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Applications of CRISPR Genome Engineering in Cell Biology

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Abstract

Recent advances in genome engineering are starting a revolution in biological research and translational applications. The CRISPR-associated RNA-guided endonuclease Cas9 and its variants enable diverse manipulations of genome function. In this review, we describe the development of Cas9 tools for a variety of applications in cell biology research, including the study of functional genomics, the creation of transgenic animal models, and genomic imaging. Novel genome engineering methods offer a new avenue to understand the causality between genome and phenotype, thus promising a fuller understanding of cell biology.

Keywords

CRISPR/Cas9; CRISPRi/a; gene editing; gene regulation; cell biology

From DNA repair pathways to CRISPR/Cas9-mediated genome editing

Eukaryotic cells use a sophisticated network of genes and genomic regulatory elements to carry out functions related to cell growth and death, organelle formation and organization, metabolite production, and microenvironment sensing. The ability to precisely manipulate the genome is essential to understanding complex and dynamic cellular processes. Broadly speaking, genome engineering defines methodological approaches to alter genomic DNA sequence (gene editing), modify epigenetic marks (epigenetic editing), modulate functional output (transcriptional regulation), and reorganize chromosomal structure (structural manipulation) (Figure 1). These goals require a toolkit of designer molecules that can be conveniently constructed and delivered into cells to perform one of the above functions.

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Naturally occurring systems and pathways have provided a rich resource for tool building. The discovery of the homology-directed repair (HDR) pathway inspired a method to modify the DNA sequence at a precise genomic locus in a targeted manner. Using the HDR pathway, a designed DNA template with flanking homologous sequences could be used to precisely recombine at the target genomic locus [1]. However, this application is usually a highly inefficient process in mammalian cells and tissues. By contrast, the presence of a double stranded DNA break (DSB) can enhance efficiency [2, 3]. Furthermore, it has been shown that in the absence of a DNA template, eukaryotic cells may generate almost random deletion or insertion indels at the site of a DSB via the alternative non-homology end joining (NHEJ) pathway, offering another approach for targeted gene knockout [4].

Since then, a major question in the field of gene editing has been how to introduce site-specific DSBs to initiate the DNA repair process. Molecules that allow sequence-specific DNA binding were of primary interest. These include programmable endonucleases engineered from zinc finger proteins (ZFNs) or transcription activator-like effectors (TALENs) [5, 6]. The peptide domains of these proteins could be designed following a simple set of rules for protein-DNA recognition. However, their utility was hindered by an often costly and tedious process to construct and by a context dependence issue in the protein design [7, 8]. Nevertheless, previous work has shown these programmable DNA binding proteins could be coupled to nuclease domains, transcriptional repressors or activators, and epigenetic modifiers to enable diverse types of genomic manipulation [9–12]. However, it remained to be understood how to precisely target a specific DNA sequence of interest via an even simpler mechanism such as Watson-Crick base pairing.

The CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR associated protein) system performs such a function. Truly a gift from Nature [13, 14], the CRISPR/Cas system was discovered initially in *Escherichia coli* in the 1980s [15], but its function remained elusive until 2007. Working in the yogurt production bacterium *Streptococcus thermophilus*, earlier work demonstrated that encoding the bacteriophage sequence from the host CRISPR locus conferred acquired resistance against the same bacteriophage [16]. Later work showed that CRISPR utilized small CRISPR-associated RNAs (crRNAs) to guide the nuclease activity of Cas proteins in *E. coli* [17]. Together, this work uncovered a RNA-guided nuclease mechanism for the CRISPR system, which also suggested a genetic system with high specificity and efficiency for DNA binding and cleavage.

The practical use of CRISPR for gene editing began with the elucidation of the mechanism of the type II CRISPR system [18]. The type II CRISPR from *Streptococcus pyogenes* encodes a RNA-guided endonuclease protein Cas9, which was shown to use only two small RNAs (a mature crRNA and a trans-acting tracrRNA) for sequence-specific DNA cleavage [18–20]. Furthermore, a chimeric single guide RNA (sgRNA) fused between crRNA and tracrRNA recapitulated the structure and function of the tracrRNA-crRNA complex, which could efficiently direct Cas9 to induce DSBs in vitro [18]. The rules used by Cas9 to search for a DNA target are strikingly elegant and simple, requiring only a 20 nucleotide (nt) sequence on the sgRNA that base pairs with the target DNA and the presence of a DNA protospacer adjacent motif (PAM) adjacent to the complementary region [18, 21].

The Cas9 complex has since been developed as a remarkably useful tool of genome editing. As demonstrated by the pioneering work in a number of cell types and organisms [22–26], the Cas9/sgRNA complex can efficiently generate DSBs, which then facilitates NHEJ-mediated gene knockout or HDR-mediated recombination. This system has since gained rapid acceptance and has been used for genome editing in essentially all organisms that can be cultured in the laboratory setting. In this review, we focus on recent applications of CRISPR-Cas9 in cell biology research using mammalian cell cultures and animal models (Figure 2).

An expanding CRISPR toolkit for RNA-guided genome editing

The different types of natural CRISPR systems encode a toolkit for genome editing. Six major types of CRISPR systems have been identified from different organisms (types I–VI), with various subtypes in each major type [27, 28]. Within the type II CRISPR system, several species of Cas9 have been characterized from *S. pyogenes*, *S. thermophilus*, *N. meningitidis*, *S. aureus*, and *F. novicida* [18, 29–34]. While these Cas9s possess a similar RNA-guided DNA binding mechanism, they often have distinct PAM recognition sequences. Similar to the toolkit of restriction enzymes for molecular cloning, a large toolkit of Cas9s expands the targetable genome sequence for gene editing and genome manipulation.

