CRISPR Handbook

Enabling Genome Editing and Transforming Life Science Research

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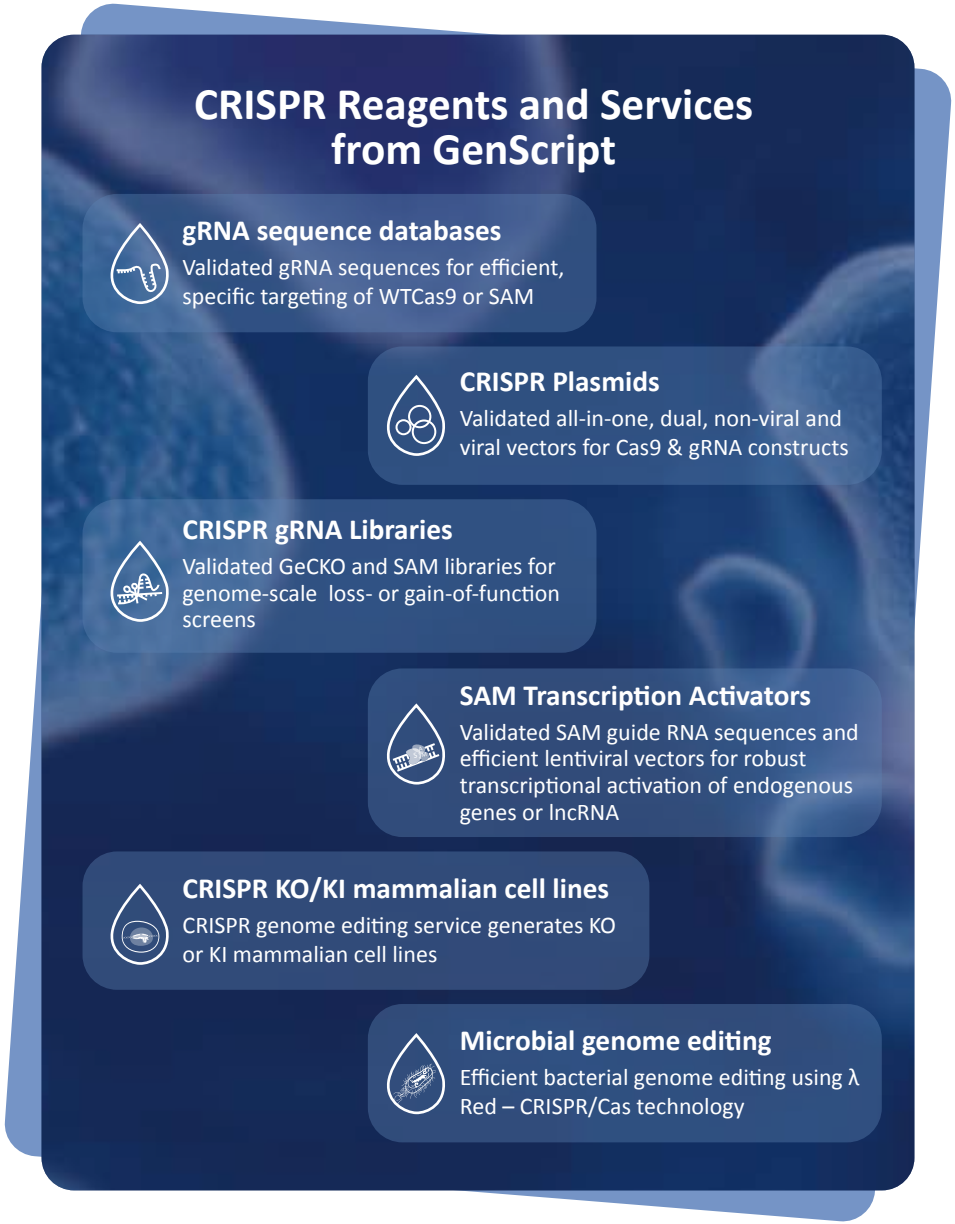

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This handbook describes CRISPR/Cas9 genome editing and other research applications for CRISPR technology.

The CRISPR Genome Editing Revolution

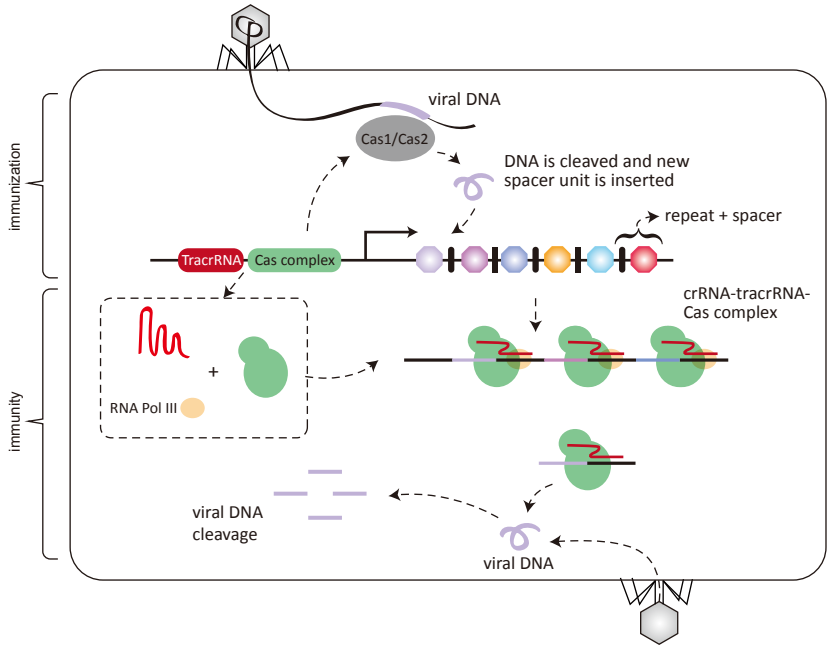
The ability to manipulate DNA has been a significant breakthrough in the scientific community – making it possible to better understand the relationship between the genome and its functions. From inhibiting gene function to altering its expression, genome editing can provide tremendous insight into the basis of disease and identification of new targets for medical intervention (Hsu *et al*., 2014). For this to become a reality, researchers need the ability to make specific, targeted changes to the genome, a simple principle that has been challenging in practice. Over the last 20 years, advances in genome editing technologies have overcome many of these challenges, allowing researchers to more precisely manipulate genomes in cell lines and animal models to more accurately model disease pathologies. Of these advances, one of the most exciting has been CRISPR/Cas, a system adapted from the bacterial immune system that is efficient, rapid, and easy to use (Doudna *et al*., 2014). In this handbook, we will discuss how CRISPR technology has fueled a genome editing revolution, as well as how it has been adapted for other biological applications and how it is expected to transform medicine.

