CRISPR Handbook

Enabling Genome Editing and Transforming Life Science Research

Second Edition

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*Updated September 2016 to include new CRISPR library and ribonucleoprotein technology and workflows

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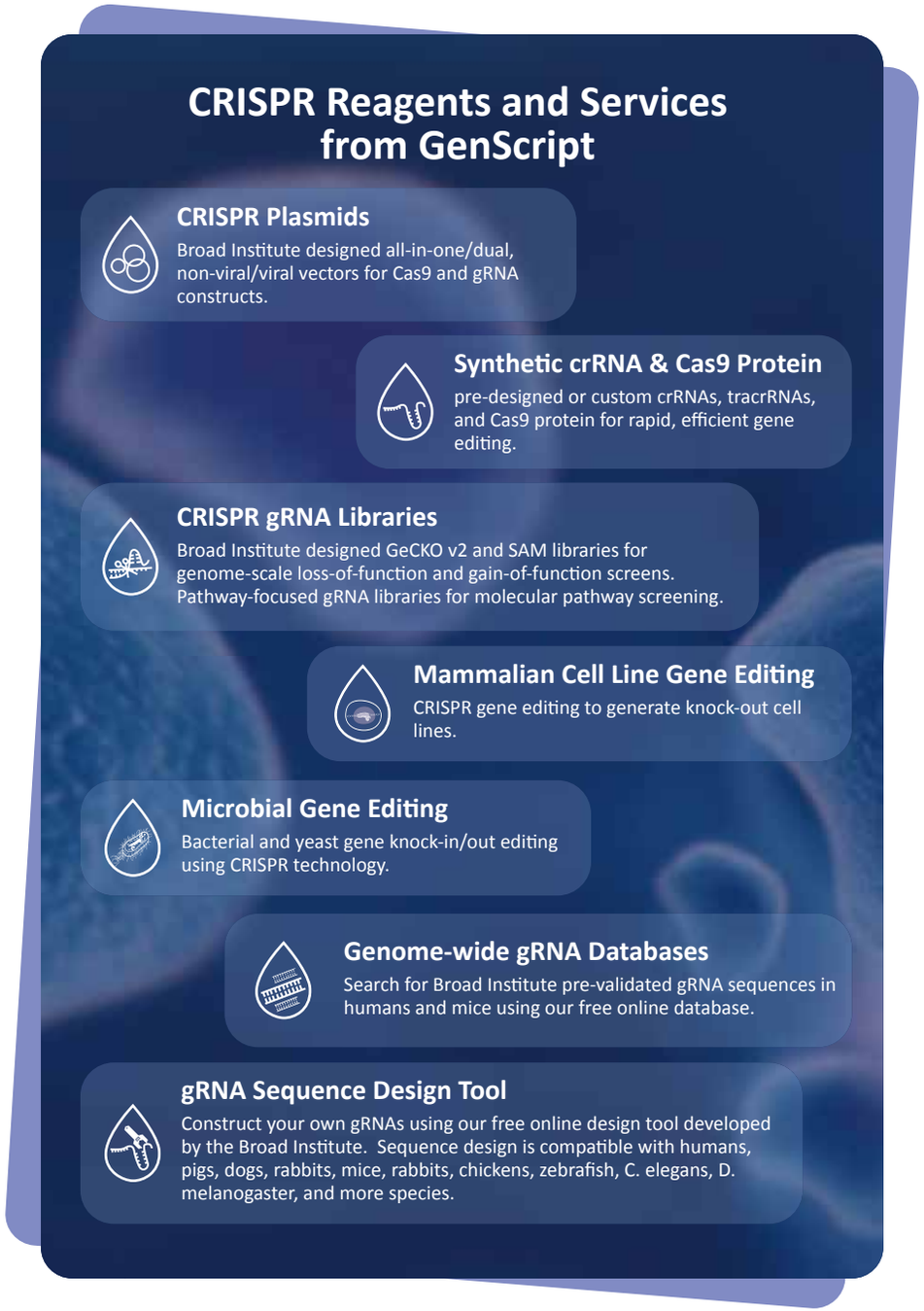

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www.genscript.com/CRISPR-handbook.html

Table of Contents

This handbook describes CRISPR/Cas9 genome editing and other research applications for CRISPR technology.

The CRISPR Genome Editing Revolution

Expanding the Research Applications for CRISPR

Putting CRISPR into Practice: Workflows and Case Studies

Part II of our CRISPR Handbook will be released next week! Our next chapter contains workflows and case studies to help you start using CRISPR/Cas9 technology in your research. Please sign up to receive a copy:

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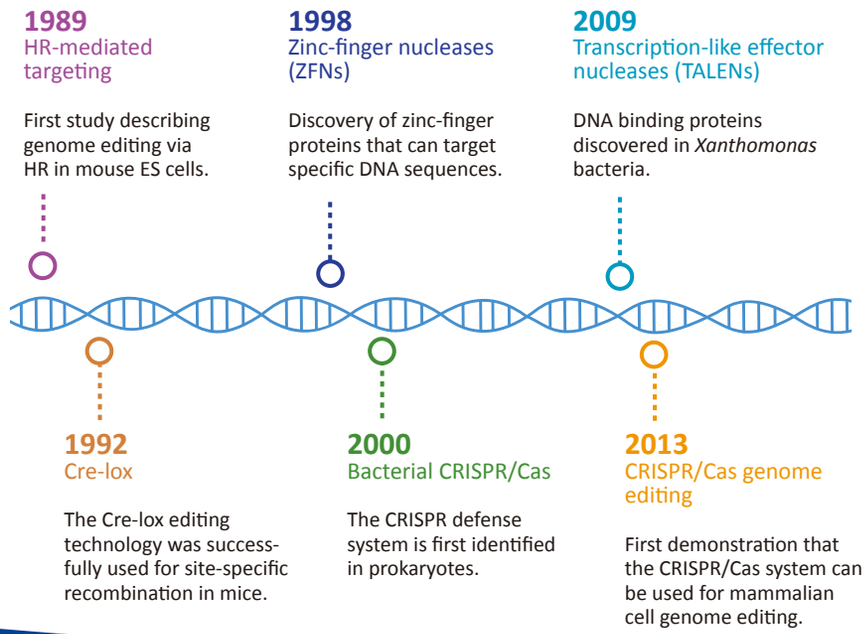
The CRISPR Genome Editing Revolution

The ability to manipulate DNA is a biologist's greatest tool – making it possible to define the relationship between a gene, its mRNA and protein products, and their functions. By inhibiting gene function or altering gene expression, genome editing can provide tremendous insight into the basis of disease or identify new targets for medical intervention. Over the last 20 years, advances in genome editing technologies have evolved to allow precise genome manipulation in cell lines and animal models. Of these new technologies, perhaps the most exciting is CRISPR/Cas9, a gene editing system adapted from the bacterial immune system that is efficient, rapid, and easy-to-use. CRISPR/Cas9 technology allows targeted knock-in and knock-out of any gene within the genome. In this handbook, we will discuss how CRISPR technology has fueled a genome editing revolution and how it has been adapted for other biological applications.