Other types of CRISPR systems may exhibit different mechanisms. For example, the Type III-B CRISPR system from *Pyrococcus furiosus* uses a Cas complex for RNA-directed RNA cleavage [35, 36], which is indicative of a mechanism for targeting and modulating RNAs in cells. The recent discovery of the protein Cpf1 from *Prevotella* and *Francisella*-1 type V CRISPR showed that Cpf1 uses a short crRNA without a tracrRNA for RNA-guided DNA cleavage [37–40]. Both biochemical and cell culture work showed that Cpf1-mediated genome targeting is effective and specific, comparable to the *S. pyogenes* Cas9. The type VI-A CRISPR effector C2c2 from the bacterium *Leptotrichia shahii* is a RNA-guided RNase, which can be programmed to knock down specific mRNAs in bacteria [41]. These results broaden our understanding of the diversity of the natural CRISPR-Cas systems, which also provides a functionally diverse set of tools.

Other enzymatic domains can also be harnessed for genome editing. For example, instead of using the endonuclease activity of Cas9, a mutation in one nuclease domain of Cas9 can create a nickase Cas9 (nCas9) that can cleave one strand of DNA [42]. With a pair of sgRNAs, the specificity of genome editing could be enhanced by using a pair of nCas9s that target each strand of DNA at adjacent sites. Furthermore, recent work demonstrated that a Cas9-fused cytidine deaminase enzyme allowed for direct conversion of a C to T (or G to A) substitution [43]. In this work, fusing the nuclease-deactivated dCas9 or the nCas9 with a cytidine deaminase domain corrected point mutations relevant to human disease without DSBs; therefore, avoiding NHEJ-mediated indel formation.

Applications of CRISPR/Cas9 for cell biological studies

The CRISPR/Cas9 technology has accelerated the discovery and mechanistic interrogation of the genome and organelles in diverse types of cells and organisms. Some examples of

utilizing CRISPR/Cas9 for studying cellular organelles are summarized in Table 1 and Figure 3. Beyond using CRISPR/Cas9 as a gene-editing tool, we describe the development of CRISPR/Cas9 as a versatile toolkit for transcriptional control and epigenetic regulation, and highlight its utilities for large-scale genetic screens, generation of animal models, genomic imaging, and lineage tracing (Figure 2).

Transcriptional regulation of the genome with CRISPR/dCas9

The nuclease-dead dCas9 has provided a broad platform for programming diverse types of transcriptional or epigenetic manipulation of the genome, without altering the genome sequence. In brief, dCas9 was created by introducing point mutations into the HNH and RuvC domains to eliminate endonuclease activity [55]. This repurposed protein became a RNA-guided DNA binding protein. In bacteria, the dCas9 protein was sufficient to induce strong sequence-specific gene repression, simply by sterically hindering the transcriptional activity of RNA polymerase [55, 56]. In eukaryotic cells, fusing dCas9 to transcriptional effector proteins allowed for more efficient RNA-guided transcriptional modulation for both gene interference (CRISPRi) and activation (CRISPRa) [12, 57–59].

By fusing dCas9 to transcriptional repressors, such as the Kruppel-associated box (KRAB) domain, CRISPRi can efficiently repress coding and noncoding genes such as microRNAs and large intergenic noncoding RNAs (lincRNAs) in mammalian cells [57, 58, 60, 61]. Compared to complete loss-of-function using Cas9, CRISPRi can use different sgRNAs that bind to different genomic loci for tunable and titratable gene repression [58]. While complete knockout is useful for studying gene function in many cases, tunable repression of a gene to different levels offers advantages when knocking out a gene leads to lethality of cells or an organism [56].

Earlier work using dCas9 fused to a peptide containing multiple VP16 domains (VP64 or VP128) could only activate endogenous genes mildly [57, 62, 63]; therefore, several strategies have been developed to improve CRISPRa efficiency. These include recruiting multiple copies of the VP64 domain via a multimeric peptide array (SunTag), wherein each peptide domain could bind to a single-chain variable fragment (scFv) fused to VP64 [64]; fusing dCas9 to a synergistic tripartite activator system containing VP64, the activation domain of p65 (p65AD), and Rta (Epstein-Barr virus R transactivator) [65]; and combining dCas9-VP64 with a modified sgRNA engineered with two copies of an MS2 RNA hairpin that could recruit p65AD and the HSF1 (human heat shock factor 1) activation domain via interaction with the MS2 binding protein [59]. A systematic comparison of the efficacy of these methods revealed that these systems perform comparable but are dependent on the genomic and cellular context [66], suggesting that activation efficiency may vary for different genes and in different types of cells. In the future, simpler yet more effective tools for RNA-guided gene activation should be further developed.

To repurpose more complex gene regulation, sgRNA was engineered as a class of “scaffold” RNAs (scRNAs) that directly recruit transcription effectors without protein fusion [67]. scRNAs are generated by fusing RNA hairpins to the sgRNA, which interact with the cognate protein to recruit activators or repressors. Using engineered scRNAs, multiple genes can be simultaneously activated and repressed in the same cells. In addition to using

scRNAs, multiple orthogonal species of dCas9s could also provide a platform for complex transcription regulation and sophisticated manipulation of the transcriptome.

Epigenetic regulation with CRISPR/dCas9

dCas9 fused to epigenetic modifying enzymes has been used to introduce locus-specific epigenetic modifications in the genome. Examples include fusing dCas9 to the core catalytic domain of the human acetyltransferase p300 (p300^{core}), which allows acetylation of histone H3 Lys27 (H3K27), and upregulation of genes when binding to proximal or distal enhancers [68]; fusing dCas9 to lysine demethylase 1 (LSD1) reduces the acetylation level of H3K27 [69]; fusing dCas9 to KRAB increases the H3K9me3 mark near the target site [70]; and fusing dCas9 to the DNA methyltransferase DNMT3A increases CpG methylation near the target site [71]. These works also demonstrated modified gene expression levels due to Cas9-mediated locus-specific epigenetic modifications. For example, in mouse embryonic stem cells, the enhancers of pluripotency factors, such as Oct4 and Tbx3 could be repressed by dCas9-LSD1 fusion, leading to loss of pluripotency [69, 72].