Discovery of CRISPR in bacterial immune systems

Microbes have adapted many strategies to evade infection by viruses and phages, from blocking virus adsorption to preventing DNA insertion. Over the past 10 years, a new bacterial immune system has been discovered, employing a novel technique to prevent infection. This immune system allows bacteria to both prevent foreign DNA from being inserted into the genome, and also target the invasive DNA for destruction (Horvath *et al*., 2010).

This system was first brought to light in 1987. Nakata and colleagues were studying the iap enzyme when they discovered curious repeat and non-repeat sequences downstream of the iap gene (Ishino *et al*., 1987). Just 5 years later, these repeat arrays would become referred to as CRISPR, or Clustered Regular Interspaced Short Palindromic Repeats (Jansen *et al*., 2002); however, their function was still a mystery. In 2005, Mojica and colleagues revealed that these sequences, or "spacers", actually contained DNA from bacteriophages (Mojica *et al*., 2005). Shortly after this discovery, Bolotin et al also observed the presence of cas genes, which encode for a DNA endonuclease, in close proximity to CRISPR structures, strongly suggesting that foreign DNA degradation may be a primary function of CRISPR/Cas (Bolotin *et al*., 2005). The specificity of this system for foreign DNA was further elucidated a few years later with the discovery of conserved motifs within the genome. Just upstream of the "protospacers," or target genomic sequences on the foreign DNA, are conserved motifs called protospacer adjacent motifs (PAM). These motifs are preferential targets for the Cas endonucleases (Horvath *et al*., 2008, Deveau *et al*., 2008), and allow the system to discern between self- and non-self DNA (Mali *et al*., 2013). Together, by the end of the early 2000s, the significance of the CRISPR as a defense strategy in bacteria was coming to light.

Figure 1: Mechanism of CRISPR-mediated immunity in bacteria



By 2010, three CRISPR systems had been identified in bacteria: Type I, II and III. Type II CRISPR interference, because of its relative simplicity, would eventually become the system adapted for genome editing in mammalian cells (Sapranauskas *et al*., 2011) (Figure 1). CRISPR-based immunity is composed of two main phases: immunization and immunity. In the immunization phase, Cas proteins (Cas1/Cas2) form a complex that cleaves the foreign, viral DNA (Jiang *et al*., 2015). This foreign DNA is then incorporated into the bacterial CRISPR loci as repeat-spacer units. In the immunity phase, following re-infection, the repeat spacer units are transcribed to form pre-CRISPR RNA (pre-crRNA). The Cas9 endonuclease and trans-activating crRNA (tracrRNA, which helps guide Cas9 to crRNA) then bind to the pre-crRNA. A mature crRNA-Cas9-tracrRNA complex is formed following cleavage by RNA polymerase. This crRNA-Cas9-tracrRNA complex is essential to target and destroy the foreign DNA.

Evolution of Genome Editing technology

Prior to the dawn of "genome editing" in the early 2000s, studying gene function was primarily limited to transgenesis. The concept of gene editing began in the late 1980s: in 1989, homologous recombination (HR) was used to target specific genes in mouse embryonic stem cells to generate knock-in (KI) and knock-out (KO) cells (Capecchi *et al*., 1989) (Figure 2). Since HR occurs rather infrequently in mammalian cells, the recombination frequency was low (1 in every 3×10^4 cells); however, this work provided new ideas for how genes can be targeted and altered in specific ways.

As the need for relevant animal disease models rose, so did the need for sophisticated and more efficient genome editing tools. The Cre-lox technology became one of the most effective gene editing tools in the early 1990s, allowing scientists to control gene expression both spatially and temporally (Utomo *et al*., 1999, Orban *et al*., 1992). Cre-lox uses a site-specific DNA endonuclease Cre, which recognizes 34-bp loci called loxP (Sauer *et al*., 1998). Recombination at these sites leads to knock-out of desired genes, which has been particularly useful for the development of transgenic mouse models. While easier to control than HR, the Cre-lox system was less efficient as the genetic distance increased between *loxP* sites (Zheng *et al*., 2000).

Figure 2: Advancements in genome editing

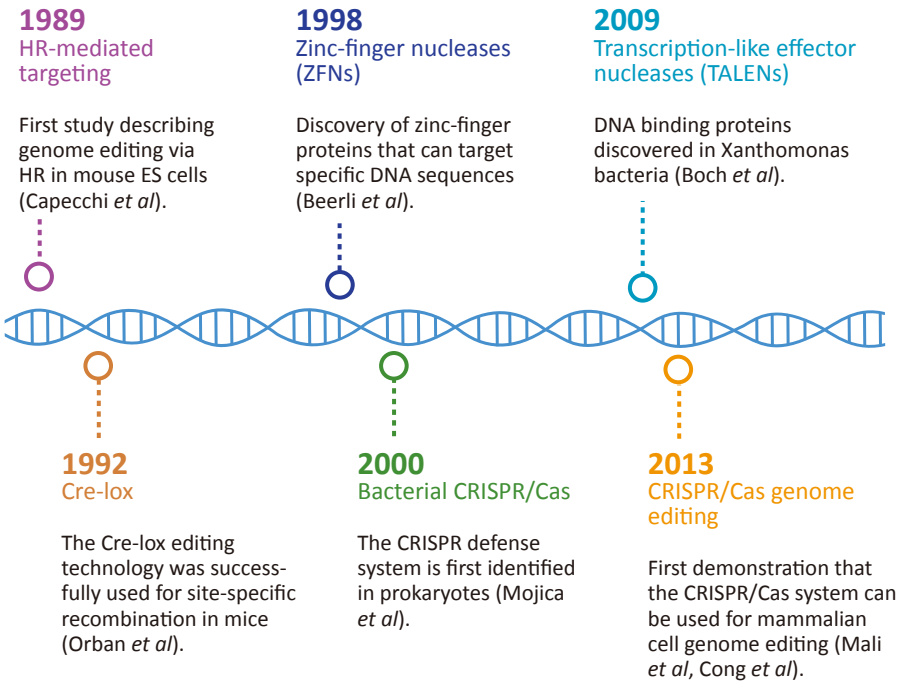
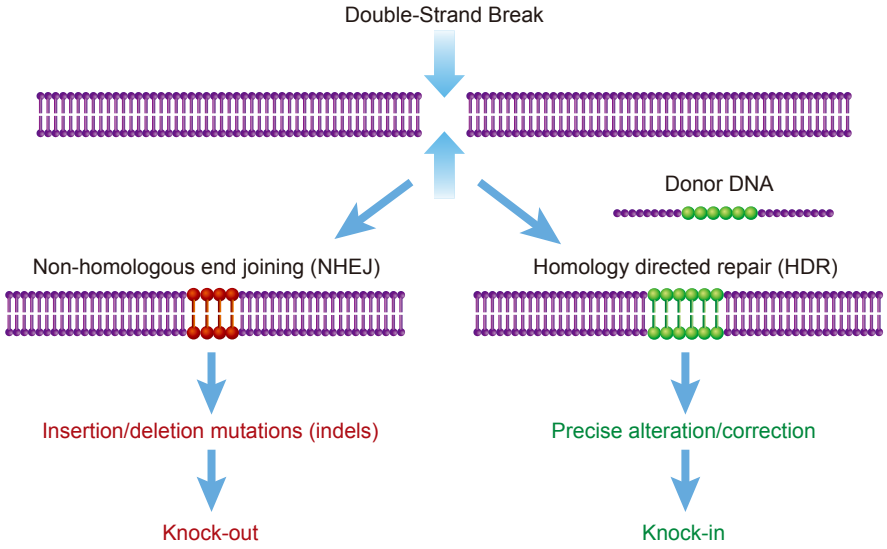