Evolution of Genome Editing Technology

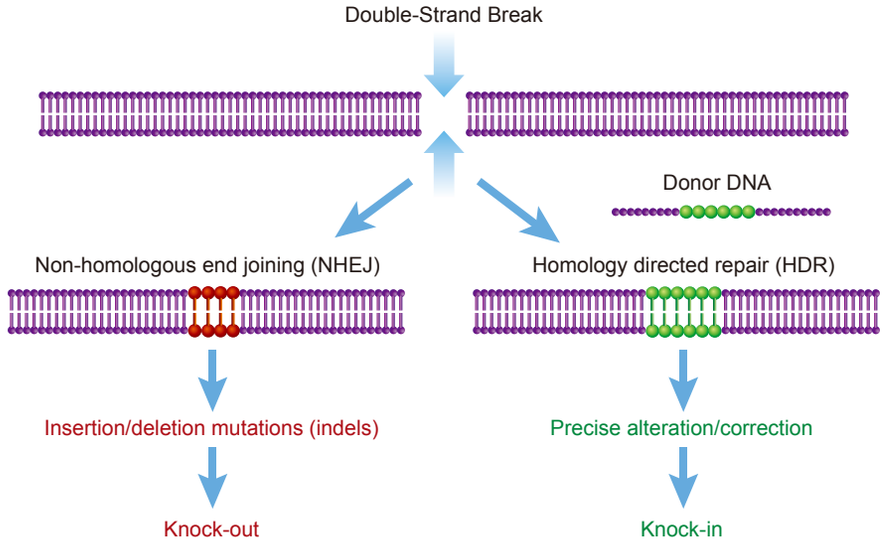
Genome editing technology allows for the direct manipulation of the genetic code, giving researchers the ability to delete, insert or replace DNA. While the use of genome editing was first widely popularized in the early 2000s, the concept of genome editing was originally born more than a decade earlier. Gene editing was first used In 1989, when researchers targeted specific genes in mouse embryonic stem cells using homologous recombination (HR) to generate knock-in and knock-out mutations[1] (Figure 1). Since HR occurs infrequently in mammalian cells, the initial targeting frequencies were low (1 per 3×10^4 cells); however, this work provided a foundation for how genes can be targeted and altered.

Figure 1: Advancements in genome editing



While HR alone rarely results in gene integration in mammalian cells, the introduction of double-strand breaks (DSBs) into the genome can increase recombination rates significantly[2]. DSB resolution occurs by either or error-prone non-homologous end joining (NHEJ) or HR (Figure 2). If no donor DNA is present, resolution will occur by NHEJ, resulting in insertion or deletion mutations (indels) that will ultimately knock-out (KO) gene function. Alternatively, if donor DNA sequences are available, the DSBs will be repaired by HR, resulting in gene knock-in (KI)[3].

Figure 2: DNA repair by targeted genome editing



As the need for relevant animal disease models rose, so did the need for more sophisticated and efficient genome editing tools. Cre-lox technology came into use in the early 1990s, contributing greatly to the development of transgenic mouse models[4,5]. The Cre-lox system allows scientists to control gene expression both spatially and temporally, using a site-specific DNA recombinase Cre, which

recognizes 34-bp loci called *loxP*[6]. Cre-mediated recombination leads to the knock-out of targeted genes between *loxP* sites. But while easier to control than homologous recombination, the Cre-lox system becomes less efficient as the genetic distance increases between *loxP* sites[7].

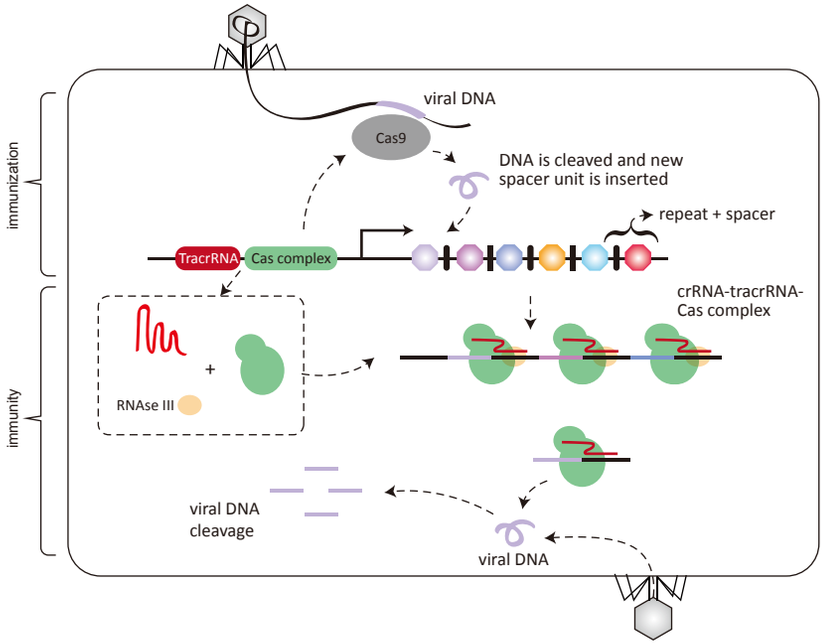
Starting in the late 1990s, newer and more effective genome editing techniques employing DSB-mediated repair came into use, including zinc-finger (ZF) and transcription activator-like effector (TALE) nucleases[8-10]. Both of these systems use DNA-binding proteins with nuclease activity that bind to DNA and create site-specific DSBs. While effective, these methods require extensive expertise in protein engineering, which has been a bottleneck for many research labs using this technology[11]. These advances in genome editing technology revealed specific prerequisites required for broader genome editing applications in research: the technology needs to be efficient, effective, affordable, and be easy-to-use.

Discovery of CRISPR in Bacterial Immune Systems

In the late 1980s, researchers discovered the clustered DNA repeats, that would become known as CRISPRs. In 1987, while studying the *iap* gene in Escherichia coli, researchers at Osaka University also accidently cloned a series of interrupted clustered repeats in the gene's 3' flanking region[12]. By 2000, additional sequencing studies had shown that these interrupted clustered repeats were widespread in both bacteria, archaea, and mitochondria[13].

In 2002, researchers dubbed these repeats, CRISPRs (clustered regularly interspaced short palindromic sequences), and identified nearby CRISPR-associated or *cas* genes[14]. These cas genes were consistently found adjacent to CRISPR loci and displayed motifs characteristic of both helicases and endonucleases[14]. The presence of multiple chromosomal CRISPR loci and invariably adjacent endonucleases suggested both that CRISPRs are mobile elements and that Cas proteins play a role in their genomic integration. In 2005, researchers from three independent groups confirmed that CRISPR spacers are derived from viral and extrachromosomal sources[15-17]. Together, these findings led researchers to conclude that theCRISPR/Cas system functions in bacterial adaptive immunity against foreign genetic elements.

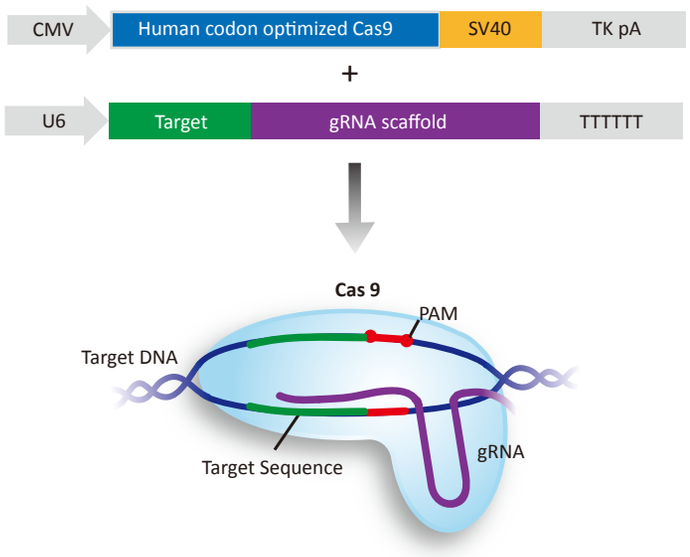
Figure 3: Mechanism of CRISPR-mediated immunity in bacteria



Experimental work in recent years has revealed the mechanism of action for CRISPR/Cas-mediated immunity. The CRISPR/Cas adaptive immune system is composed of two main phases: immunization and immunity (Figure 3). In the immunization phase, Cas proteins form a complex which cleaves foreign, viral DNA[18]. This foreign DNA is then incorporated into the bacterial CRISPR loci as repeat-spacer units18. In the immunity phase, following re-infection, the repeat spacer units are transcribed to form CRISPR RNA (crRNA) precursors[19]. The Cas9 endonuclease is then guided by trans-activating crRNAs (tracrRNA) to bind with crRNA precursors[20]. A mature crRNA-Cas-tracrRNA complex is formed following cleavage by RNAse III[20]. The mature crRNA serves as a small guide RNA, which upon infection will pair with viral DNA, triggering Cas cleavage and interference of the viral DNA[19,21,22].