While these examples provide an approach to edit the epigenetic states of essentially any locus in the genome, a largely unexplored question is the fate of the synthetic epigenetic marks, and whether or not they can be stably inherited when cells proliferate. Furthermore, given the diverse types of epigenetic modifications and their mutual interactions, a comprehensive toolkit consisting of multiple orthogonally acting dCas9s and their cognate sgRNA that allows flexibly editing multiple epigenetic (histone or DNA) marks simultaneously is needed. The toolkit is useful for understanding the function of diverse epigenetic marks, their interactions, and their relationship to genomic and cellular functions.

Large-scale functional genomic studies using CRISPR/Cas9

One of the powerful applications of the CRISPR/Cas9 technology is the high-throughput screening of genomic functions. The oligo libraries encoding hundreds of thousands of sgRNAs can be computationally designed and chemically synthesized to target a broad set of genome sequences. Pairing with Cas9 or dCas9 fusion proteins, this provides an approach to systematically knock out, repress, or activate genes on a large-scale. The technique requires a delicate delivery method that ensures every cell only receives a single sgRNA, usually via lentiviral or retroviral delivery into mammalian cells. The screens are frequently performed in a pooled manner, as cells transduced with the lentiviral library as a mixed population are cultured together. Via deep sequencing and analysis of the sgRNA features in the pooled cells, genes causing changes in cell growth and death can be inferred with bioinformatics. Indeed, CRISPR screens can easily identify genes, their regulatory elements, and protein domains in the mammalian genome responsible for cell growth and drug resistance [73]. A genomic tiling screen using CRISPR/Cas9 precisely mapped functional domains within enhancer elements and found that a p53-bound enhancer of the p53 effector gene CDKN1A is required for oncogene-induced senescence in immortalized human cells [74].

Using the endonuclease Cas9, loss-of-function genome-wide knockout screens have been performed in cultured or primary mammalian cells with sgRNA libraries (usually 3–10 sgRNAs per gene) to investigate a range of phenotypes including cell growth, cancer cell

drug resistance, and viral susceptibility [75–77]. A genome-scale sgRNA library can also be used to manipulate cultured cells that are later introduced in vivo. Indeed a genome-scale sgRNA library was created to mutagenize a non-metastatic mouse cancer cell line for the study of metastasis in a mouse model [78]. The mutant cell pool rapidly generated metastases when transplanted into immunocompromised mice in vivo. Sequencing of the metastatic cells suggested genes that accelerate lung cancer metastases and development of late-stage primary tumors. Moreover, this screening method can be extended to use in primary cells, which can lead to novel findings that are often overlooked using cell lines. Indeed, introducing a genome-wide sgRNA library into primary dendritic cells (DCs) allowed for the identification of genes related to cell growth that induce tumor necrosis factor (TNF) in response to bacterial lipopolysaccharide (LPS), an essential host response to pathogens [79], which would otherwise be technically challenging with other genome editing tools.

Cas9-mediated loss-of-function screens have also performed to knock out pairs of genes in combination [80]. A library of 23,409 barcoded dual sgRNA combinations were created and a pooled screen was performed to identify gene pairs in human cells that inhibit ovarian cancer cell growth in the presence of small molecule drugs. While further work is needed to characterize the efficacy and accuracy of multiplex genetic screening, this work highlights the potential of more sophisticated functional screening studies using CRISPR.

Beyond Cas9-based complete loss-of-function screens, the invention of CRISPRi and CRISPRa further enables both partial loss-of-function and gain-of-function genetic screens [58, 59]. Growth-based screens using CRISPRi/a have been used to identify essential genes, tumor suppressor genes, and potential mechanisms that confer cytotoxicity induced by a cholera-diphtheria toxin [58]. Using a library consisting of ~70,000 guides targeting the human RefSeq coding isoforms, a CRISPRa-based screen identified genes that, upon activation, conferred resistance to a BRAF inhibitor [59].

In addition to the use of pooled screens, multi-well plates have been used in combination with the partial repression feature of CRISPRi to study the function of the full set of essential genes in the gram-positive bacterium *Bacillus subtilis* [56]. As knocking out essential genes results in lethality that prevents further assay of the phenotype, partial knockdown of essential genes becomes a powerful approach. A mutant *B. subtilis* library was created to include gene partial knockdowns (~3-fold) of all essential genes using CRISPRi, which was tested for the growth phenotype under 35 unique compounds. Using this chemical genomic approach, a comprehensive interconnecting essential gene network was identified, as well as targeted genes that interact with uncharacterized antibiotics. Inducible knockdown of essential genes also allowed for systematic characterization of cell morphology and terminal death phenotypes.

An important question is how these screens compare to each other and to other existing approaches. Several works compared different screens based on CRISPR, CRISPRi and RNA interference (RNAi). One work performed comparative screens of 46 essential and 47 nonessential genes, and concluded that the CRISPR/Cas9 nuclease system outperformed the shRNA- and CRISPRi/dCas9-based gene regulation systems for the sets of essential and

nonessential genes [81]. From the CRISPR screening data, they observed less variation across the data, and detected more functional constructs with less off-target effects. Another work concluded that CRISPR could identify more essential gene targets compared to RNAi [82]. Since similar precision was observed between the two approaches, it was suggested that combining data from both screens could improve the predictive accuracy. The systematic comparison of different approaches may suggest that a comparative screening approach will be more powerful for studying complex cell biology phenotypes.