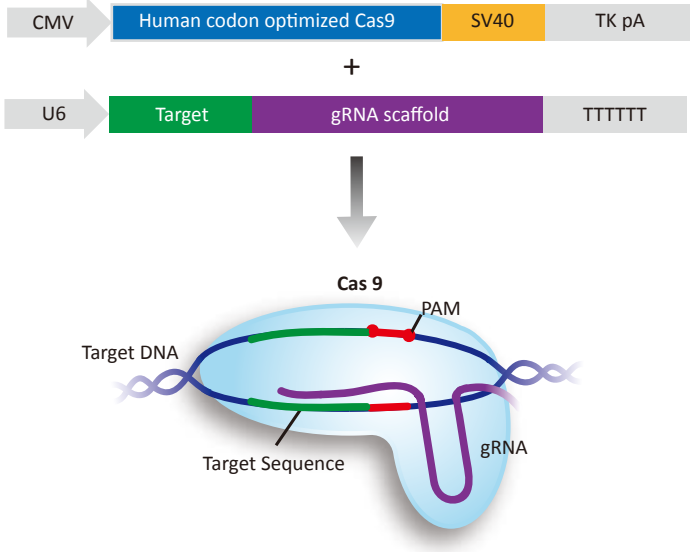
Figure 3: DNA repair by targeted genome editing



Since HR alone rarely results in gene integration in mammalian cells, the introduction of double strand breaks (DSB) into the genome can increase recombination significantly (Choulika *et al*., 1995). DSB resolution occurs by either HDR or error-prone nonhomologous end joining (NHEJ) (Figure 3). If there is no donor DNA present, resolution will occur by NHEJ, resulting in insertion/deletions (indels) that will ultimately knock-out gene function. Alternatively, if donor DNA sequences are available, the DSBs will be repaired by HDR, resulting in gene knock-in (Bibikova *et al*., 2002). Combined, these strategies represented new and more effective approaches for modifying the eukaryotic genome (Hsu *et al*., 2014).

CRISPR aside, the most effective genome editing techniques employing DSB-mediated repair have been zinc-finger (ZF) domains (Beerli *et al*., 1998) and transcription activator-like effectors (TALEs) (Moscou and Bogdanove, 2009; Boch et al., 2009). Both of these systems use DNA binding proteins with nuclease activity that bind to DNA and create site-specific DSBs. While effective, both of these methods require extensive expertise in protein engineering, which has been a bottleneck for many research labs' use of this technology (Perez-Pinera *et al*., 2012).

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



The use of CRISPR/Cas as a gene editing tool began in 2013, with the observation that type II CRISPR systems from *S. Thermophilus* and *S. Pyogenes* (SpCas) could be engineered to edit mammalian genomes (Mali *et al*., 2013, Cong *et al*., 2013). To further adapt the system for mammalian cells, a two-vector system was optimized (Mali et al., 2013). The two major components include (1) a Cas9 endonuclease and (2) the crRNA-tracrRNA complex; when co-expressed, they form a complex that is recruited to the target DNA sequence. The crRNA and tracrRNA can be combined to form a chimeric guide RNA (gRNA) with the same function – to guide Cas9 to target gene sequences (Jinek *et al*., 2012). These components can then be delivered to mammalian cells via transfection or lentiviral transduction.

Advantages of CRISPR genome editing

The adaptation of CRISPR for mammalian cells has revolutionized genome editing – not only for its accuracy but also for its ease of use in any lab regardless of molecular biology expertise. Unlike ZF and TALE nucleases, CRISPR/Cas does not require protein engineering for every gene being targeted. The CRISPR system only requires a few simple DNA constructs to encode the gRNA and Cas9, and, if knock-in is being performed, the donor template for HR. In addition, multiple genes can be edited simultaneously. The table below summarizes the key differences and advantages between the most common DSB-mediated genome editing technologies.

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
References	Moscou and Bogdanove, 2009 Boch *et al*., 2009 Gaj *et al*., 2013	Beerli *et al*., 1998 Perez-Pinera *et al*., 2012 Gaj *et al*., 2013	Mali *et al*., 2013 Cong *et al*., 2013 Jiang *et al*., 2015

Improving the specificity of CRISPR genome editing

Several systematic efforts have been undertaken to **empirically determine the rules governing gRNA efficiency and specificity.** One study looked at all possible targetable sites tiling across 6 mouse and 3 human genes -- 1,841 sgRNAs in total – and quantified their ability to create null alleles as assayed by antibody staining and flow cytometry. The results were used to construct a predictive model of sgRNA activity to improve sgRNA design for gene editing and genetic screens. (Doench *et al*. 2014) The gRNA design tool, which returns a score predicting the activity of any sgRNA based on empirical rules determined by this study, is freely available at http://www.broadinstitute.org/rnai/public/analysis-tools/sgrna-design.

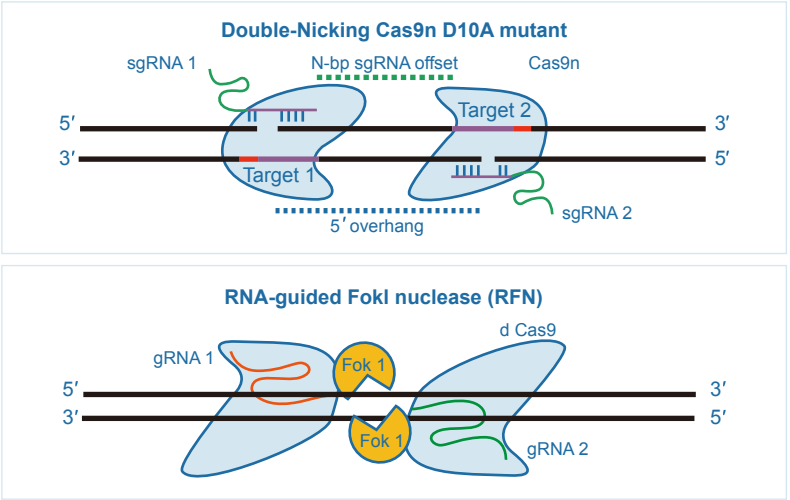
Another more recent study measured sgRNA activity across ~1,400 genomic loci, across multiple human cell types, using two Cas9 orthologs with different PAMs (*S. Pyogenes* and *S. Thermophilus*), to uncover parameters that govern gRNA efficiency based not only on the nucleotide sequences but also on epigenetic status (Chari *et al*., 2015). These results power an interactive web tool that can identify putative CRISPR/Cas9 sites) and assign a predicted activity, freely available at http://crispr.med.harvard.edu/sgRNAScorer.