The specificity of CRISPR/Cas for foreign DNA is triggered by the presence of a short 3-5 base pair sequence known as a protospacer adjacent motif (PAM)[23]. PAM sequences lie adjacent upstream of "protospacers," the foreign DNA genomic target sequences[23]. These motifs allow Cas endonucleases to discern between bacterial self DNA and invading non-self DNA, and are essential for Cas-mediated cleavage and targeting[24,25].

Figure 4: CRISPR/Cas system for genome editing in mammalian cells



Since their initial discovery, five different CRISPR/Cas system types have been characterized in bacteria[26]. In 2013, researchers adapted type II *Streptococcus pyogenes* Cas9 (SpCas9) for genome editing in mammalian cells[25,27]. But, instead of interfering with invading DNA, the CRISPR/Cas9 system can be used to create DSBs in endogenous genes to either trigger a KO mutation via NHEJ, or if cleaved in the presence of donor DNA, a KI mutation via HR.

The CRISPR/Cas9 system is relatively simple and requires only two components, the Cas9 endonuclease and a fused crRNA-tracrRNA transcript, known as a guide RNA (gRNA)[25]. The gRNA combines the functionality of its crRNA-tracrRNA components, acting as both a targeting apparatus to DNA target sequences

GenScript offers Broad Institute-validated **gRNA/SpCas9 Vectors** for gene editing in mammalian cells. SpCas9 editing allows for knock-out or knock-in mutation of any gene.

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Expanding CRISPR/Cas9 Recognition Sequences

One limitation of the first CRISPR genome editing protocol was the constraint on genomic sequences that could be targeted. The SpCas9 enzyme requires the presence of a PAM sequence "NGG" at the end of the gRNA 20-mer recognition sequence[29]. Guide RNA expression was driven by the U6 human pol III promoter due to its transcription initiation efficiency. But U6 initiates transcription from a guanosine (G) nucleotide, forcing U6-expressed gRNAS to be selected from genomic sequences that fit the pattern GN19NGG – which might occur infrequently in a gene of interest.

One strategy for expanding CRISPR/Cas9 sequence recognition was to drive gRNA expression using a different promoter. The H1 promoter can initiate transcription from A or G; therefore, H1-driven gRNAs can also target sequences of the form AN19NGG, which occur 15% more frequently than GN[19]NGG within the human genome[29]. This small change in the gRNA expression cassette more than doubles the number of targetable sites within the genomes of humans and other eukaryotes.

Another strategy has been to remove restrictions on the PAM sequence, as the SpCas9 requirement for NGG presents a tight constraint. One approach has been to use protein engineering techniques to create novel engineered Cas mutants that recognize alternative PAM sequences[30]. By analyzing structural information, bacterial selection-based directed evolution, and combinatorial design, researchers developed several Cas9 variants that can recognize alternative PAM sequences, including NGA and NGCG, NNGRRT, and NNHRRT[30].

In addition to alternative PAM variants, there has also been increased interest in Cas9 alternatives for CRISPR-mediated gene editing. One example is the exploration of *Francisella* Cpf1 (FnCpf1), a type 2 nuclease that lacks the HRH domain present in SpCas931. Unlike traditional Cas9 nucleases, this enzyme recognizes a different PAM sequence (5'-TTN-3'), features a shorter crRNA, and does not require a tracrRNA31. In addition, Cpf1 cleaves DNA is a staggered pattern, which can be beneficial for primary cell editing[31]. And due to the smaller size of Cpf1, the enzyme is easier to package into vectors, making it ideal for in vivo gene editing applications[31].

Improving Cleavage Specificity of the CRISPR/Cas9 System

While specificity of the CRISPR/Cas9 system is governed by gRNA sequence complementation, CRISPR/Cas9 complexes are tolerant of several mismatches with their targets[32]. DSBs have been observed at sites containing five or more mismatched nucleotides relative to the guide RNA sequence[33]. Due to the variable activity of the CRISPR/Cas9 system major efforts have been undertaken in recent years to improve cleavage specificity.

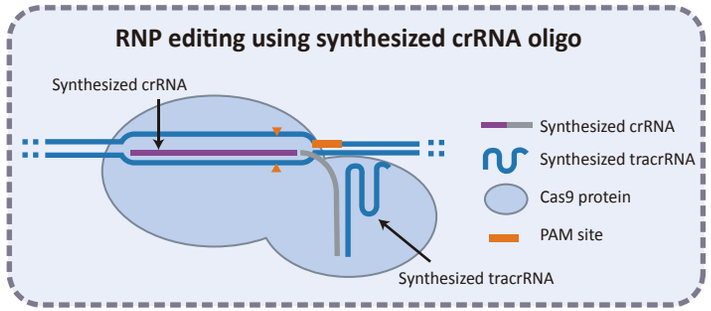
In 2014, the Broad Institute of MIT and Harvard pioneered one of the earliest studies for modeling gRNA binding specificity[34]. Researchers examined all possible targetable sites present in 6 mouse and 3 human genes, creating 1,841 gRNAs in total, and quantified their ability to knock out gene expression[34]. Based on the results a predictive model of gRNA activity was constructed. This model enabled the development of a gRNA design tool publicly available at: http://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design.

Later in 2016, a second generation model was designed by the Broad Institute in collaboration with Microsoft[35]. The second generation model utilizes an algorithm that better models on-target activity, as well as a Cutting Frequency Determination (CFD) score to predict off-target activity[35]. These new tools are also publicly available at: http://portals.broadinstitute.org/gpp/public/software/sgrna-scoring

Another strategy used to improve the cleavage specificity of the CRISPR/Cas9 system is the direct transfection of crRNA/Cas9 ribonucleoproteins (RNPs) into the cell. Recent experiments have observed that off-target binding of Cas9 is concentration-dependent36. By delivering optimized concentrations of Cas9 protein, as opposed to ubiquitously or differentially expressing DNA plasmids, the persistence and expression levels of Cas9 can be limited. When Cas9 is combined with duplexed crRNA:tracrRNA and delivered into cells the RNP complex is able to cleave chromosomal target DNA immediately after delivery (Figure 5)[37,38]. The RNP complex is then quickly removed by the cell's endogenous degradation machinery, further limiting off-target cleavage.

GenScript's **synthetic crRNA/Cas9** service delivers pre-duplexed crRNA:tracrRNA oligos and Cas9 protein ready-for-use. This service offers high-efficiency gene editing, with low off-target effects.