In addition, new methods to generate CRISPR libraries may help reduce the overall cost associated with this technique and extend its uses to screen a larger chromosomal region (e.g., the tiling along a whole chromosome). While most CRISPR libraries are generated via chemical synthesis of large pools of oligos, a new method termed CRISPR EATING (Everything Available Turned Into New Guides) can inexpensively generate large quantities of sgRNAs for whole genome targeting [83]. In this approach, PAM-proximal sequences are extracted by digesting input DNA with restriction enzymes that target immediately 5' to an NGG or NAG (the PAM sequences for *S. pyogenes* Cas9, N = any nucleic acid). In this study, one library was generated and used to label the whole 3.4-mb region on X. laevis chromosome 4 in the egg extracts. The method allows for the generation of complex and customized libraries from any source of DNA via routine molecular biology methods.

CRISPR/Cas9 for generating animal models

Genetically engineered animal models are crucial for the study of complex cellular and physiological processes. While mouse models have been widely used, the CRISPR/Cas9 gene editing approach has been established in many other animal models including worm [84], fly [85], fish [86, 87], rat [88], rabbit [89, 90], goat [91], sheep [92], dog [93], pig [94], and monkeys [95]. The expansion of transgenic animal models beyond mouse is advantageous to biomedical research that accelerates the development of new therapeutic strategies.

CRISPR provides a much easier approach to establish these transgenic animal models compared to previous gene-editing tools. Traditional approaches to construct transgenic mice via insertional mutagenesis or TALEN-mediated gene editing are time-consuming, costly, and inefficient. The robustness and high efficiency of CRISPR-Cas9 simplifies the process for creating model systems [96, 97]. Moreover, nucleic acids encoding the Cas9 protein and target-specific sgRNAs can be conveniently injected into embryos to generate gene-modified mice with deletions of multiple genes, mutations in defined genes, or insertions of fluorescence reporters or other peptide tags to endogenous genes. For example, coinjection of Cas9 mRNA and sgRNAs targeting Tet1 and Tet2 into zygotes generated mice with biallelic mutations in both genes with an efficiency of 80% [96]. Furthermore, coinjection of Cas9 mRNA and sgRNAs with mutant oligos generated precise point mutations simultaneously in two target genes, while coinjecting Cas9 mRNA and sgRNAs into one-cell-stage cynomolgus monkey embryos generated founder animals harboring two gene modifications [95].

The establishment of a Cre-conditional Cas9 knockin mouse has broadened the applications of Cas9 *in vivo* [98]. The Cas9 knockin mouse is a great resource to rapidly generate

mutations in a subpopulation of cells in vivo, and test how mutations cause disease phenotypes. Different methods based on adeno-associated virus (AAV), lentivirus, or nanoparticles can be used to deliver sgRNAs into multiple cell types such as neurons, immune cells, and endothelial cells in a Cas9 knockin mouse to model the dynamics of significantly mutated genes in lung adenocarcinoma [98]. Another work demonstrated that the Cre-conditional Cas9 knockin mouse phenocopied Cre-mediated genetic deletion of genes in Cre/LoxP mouse models in studying pancreatic ductal adenocarcinoma [99]. Via retrograde pancreatic ductal injection of lentiviral vectors expressing Cre and an sgRNA into Cre-conditional Cas9 knockin mice, the authors showed knockout of *Lkb1* together with manipulated expression of oncogenic *Kras*. However, due to the heterogeneity of delivery and Cas9-mediated gene editing, caution is required when interpreting results.

In addition to using a Cas9 knockin mouse model, viral vectors encoding Cas9 and an sgRNA can be directly delivered into wildtype mice or Cre/loxP mouse models to probe gene function. One work used adeno-associated viral (AAV) vectors encoding Cas9 and sgRNAs to target a single gene or multiple genes in the normal adult mouse brain in vivo [100]. Characterizing the effects of gene modifications in postmitotic neurons revealed similar phenotypes as observed in gene knockout mice. Another work used a lentiviral system that delivers both the CRISPR system and Cre recombination to examine CRISPR-induced mutation of genes in the context of well-studied conditional Cre/*loxP* mouse models of lung cancer and other cancer types [101]. In another work to study cancer genes in the mouse liver, a hydrodynamic injection delivered a plasmid DNA expressing Cas9 and sgRNAs that directly targeted the tumor suppressor genes (*p53* or *PTEN*) alone and in combination into the liver. The authors demonstrated the feasibility of Cas9-mediated mutation of tumor suppressor genes in the liver as an avenue for rapid development of liver cancer models [102]. However, similar to the Cas9 knockin mouse, the virally delivered Cas9 may only edit genes in a fraction of cells, and the approach may be most effective for studying the effects of loss-of-function mutations on cell autonomous properties.

Genome imaging using CRISPR/Cas9

Imaging offers a direct approach for studying the spatial and temporal behavior of the genome in living cells [103]. The ability of Cas9 to target specific sequences in the genome makes it a promising imaging tool for directly observing genomic organization and dynamics in cells. The first proof-of-concept work fused the *S. pyogenes* dCas9 to EGFP and used the fusion protein to visualize the dynamics of coding or non-coding sequences in living human cell lines [104]. The authors tracked the dynamics of telomeres, and the repetitive and nonrepetitive sequences of coding genes (*MUC4*, *MUC1*) in a short time frame (~minutes) and throughout the whole cell cycle. In addition, dCas9 fused to EGFP has been used to label endogenous centromeres and telomeres loci in live mouse embryonic stem cells [105]. The development of the SunTag system, a repeating peptide array that can recruit multiple copies of an antibody-fusion protein, enhanced the sensitivity to amplify the dCas9 fluorescent signal in the genome [64]. Using dCas9 orthologs tagged with different fluorescent proteins, it was shown that the dynamics of multiple repetitive genomic loci could be tracked in living cells [106]. A method termed CASFISH (Cas9-mediated fluorescence in situ hybridization) further combined dCas9 with FISH (fluorescence in situ

hybridization) [107]. Due to the specific DNA targeting and unwinding activity of dCas9, CASFISH allowed a fast and convenient process for labeling DNA elements while avoiding treatment of heat and disruptive chemicals that distort the natural organization of the nucleus, which is normally seen in FISH. Thus the approach preserves the spatial relationships of the genetic elements that are important for studying gene expression.

