryos, although this was done in nonviable, triploid zygotes (164). There is much debate among scientists, bioethicists, policymakers, and the public about how to ethically and responsibly use gene editing technology in a way that does not hamper beneficial scientific research and discovery (165–171). Regarding the editing of human cells, major questions include, but are not limited to, should editing of human somatic tissues or germ-line cells be regulated and monitored? Should this technology be used in human embryos? If the answer to these questions is yes, in which cases would this be applicable?

In addition to the ability to edit the human genome, Cas9 also offers the possibility of drastically altering ecosystems by editing the genomes of plants and animals. By editing the genomes of crops and livestock, there is a potential to greatly increase the yield of food production. Cas9-based genome editing technology also has been proposed as a possible method for controlling the populations of disease transmitters, such as mosquitoes that transmit malaria (172). This could be accomplished through the use of gene drive technology, which facilitates the rapid spread of genomic alterations in wild populations (reviewed in 173). Although there is potential benefit to using Cas9-based gene drives, there is still much debate about how or if this technology should be used. Major concerns include doubts about our ability to predict the full ecological impact of such genetically modified organisms and our ability to contain or control them once released into the wild.

The rapid development of Cas9 technology underscores our need as a scientific community and as a society for a comprehensive policy regarding the use of genome editing technology. As the rapid pace of biological discovery continues, discussion will be necessary to ensure that genome editing technologies, such as Cas9, will be used in a safe and responsible manner.

Cas9 FOR GENE REGULATION: CRISPR INTERFERENCE (CRISPRi) AND CRISPR ACTIVATION (CRISPRa)

Nuclease-Deactivated Cas9 (dCas9): A Programmable Platform for Sequence-Specific Gene Regulation

In addition to its nuclease activity, Cas9 can serve as a unique platform to recruit protein and RNA factors to a targeted DNA site, and it has been engineered into powerful tools for sequence-specific gene regulation (37, 41, 174, 175). To achieve this, transcriptional activators and repressors are fused to dCas9; dCas9 maintains its ability to bind both the sgRNA and targeted DNA, but it lacks nuclease activity and, thus, can serve as a sequence-specific RNA-guided DNA-binding platform.

In bacterial cells, dCas9 alone can efficiently inhibit the transcription of targeted genes through steric hindrance of transcriptional machinery (**Figure 2*a***) (41, 176). This novel technique is

termed CRISPR interference (CRISPRi), as it interferes with the transcription of RNA. Although CRISPRi is generally highly efficient in prokaryotes, the dCas9–sgRNA complex alone may not be very efficient at silencing gene expression in mammalian cells (41). However, CRISPRi in mammalian cells can be enhanced by fusing dCas9 to a transcriptional repressor domain (e.g., the KRAB domain of Kox1), which leads to successful suppression of reporter and endogenous genes (**Figure 2*a***) (37, 177).

In addition to CRISPRi, CRISPR activation (CRISPRa) has been created by fusing dCas9 to transcriptional activators, such as VP64 and p65AD in mammalian cells (**Figure 2*b***) (37, 76, 175, 177) and the ω subunit of RNA polymerase in bacteria (176). These dCas9 fusions are able to upregulate gene expression in host cells. In this review, we focus on the use of CRISPRi and CRISPRa (CRISPRi/a) in eukaryotic cells. CRISPR/Cas9-based prokaryotic activation and repression is reviewed in Reference 178.

Multimodal CRISPRi/a Function

In addition to direct fusions of an activator or repressor to dCas9, the sgRNA can be modified and turned into a scaffold to recruit transcriptional regulators (76, 179–181). The sgRNAs can be fused to orthogonal protein-interacting RNA aptamers, which recruit specific RNA-binding proteins (RBPs) (**Figure 2*c***). These aptamer-modified sgRNAs are termed scaffold RNAs (scRNAs) (179). Transcriptional activators and repressors can be fused to these RBPs in lieu of dCas9. When orthogonal RNA aptamer–RBP pairs (e.g., MS2–MCP, PP7–PCP, com–Com) are coupled to different sgRNAs, distinct RBP transcriptional modules can be recruited to different genes to achieve multimodal regulation (i.e., simultaneous activation and repression) (179). For example, in the presence of Sp dCas9, one gene can be targeted by an scRNA with an aptamer that will recruit VP64 and cause activation. Another gene can simultaneously be targeted by an scRNA with an aptamer that will recruit KRAB and cause repression (**Figure 2*c***) (179). Thus, this system allows for multimodal regulation of different genes within the same cell using a single Sp dCas9 protein (179).

Strategies to Improve CRISPRa Efficiency

The efficiency of CRISPRa can be dramatically enhanced by recruiting multiple transcriptional activators to upregulate gene transcription. In addition to using multiple sgRNAs tiled along the promoter to recruit multiple dCas9 activators (182–184), other strategies have been developed to recruit multiple transcriptional activators to a dCas9-binding site (180, 184, 185). For example, the synergistic activation mediator (SAM) system uses both dCas9 and sgRNA as scaffolds to recruit multiple activators that function synergistically to enhance the activation of endogenous genes (180). In this system, dCas9-VP64 is combined with a modified sgRNA containing two MS2 RNA aptamers. Each MS2 aptamer recruits a pair of cognate RNA-binding proteins, MCPs, which are fused with the activating domains of p65 and HSF1 (MCP-p65-HSF1) (**Figure 2*b***) (180). This system increases activation efficiency and has been applied to large-scale genomic screening (180).

Another strategy to enhance activation was developed by Tanenbaum et al. (185) and Gilbert et al. (40). In these two articles, the authors combined the dCas9 system with a recently developed multipeptide array, SunTag, to recruit multiple VP64 activator modules to a single dCas9-binding site. Specifically, dCas9 was fused to an array of polypeptides (GCN4s) that can recruit multiple copies (e.g., 10 or 24 copies) of its cognate single-chain variable fragment (scFv, an engineered portion of an anti-GCN4 antibody). The scFv was then fused to VP64, leading to the recruitment