Figure 5. Increasing specificity through paired guides: Nickase or RFN

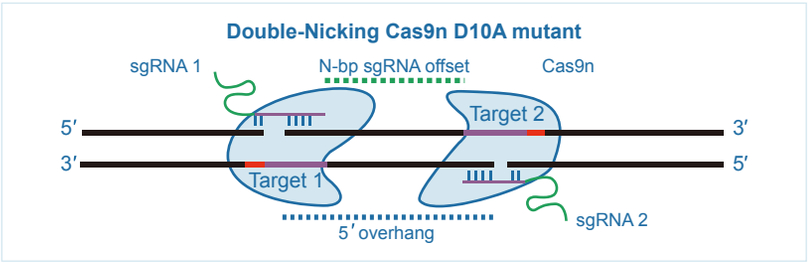


New strategies to improve CRISPR efficiency and specificity involve modifications to the Cas9 endonuclease itself. Cas9 contains two catalytic domains, RuvC and HNH. Broad Institute researchers have recently shown that mutations to catalytic residues D10A in RuvC and H840A in HNH, cause Cas9 to create single strand nicks as opposed to double strand breaks[28].

By utilizing nickase mutants (Cas9n) with paired gRNAs, it is possible to obtain target specific knock out mutants (Figure 6). Targeting Cas9n to two loci within close proximity, but on opposite strands, causes the endonuclease to nick both strands and create a DSB, eventually resulting in a mutation. Although each gRNA used might have off-target binding sites throughout the genome, Cas9n only catalyzes single strand breaks (SSBs) at each of those locations. SSBs are preferentially repaired through HR rather than NHEJ, which will decrease the frequency of unwanted indel mutations. Paired nicking has been demonstrated to reduce off-target activity by 50-1,000 fold in cell lines[39].

GenScript offers Broad Institute-validated **gRNA/Cas9 Nickase Vectors** for gene editing in mammalian cells. Use paired nicking for maximized

Figure 6. Increasing cleavage specificity through paired nickase



Upon further analysis of the Cas9 protein structure, Broad Institute researchers discovered a positively charged groove between the HNH and RuvC domains, which likely stabilizes non-target DNA within the enzyme and ultimately increases the likelihood of off-target cleavage[40]. Working under the hypothesis that neutralizing this interaction would reduce the attraction between Cas9 and non-target DNA, the group replaced the positively charged amino acids within to groove to alanine[40]. This Cas9 mutant, named "enhanced" SpCas9 or eSpCas9, was also able to reduce off-target effects without compromising on-target cleave efficiency[40].

Inducible Cas9 Expression

In order to make Cas9 active only at specific times or in specific tissues, several research groups have engineered CRISPR/Cas9 systems that are inducible or conditional. For example, spatial and temporal control of genome editing can be accomplished using a photoactivatable Cas9 (paCas9), created by splitting Cas9 into two fragments each fused to a photoinducible dimerization domain[41]. Upon blue light irradiation, paCas9 dimerizes and becomes active, creating targeted genome edits via NHEJ or HDR only while the optical stimulus is present[41].

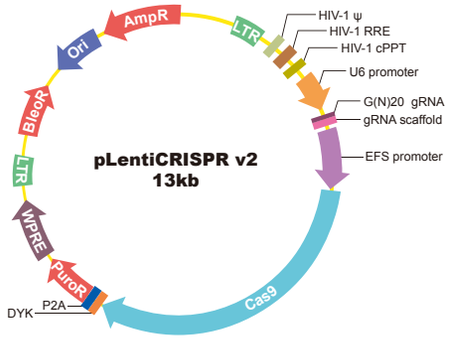
Tissue-specific genome editing can be accomplished by using tissue-specific promoters to drive Cas9 expression. Many mouse strains have been developed that stably express Cre recombinase under the control of tissue-specific specific promoters. These can easily be crossed with mice harboring a Cre-driven Cas9 cassette to enable tissue-specific genome editing upon delivery of guide RNAs[42]. Heritable tissue-specific Cas9 expression has also been achieved in a variety of model animals other than mice, including zebrafish, sea squirts, and fruit flies[43-46]. Tissue-specific promoters are also useful for constraining Cas9 activity after in vivo delivery via AAV vectors, which can infect many different cell types[47].

Cas9 & gRNA Vector Delivery Options

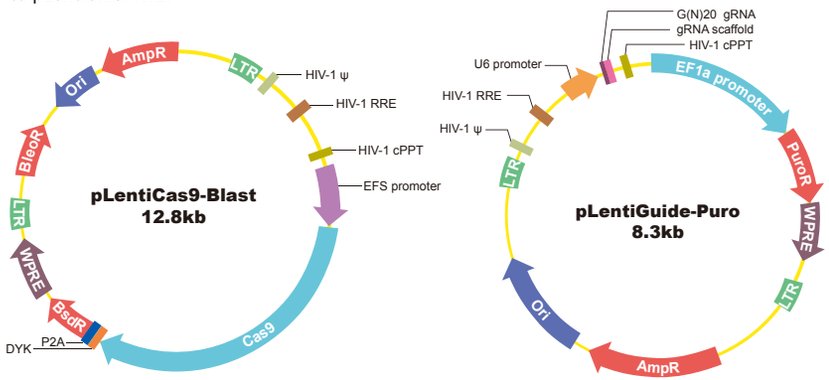
CRISPR/Cas9 system components can be delivered *in vivo* using modified viral vectors or any number of non-viral drug delivery systems. Some of the most widely-used model systems for biomedical research are primary mammalian cell cultures or hard-to-transfect cell lines in which transfection efficiency can be quite low. For these cell types lentiviral vectors are preferred (Figure 7).

Figure 7. Optimized lentiviral vectors for CRISPR genome editing inmammalian cells

Option 1: An all-in-one vector, pLentiCRISPRv2, enables CRISPR editing in any cell type of interest without generating stable Cas9-expressing cell line first.



Option 2: A two-vector system; sequential transduction with, and selection for, pLenti-Cas9-Blast followed by pLentiGuide-Puro, shows 10-fold higher efficiency compared to pLentiCRISPRv2.



Modified recombinant AAV particles are attractive for transduction because of their low immunogenicity and capability to infect both quiescent and dividing cells48. While AAV vectors are a preferred vehicle for in vivo gene delivery the size of the SpCas9 gene (>4 kb) exceeds the typical cargo limit of AAV vectors. Solutions that have been developed to date include:

- Creating transgenic animal lines that express Cas9, either constitutively or in an inducible manner, and then to deliver only the guide RNAs and any necessary inducer at the time of the experiment[42].

GenScript offers Broad Institute-validated **gRNA/SaCas9 Vectors** for gene editing in mammalian cells. Use SaCas9 for AAV packaging and transduction.

- Developing a split-Cas9 system using split-inteins[49].
- Use smaller Cas9 orthologues from other species, such as *Staphylococcus aureus* (SaCas9), which are small enough to be packaged along with a single guide RNA expression cassette into a single AAV vector[50].

Advantages of CRISPR Genome Editing

The advent of CRISPR has revolutionized genome editing – not only for its cost effective specificity, but also for its ease-of-use in any lab, regardless of molecular biology expertise. Unlike ZF and TALE nucleases, CRISPR/Cas9 does not require protein engineering for each gene being targeted. The CRISPR/Cas9 system requires only a DNA construct encoding the target specific gRNA and Cas9, and if knock-in is being performed, the donor template for HR. In addition, multiple genes can be edited simultaneously with CRISPR, vastly increasing the efficiency of your experiments. The key differences and advantages between the most common DSB-mediated genome editing technologies are summarized below (Table 1).