Recent work also established a CRISPR approach to facilitate super-resolution imaging in living mammalian cells [108]. Current live cell super-resolution imaging normally relies on the overexpression of a host protein fused to a fluorescent protein, which results in artifacts that may obscure the interpretation of imaging results. Using CRISPR/Cas9 to fluorescently tag the endogenous genes that are expressed from their native genomic loci could allow genes to be expressed at close to endogenous levels, thus avoiding artifacts. Based on the idea, a method termed RESOLFT (reversible saturable optical fluorescence transitions) was developed, wherein heterozygous and homozygous Cas9-edited human knockin cell lines were generated that expressed the reversibly switchable fluorescent protein rsEGFP2 from their respective native genomic, which prevent the appearance of typical overexpression-induced artifacts in these cells.

To enhance signals for endogenous proteins imaging, one work adapted self-complementing split fluorescent proteins, GFP11 and sfCherry11, derived from the sfGFP and sfCherry [109]. The small sizes of these split fluorescent domains (16–18 amino acids) allow easily inserting them into endogenous genomic loci via CRISPR gene editing. Tandem arrays of these domains further amplify fluorescence signals in imaging, such as for tracking intraflagellar transport particles.

In addition to DNA imaging, *S. pyogenes* dCas9 can also allow for endogenous RNA imaging in living cells [110]. In the presence of sgRNAs targeting mRNA and a stabilized PAMmer oligonucleotide that contains the PAM domain for dCas9 binding, specifically targeted RNA can be visualized. Indeed, it was observed that nuclear localized dCas9 could be exported to the cytoplasm. Furthermore dCas9 allowed for tracking of RNA during induced RNA/protein accumulation in the presence of oxidative stress.

Lineage tracing using CRISPR/Cas9

It is worth noting that gene editing has been used as tools for cell lineage tracing. One recent work demonstrated a lineage tracing method termed genome editing of synthetic target arrays for lineage tracing (GESTALT) [111]. This method uses CRISPR/Cas9 gene editing to generate a combinatorial diversity of mutations that accumulate over cell divisions within a series of DNA barcodes. Via deep sequencing, lineage relationship between many cells can be inferred using patterns of the edited barcodes. The approach was developed in both cell culture and zebrafish, by editing synthetic arrays of approximately a dozen CRISPR/Cas9 target sites. The approach generated thousands of unique edited barcodes in cell lines, which could be sequenced from either DNA or RNA. By injecting fertilized eggs with editing reagents that targeted a genomic barcode with 10 target sites, the authors observed the accumulation of hundreds to thousands of uniquely edited barcodes per animal, and further inferred the lineage relationship between ancestral progenitors and organs based on mutation

patterns. This proof-of-principle work showed that combinatorial and cumulative genome editing is a powerful approach to record lineage information in multicellular systems.

In another work, the type I-E CRISPR/Cas system of *Escherichia coli* was harnessed to generate records of specific DNA sequences in bacterial genomes [112]. Unlike gene editing, the work is based on the native adaptive immunity acquisition ability of CRISPR, as new spacer sequences can be acquired and integrated stably into the CRISPR crRNA array. Using this feature, it was demonstrated that the Cas1–Cas2 complex enables recording of defined sequences over many days and in multiple modalities. The work elucidated fundamental aspects of the CRISPR acquisition process. The recording system developed could be useful for applications that require tracing long histories of *in vivo* cellular activity.

While optimization of these methods is required for more robust performance, genome editing and the unique features (i.e., adaptation) of the CRISPR system provide promising approaches to record biological information and history in living cells and tissues. One can envision that these tools may enable mapping of the complete cell lineage in multicellular organisms as well as linking cell lineage information to molecular profiles (e.g., transcription, epigenetics, and proteomics) such as those in single cells.

Concluding remarks

CRISPR/Cas9 technology has revolutionized cell biology research. The system is versatile, enabling diverse types of genome engineering approaches. While most of the work has used Cas9-mediated knockout or dCas9-mediated repression and activation to study gene function, we expect expansion of these tools to study the epigenome and 3-dimensional chromosomal organization in greater detail in the future. Furthermore, studies have used CRISPR to model complex genomic rearrangements *in vitro* and *in vivo*, which brought breakthroughs in studying chromosomal translocations [113, 114]. Most research has been performed in cell lines, and future work related to the interrogation of cellular functions should be carried out in primary cells derived from animals or human patients or *in vivo* using relevant animal models.

CRISPR/Cas9 is emerging as a major genome-manipulation tool for research and therapeutics, yet there are remaining challenges to improve its specificity, efficiency, and utility (see Outstanding Questions). One major concern is the off-target effects, since Cas9 can tolerate mismatches between sgRNA and target DNA [115–117]. Methods have been developed to profile the off-target effects such as GUIDE-seq [118]. To improve specificity, several strategies have been developed including using paired nickase variants of Cas9 [32, 42], paired dCas9-FokI nucleases [119, 120], truncated sgRNAs ((17 to 18 bp) that are more sensitive to mismatches [121], and controlling acting concentration of the Cas9/sgRNA complex [122]. Using structure-guided protein engineering approaches, a few work recently created *S. pyogenes* Cas9 variants with improved specificity [123, 124]. For example, a high fidelity variant of Cas9 harboring designed alterations showed reduced non-specific DNA contacts, while retaining robust on-target activities comparable to wild-type Cas9 [124]. Combinations of these methods could provide a route to its ultimate use for gene therapy.