Although it is rare for a 20 bp gRNA sequence to have 100% homology at multiple sites throughout the genome, sgRNA-Cas9 complexes are tolerant of several mismatches in their targets. Cas9 binds to many locations throughout the genome that display several mismatches to the guide (Kusco *et al*., 2014), but the enzyme only creates DSBs at a small subset of those locations. Still, DSBs have been observed at sites containing five or more mismatched nucleotides relative to the guide RNA sequence (Tsai *et al*., 2015). Therefore, there has been a major effort to develop modified CRISPR/Cas9 systems with improved specificity.

One strategy for improving gRNA-Cas9 targeting specificity is to require a pair of guides that target very nearby regions. Feng Zhang's laboratory at the Broad Institute and Keith Joung's laboratory at Harvard/MGH both developed systems that implement this strategy in slightly different ways.

The Zhang lab observed that an aspartate-to-alanine (D10A) mutation in the RuvC catalytic domain of Cas9 causes it to create single strand breaks (nicks) instead of double strand breaks. Targeting this nickase mutant (Cas9n) to two loci within close proximity, but occurring on opposite strands of the genomic DNA, causes Cas9n to effectively nick rather than cleave DNA to yield single-stranded breaks. Appropriately offset sgRNA pairs can guide Cas9n to simultaneously nick both strands of the target locus to mediate a DSB, thus effectively increasing the specificity of target recognition. Although each gRNA might have off-target binding sites throughout the genome, the Cas9n would cause only single strand breaks (SSB) at those locations; SSBs are preferentially repaired through HDR rather than NHEJ, which can potentially decrease the frequency of unwanted indel mutations from off-target DSBs.

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



Another strategy to improve specificity has focused on the gRNA itself. Although 20 bp regions were initially used, it was observed that mismatches were tolerated most often in the 3' end of the gRNA, and some wondered if these final nucleotides were necessary. Researchers in the Joung lab found that gRNAs with 17 or 18 nucleotides of complementarity functioned as efficiently as (or, in some cases, more efficiently than) 20 bp sequences to introduce mutations by means of NHEJ or HDR at on-target sites, and they showed reduced mutagenic effects at closely matched off-target sites (Fu *et al*., 2014). These truncated gRNAs (tru-gRNAs) can be used with WT SpCas9 or in combination with the RNA-Fok1 nuclease described above (Wyvekens *et al*., 2015).

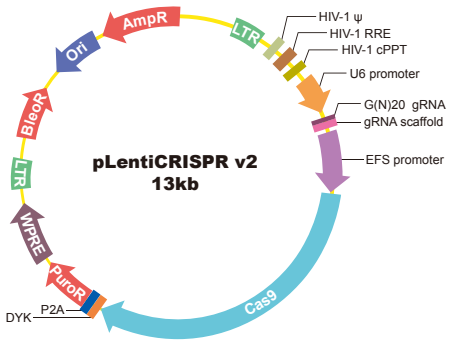
Off-target binding of Cas9 throughout the genome has been observed to be concentration-dependent (Wu *et al*., 2014) This finding spurred investigations of whether the frequency of off-target cleavage events could be reduced by delivering a short-lived Cas9 protein rather than plasmid that would drive expression of Cas9 for a longer period of time than was strictly necessary. A purified Cas9 protein can be complexed to its guide RNA *in vitro* to form a ribonucleoprotein (RNP), which will cleave chromosomal DNA almost immediately after delivery and then be degraded rapidly in cells, reducing off-target effects. RNPs can be efficiently delivered to hard-to-transfect cells such as human fibroblasts and pluripotent stem cells. Another advantage is that RNP delivery may be less stressful for cells than plasmid transfection (Kim *et al*., 2014).

Improving gRNA & Cas9 delivery efficiency

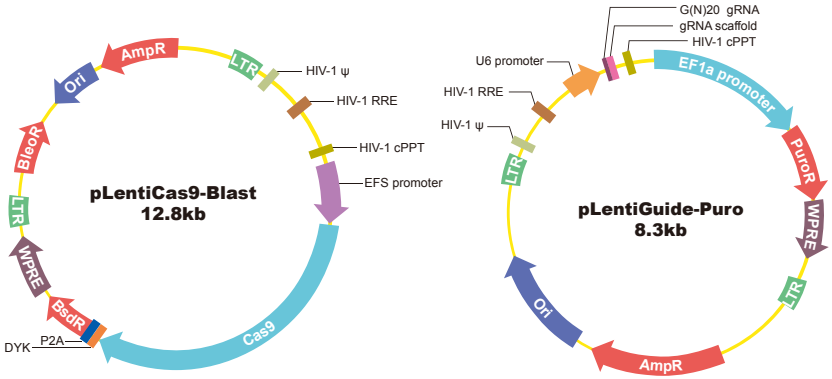
Some of the most widely-used model systems for biomedical research are primary mammalian cell cultures or hard-to-transfect cell lines in which transfection efficiency via lipofection or electroporation can be quite low. Lentiviral vectors are preferred for these cell types (Figure 6).

Figure 6 : Optimized Lentiviral Vectors for CRISPR genome editing in mammalian 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.



CRISPR/Cas9 system components can be delivered *in vivo* using modified viral vectors or any number of non-viral drug delivery systems. Modified recombinant adeno-associated virus (rAAV) particles are a preferred vehicle for *in vivo* gene delivery, but the size of the SpCas9 gene (> 4 kb) exceeds the typical cargo limit of AAV vectors. Solutions that have been developed to date include:

- Create 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 (Platt *et al*., 2014).
- Develop a split-Cas9 system using split-inteins (Truong *et al*., 2015).
- 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 (Ran *et al*., 2015)

Expanding the applicability of CRISPR genome editing

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 the PAM sequence "NGG" at the end of the ~20-mer. Guide RNA expression was typically driven by the U6 human pol III promoter due to its efficiency at initiating transcription, which initiates transcription from a guanosine (G) nucleotide. Therefore, U6-driven gRNAs used with SpCas9 needed to be selected from genomic sequences that fit the pattern GN19NGG – which might occur infrequently in a gene of interest.