Table 1: Key differences between TALENs, ZFNs, and CRISPR/Cas

	TALEN (transcription activator-like effector nucleases)	ZFN (zinc finger nucleases)	CRISPR/Cas
Target	Protein: DNA	Protein: DNA	(gRNA-Cas9): DNA
Construct	Proteins containing DNA-binding domains that recognize specific DNA sequences down to the base pair	Zinc finger DNA binding motifs in a ββα configuration, the α-helix recognizes 3 bp segments in DNA	20nt crRNA (CRISPR RNA) fused to a tracrRNA and Cas9 endonuclease that recognize specific sequences to the base pair
Design feasibility	**Difficult:** -Need a customized protein for each gene sequence -Low delivery efficiency		**Easy:** - all-in-one gRNA-Cas9 vector system - multigene editing is feasible

Genome-wide and Pathway-specific Screens Using CRISPR Libraries

In addition to single- and multi-gene targeting, CRISPR has been adapted for genome-wide screening to discover genes whose inhibition or aberrant activation can drive phenotypes implicated in disease, development, and other biological processes.

Genome-scale CRISPR knock out libraries (GeCKO v2 libraries) in mouse and humans enable rapid screening of loss-of-function mutations51. GeCKO v2 libraries express a mixed pool of CRISPR guide RNAs that target every gene and miRNA in the genome. Each gRNA is cloned into a lentiviral vector optimized for high-titer virus production and high efficient transduction of primary cells or cultured cell lines. After transduction, deep sequencing can be performed to assess gRNA representation in the cell pool prior to screening. After selection, a second round of sequencing is performed to identify the gRNAs that were lost or enriched over the course of the screen. Genes identified with multiple gRNAs enriched represent positive hits. A detailed GeCKO screening protocol may be found on the GeCKO Genome Engineering website: http://genome-engineering.org/gecko/

GenScript offers Broad Institute sequence validated **GeCKO v2 Libraries** to accelerate your genome-wide screening efforts.

GeCKO v2 libraries were designed to contain 6 gRNAs targeting each gene within the genome, 4 gRNAs targeting each miRNA within the genome, and 1,000 control (non-targeting) gRNAs. GeCKO v2 libraries are also available as two half-libraries, A and B, each containing 3 gRNAs per gene. The gRNA sequences are distributed over three or four constitutively expressed exons for each gene and are selected to minimize off-target genome modification.

Pathway-focused gRNA libraries have also been developed for targeted screening of specific molecular pathways. Pathway-focused gRNA libraries were designed using gene targets identified through the Drug Gene Interaction Database by the McDonnell Genome Institute at Washington University in St. Louis. All gRNA sequences have been pre-designed and validated by the Broad Institute.

GenScript offers Broad Institute sequence validated **Pathway-focused gRNA Libraries** to assist in screening specific molecular pathways.

Expanding the Research Applications for CRISPR

CRISPR/Cas9 technology has been adapted for many research applications beyond than genome editing, such as:

- CRISPR/Cas9-mediated Chromatin Immunoprecipitation
- CRISPR Technologies for Transcriptional Activation and Repression
- Epigenetic Editing with CRISPR/Cas9
- Live Imaging of DNA/mRNA with CRISPR/Cas9
- CRISPR/Cas9 Therapeutic Applications

CRISPR/Cas9-mediated Chromatin Immunoprecipitation

Purification of specific genomic loci is vital for the characterization of chromatin-associated proteins and RNAs. Modifications to the CRISPR/Cas9 system allow for flexible targeting and isolation of these genomic regions[52]. A nuclear localization signal and epitope tag can be introduced into catalytically inactive Cas9 (dCas9) to create a DNA-binding protein that can be targeted by CRISPR guide RNAs[52]. Existing CRISPR gRNA databases and design tools allow for targeting to any gene of interest. The CRISPR/Cas9-chromatin complex can then be purified with traditional chromatin immunoprecipitation (CHiP) techniques and exposed to mass spectrometry for further characterization.

CRISPR-mediated CHiP techniques have been utilized to identify proteins associated with the interferon regulatory factor-1 (IRF-1) promoter region in response to interferon γ stimulation53. In this study, researchers purified 15 associated proteins including histone deacetylase complex proteins, which have previously been implicated in interferon γ-mediated gene expression, as well as transcription factors, histones and other DNA-associated proteins[53].

CRISPR-mediated CHiP holds a number of advantages over traditional CHiP methods. While large scale assays require the use of multiple antibodies against each DNA-binding protein or the creation and expression of epitope-tagged proteins, the modular nature of the CRISPR/Cas9 system requires only a single antibody against the tagged-Cas9 protein for purification. In addition, the CRISPR/Cas9 system is unaffected by issues stemming from low, differential or toxic gene expression.

CRISPR Technologies for Transcriptional Activation and Repression

Several research groups have harnessed the specificity and easy re-programmability of the CRISPR/Cas9 system to create targetable CRISPR/Cas9 ribonucleoprotein complexes that can either activate (CRISPRa) or interfere (CRISPRi) with transcription of any desired coding region within a genome[54-56].

These systems fuse dCas9 to a well-characterized transcription-regulatory domain, using pre-designed guide RNAs to direct the complex upstream of the transcription start site. By using inactivated dCas9 protein, the complex can be targeted to specific loci without cleaving or altering the genomic DNA. After Cas9 binds the targeted DNA sequence, the fused transcription-regulatory domains are then able to recruit repressive or activating effectors to modify gene expression.

The laboratory of Feng Zhang at the Broad Institute has pioneered use of the CRISPR/Cas9 Synergistic Activation Mediator (SAM) system in gene activation assays. The SAM system enables robust transcriptional activation of endogenous genes targeted by guide RNA that binds within 200 bp upstream of the transcription start site[57].

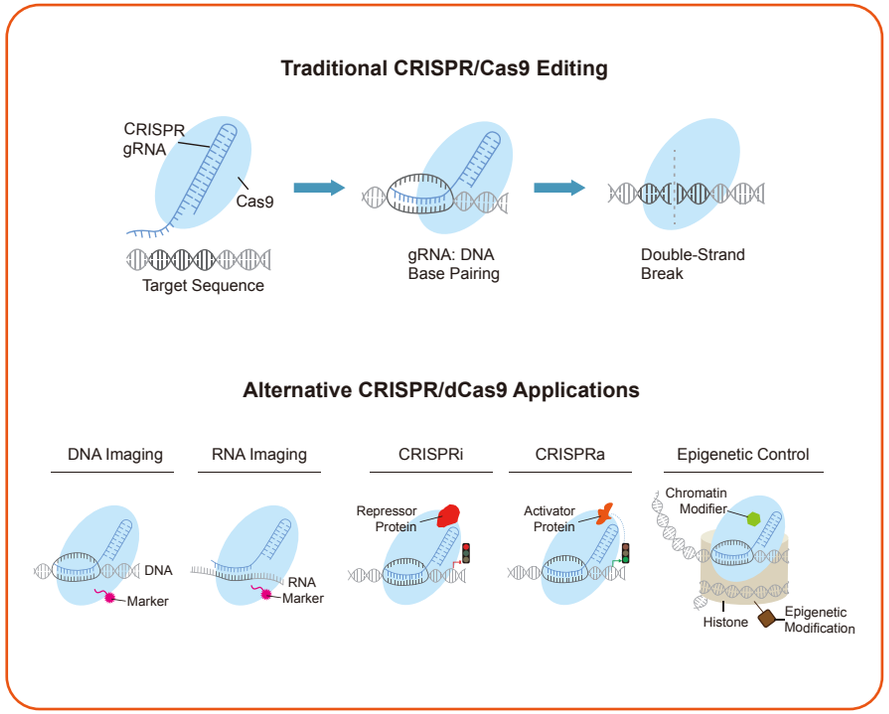
GenScript offers Broad Institute-validated **SAM Vectors** for transcriptional activation in mammalian cells.

Studies using the SAM system to activate gene expression show an increase in transcription of up to 3,000[57,58]. The SAM system has multiplexing gene activation ability, and has been shown to activate the transcription of as many as 10 genes concurrently[57]. In addition, SAM has been demonstrated to activate non-coding elements, such as long intergenic non-coding RNAs[57].