As a powerful yet versatile gene editing and regulation tool, the CRISPR-Cas9 technology is already accelerating both research and therapeutics. We believe its broad applications in genomics research and cell biology research will greatly advance our knowledge of both basic biology and diseases in the years to come.

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Outstanding questions

- How can the off-target effects of CRISPR/Cas9 be avoided in mammalian cells and whole organisms?
- Can CRISPR/Cas9 technology be developed to insert a large gene fragment into the mammalian genome for gene knockin studies with similar efficiency to that of gene knockout studies?
- Will CRISPR/Cas9 technology be able to efficiently modulate different types of epigenetic modifications? Can it control the fate of synthetic epigenetic marks, and whether or not they can be stably inherited when cells proliferate?
- Can CRISPR/Cas9-mediated genetic screens be performed on non-proliferation based phenotypes such as differentiation?
- Can CRISPR/Cas9 technology enable more robust transgenic animal generation by deleting, mutating, and inserting any gene of interest?
- Beyond gene editing, how can CRISPR/Cas9 be used to help advance cell biology research?

Trends

- The RNA-guided CRISPR/Cas9 endonuclease and the endonuclease-dead dCas9 protein are powerful genomic manipulations tools for gene editing, transcriptional regulation, and epigenetic modifications.
- Both Cas9 and dCas9 enable diverse types of high-throughput screenings of gene functions in cell lines and in vivo
- The CRISPR/Cas9 accelerates the establishment of many useful transgenic animal models for biomedical research.
- The CRISPR/Cas9 is repurposed for genomic imaging and lineage tracing in living cells and tissues.

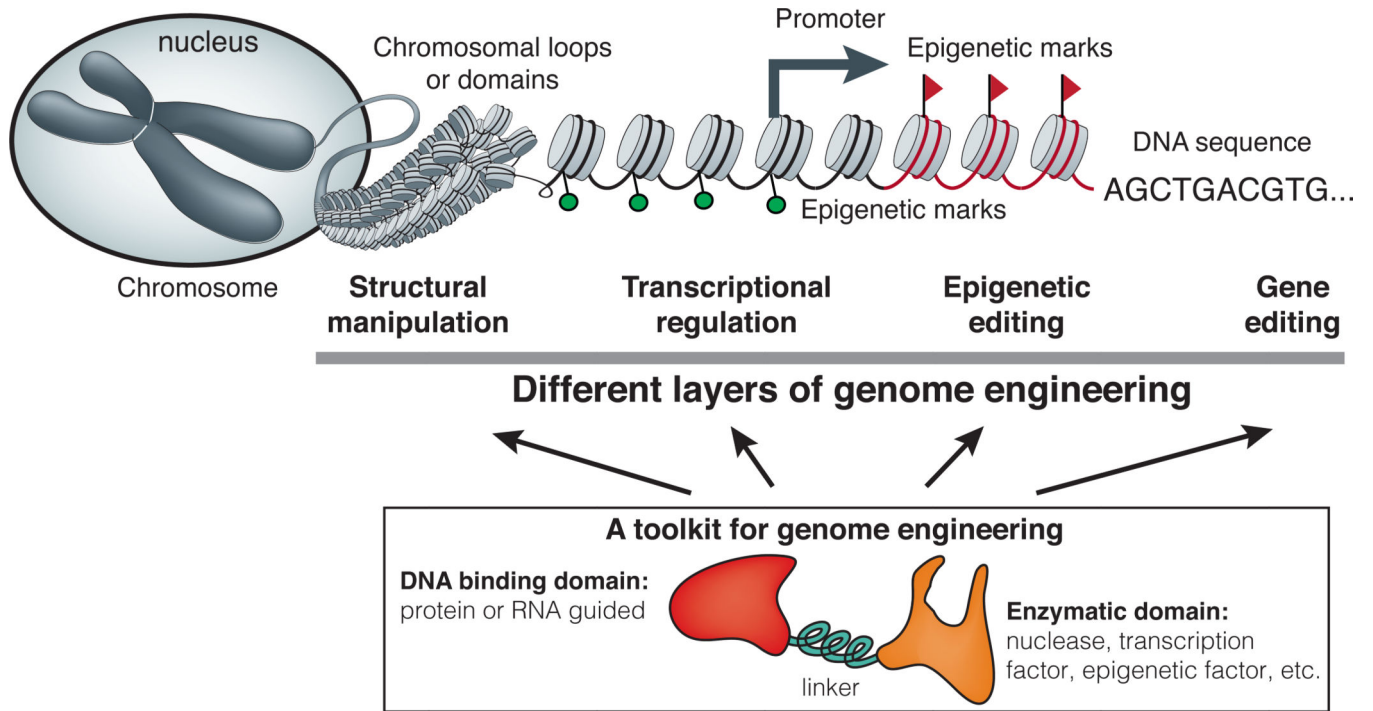


Figure 1. A schematic view of the diverse goals of genome engineering
 Genome engineering defines methodological approaches to alter the DNA sequence (gene editing), modify the epigenetic marks (epigenetic editing), modulate the functional output (transcriptional regulation), and reorganize the chromosomal structure (structural manipulation).