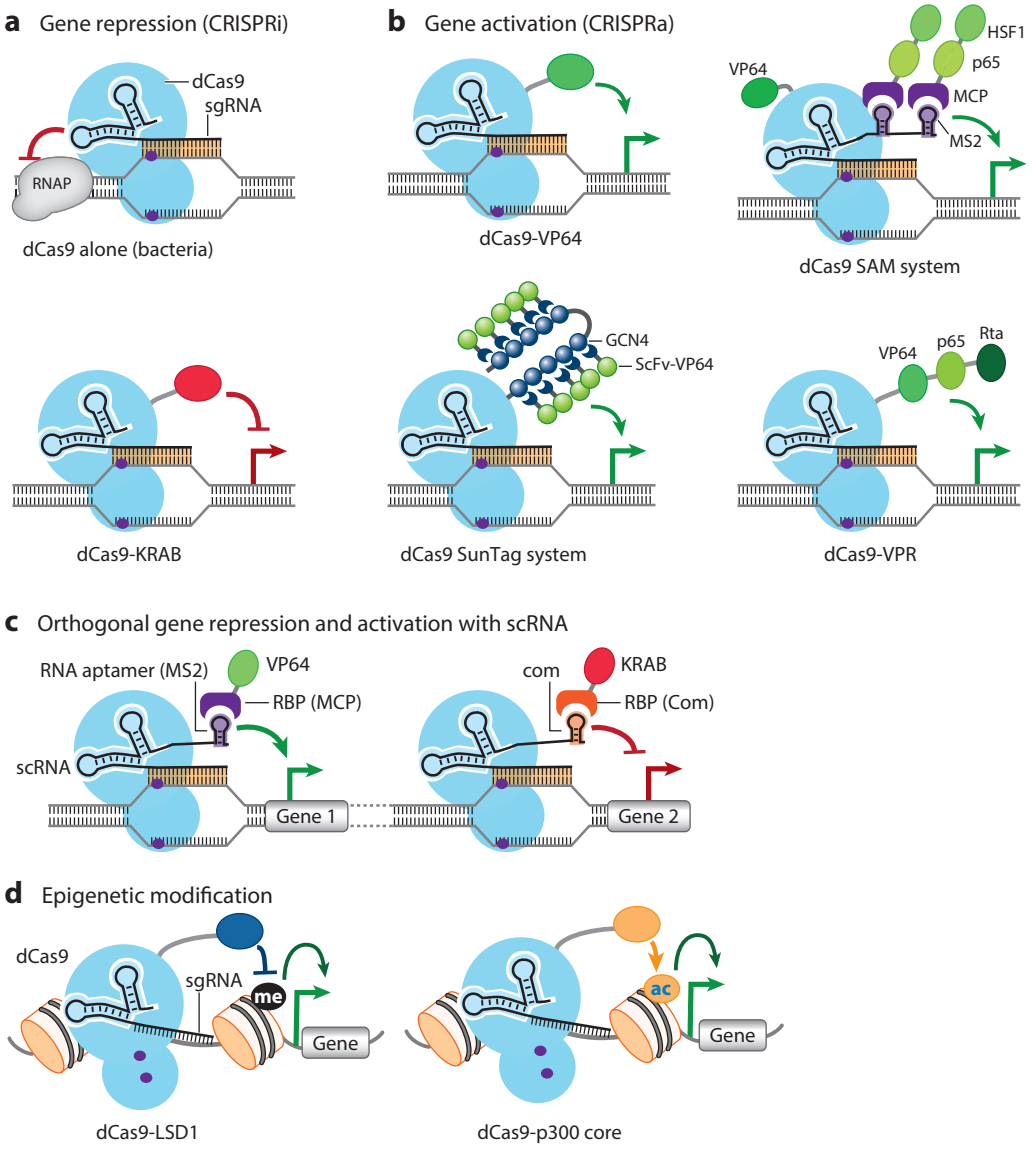


Figure 2

Nuclease-deactivated Cas9 (dCas9)-mediated sequence-specific gene regulation. (*a*) CRISPR interference (CRISPRi) strategies. Repression can occur with dCas9 alone in bacteria, which sterically blocks transcriptional elongation of RNA polymerases (RNAPs) (41). Alternatively, dCas9 can be fused to a repressor domain such as KRAB to enhance repression (37). (*b*) CRISPR activation (CRISPRa) strategies. Activation can be achieved by directly fusing dCas9 to a transcriptional activator (e.g., VP64) or by recruiting multiple transcriptional activators using the synergistic activation mediator (SAM), SunTag, or VP64-p65-Rta (VPR) systems (180, 184, 185). Note for the SAM system, each MS2 aptamer can recruit a pair of MCP-p65-HSF1, but only one is shown for simplicity. (*c*) Gene activation and repression can occur simultaneously in the same cell using the scaffold RNA (scRNA) system. RNA aptamers (e.g., MS2, com, PP7) are fused to single guide RNA (sgRNA), creating an scRNA that is localized to a specific genomic locus with dCas9. The scRNAs can recruit RNA-binding proteins (RBPs; e.g., MCP, Com, PCP) fused to an activator (e.g., VP64, *left*) or a repressor (e.g., KRAB, *right*) (179). (*d*) CRISPR-mediated epigenetic modification. The epigenetic landscape can be altered in a site-specific manner by fusing epigenetic modifying enzymes such as p300 or LSD1 to dCas9. For example, dCas9-LSD1 decreases H3K4me2 near the targeted enhancer region, resulting in repression of related genes, whereas dCas9-p300 increases H3K27 acetylation at the promoter or enhancer regions and activates the expression of downstream genes (38, 39).

of multiple copies of VP64 to each dCas9 (**Figure 2*b***) (185). This system has been used to strongly upregulate chemokine (C-X-C motif) receptor 4 (known as CXCR4), thus enhancing cell migration in K562 cells. Additionally, Tanenbaum et al. (185) upregulated cyclin-dependent kinase inhibitor 1B (known as CDKN1B) using the SunTag system, leading to a reduction in cell growth. The system has also been used for genome-scale gain-of-function screening (40).

In a third strategy, used by Chavez et al. (184), dCas9 was fused to three different activators in tandem, VP64-p65-Rta (VPR), resulting in a tripartite activator. dCas9-VPR was able to achieve greater activation of endogenous genes than dCas9-VP64 (**Figure 2*b***). This system has been used to direct the neuronal differentiation of induced pluripotent stem cells with multiple coexpressed sgRNAs (184). All of these studies show that synergistically recruiting multiple activators to the dCas9 target locus enhances the activation of the CRISPRa system (180, 184, 185). These engineered systems likely mimic intrinsic cellular gene activation mechanisms, which work by coordinating the recruitment of multiple activators (186, 187).