SAM can also be used for discovery research to identify the genes that drive phenotypes of interest in disease models or developmental/differentiation process by using a genome-wide SAM gRNA library for gain-of-function screening[57]. The screening process is similar to the GeCKO loss-of-function library screening techniques, but the library is designed to activate transcription rather than edit the genome. The human genome-wide SAM library contains 3 distinct guide RNAs each targeting one of 23,430 coding gene isoforms with a unique transcription start site in the human reference genome, for a total of 70,290 guides.

GenScript offers Broad Institute sequence-validated **SAM Libraries** to promote your genome-wide activation screening efforts.

Figure 8. CRISPR/Cas9 *In Vivo* Applications



Epigenetic Editing with CRISPR/Cas9

Epigenetic modifications to genomic DNA and histone proteins have been shown to play increasingly critical roles in biological processes. Epigenetic marks, such as methylation or acetylation, at specific genomic loci and histone residues can either be inherited or acquired, and can influence gene expression. Recent studies have used CRISPR/Cas9 genome editing to investigate the roles and targets for these epigenetic marks. In one such study, researchers performed CRISPR-mediated knock out of all three active DNA methyltransferases present in human embryonic stem cells, to characterize viable, pluripotent cell lines and study the distinct effects on the DNA methylation landscape[59].

But researchers increasingly need methods for introducing epigenetic modifications at desired genomic loci, in order to model diseases and test hypotheses regarding potential therapeutic strategies. For example, specific epigenetic alterations are often necessary and sufficient to drive the transformation of normal cells into cancerous cells, and play roles in later steps of carcinogenesis[60].

Using the CRISPR/Cas9 system, epigenetic editing has now become feasible. Utilizing inactive dCas9 as a DNA-binding domain platform, fused enzymes such as DNA methylases, histone acetyltransferases, and deacetylases (HATs or HDACs), can be targeted to alter the epigenetic state at precise locations within the genome. Researchers have used this approach, to fuse the catalytic core of human acetyltransferase p300 with dCas9, and shown this system to be sufficient for acetylation of histone H3 lysine 27 at specific target sites and to robustly activate transcription of target genes[61]. Cas9 epigenetic effectors (epiCas9s) can also be used for genome-wide screening to discover novel relationships between epigenetic modifications, chromatin states, and phenotypes such as, cellular differentiation or disease progression[62].

Live Imaging of DNA/mRNA with CRISPR/Cas9

DNA visualization is an important application in understanding a variety of cellular processes, such as replication, transcription, and recombination, and the interactions between DNA and associated proteins and RNA. Two techniques are commonly used for DNA imaging, fluorescence in situ hybridization (FISH) and fluorescent tagging of DNA-binding proteins. FISH uses fluorescently tagged nucleic acid probes to bind and visualize DNA[63,64]. While this technique offers the flexibility to target specific sequences through base pairing of the nucleic acid probes, it cannot be used for live imaging because of the requirement for sample fixation. Conversely, proteins tagged with a fluorescent label can be used for live imaging, but are limited by their fixed target sequences, restricting their use mostly to repetitive DNA sequences, such as telomeres[65].

New advances in CRISPR/Cas9 technology offer the benefits of both live imaging and easy target sequence customization and flexibility. Inactivated dCas9 can be tagged with fluorophores for imaging both repetitive DNA elements and protein-encoding genes, enabling us to observe chromatin organization throughout the cell cycle[66]. In addition to live DNA imaging, the CRISPR/Cas9 system can be used for live RNA imaging as well. Modifications to the gRNA sequence allow for mRNA recognition and tracking[67]. Using CRISPR-mediated RNA imaging techniques, researchers have been able to visualize the accumulation of ACTB, CCNA2 and TRFC mRNAs in RNA granules[67]. These new applications improve existing methodologies

CRISPR/Cas9 Therapeutic Applications

Both well-established pharmaceutical companies and new start-up biotech companies are racing to create CRISPR-based therapeutics. Compared to other strategies for gene therapy, CRISPR genome editing is thought to be faster, less expensive, and potentially far safer. Autologous CRISPR cell therapies that use genome editing to correct a mutation in a patient's own cells hold promise in circumventing the rejection issues present with transplant therapies that require donor matching. CRISPR genome editing is especially promising for diseases that can be tackled by modifying cells that can easily be removed from a patient, which allows for additional screening to ensure no off-target genome modifications during genome-editing.

Cancer Immunotherapy	New studies using CRISPR/Cas9-mediated immunotherapy are being used to combat metastatic lung cancer at Sichuan University and myeloma at the University of Pennsylvania[68,69]. Researchers will be using the CRISPR/Cas9 system to knock out the PD-1 gene in T-cells extracted from patients. PD-1 is an important down-regulator in T-cell activation and functions as an immune checkpoint. After knockout of PD1, the modified T-cells will be released into the bloodstream to target cancer. PD-1 inhibition offers a promising approach for cancer treatment. Last year alone, the FDA approved two new antibody-based therapies which target PD-1, nivolumab and pembrolizumab.
Tissue Regeneration	Recent reports indicate that CRISPR/Cas9 may be an essential tool to improve cell differentiation. CRISPR technology and has been used to derive a variety of cell types for transplantation, including muscle cells for the treatment of muscular dystrophy and hematopoietic stem cells for the treatment of sickle cell anemia[70,71]. Together these results demonstrate that CRISPR/Cas9 technology can be applied for directed cell differentiation and implantation.
Gene Therapy	Huntingtons disease is an inherited neurological condition caused by accumulation of mutant Huntingtin protein within the brain which results in cognitive impairment, dementia and death. Using mouse models, researchers have shown that CRISPR/Cas9 gene editing can knock-out production of these mutant proteins[72]. CRISPR/Cas9 editing is sufficient to reduce mutant protein production by up to 90% and shows promise as a therapeutic solution. Additional studies are ongoing using humanized huntingtin genes.
Malaria and Insect-borne Diseases	Insect-borne diseases such as malaria and zika pose enormous health concerns across the world. To combat the spread of insect-borne diseases, researchers have modified CRISPR/Cas9 into highly efficient "gene drive" systems which can spread disease resistance genes to entire populations. To create a gene drive, researchers have package disease resistance genes together with CRISPR gRNA and Cas9 components into a single DNA construct. After insertion, the gene drive autonomously replicates into both parental chromosomes, and is inherited by ~99.5% of progeny[73,74]. Advances in gene drive technology offer immediate solutions for the eradication of these diseases.
HIV and Viral Diseases	Gene editing can provide new strategies and therapeutic applications against infectious viral diseases. HIV has been effectively eliminated in patients using gene therapy to delete receptors essential for viral cell entry and infection. Recent studies using CRISPR technology have shown that mutations in CCR5 and CXCR4 receptors in both induced pluripotent stem cells (iPSCs) and primary CD4+ cells can lead to HIV resistance in lineages derived from these cells[75,76].
Obesity and Metabolism	FTO is one of the most strongly linked genes to obesity. Certain FTO genetic variants correlate significantly with obesity and heavier weight. Researchers have shown that CRISPR/Cas9-mediated knock-in techniques can convert obesity-promoting FTO variants to normal variants in adipocyte precursor cells[77]. Treated cells display increased metabolic activity and reduced expression of IRX3 and IRX5, genes which determine cell fate as white adipocytes for fat storage.

References

1. Capecchi, M. R. Altering the genome by homologous recombination. *Science* **244**, 1288-1292 (1989).

2. Choulika, A., Perrin, A., Dujon, B. & Nicolas, J. F. Induction of homologous recombination in mammalian chromosomes by using the I-SceI system of Saccharomyces cerevisiae. *Mol Cell Biol* **15**, 1968-1973 (1995).