One strategy to expand the possibilities for CRISPR-mediated genome editing was to drive gRNA expression from 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 GN19NGG within the human genome. 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 search for ways to relax the restriction on the PAM sequence, as SpCas9's requirement for NGG presents a tight constraint. One approach to this has been to use protein engineering techniques to create novel engineered Cas mutants that recognize alternative PAM sequences (Kleinstiver *et*

al., 2015). Through a painstaking process that used structural information, bacterial selection-based directed evolution, and combinatorial design, researchers developed several mutant Cas9 that could recognize alternative PAMs. Engineered Cas9 nucleases can cleave at PAM sites consisting of NGA and NGCG, which allows targeting 50% more sites than can be reached with the NGG PAM alone. Additionally, there is data showing that these newly engineered Cas9s have lower off-target activity compared to wt SpCas9.

Regulating 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) that was created by splitting Cas9 into two fragments each fused to a photoinducible dimerization domain; upon blue light irradiation, paCas9 dimerizes and becomes active, creating targeted genome edits via NHEJ or HDR only while the optical stimulus is present (Nihongaki *et al*., 2015).

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 (cre-driver mice); these can easily be crossed with mice harboring a CRE-driven Cas9 cassette to enable tissue-specific genome editing upon delivery of guide RNA (Platt *et al*. 2014). Heritable tissue-specific Cas9 expression has also been achieved in diverse species other than mice, including zebrafish (Ablain *et al*., 2015; Yin *et al*., 2015), sea squirt *Ciona intestinalis* (Stolfi *et al*., 2014), and drosophila (Xue *et al*. 2014). Tissue-specific promoters are also useful for constraining Cas9 activity after *in vivo* delivery via AAVs, which can infect many different cell types (Cheng *et al*. 2014).

Putting CRISPR into Practice: Workflows and Case Studies

With CRISPR genome editing, modified clonal cell lines can be derived within 2–3 weeks starting from the guide RNA design stage; transgenic animal strains can be created in a single generation; and clinically relevant animal models of disease can be rapidly created through introducing somatic mutations *in vivo*. To jump-start your CRISPR experiments, the workflow and references below may help.

Design guide RNA and generate expression constructs

To perform CRISPR/Cas9-mediated gene editing, the first step is to select the nuclease you will use (e.g. WT SpCas9, Paired-nickase with Cas9D10A, *etc*) and then to design, or select from a pre-existing database, the guide RNA sequences appropriate for your nuclease.

Gene sequence analysis: It is advisable to sequence the region of interest within the host genome of the cell line or animal model you are using, rather than assuming that it will perfectly match the NCBI ref seq for your species/strain.

GenScript offers custom gRNA design services for any target in any species, as well as searchable online databases of validated gRNAs for human and mouse

Designing gRNA for single DSB-induced gene KO: Designing gRNAs against early exons tends to disrupt expression, reducing the chance of having truncated forms of the protein expressed. Alternatively, targeting a functional site can generate a loss-of-function mutant. For genes with multiple splice variants, care should be taken to ensure that a constitutive exon is targeted if the goal is to knock out all splice variants.

Designing guides for paired nickase: Guide RNA for use with Cas9n should be designed to target opposite strands of the genomic DNA with an offset of 0-20 bp from the 5' ends of the gRNA (i.e. a 40-60bp offset between PAM sequences).

Designing constructs for knock-in: As a general rule, WT Cas9 is more efficient at mediating homologous recombination than Cas9 nickase; although using a paired nickase strategy can reduce the risk for off-target activity, the efficiency of HDR mediated by Cas9 nickase is highly dependent on the cell type (Ran *et al*., 2013).

To introduce a specific change within the genome, for example a point mutation that will cause a specific amino acid substitution in the protein product, it is

necessary to supply a donor template that can be used for HDR after Cas9 creates a DSB. HDR templates may be delivered in plasmids or as single-stranded oligos (ssODN). To assist in detecting successful HDR and quantifying knock-in efficiency, donor templates are often designed to include several synonymous mutations so that sequencing can easily distinguish between the donor and the wild-type sequences. To prevent the cleavage of donor templates or of the genomic DNA after successful HDR, the donor template should be designed with mutations in the PAM sequence.

GenScript offers custom gRNA constructs built from vectors developed in Feng Zhang's laboratory, offered through a license with the Broad Institute.

Making gRNA and Cas9 Constructs

Once you have designed your gRNA, you need to synthesize them and clone them into your vector of choice. The plasmid vector you choose will depend upon your host and delivery method (Table 2).

Deliver CRISPR reagents to target cells

CRISPR/Cas9 technology for precise genome editing has already proven successful in many cell lines and species, including *C. elegans* (Friedland *et al*., 2013; Waaijers *et al*., 2013), *Xenopus tropicalis* (Guo *et al*., 2014), plants (Jiang *et al*., 2013), and even monkeys (Niu *et al*., 2014). Although the basic components are the same regardless of the target organism, the delivery method varies widely, and choosing the most appropriate vector for your host is critical for success.

***In vitro* genome editing:**

For easy-to-transfect cell lines, plasmids encoding gRNA and Cas9 can be delivered with high efficiency via lipofection. CRISPR plasmids typically contain selection markers such as genes conferring antibiotic resistance, or fluorescent proteins for easy visualization or FACS. For difficult-to-transfect cell lines or primary cells, lentiviral vectors are preferred. gRNA may be delivered either via an all-in-one plasmid that also encodes the Cas9 nuclease, or a separate plasmid that can be delivered into cells already expressing Cas9. Alternatively, gRNA may be introduced via a PCR-generated U6-sgRNA expression cassettes expression. Cleavage efficiency is typically lower than when gRNA is expressed from a plasmid; however, PCR-generated cassettes may be used for rapid comparison of sgRNA efficiencies so that the most optimal sgRNA, in terms of both efficiency and specificity, can be identified before subsequent cloning into pSpCas9 (Ran *et al*., 2013).

***In vivo* genome editing:**

As with prior methods for creating transgenic animal strains, CRISPR/Cas9 system components can be delivered to germ line cells to create heritable mutations; stable, homozygous mutations at multiple loci can be achieved in a single generation in mice (Wang *et al*., 2013). CRISPR genome editing can also be used to generate precise mutations in somatic tissues of adult animals, and to modify multiple genes at once in the same cells (Cong *et al*., 2013, Mali *et al*., 2013). This is especially valuable for creating clinically relevant *in vivo* cancer models, because human tumors often contain a combination of gain-of-function mutations in oncogenes and loss-of-function mutations in tumor suppressor genes (Platt *et al*., 2015).

In addition, CRISPR can be used to generate chromosomal rearrangements seen in human cancers, such as the EML4-ALK inversion observed in human non-small cell lung cancer. Viral-mediated delivery of CRISPR/Cas9 system to somatic cells in the lung of adult mice yielded a new clinically faithful mouse model of Eml4-Alk human lung cancer and presents a new paradigm for accurately modeling human cancers in mice (Maddalo *et al*., 2014).