Advantages of CRISPRi/a and Their Applications

Compared with RNAi and other established methods of regulating gene expression (e.g., gene overexpression, TALE- or ZF-mediated regulations), CRISPRi/a combine the advantages of design simplicity (37, 174, 188) with high specificity (37, 175, 180, 189), directly control endogenous gene expression at the transcriptional level, and can act on both coding and noncoding sequences (40, 180, 190) (reviewed in 191, 192). CRISPRi/a have been applied in large-scale genomic screening, showing minimal off-target effects in different systems (40, 180). The high specificity of CRISPRi/a may be attributed to their narrow regions of activity (i.e., around the transcriptional start site, or TSS; discussed in the section Use and Delivery of Cas9) and their high sensitivity to sgRNA–DNA mismatches (40). In addition, CRISPRi/a have also been used to regulate the transcription of non-protein coding RNAs, such as long noncoding RNAs (40, 180) and micro RNAs (190). Other work has also expanded the applications of CRISPRi/a to the regulation of gene expression in multicellular organisms, such as *Drosophila* (193) and plants (194, 195), and to the reactivation of latent reservoirs of HIV-1 for its permanent elimination (196).

Cas9 FOR EPIGENOME EDITING

In a manner similar to that used to regulate transcription, sequence-specific DNA-binding proteins can recruit epigenetic modifiers to reshape the epigenome at a given locus. It has been shown that ZFs and TALEs fused with epigenetic modifiers can alter epigenetic marks at their target DNA sites, which can lead to changes in relevant gene expression (197–202). Some studies have also reported the use of dCas9 systems to achieve site-specific epigenome editing (38, 39, 203). Kearns et al. (39) fused Nm dCas9 with the histone demethylase LSD1 (**Figure 2*d***). They then targeted enhancers of genes (e.g., Oct4, Tbx3) that are crucial for maintaining pluripotency in mouse embryonic stem cells (mESCs). They demonstrated that Nm dCas9-LSD1 efficiently suppressed the expression of genes controlled by the targeted enhancers, decreased the level of the epigenetic marks H3K4me2 and H3K27ac near the targeted Tbx3 enhancer region, and also caused changes in cell morphology. Another study revealed that the dCas9-KRAB fusion can induce H3K9 trimethylation (H3K9me3) when targeted to the HS2 enhancer and suppress the expression of globin genes that is regulated by the HS2 enhancers (203).

Using a slightly different approach than those described above, Hilton et al. (38) fused Sp dCas9 and Nm dCas9 to the catalytic core domain of the histone acetyltransferase p300 (Sp dCas9-p300 core and Nm dCas9-p300 core, respectively) (**Figure 2*d***). The authors were able to activate the

expression of several endogenous genes by targeting the promoter or enhancer regions, and they showed that the activation was specific using genome-wide RNA sequencing. When targeted to the HS2 enhancer, dCas9-p300 core increased the level of H3K27 acetylation at both the targeted enhancer and the promoters of its downstream genes.

The studies (38, 39, 203) described above have demonstrated that dCas9 fusion proteins can act as sequence-specific, synthetic epigenome modifiers, which not only change local epigenetic status but also change the gene expression of relevant genes. Given the broad expanse of functional epigenetic marks—from DNA methylation to histone modifications—future studies are needed to develop a full toolkit of dCas9-based epigenetic modifiers. Given the possible off-target effects of dCas9 when using dCas9-mediated epigenome editing systems to target a specific locus, the specificity and toxicity of such tools should also be assessed.

Cas9 FOR GENOMIC IMAGING

In the postgenomic era, another challenge for scientists is to understand the correlations between the linear genetic information within DNA and its three-dimensional organization within the cell nucleus. Many studies have revealed that the three-dimensional organization of genomic structure may play an important part in regulating gene expression and controlling cell differentiation (204–207). Further research into the correlations between genomic architecture, gene expression, and cell behavior is hindered by the lack of tools for visualizing sequence-specific genomic dynamics in living cells. Cas9's ability to localize to specific sequences within the genome and the ease of redirecting it to different genomic loci have made it a promising candidate for studying genomic organization and dynamics in living cells.

Sequence-Specific Genomic Imaging Tools Based on Nucleotide Base-Pairing Interactions

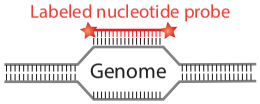
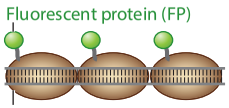
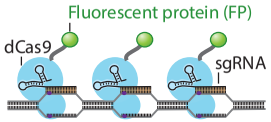
In principle, the labeling of a specific genomic locus in the nucleus can be achieved through either nucleotide base-pairing interactions or sequence-specific protein–DNA interactions (**Table 1**). Labeling techniques that rely on nucleic acid interactions, such as in situ hybridization (ISH) assays, have been developed and used extensively in genomic research and for the clinical diagnosis of genetic diseases. ISH uses in vitro synthesized and labeled nucleotide probes to visualize complementary endogenous genomic loci (208–210). For example, multicolor, fluorescent in situ hybridization (FISH) uses fluorescently labeled nucleotide probes to simultaneously detect the localization of multiple loci (211–215). Likewise, electron microscopy in situ hybridization (known as EM-ISH) uses radioactively labeled probes or biotin and digoxigenin labeled probes to detect genomic ultrastructure (216, 217).

Although powerful, these ISH assays are generally restricted to fixed samples because cells need to be fixed, permeabilized, and their DNA denatured before the labeled probes can bind. One exception to this, reported by Molenaar et al. (218), demonstrated that a peptide nucleic acid (PNA) probe can be introduced into the cells by glass beads to track the dynamics of telomeres in living cells. Whether a similar system can be used to label other genomic loci remains untested.

Sequence-Specific Genomic Imaging Tools Based on Protein–DNA Interactions

An alternative to ISH techniques is the labeling of genomic loci through sequence-specific protein–DNA interactions. This technique is feasible for live cell imaging, given the ease of fusing DNA-binding proteins with FPs and expressing these constructs in living cells. Moreover,

Table 1 Comparison of sequence-specific genomic imaging methods: Nucleic acid probes (*left*), imaging based on protein–DNA interactions (*middle*), and CRISPR/dCas9-based genomic imaging (*right*) have different advantages and disadvantages based on the given application

Mechanisms	Nucleotide base-pairing interactions		Protein–DNA interaction			Combined protein–DNA and nucleotide base-pairing interactions
						