3. Bibikova, M., Golic, M., Golic, K. G. & Carroll, D. Targeted chromosomal cleavage and mutagenesis in Drosophila using zinc-finger nucleases. *Genetics* **161**, 1169-1175 (2002).

4. Utomo, A. R., Nikitin, A. Y. & Lee, W. H. Temporal, spatial, and cell type-specific control of Cre-mediated DNA recombination in transgenic mice. *Nat Biotechnol* **17**, 1091-1096, doi:10.1038/15073 (1999).

5. Orban, P. C., Chui, D. & Marth, J. D. Tissue- and site-specific DNA recombination in transgenic mice. *Proc Natl Acad Sci U S A* **89**, 6861-6865 (1992).

6. Sauer, B. Inducible gene targeting in mice using the Cre/lox system. *Methods* **14**, 381-392, doi:10.1006/meth.1998.0593 (1998).

7. Zheng, B., Sage, M., Sheppeard, E. A., Jurecic, V. & Bradley, A. Engineering mouse chromosomes with Cre-loxP: range, efficiency, and somatic applications. *Mol Cell Biol* **20**, 648-655 (2000).

8. Beerli, R. R., Segal, D. J., Dreier, B. & Barbas, C. F., 3rd. Toward controlling gene expression at will: specific regulation of the erbB-2/HER-2 promoter by using polydactyl zinc finger proteins constructed from modular building blocks. *Proc Natl Acad Sci U S A* **95**, 14628-14633 (1998).

9. Boch, J. *et al*. Breaking the code of DNA binding specificity of TAL-type III effectors. *Science* **326**, 1509-1512, doi:10.1126/science.1178811 (2009).

10. Moscou, M. J. & Bogdanove, A. J. A simple cipher governs DNA recognition by TAL effectors. *Science* **326**, 1501, doi:10.1126/science.1178817 (2009).

11. Perez-Pinera, P., Ousterout, D. G. & Gersbach, C. A. Advances in targeted genome editing. *Curr Opin Chem Biol* **16**, 268-277, doi:10.1016/j.cbpa.2012.06.007 (2012).

12. Ishino, Y., Shinagawa, H., Makino, K., Amemura, M. & Nakata, A. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. *J Bacteriol* **169**, 5429-5433 (1987).

13. Mojica, F. J., Diez-Villasenor, C., Soria, E. & Juez, G. Biological significance of a family of regularly spaced repeats in the genomes of Archaea, Bacteria and mitochondria. *Mol Microbiol* **36**, 244-246 (2000).

14. Jansen, R., Embden, J. D., Gaastra, W. & Schouls, L. M. Identification of genes that are associated with DNA repeats in prokaryotes. *Mol Microbiol* **43**, 1565-1575 (2002).

15. Pourcel, C., Salvignol, G. & Vergnaud, G. CRISPR elements in Yersinia pestis acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies. *Microbiology* **151**, 653-663, doi:10.1099/mic.0.27437-0 (2005).

16. Mojica, F. J., Diez-Villasenor, C., Garcia-Martinez, J. & Soria, E. Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *J Mol Evol* **60**, 174-182, doi:10.1007/s00239-004-0046-3 (2005).

17. Bolotin, A., Quinquis, B., Sorokin, A. & Ehrlich, S. D. Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. *Microbiology* **151**, 2551-2561, doi:10.1099/mic.0.28048-0 (2005).

18. Barrangou, R. *et al*. CRISPR provides acquired resistance against viruses in prokaryotes. *Science* **315**, 1709-1712, doi:10.1126/science.1138140 (2007).

19. Brouns, S. J. *et al*. Small CRISPR RNAs guide antiviral defense in prokaryotes. *Science* **321**, 960-964, doi:10.1126/science.1159689 (2008).

20. Deltcheva, E. *et al*. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature* **471**, 602-607, doi:10.1038/nature09886 (2011).

21. Jinek, M. *et al*. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816-821, doi:10.1126/science.1225829 (2012).

22. Marraffini, L. A. & Sontheimer, E. J. CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. *Science* **322**, 1843-1845, doi:10.1126/science.1165771 (2008).

23. Mojica, F. J., Diez-Villasenor, C., Garcia-Martinez, J. & Almendros, C. Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiology* **155**, 733-740, doi:10.1099/mic.0.023960-0 (2009).

24. Deveau, H. *et al*. Phage response to CRISPR-encoded resistance in Streptococcus thermophilus. *J Bacteriol* **190**, 1390-1400, doi:10.1128/JB.01412-07 (2008).

25. Mali, P. *et al*. RNA-guided human genome engineering via Cas9. *Science* **339**, 823-826, doi:10.1126/science.1232033 (2013).

26. Makarova, K. S. *et al*. An updated evolutionary classification of CRISPR-Cas systems. *Nat Rev Microbiol* **13**, 722-736, doi:10.1038/nrmicro3569 (2015).

27. Cong, L. *et al*. Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819-823, doi:10.1126/science.1231143 (2013).

28. Ran, F. A. et al. Genome engineering using the CRISPR-Cas9 system. *Nat Protoc* **8**, 2281-2308, doi:10.1038/nprot.2013.143 (2013).

29. Ranganathan, V., Wahlin, K., Maruotti, J. & Zack, D. J. Expansion of the CRISPR-Cas9 genome targeting space through the use of H1 promoter-expressed guide RNAs. *Nat Commun* **5**, 4516, doi:10.1038/ncomms5516 (2014).

30. Kleinstiver, B. P. *et al*. Engineered CRISPR-Cas9 nucleases with altered PAM specificities. *Nature* **523**, 481-485, doi:10.1038/nature14592 (2015).

31. Zetsche, B. *et al*. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* **163**, 759-771, doi:10.1016/j.cell.2015.09.038 (2015).

32. Kuscu, C., Arslan, S., Singh, R., Thorpe, J. & Adli, M. Genome-wide analysis reveals characteristics of off-target sites bound by the Cas9 endonuclease. *Nat Biotechnol* **32**, 677-683, doi:10.1038/nbt.2916 (2014).

33. Tsai, S. Q. et al. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nat Biotechnol* **33**, 187-197, doi:10.1038/nbt.3117 (2015).

34. Doench, J. G. *et al*. Rational design of highly active sgRNAs for

CRISPR-Cas9-mediated gene inactivation. *Nat Biotechnol* **32**, 1262-1267, doi:10.1038/nbt.3026 (2014).

35. Doench, J. G. *et al*. Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. *Nat Biotechnol* **34**, 184-191, doi:10.1038/nbt.3437 (2016).

36. Wu, X. *et al*. Genome-wide binding of the CRISPR endonuclease Cas9 in mammalian cells. *Nat Biotechnol* **32**, 670-676, doi:10.1038/nbt.2889 (2014).

37. Kim, S., Kim, D., Cho, S. W., Kim, J. & Kim, J. S. Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. *Genome Res* **24**, 1012-1019, doi:10.1101/gr.171322.113 (2014).

38. Liang, X. *et al*. Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection. *J Biotechnol* **208**, 44-53, doi:10.1016/j.jbiotec.2015.04.024 (2015).

39. Ran, F. A. *et al*. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell* **154**, 1380-1389, doi:10.1016/j.cell.2013.08.021 (2013).

40. Slaymaker, I. M. *et al*. Rationally engineered Cas9 nucleases with improved specificity. *Science* **351**, 84-88, doi:10.1126/science.aad5227 (2016).