Table 2: gRNA & Cas9 Delivery Methods used for different hosts

Host	Delivery Method	Reference
Mammalian cells	- Lipofection-based transfection of DNA plasmids - Electroporation of DNA plasmids or RNP - Lentiviral transduction of DNA plasmids	Cong *et al*., 2013, Mali *et al*., 2013 Schumann *et al*., 2015 Shalem *et al*., 2014
Microbial organisms	- Transformation of plasmids into competent cells	Jiang *et al*., 2015 Pyne *et al*., 2015
Plants	- Agrobacterium mediated transformation of sgRNA and Cas9 vector	Gao *et al*., 2014, Zhou *et al*., 2014
Mouse: heritable mutations	- Direct injection into embryos - Electroporation into zygotes	Wang *et al*., 2014 Qin *et al*., 2015
Mouse: mutations to adult somatic tissue	- Direct injection of AAV into tissue of interest	Cheng *et al*., 2014 Maddalo *et al*., 2014
Yeast	- Electroporation of plasmids and galactose induction of Cas9	DiCarlo *et al*., 2013

Check for intended KO / KI and off-target effects

To identify successful cases of CRISPR-mediated KO, the target site should be sequence to confirm a frame-shift mutation has occurred. You should also confirm that the mRNA and protein are significantly depleted or absent, such as by qPCR and Western blot on genome-edited samples versus unedited (parental) controls.

In some cases, such as in populations of primary cells, you may simply want to show that you achieved high KO or KI efficiency, without isolating clones for confirmation. Genome editing efficiency is typically determined via Surveyor assay (T7E1 assay) or assayed with next-generation sequencing (NGS). Many unique insertions and deletions will likely be observed.

To determine off-target effects, you may sequence around regions that are predicted to be likely sites for off-target cleavage based on sequence similarity to the on-target site, particularly in the "seed" region. A more rigorous measure of off-target cleavage can be performed using whole-genome sequencing.

Whole genome sequencing is often not practical for low frequency events. In addition, targeted sequencing only of computationally predicted off-target sites introduces a strong observational bias. Therefore, researchers in Keith Joung's lab developed a technique called Genome-wide Unbiased Identification of DSBs Enabled by sequencing (GUIDE-seq) to better quantify off-target activity of Cas9 throughout the genome (Tsai *et al.*, 2015). GUIDE-seq introduces a tag any time a DSB occurs, and then sequences around the tags to determine all off-target cleavage locations. They found surprising results, including that the majority of cleavage sites identified by GUIDE-seq were not of GUIDE-seq OT sites were not predicted by any algorithm, because they contain up to 6 mismatched nucleotides and in many cases include non-canonical PAMs.

How to ensure that off-target Cas9 activity won't confound your experiments:

- For each guide RNA you use, isolate multiple, independent clonal cell populations or founder individuals. The likelihood off-target DSBs occur in the same place in independent clones is very low.
- Use at least two independent gRNA sequences in parallel to derive distinct clones or founder individuals. Models created through genome editing with distinct guideRNA that share an on-target locus but do not share off-target loci are an excellent way to create independent replicates.
- Although few labs have the resources to do statistically powerful whole genome sequencing verification protocols such as gUIDEseq, it is relatively easy to select the few predicted off-target sequences for each gRNA you use and then sequence around those loci to ensure that off-target indels have not been introduced.

If you use most or all of these tips in combination, you can have confidence that your experiments will reveal true genotype/phenotype relationships.

Case Study 1: Generating K-Ras knock-out cell lines using CRISPR genome editing

The KRAS gene encodes for a protein called K-Ras, which is an important regulator of cell division. This gene, when mutated, can cause cells to become cancerous. In this case study, the *K-Ras* locus was knocked-out in the human colon cancer cell line, HCT116 (Figure 7).

Using GenScript's **GenCRISPR™ cell line services**, any gene can be targeted in any mammalian cell. All clones are target sequence validated and a detailed report on clone generation is provided.

To knock-out the K-Ras locus, gRNA and Cas9 vectors were encapsulated into a virus. In this case, exon 4 was targeted by the gRNA-Cas9 complex to generate a DSB. In the absence of donor DNA, the DSB was repaired by NHEJ to create an indel. Sanger sequencing (Figure 8A) and a western blot (Figure 8B) were used to confirm successful knock-out of the KRAS gene.

Figure 7: Knock-out targeting strategy for K-Ras

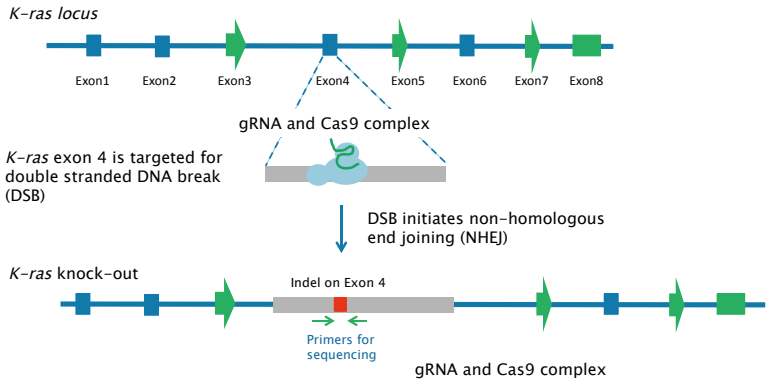
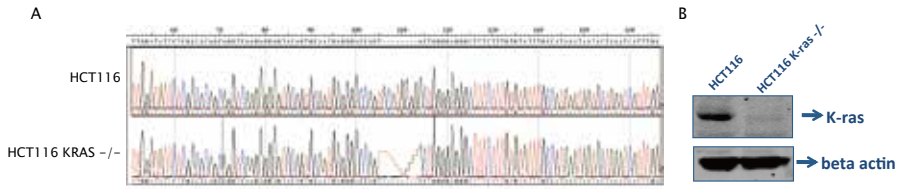


Figure 8: Sanger sequencing (A) and western blot (B) results for HCT116 KRAS -/-



Case Study 2: Using CRISPR to generate GLP-1R knock-in cell lines

Glucagon-like peptide 1 receptor (GLP-1R) is expressed in pancreatic cells and when stimulated increases insulin synthesis and release (Drucker *et al*., 1987). Consequently, it is a common target for the development of therapeutics for diabetes. In this study, a knock-in cell line was generated using GLP-1R donor DNA (containing the gene of interest and a puromycin selectable marker) and HEK 293T cells. The AAVS1 locus was targeted as the knock-in region (Figure 9). The cells were co-transfected with the donor DNA, Cas9 and gRNA, and positive clones were selected from the cell pools by Sanger sequencing and PCR.