Technique	**ISH**	**PNA probe live imaging**	**LacO/TetO**	**Native DNA-binding proteins**	**ZF/TALE**	**CRISPR/Cas9**
Probe	Labeled nucleic acids strand (DNA, RNA, and PNA, etc.)	Labeled PNA probe	Lac/Tet repressors-FP	Endogenous DNA-binding protein-FP	ZF/TALE-FP	dCas9-FPs + sgRNAs
Genomic target	Any genomic loci	Telomeres	Genomically integrated LacO or TetO arrays	Genomic loci containing repetitive native protein-binding sites (telomeres, etc.)	Repetitive genomic loci	Essentially any genomic locus (repetitive or nonrepetitive sequences)
Cells	Fixed cells	Living cells	Living and fixed cells			Living or fixed cells (i.e., CASFISH)
Advantages	■ Easy probe design ■ Allows multiple color and high-resolution imaging	■ Enables live cell imaging	■ Enables live cell imaging ■ Allows multiple color imaging			■ Enables live cell imaging ■ Easy sgRNA probe design ■ Multiple color imaging with orthogonal dCas9s or CASFISH
Disadvantages	■ Restricted to fixed samples ■ Lacks dynamic information	■ Only shown for telomere live cell imaging ■ Challenges in delivery	■ Laborious to create and characterize insertions ■ Cannot directly label endogenous genomic loci	■ Restricted to genomic loci that have natural sequence-specific binding proteins	■ Laborious to construct many ZF/TALE proteins ■ So far only restricted to repetitive sequences	■ Requires multiple sgRNAs to image a nonrepetitive sequence Requires a PAM
References	208–217	218	219–221	222–224	42–46	47, 72, 185, 225, 226

Abbreviations: Cas9, CRISPR-associated protein 9; FP, fluorescent protein; ISH, in situ hybridization; PAM, protospacer-adjacent motif; PNA, peptide nucleic acid; sgRNA, single guide RNA; TALE, transcription activator-like effector; ZF, zinc finger.

some DNA-binding proteins have high binding specificity for cognate DNA sequences in living cells, and these protein–DNA interactions do not require DNA denaturation.

Initial work to visualize genomic dynamics involved the insertion of Lac/Tet operator tandem repeats into a specific genomic locus. These exogenously added repeats were then visualized using their binding proteins fused with FPs (219–221). This method allows us to understand the dynamics of genomic loci in living cells. However, it is labor intensive to insert repetitive tandem repeat sequences into a specific genomic locus, and this method cannot be used to directly label endogenous sequences.

One way to visualize endogenous loci is to co-opt endogenous DNA-binding proteins and label them with FPs. Some repetitive genomic loci, such as telomeres and centromeres, have native sequence-specific binding proteins and, thus, can be easily visualized by fusing these binding proteins to FPs or by immunostaining with related antibodies (222–224). However, the majority of the human genome sequence lacks unique binding proteins. For this reason, the use of programmable DNA-binding proteins, such as ZFs, TALEs, and Cas9s, offers a powerful approach for imaging these genomic loci.

Several groups have used ZFs or TALEs to image endogenous genomic loci. Initial efforts were made by fusing green fluorescent protein (GFP) to ZF proteins to image repetitive sequences at pericentric regions in living cells (42). Several studies (43–46) have also imaged repetitive genomic elements by fusing FPs to TALEs. Miyanari et al. (43) reported a TALE-mediated genome visualization method used to track the dynamics of centromeres and telomeres in living mESCs and mouse embryos, and they also used this method to efficiently distinguish two parental chromosomes with distinct single nucleotide polymorphisms, suggesting a high specificity. Ma et al. (44) published similar and complementary results showing that two TALEs tagged with different colors can simultaneously track the dynamics of centrosomes and telomeres in living cells. They also showed in vitro purified TALE proteins could label genomic loci in fixed cells by using a protocol simpler than FISH. Another work by Thanisch et al. (45) used a FP-TALE to track the dynamics of satellite repeats throughout the cell cycle in mESCs.

Cas9-Based Genomic Imaging: A Combination of Nucleotide Base-Pairing and Protein–DNA Interactions

Cas9-based imaging approaches combine the advantages of nucleotide interactions and protein–DNA interactions to label endogenous genomic loci (**Table 1**). Soon after TALE-mediated genomic imaging was reported, the first exciting work using dCas9 for genomic imaging was published (47). In this work, Chen et al. (47) fused Sp dCas9 to enhanced GFP to visualize the dynamics of the genomic loci of coding and noncoding sequences in living human cells. In this study, repetitive genomic loci were dynamically tracked throughout the cell cycle using a single sgRNA. A nonrepetitive genomic locus can also be labeled by co-delivering multiple sgRNAs that tile the locus (47). Another group used a similar strategy to label endogenous centromeres, pericentric regions, and telomeres in living mESCs (225).

There have been several additions made to improve and expand Cas9-based genomic imaging. Tanenbaum et al. (185) combined dCas9-mediated genomic imaging with the SunTag peptide array to amplify the fluorescent signal generated by each dCas9. Additionally, Ma et al. (72) developed multicolor genomic imaging using orthogonal dCas9s tagged with different FPs. In this work, the Sp dCas9, Nm dCas9, and the St1 dCas9 were individually tagged with differently colored FPs. The different dCas9-FP fusions were targeted to distinct genomic loci by their corresponding sgRNAs. The authors demonstrated simultaneous tracking dynamics of multiple, repetitive genomic loci in living cells and that two orthogonal dCas9s may distinguish two genomic

loci separated by around 2 mega bases (72). Because the three dCas9s recognize distinct sgRNAs and PAM sequences, this system provides a tool for tracking the dynamics of multiple genomic loci simultaneously.

In a method termed CASFISH, fixed cells and tissues can also be efficiently labeled by fluorescently labeled dCas9–sgRNA complexes assembled in vitro (226). Compared with traditional FISH, which uses high temperature and formamide for denaturation prior to probe hybridization, CASFISH allows rapid sequence-specific labeling using the dCas9-mediated enzymatic reactions and, thus, may help to preserve cellular and genomic structure. The authors were able to image repetitive sequences with a single sgRNA and to image nonrepetitive sequences with an array of sgRNAs. Given that preassembled Cas9–sgRNA complexes are stable, the authors were also able to demonstrate multicolor imaging using separately preassembled Cas9–sgRNA complexes targeting different loci with different colors.

Visualizing the dynamics of fluorescently labeled dCas9 also provides insights into the mechanism of how Cas9 searches the genome for its target sites in living cells. Fusing dCas9 to a HaloTag system and using a single-particle tracking method, Knight et al. (92) found that dCas9 mainly surveys the genome through three-dimensional diffusion, with transient off-target binding. They also found that dCas9 on-target binding lasts much longer than off-target binding and observed reduced searching efficiency in heterochromatic regions.