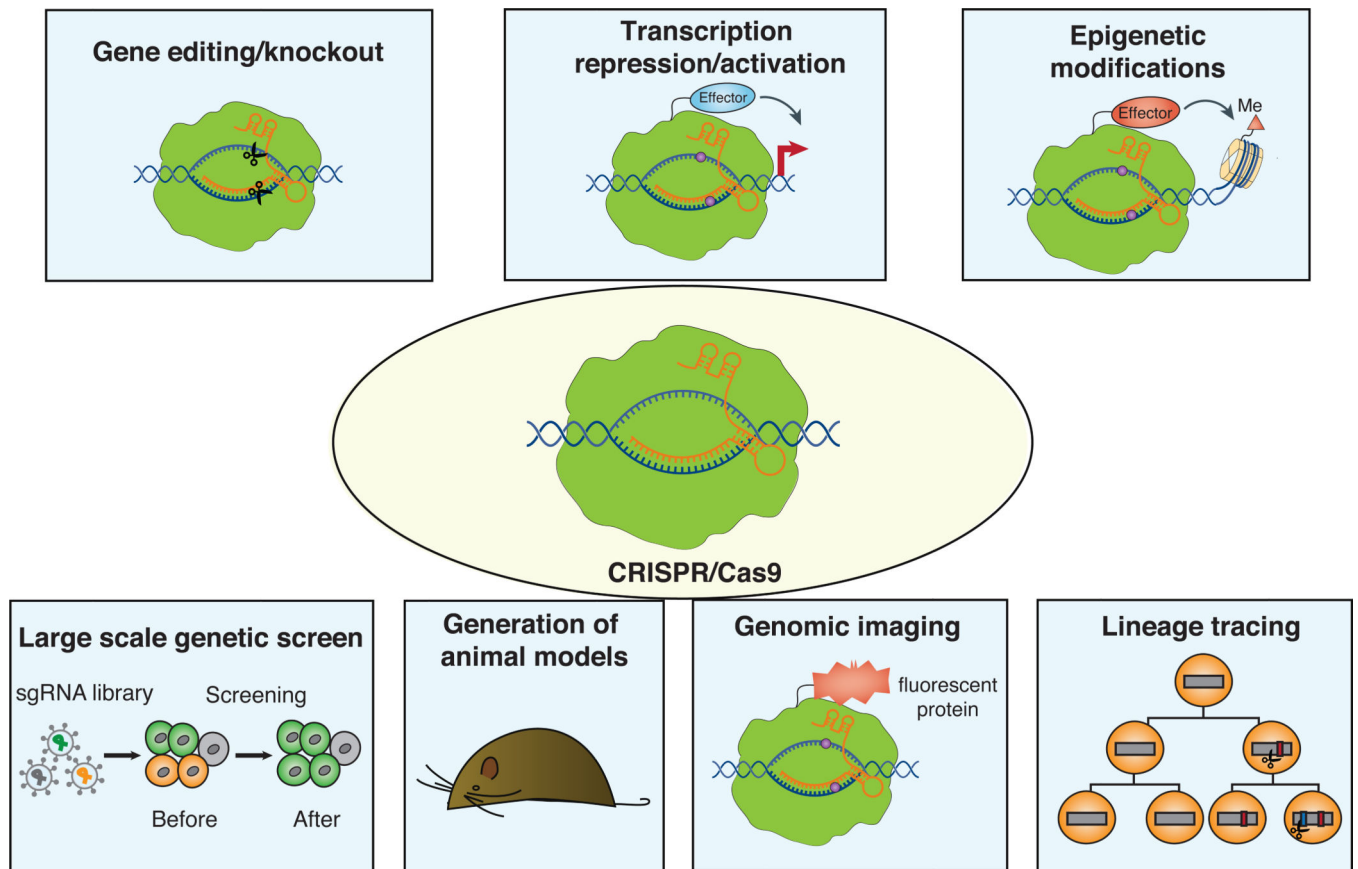


Figure 2. Applications of CRISPR/Cas9 to the cell biology research

The CRISPR/Cas9 technology has been used for gene editing, transcriptional regulation, epigenetic regulation, large-scale genetic screens, generation of animal models, and genomic imaging.