Figure 9: Integration of GLP-1R into HEK 293T cells

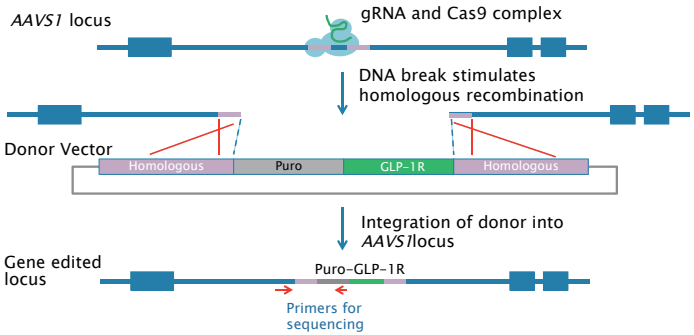
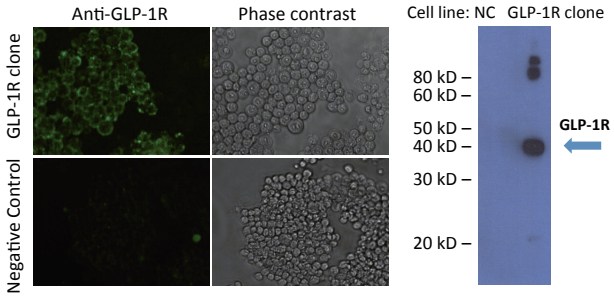


Figure 10: Immunocytochemistry (left) and western blot (right) analysis of GLP-1R clones



After 2 weeks of maintenance under puromycin selection, surviving cells were isolated and PCR analyzed for the Puro-GFP insert, which indicated GLP-1R was successfully inserted into the AAVS1 locus. Along with the Sanger sequencing results, immunocytochemistry and western blot analysis confirmed that the transfection was successful (Figure 10).

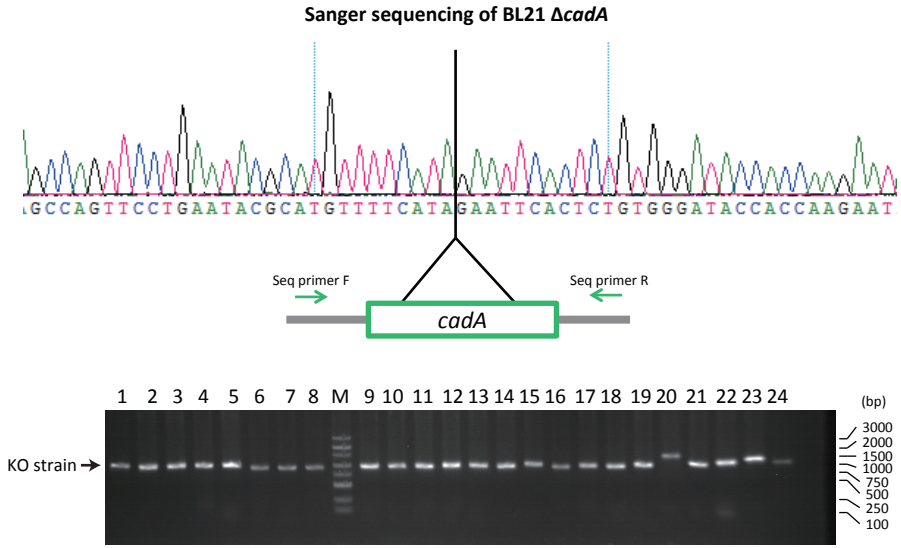
Case Study 3: Microbial Genome Editing

GenScript's **Microbial Genome Editing service** uses λ Red – CRISPR/Cas editing technology. This technique is the most precise, efficient, and cost effective recombineering method on the market!

Microbial genome editing has many applications in both pharma and industry – from studying gene function to the production of recombinant proteins for drug discovery and development. CRISPR/Cas can also be used to generate knock-in and knock-outs in microbes, such as *E. coli*. Since HR frequency is generally lower in microbes than mammalian cells, CRISPR/Cas can be combined with other recombination techniques to improve gene editing efficiency (Jiang *et al*., 2015). In this example, λ Red recombineering, one of the most effective recombination techniques in bacteria, is combined with CRISPR/Cas for efficient, seamless genome editing in *E. coli*.

In this case study, λ Red – CRISPR/Cas is used to knock-out *cadA* in the BL21 *E. coli* strain. The CasA protein is a component of lysine decarboxylase, an enzyme that helps bacteria survive in acidic environments (Lee *et al*., 2007). After the reaction, Sanger sequencing and colony PCR screening was used to confirm knock-out was successful (Figure 11).

Figure 11: Seamless knock-out of cadA in BL21 *E. coli*



CRISPR genome editing powers novel findings across disciplines

CRISPR/Cas genome editing has been used to accelerate research in many different arenas of basic life science and biomedical research.

Table 3: Research applications for CRISPR/Cas9 genome editing

Neuroscience	**A novel rat model for muscular dystrophy reveals new treatment targets.** Muscular Dystrophy is a condition associated with a loss of the protein Dystrophin, which is deadly when it affects the cardiac muscle. The lack of appropriate animal modes has made therapeutic discovery challenging; however, in a recent study by Nakamura *et al* (2014), CRISPR/Cas was used to knock-out the Dystrophin gene (Dmd) in rats. These mutations were heritable, thus presenting a new animal model to study new therapeutic targets for muscular dystrophy.
Cancer Biology	**CRISPR/Cas identifies novel tumor suppressor genes and new animal models for brain tumors.** Mutations to tumor suppressor genes are often causes for cancer progression, and developing animal models for these transformations is a very time-intensive. To address this, Zimermann *et al* (2015), used CRISPR/Cas to somatically induce loss-of-function (LOF) mutations in genes in the Sonic Hedgehog (Shh) signaling pathway: in previous studies, the authors found that SHH regulates proliferation of neural cells in the brain that can lead to malignant brain tumors. The results of this study confirmed that CRISPR/Cas could successfully induce these LOF mutations for the development of new, relevant brain tumor models.
Vaccines/ Virology	**T cell engineering with CRISPR/Cas reveals a new therapeutic strategy for HIV.** While successful T cell editing has historically been challenging, Schumann *et al* (2015) reported that the CRISPR/Cas editing tool can be used to successfully knock-out CXCR4, a co-receptor that HIV uses to infect cells. Using this technology, the authors reported that approximately 40% of CD4+ T-cells are CXCR4- following transfection with Cas9: gRNA ribonucleases.
Plant Biology	**Successful adaptation of the CRISPR/Cas editing system in rice.** Targeted mutagenesis has many implications for developing new traits in plants; however, mutation frequencies have varied significantly between species and delivery in plants can be particularly difficult. In an effort to optimize the process in rice, Mikami *et al* (2015) tested the efficiency of multiple gRNA and Cas9 vectors in rice calli. From this study they identified two Cas9 vectors, MMCas9 and FFCas9, as being the most effective for rice plants.
Immunology	**Knock-out nasal airway epithelial cells reveal a new pro-inflammatory function of the MUC18 gene.** Genome editing in primary cell lines has been a persistent challenge; however, Chu *et al* (2015) demonstrated that CRISPR/Cas could be used to knockout Muc18, a gene known to promote tumor metastasis, to better understand its function. In this study, the group showed that MUC18 KO has a pro-inflammatory role in the airway epithelium following exposure to viral and bacterial stimuli.