Challenges in Live Cell Genomic Imaging

These programmable sequence-specific DNA-binding proteins are promising tools for monitoring genomic dynamics in living cells. However, the visualization of a specific genomic locus requires recruiting many copies of labeled proteins within a nearby region. How site-specific binding affects the local chromatin structure and transcriptional activity requires more investigation. To minimize the perturbation of local chromatin structure, future efforts should focus on reducing the number of Cas9–sgRNA complexes recruited to a given locus.

Moreover, these techniques require further optimization to efficiently track the dynamics of any nonrepetitive sequences in living cells. It is challenging to visualize nonrepetitive sequences using ZF and TALE systems because they require delivery, expression, and recruitment of multiple DNA-binding proteins, each of which must be directed to a unique sequence. Cas9-based genomic imaging is more likely to overcome this challenge because it is easier to deliver and express multiple small-sized sgRNAs. However, the current dCas9 system requires at least 26–36 sgRNAs to efficiently visualize a nonrepetitive genomic locus (47), which is not trivial in terms of design, cloning, and delivery and, thus, limits the applications of dCas9 imaging in diverse cell types and tissues. To improve this system, it is necessary to reduce the required number of sgRNAs and achieve more sensitive genomic labeling. In addition, more studies are required to evaluate the specificity and stability of the dCas9-mediated labeling of genomic loci.

Cas9 FOR STUDYING ENDOGENOUS PROTEIN–GENOME INTERACTIONS AT SPECIFIC LOCI

An affinity-tagged dCas9 can be used in chromatin immunoprecipitation (ChIP) assays to study proteins that interact with specific portions of the genome (48, 49). The tagged dCas9 can be targeted to a specific locus and used to pull down the proteins associated with that region. Associated proteins can then be identified via mass spectrometry or other methods. This CRISPR-based engineered DNA-binding molecule-mediated ChIP (enChIP) method allows for the study of endogenous protein–genome interactions at specific genomic loci. The authors have successfully

used this method to characterize proteins interacting with an interferon-γ-responsive promoter (48, 49).

This CRISPR enChIP method may provide an inexpensive and convenient way of characterizing regional DNA–protein interactions. However, it is necessary to carefully set up proper controls and compare its results with complementary experiments for validation, given that many studies have reported extensive genome-wide off-target dCas9-binding events (89, 90, 189, 227, 228). Indeed, ChIP sequencing and other assays have shown that the Cas9–sgRNA complex may bind from tens to thousands of off-target sites in addition to its target, although most of the complexes bind to the off-target sites with much weaker affinity (89, 90, 189, 227, 228). Of note, only a small subset of these off-target sites is actually cleaved by Cas9. Detailed analyses have revealed that the probability of off-target binding events largely depends on chromatin accessibility (89, 90). In this regard, the specificity and efficacy of enChIP pull-down assays may greatly depend on the choice of sgRNAs and the chromatin accessibility of targeted regions and, therefore, it is necessary to use additional assays to validate the results from CRISPR enChIP.

SPATIOTEMPORAL REGULATION OF Cas9 IN GENOMIC EDITING, REGULATION, AND TARGETING

Tight regulation of Cas9 expression and activity may potentially reduce its off-target effects and, thus, this is a prerequisite for the future use of Cas9 in clinical applications. Precise spatiotemporal control of its expression and activity can be achieved at the transcriptional and posttranslational levels by combining Cas9 with chemical or light-inducible systems. At the transcriptional level, the expression of Cas9 and nCas9 can be controlled under doxycycline-inducible promoters to achieve inducible genome editing in cells and mice, although the promoter leakiness may be a concern in many cell types (138, 229, 230). At the posttranslational level, Davis et al. (158) created intein-Cas9 fusion proteins that produce active Cas9s when a cell-permeable ligand is present. In this system, a chemical-responsive intein is inserted into Cas9 to interfere with its nuclease activity. Upon chemical addition, active Cas9 endonuclease can be produced after intein cleavage through inducible protein splicing, thereby achieving inducible genome editing at the posttranslational level. This system enhances the specificity of Cas9-mediated genome editing. However, it should be noted that in both systems, once active Cas9s are produced, they cannot be removed until intrinsic protein degradation occurs.

The two components (dCas9 and effector) of the CRISPRi/a gene regulation systems can also be coupled with chemical and light-inducible systems for spatial and temporal regulation. For example, Nihongaki et al. (231) and Polstein & Gersbach (232) developed light-inducible CRISPRa systems to activate endogenous gene expression when cells are exposed to blue light, thus allowing rapid and reversible control of gene expression. The authors fused light-inducible heterodimerizing proteins CIB1 and CRY2 to, respectively, dCas9 and a transcriptional activator (e.g., VP64). Blue-light-induced CIB1–CRY2 dimerization recruits the transcriptional activator to dCas9 at its target locus, activating gene expression.

Split Cas9s

In addition to light-inducible two-component systems, different versions of split Cas9 have been created to allow inducible control of gene editing and regulation. For example, Zetsche et al. (233) split Cas9 into N-terminal and C-terminal fragments and used a rapamycin-binding dimerization system to induce fragment assembly into active Cas9. Due to their autoassembly, the two split Cas9 fragments need to be spatially separated by tagging with, respectively, nuclear export sequences and

nuclear localization sequences. These systems then make use of rapamycin-induced dimerization to shift the whole complex to the nucleus for DNA targeting. In addition to genomic editing, inducible gene activation is also achieved with this system by fusing split dCas9 to VP64. A similar approach has been used to construct split Sa Cas9 for inducible genomic editing (70). Another strategy reported by Wright et al. (234) uses a split Cas9 that is separated into a nuclease lobe peptide and an α-helical lobe peptide. An sgRNA is sufficient to induce their assembly into a whole Cas9 nuclease, although the efficiency of the split Cas9 for genome editing is much lower than for wild type Cas9.

Nihongaki et al. (235) created a photoactivatable Cas9 system by fusing split Cas9 fragments (N713 and C714) with light-inducible dimerization domains. Blue-light-induced dimerization allowed split Cas9 fragments to reconstitute nuclease activity. This system also allows for optogenetic control of nCas9 activity as well as dCas9-mediated transcriptional repression. By tuning the region and timing of blue light excitation, spatiotemporal and reversible control of Cas9 nuclease activity can be achieved.

Truong et al. (236) developed an intein-mediated split Cas9 system to potentially facilitate in vivo delivery of Cas9. Cas9 was split into N-terminal and C-terminal fragments that were fused to each of the two components of intein, respectively. The two fragments of Cas9-intein fusions can be delivered into cells in separate vectors. After intein self-splicing, a full length Cas9 forms, exhibiting comparable gene targeting efficiency to wild type Cas9 in cells. This method allows the large Sp Cas9 to easily fit into the packaging limit of the recombinant adeno-associated virus (AAV) system, although its in vivo effectiveness still needs to be assessed.