41. Nihongaki, Y., Kawano, F., Nakajima, T. & Sato, M. Photoactivatable CRISPR-Cas9 for optogenetic genome editing. *Nat Biotechnol* **33**, 755-760, doi:10.1038/nbt.3245 (2015).

42. Platt, R. J. *et al*. CRISPR-Cas9 knockin mice for genome editing and cancer modeling. *Cell* **159**, 440-455, doi:10.1016/j.cell.2014.09.014 (2014).

43. Ablain, J., Durand, E. M., Yang, S., Zhou, Y. & Zon, L. I. A CRISPR/Cas9 vector system for tissue-specific gene disruption in zebrafish. *Dev Cell* **32**, 756-764, doi:10.1016/j.devcel.2015.01.032 (2015).

44. Yin, L., Jao, L. E. & Chen, W. Generation of Targeted Mutations in Zebrafish Using the CRISPR/Cas System. *Methods Mol Biol* **1332**, 205-217, doi:10.1007/978-1-4939-2917-7_16 (2015).

45. Stolfi, A., Gandhi, S., Salek, F. & Christiaen, L. Tissue-specific genome editing in Ciona embryos by CRISPR/Cas9. *Development* **141**, 4115-4120, doi:10.1242/dev.114488 (2014).

46. Xue, Z. *et al*. CRISPR/Cas9 mediates efficient conditional mutagenesis in Drosophila. *G3 (Bethesda)* **4**, 2167-2173, doi:10.1534/g3.114.014159 (2014).

47. Cheng, R. *et al*. Efficient gene editing in adult mouse livers via adenoviral delivery of CRISPR/Cas9. *FEBS Lett* **588**, 3954-3958, doi:10.1016/j.febslet.2014.09.008 (2014).

48. Deyle, D. R. & Russell, D. W. Adeno-associated virus vector integration. *Curr Opin Mol Ther* **11**, 442-447 (2009).

49. Truong, D. J. *et al*. Development of an intein-mediated split-Cas9 system for gene therapy. *Nucleic Acids Res* **43**, 6450-6458, doi:10.1093/nar/gkv601 (2015).

50. Ran, F. A. *et al*. *In vivo* genome editing using Staphylococcus aureus Cas9. *Nature* **520**, 186-191, doi:10.1038/nature14299 (2015).

51. Sanjana, N. E., Shalem, O. & Zhang, F. Improved vectors and genome-wide libraries for CRISPR screening. *Nat Methods* **11**, 783-784, doi:10.1038/nmeth.3047 (2014).

52. Fujita, T. & Fujii, H. Efficient isolation of specific genomic regions and identification of associated proteins by engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP) using CRISPR. B*iochem Biophys Res Commun* **439**, 132-136, doi:10.1016/j.bbrc.2013.08.013 (2013).

53. Fujita, T. & Fujii, H. Identification of proteins associated with an IFNgamma-responsive promoter by a retroviral expression system for enChIP using CRISPR. *PLoS One* **9**, e103084, doi:10.1371/journal.pone.0103084 (2014).

54. Bikard, D. *et al*. Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system. *Nucleic Acids Res* **41**, 7429-7437, doi:10.1093/nar/gkt520 (2013).

55. Gilbert, L. A. *et al*. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* **154**, 442-451, doi:10.1016/j.cell.2013.06.044 (2013).

56. Perez-Pinera, P. *et al*. RNA-guided gene activation by CRISPR-Cas9-based transcription factors. *Nat Methods* **10**, 973-976, doi:10.1038/nmeth.2600 (2013).

57. Konermann, S. *et al*. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature* **517**, 583-588, doi:10.1038/nature14136 (2015).

58. Zhang, Y. *et al*. CRISPR/gRNA-directed synergistic activation mediator (SAM) induces specific, persistent and robust reactivation of the HIV-1 latent reservoirs. *Sci Rep* **5**, 16277, doi:10.1038/srep16277 (2015).

59. Liao, J. *et al*. Targeted disruption of DNMT1, DNMT3A and DNMT3B in human embryonic stem cells. *Nat Genet* **47**, 469-478, doi:10.1038/ng.3258 (2015).

60. Yao, S., He, Z. & Chen, C. CRISPR/Cas9-Mediated Genome Editing of Epigenetic Factors for Cancer Therapy. *Hum Gene Ther* **26**, 463-471, doi:10.1089/hum.2015.067 (2015).

61. Hilton, I. B. *et al*. Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. *Nat Biotechnol* **33**, 510-517, doi:10.1038/nbt.3199 (2015).

62. Hsu, P. D., Lander, E. S. & Zhang, F. Development and applications of CRISPR-Cas9 for genome engineering. *Cell* **157**, 1262-1278, doi:10.1016/j.cell.2014.05.010 (2014).

63. Lichter, P. *et al*. High-resolution mapping of human chromosome 11 by in situ hybridization with cosmid clones. *Science* **247**, 64-69 (1990).

64. Pinkel, D. *et al*. Fluorescence in situ hybridization with human chromosome-specific libraries: detection of trisomy 21 and translocations of chromosome 4. *Proc Natl Acad Sci U S A* **85**, 9138-9142 (1988).

65. Wang, X. *et al*. Rapid telomere motions in live human cells analyzed by highly time-resolved microscopy. *Epigenetics Chromatin* **1**, 4, doi:10.1186/1756-8935-1-4 (2008).

66. Chen, B. *et al*. Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. *Cell* **155**, 1479-1491, doi:10.1016/j.cell.2013.12.001 (2013).

67. Nelles, D. A. *et al*. Programmable RNA Tracking in Live Cells with CRISPR/Cas9. *Cell* **165**, 488-496, doi:10.1016/j.cell.2016.02.054 (2016).

68. Cyranoski, D. Chinese scientists to pioneer first human CRISPR trial. *Nature* **535**, 476-477, doi:10.1038/nature.2016.20302 (2016).

69. Reardon, S. First CRISPR clinical trial

gets green light from US panel, 2016).

70. Loperfido, M., Steele-Stallard, H. B., Tedesco, F. S. & VandenDriessche, T. Pluripotent Stem Cells for Gene Therapy of Degenerative Muscle Diseases. *Curr Gene Ther* **15**, 364-380 (2015).

71. Song, B. *et al*. Improved hematopoietic differentiation efficiency of gene-corrected beta-thalassemia induced pluripotent stem cells by CRISPR/Cas9 system. *Stem Cells Dev* **24**, 1053-1065, doi:10.1089/scd.2014.0347 (2015).

72. Armitage, H. *Gene-editing method halts production of brain-destroying proteins*, 2015).

73. Gantz, V. M. & Bier, E. Genome editing. The mutagenic chain reaction: a method for converting heterozygous to homozygous mutations. *Science* **348**, 442-444, doi:10.1126/science.aaa5945 (2015).

74. Gantz, V. M. *et al*. Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito Anopheles stephensi. *Proc Natl Acad Sci U S A* **112**, E6736-6743, doi:10.1073/pnas.1521077112 (2015).

75. Hou, P. *et al*. Genome editing of CXCR4 by CRISPR/cas9 confers cells resistant to HIV-1 infection. *Sci Rep* **5**, 15577, doi:10.1038/srep15577 (2015).

76. Ye, L. *et al*. Seamless modification of wild-type induced pluripotent stem cells to the natural CCR5Delta32 mutation confers resistance to HIV infection. *Proc Natl Acad Sci U S A* **111**, 9591-9596, doi:10.1073/pnas.1407473111 (2014).

77. Claussnitzer, M. *et al*. FTO Obesity Variant Circuitry and Adipocyte Browning in Humans. *N Engl J Med* **373**, 895-907, doi:10.1056/NEJMoa1502214 (2015).