Recent examples of applying CRISPR/Cas9 technology to study cellular organelles

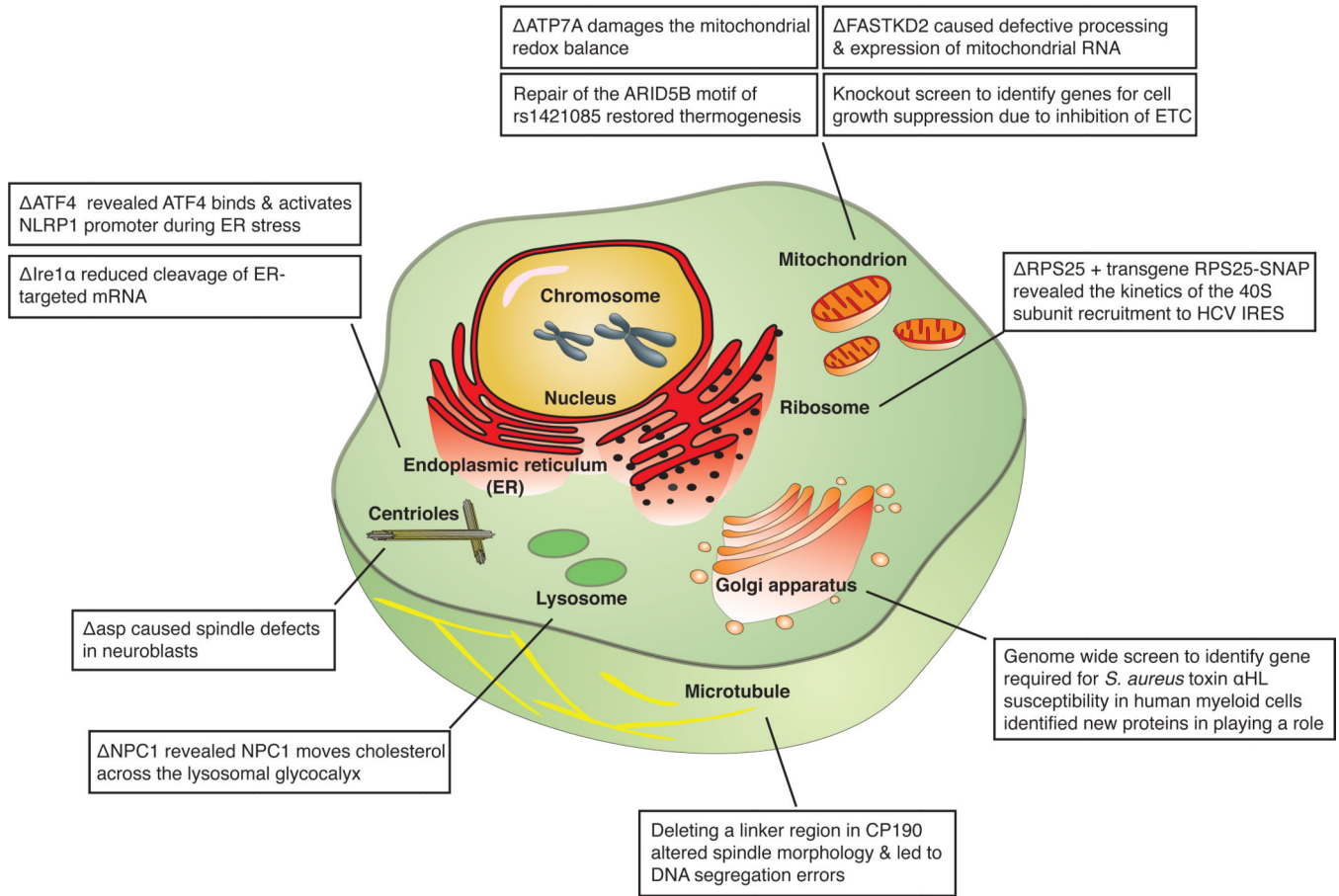


Figure 3. Examples of applying the CRISPR/Cas9 technology to study cellular organelles
 The figure illustrates exemplar studies in particular organelles, with more details listed in Table 1.

Table 1

Examples of CRISPR/Cas9 being used for cell biology research

| Organelle | CRISPR-Cas9 target | Finding | Ref |
|-----------------------|--|--|------|
| Microtubule | CRISPR/Cas9 generation of mutant flies by deleting a linker region in the centrosome protein CP190 | Identified a centrosome and microtubule targeting region in CP190 for spindle localization Deletion of the linker region altered spindle morphology and led to DNA segregation errors | [44] |
| Mitochondria | CRISPR/Cas9 knockout of the copper transporting ATPase ATP7A in mouse 3T3-L1 cells and in Menkes Disease (MD) patient fibroblasts | ATP7A dysfunction damages the mitochondrial redox balance | [45] |
| | CRISPR/Cas9 knockout of FASTKD2, a RNA binding protein of the FAS-activated serine/threonine kinase family | Defective processing and expression of mitochondrial RNA Cellular respiration damage with depressed activities of respiratory complexes | [46] |
| | CRISPR/Cas9 mediated repair of the ARID5B motif of rs1421085 in primary adipocytes from a patient carrying the risk allele | IRX3 and IRX5 repression restored Browning expression programs activated Thermogenesis restored | [47] |
| | CRISPR/Cas9-based genetic screen to study cell proliferation suppression due to inhibition of the mitochondrial electron transport chain (ETC) | Identified the cytosolic aspartate aminotransferase (GOT1) as the key gene GOT1 loss of function kills cells upon ETC inhibition. | [48] |
| Endoplasmic Reticulum | CRISPR/Cas9 knockout of ATF4 or NLRP1 | NLRP1 is up-regulated during severe ER stress ATF4 binds and activates NLRP1 promoter during ER stress | [49] |
| | CRISPR/Cas9 mediated deletion of transmembrane endoribonuclease Ire1 α in HEK293 cells | Ire1 α forms a complex with the Sec61 translocon to cleave its mRNA substrates Disruption of Ire1 α complex caused reduced cleavage of ER-targeted mRNA | [50] |
| Centrosome | CRISPR/Cas9 dual-sgRNA to generate a null abnormal spindle (<i>asp</i>) allele by excising a 750-bp fragment that included the promoter, 5' UTR, and the first exon in <i>Drosophila</i> neuroblasts | <i>Asp</i> null mutations cause spindle defects in neuroblasts <i>Asp</i> is regulated by <i>Drosophila melanogaster</i> calmodulin (CaM) to cross-link spindle microtubules | [51] |
| Lysosome | Generation of Niemann-Pick type C 1 (NPC1)-deficient cell line using CRISPR/Cas9 | NPC1 moves cholesterol across the lysosomal glycocalyx | [52] |
| Ribosome | CRISPR/Cas9 knockout of nonessential gene of ribosomal protein eS25 (RPS25) in Hap1 cell line. | Demonstrated an approach to create fluorescently labeled 40S ribosomal subunits from human cells | [53] |

| Organelle | CRISPR-Cas9 target | Finding | Ref |
|-----------------|--|--|------|
| | RPS25-SNAP (mutant O6-alkylguanine DNA alkyltransferase) transgene was transduced into RPS25-KO Hap1 cells to be the only source of the protein. | Studied kinetics of the 40S subunit recruitment to the hepatitis C virus (HCV) internal ribosome entry site (IRES) | |
| Golgi apparatus | Genome-wide CRISPR/Cas9 loss-of-function screen to identify host targets required for <i>S. aureus</i> toxin alpha hemolysin (α HL) susceptibility in human myeloid cells. | Identified new proteins (SYS1, ARFRP1, TSPAN14) in regulating the presentation of ADAM10 on the plasma membrane post-translationally Cells lacking sphingomyelin synthase 1 (SGMS1) resist α HL intoxication | [54] |

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