The development of split Cas9 systems provides new strategies for regulating Cas9 activity, and it may facilitate the delivery of Cas9 by bypassing the size limitations of some delivery systems. However, with the exception of intein-mediated split Cas9 (236), most split Cas9 systems have shown reduced nuclease activity compared with full-length Cas9 (233–235). Additionally, the background activity of split Cas9 due to autoassociation needs to be critically evaluated.

Cas9 FOR TARGETING RNA

Although most current applications of Cas9 make use of its sequence-specific DNA editing and targeting capabilities, some articles have opened up the exciting possibility of using Cas9 to target RNA sequences. Cas9 from the pathogenic *Francisella novicida* (Fn) can target and degrade mRNA transcripts for a bacterial lipoprotein, leading to suppression of its host's immune response (237, 238). For such mRNA recognition, Fn Cas9 forms a complex with its tracrRNA and a novel, small, CRISPR/Cas-associated RNA (termed a scaRNA) instead of the crRNA. Based on these observations, Price et al. (50) further engineered this Fn Cas9 system to retarget RNA from the hepatitis C virus (HCV) in eukaryotic cells. The authors showed that introducing Fn Cas9 together with a synthetically designed, RNA-targeting guide RNA (rgRNA) into cells inhibited HCV activity, and they suggested that the Fn Cas9–rgRNA complex directly bound to HCV RNA to inhibit its translation and replication. Interestingly, such Fn Cas9-mediated RNA inhibition seems to be independent of its nuclease activity and does not require a PAM sequence. Although the detailed molecular mechanisms of how Fn Cas9–rgRNA binds to and suppresses RNA activity remain unclear, these findings raise hope that the Cas9 system can be used to target endogenous RNAs.

Purified Sp Cas9–sgRNA complexes can also target and cleave ssRNA in vitro (51), but this depends on the presence of a DNA-based PAM-presenting oligonucleotide (PAMmer) that hybridizes with the targeted ssRNA. This system is distinct from the Fn Cas9–rgRNA system, which does not need a PAM sequence to target RNA.

The exciting potential of using Cas9 to target RNA may inspire further development of Cas9-based tools for RNA manipulation. For example, by coupling RNA-targeting Cas9 to different modulators, it may be possible to regulate the stability, localization, and splicing of the targeted RNA and to track the real-time dynamics of RNA processing (239).

USE AND DELIVERY OF Cas9

Designing Single Guide RNAs

Designing highly active sgRNAs depends on conforming to a certain set of design rules. To further improve sgRNA design for nuclease Cas9, Doench et al. (142) made a library of all possible sgRNAs, tiling across a handful of genes. The authors were able to greatly improve the design of CRISPR knockout sgRNAs. For example, a typical CRISPR knockout library design before this study would create 0–2 highly active sgRNAs out of a pool of 6 for 90% of genes; this was improved to 3 or more out of 6 sgRNAs for 90% of genes. Gilbert et al. (40) used a similar technique to define the rules for effective CRISPRi/a sgRNA, tiling thousands of sgRNAs in a 10 kb window around 49 genes. They defined a set of rules from which it is possible to obtain at least 1–2 highly active (80–99% repression) sgRNAs out of a pool of 5–10. Currently, it is usually necessary to clone a handful of sgRNAs per target gene, as there are other factors affecting sgRNA function that have not yet been defined.

Although there are several online tools available to aid in designing sgRNAs, it is also possible to design sgRNAs manually. Several groups have distilled information from large data sets to come up with a set of simple rules for what makes an sgRNA most effective in various contexts. Design rules include, for example, the following:

- All sgRNAs must be adjacent to a PAM site: Sp Cas9 uses NGG or a less efficient NAG (3, 68). Sa Cas9's PAM is NNGRRT (69).
- When using a U6 promoter for sgRNA expression in mammalian cells, the first nucleotide of the sgRNA must be a G for effective expression, although it has been suggested that an A may work in some contexts (240). It has been shown with Cas9 nuclease that an sgRNA can still function if the 5′ G of the sgRNA is mismatched with the target site, which is useful if no target site can be found where a G is 18–25 bp upstream of NGG (139, 241).
- An sgRNA expressed from a U6 promoter in mammalian cells should not contain a stretch of four or more uracils (U's) in a row or it will be terminated prematurely due to the activity of RNA polymerase III (242). A stretch of U's near the 3′ end of the guide sequence is unfavorable for Cas9–sgRNA binding (138).
- There are mixed reports about the effect of GC content. An article by Wang et al. (138) suggested that sgRNAs with a very high or low GC content were less effective when combined with nuclease Cas9. Another study, by Gilbert et al. (40), used dCas9 fused to effectors and found that variations in GC content did not significantly change sgRNA effectiveness.
- Long stretches of the same nucleotide greatly decrease sgRNA activity (40).

Although great strides have been made in finding ever better sgRNAs, the design rules for both Cas9-based genome editing and gene regulation will no doubt benefit from further optimization in the future.

Choosing Target Sites

When using nuclease Cas9 to knock out a gene through the creation of indels, it is most common to target an early exon in the coding sequence, to disrupt as much of the protein as possible by

a frameshift mutation (138, 139). To determine the most potent targeting sites for CRISPRi/a, Gilbert et al. (40) and Konermann et al. (180) have tiled sgRNAs around the promoters of various genes. By tiling a library of sgRNAs in a 10 kb window around the TSS of 49 genes (approximately 55,000 sgRNAs total), Gilbert et al. (40) systematically examined which sites allowed for the most active sgRNAs for both CRISPRa and CRISPRi. CRISPRi is most effective with sgRNAs in the −50 to +300 bp window around the TSS, with the absolute highest repression occurring in approximately the 50–100 bp window downstream of the TSS. The most effective sgRNAs for CRISPRa are targeted to a window −400 to −50 bp upstream of the TSS. Both of these activity windows are consistent with data regarding the mechanism of action for the KRAB repressor (243, 244) and VP64 activator (245).

Although these studies have been greatly informative for the creation of effective sgRNAs, much work still remains for figuring out the full set of rules, particularly for different cell types and organisms. Future work should systematically determine how local chromatin, transcriptional, and epigenetic status (i.e., local histone marks, methylated DNA, or actively transcribing DNA) affect genome targeting, binding, nuclease, and regulatory activities. For example, there is a positive correlation between the level of sgRNA expression in mammalian cells and the regulatory function of Cas9, suggesting that the sgRNA expression level is likely a limiting factor for the dCas9 function (7). For CRISPRi/a, the absolute dosage of sgRNAs and dCas9, as well as the most effective ratio of the two, also require future study.

Delivery Methods

There are many vectors available on Addgene (**https://www.addgene.org/**) and through commercial vendors that encode various CRISPR components. The particular components and their delivery will depend on the assay and cell type. After picking an appropriate Cas9-encoding vector, a complementary sgRNA-encoding vector can also be adopted. Directing a dCas9 effector to a sequence of interest requires the simple cloning of a short sgRNA (approximately 100 bp) and then introducing both components into the cell type of interest (141, 174, 188). For CRISPRi/a, it is standard to also use one or multiple nontargeting sgRNAs—which should not target anywhere in the genome or introduced DNA—as a negative control and as a way to normalize results.

Depending on the desired application, there are various ways in which the Cas9 system can be introduced. For an application such as a pooled screen, it is often desirable to make a stable Cas9 or dCas9 effector cell line using lentiviral or retroviral vectors before the introduction of a lentiviral sgRNA pool (138). This method may be problematic when working with primary cells. Alternatively, it is possible to use vectors that encode both Cas9 and a single sgRNA (139, 141). It is necessary to have just one sgRNA present per cell in pooled screens because this gives the highest signal-to-noise ratio when correlating an observed phenotype with the effect of a given sgRNA. Thus, viral delivery is ideal for pooled screens because the viral particles can be titered such that, on average, there is less than one sgRNA-containing viral particle per cell in the infected pool (giving a multiplicity of infection of less than 1).

For shorter-term or smaller-scale experiments in cell lines, it is also possible to use transient transfection to introduce plasmids containing Cas9 or dCas9 effectors and sgRNAs. When knocking out or editing a gene or handful of genes, this may be ideal, as continued expression of the CRISPR components is not necessary once the desired genome modification has occurred. For CRISPRi/a, transfection is best suited to activation assays, which can often be assessed after 24–48 hours (37). Repression assays may be less effective when using transfection because CRISPRi affects transcriptional efficiency and not mRNA or protein stability. The already-present mRNA and protein for the target gene must be degraded at its normal turnover rate, which can vary from

gene to gene, before the full effects of repression are seen. It may take several days to see repression, but by that time the CRISPRi plasmids may have been diluted out of the cells as they divide and, thus, CRISPRi may become less effective. Thus, any studies using CRISPRi may be better suited to viral delivery to maintain CRISPRi component expression over a longer time scale. With an eye toward the technical aspects of Cas9–sgRNA component expression and delivery, it is quite straightforward to design an effective assay.

For gene therapy, the delivery of the Cas9 system is more challenging and raises more safety concerns. AAV-based vectors are the preferred candidates for somatic gene therapy due to their mild immune response, lack of pathogenicity, and ability to target nondividing cells (246). However, the coding sequences of Sp Cas9, the most widely used Cas9, and its sgRNA are already approaching the packaging limit of AAV-based gene therapy vectors. The large size of Sp Cas9 makes its usage in AAV-based gene therapy unfavorable, especially when promoter sequences, localization signals, donor DNA, or additional sgRNAs are needed. The Sa Cas9 has an approximately 1 kb shorter coding sequence than Sp Cas9, allowing it to be easily packaged into AAV-based vectors for gene editing (69, 247). Using this AAV Sa Cas9 approach, Ran et al. (69) efficiently induced indel formation of more than 40% of the cholesterol regulatory *Pcsk9* (proprotein convertase subtilisin/kexin type 9) gene in mouse liver after 1 week of treatment, causing a 95% drop in serum Pcsk9 and a 40% drop in total cholesterol.

Besides viral systems, nonviral methods have been used to deliver Cas9-encoding plasmids or Cas9 mRNA into animal cells and tissues. For example, coinjection of Cas9-encoding mRNA and an sgRNA into zygotes or embryos can generate genome-edited animals (36, 128, 248, 249). Cas9-containing vectors can also be introduced into adult animals through hydrodynamic injections, leading to efficient gene corrections or mutations (125, 250).

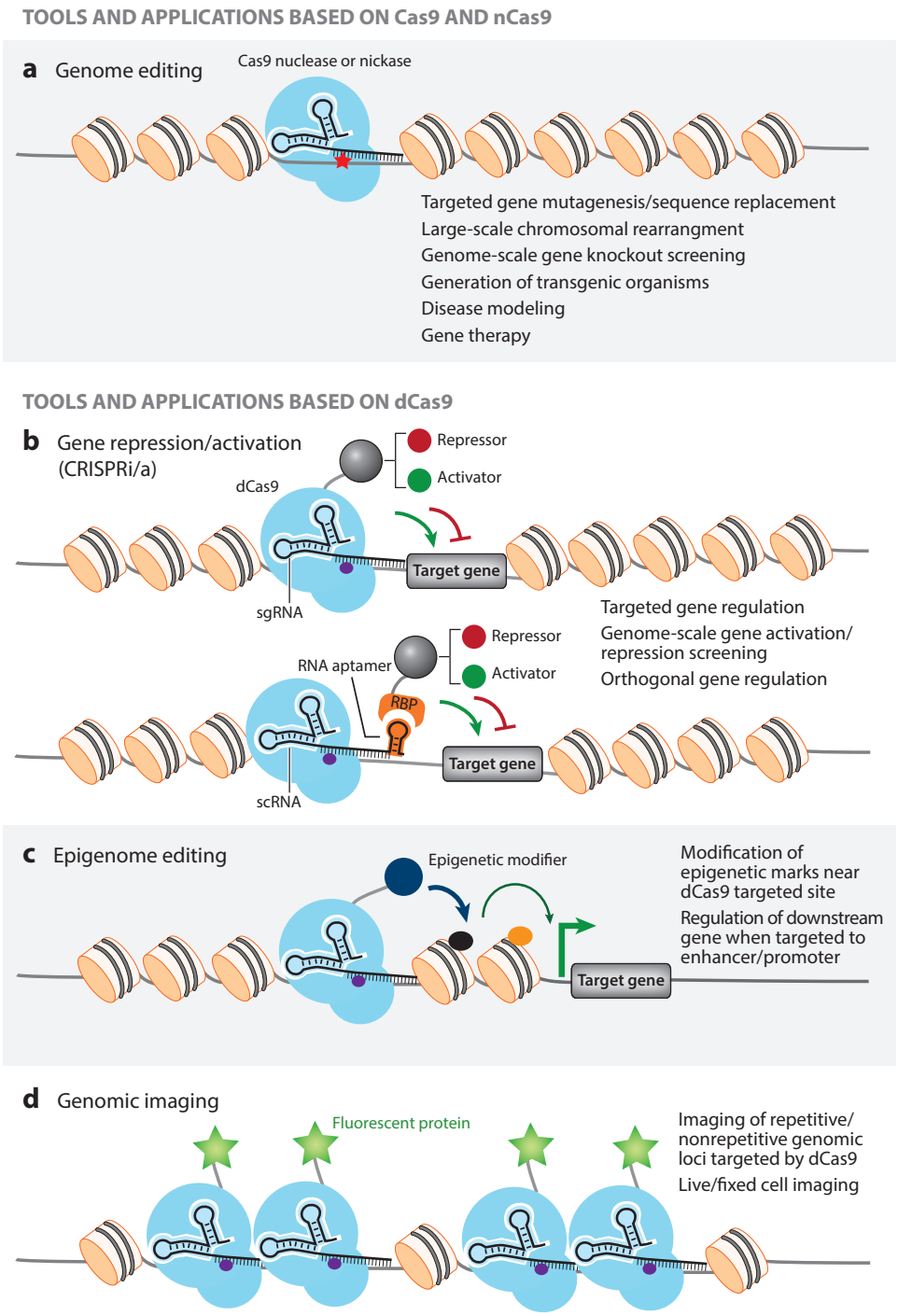
The use of purified Cas9–sgRNA ribonucleoproteins (RNPs) is another option for cellular delivery. Compared with a viral or nonviral nucleotide delivery method, the RNP delivery systems allows for fast action of the RNP complex in the nucleus and a shorter duration of the Cas9 nuclease presence in the cells and, thus, it may increase the efficiency and reduce the off-target effects. RNP delivery also avoids undesired genomic alterations that can occur when using other nucleotide delivery methods.

RNP delivery of Cas9 and sgRNAs can be achieved through a variety of strategies. Many methods traditionally used for nucleotide transfection have been proven useful for Cas9 RNP delivery, including microinjections, electroporation, and lipid-mediated transfection. Microinjection of purified Cas9 RNP complexes into animal embryos successfully generated genome-edited animals (251, 252). Electroporation methods have been established to introduce Cas9–sgRNA complexes into primary cells and embryonic stem cells, and these can induce targeted gene mutations and large chromosome deletions while minimizing the off-target effects (108, 161, 253–256). Commercially available nucleotide transfection reagents have also been adapted to deliver Cas9–sgRNA RNP complexes (256, 257). Given that the Cas9–sgRNA complex is inherently anionic, Zuris et al. (257) adapted cationic lipid transfection reagents to deliver the Cas9 nuclease, nCas9, and the dCas9-VP64 transcriptional activator. This method allowed for gene modification of up to 80% in cultured cells and approximately 20% in mouse inner ear hair cells in vivo (257).

Other approaches have also been explored for delivering RNP complexes. For example, Ramakrishna et al. (258) used cell-penetrating peptides (CPPs) to induce gene editing through Cas9 conjugated with CPPs and through the sgRNA in complex with CPPs. These CPP complexes entered cells in the form of positively charged nanoparticles to achieve gene editing in a variety of human cell types. D'Astolfo et al. (259) developed a method of induced transduction by osmocytosis and propanebetaine (termed iTOP) to deliver Cas9–sgRNA complexes, leading to highly efficient gene editing in primary cells. This method allows uptake and release of proteins

Figure 3

Applications of CRISPR-associated protein 9 (Cas9) in gene editing, gene regulation, epigenome editing, and genomic imaging. (*a*) Cas9-mediated site-specific genome editing has a variety of applications. (*b–d*) Tools based on nuclease deactivated Cas9 (dCas9) are built to achieve (*b*) site-specific gene repression or activation [i.e., CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa)], (*c*) epigenetic modification, or (*d*) genomic imaging. Their applications are summarized. Transcriptional activators and repressors, epigenetic modifiers, and fluorescent proteins can be fused or directed to either (*b–d*) the dCas9 or (*b*) the single guide RNA (sgRNA) bound to their target DNA site. Abbreviations: RBP, RNA-binding proteins; scRNA, scaffold RNA.



and other molecules in a variety of primary cells through the macropinocytosis pathway. In addition, Sun et al. (260) made use of a DNA nanoparticle, termed a nanoclew, to deliver a Cas9 RNP complex into U2OS cells for gene editing. They also used nanoclews to deliver the Cas9–sgRNA complex in vivo, successfully editing a genome-integrated GFP reporter in 25% of U2OS tumor cells, which had been xenografted in mice.

Compared with vector-mediated nucleotide delivery methods, Cas9-mediated genome editing via RNP delivery methods (108, 161, 253, 257, 258) exhibits higher fidelity and lower cell toxicity, bypasses the safety problems of introducing foreign DNA into the host genome and, thus, may provide a platform for developing gene therapy tools.

CONCLUSIONS AND FUTURE PERSPECTIVES

Although we have not yet harnessed the full potential of CRISPR/Cas9, this technology has brought forth revolutionary changes in genomic research, including genome editing, regulation, and imaging (**Figure 3**). As we look to the future, we can envision the advances that CRISPR/Cas9 technology will bring to basic bioscience researchers and clinicians. Basic scientists are already making great strides in understanding and manipulating biology using CRISPR/Cas9 technology. In the clinic, we can look forward to new therapies for genetic diseases (using Cas9 genome editing or CRISPRi/a) and new diagnostic techniques (using dCas9-based imaging).

Basic research similar to that which uncovered CRISPR/Cas9 allows us to make use of nature's technological toolbox, which has been honed for billions of years through evolution. Although most research thus far has focused on the type II Cas9 proteins, there is much still to be discovered about the broader CRISPR systems from other diverse species of bacteria and Archaea. New CRISPR systems, hidden in plain sight in the genomes of the organisms around us, may continue to surprise us in the future with their elegant mechanisms and function, offering powerful and groundbreaking technologies.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

The authors thank all the members of the Qi lab, as well as Dr. Fuguo Jiang and Arjun Aditham for advice and helpful discussions. Thanks also to Matias Kaplan for preparing the structure comparison of Cas9s. L.S.Q. acknowledges support from National Institutes for Health's Office of the Director and the National Institute of Dental and Craniofacial Research. M.L. acknowledges support from an Agilent University Relations grant. This work was supported by NIH grants R01 DA036858 and U01 EB021240 (H.W., L.S.Q.) and DP5 OD017887 (M.L., L.S.Q.).

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