Expanding the Research Applications for CRISPR

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

- in situ functional assays in mouse tumor models (Malina *et al*., 2013),
- targeting functional long noncoding RNAs (lncRNA) or ribonucleoprotein (RNP) complexes to specific genomic loci (Shechner *et al*., 2015)
- Studying genome architecture and long-distance gene-enhancer interactions by disrupting megabase-scale topological chromatin domains (Lupiáñez *et al*., 2015)

Genome-wide screens using CRISPR libraries

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

GenScript offers amplified, NGS validated GeCKO and SAM libraries to accelerate your genome-wide screening efforts.

Genome-scale CRISPR knock-out libraries (GeCKO v2) libraries for mouse and human genomes enable rapid screening for loss-of-function mutations, as described by Sanjana *et al* (2014). GeCKO libraries are 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 to produce high-titer virus for efficient lentiviral transduction of primary cells or cultured cell lines. Either a single-vector or dual-vector system may be used (see Figure 6 on page 11). A cell population should be transduced with the GeCKO library pool at a low MOI ensuring no more than one gRNA enters any given cell. After transduction, deep sequencing with NGS should be performed to assess gRNA representation in the cell pool before beginning a screening protocol. At the end of the screen, after a second round of NGS, data analysis should be performed to identify the guides that were lost or enriched over the course of the screen. In order to identify true positive hits from a GeCKO library screen, you should identify genes for which multiple guides were enriched. A detailed GeCKO screening protocol may be found on the Genome Engineering website.

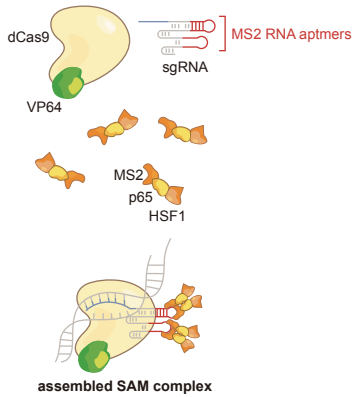
GeCKO libraries were designed to contain 6 single guide RNA (sgRNA) molecules targeting each gene within the human or mouse genome, as well as 4 sgRNA targeting each miRNA, and 1000 control (non-targeting) sgRNAs. The gRNA sequences are distributed over three or four constitutively expressed exons for each gene and were selected to minimize off-target genome modification.

Each library was divided into two sublibraries, A and B, containing 3 unique sgRNA for each gene; only library A contains 4 sgRNA targeting each of 1,864 miRNAs; both A and B contain the same 1,000 nontargeting control sgRNAs. The use of a single sublibrary maintains comprehensive genome-scale coverage but reduces the number of cells required to perform a screen, which is useful when cell numbers are limiting (for example, with primary cells or *in vivo* screens); alternatively, larger screens can be performed by combining both sublibraries.

The GeCKO library can be used in place of RNAi libraries for loss-of-function screening for any phenotype of interest, for example, to identify genes whose loss of function enables drug resistance in cancer cells (see box on page 23). As a complimentary approach, a CRISPR-based gene activation library can be used in place of a cDNA overexpression library for gain-of-function screening, as described below.

Adapting CRISPR for Transcriptional Regulation

Several research groups have harnessed the specificity and easy re-programmability of the CRISPR/Cas9 system to create programmable transcription factors that can activate or repress transcription of any desired coding region within a genome (Gilbert *et al*., 2013; Bikard *et al*., 2013; Cheng *et al*., 2013; Perez-Pinera *et al*., 2013). These systems use a nucleolytically inactive Cas9 protein (typically denoted as "dead" or dCas9) in order to target the Cas9-gRNA complex to the right position in the genome without cleaving or altering genomic DNA. They fuse the Cas9 to a well-characterized transcription-regulating domain, and then design guide RNA to direct the complex to just upstream of the transcription start site. Several light-inducible CRISPR-based transcription factors have been designed to allow precise spatial and temporal control of endogenous gene activation (Polstein *et al*., 2015; Nihongaki *et al*., 2015). One CRISPR-based transcriptional activator that has been used not only to target single genes but also for genome-wide gain-of-function screening is the CRISPR/Cas9 Synergistic Activation Mediator (SAM) system developed in the laboratory of Feng Zhang at the Broad Institute. SAM enables robust transcriptional activation of endogenous genes targeted by guide RNA that binds within 200 bp upstream of the transcription start site. SAM can be used to activate transcription of a single gene or up to 10 genes at once in the same cell. They can also be used to interrogate the function of long intergenic non-coding RNA (lincRNA) transcripts in addition to genes. Stable expression of SAM components via lentiviral transduction generates cell lines show stable and robust transcriptional activation, even of genes that are normally transcriptionally silent. These cell lines can be ideal research tools to characterize the function of specific candidate genes or groups of genes.



SAM can also be used for discovery research to identify the genes that drive phenotypes of interest in any disease model or developmental/differentiation process by using a genome-wide SAM gRNA library for gain-of-function screening (Konermann *et al*., 2015). The screening process is similar to the GeCKO library screening experimental protocol described above, but the library is designed to activate transcription rather than edit the genome. The human genome-wide SAM library contains 3 guide RNA targeting within 200 bp upstream of each of 23,430 coding gene isoform with a unique transcription start site in the human reference genome, for a total of 70,290 guides. This mixed pool of SAM guide RNAs is delivered along with the other SAM components using lentiviral vectors.

CRISPR libraries yield insights into Cancer Biology

An oncogenic mutation observed in melanoma cells, BRAF(V600E), makes cells susceptible to therapeutic treatment with BRAF inhibitors. However, some melanoma cells are able to develop resistance to these drugs over time. Genome-wide CRISPR libraries were used to identify genes whose up- or down-regulation within melanoma cells could confer resistance to BRAF inhibiting drugs (Shalem *et al*., 2014; Konermann *et al*., 2015)

Both GeCKO and SAM libraries were used to screen A375 (BRAF(V600E)) melanoma cells, by transducing a cell pool with the library and performing NGS to quantify sgRNA representation before and after a 14-day drug treatment. After treatment, most gRNA were substantially reduced, while a small set were highly enriched. The gene expression signature based on the top screening hits correlated with markers of BRAF inhibitor resistance in cell lines and patient-derived samples, enhancing confidence in the clinical relevance of these results. Genes for which several unique gRNA were enriched were considered top hits; these included genes previously known to confer resistance, such as EGFR and other genes in the ERK pathway, as well as numerous novel candidate genes, which can be subsequently validated using individual sgRNA and cDNA overexpression.

Epigenetic Modifications

Epigenetic modifications to genomic DNA and to the histone proteins that help organize chromosomes are increasingly shown to play critical roles in biological processes. Epigenetic marks such as methylation or acetylation at specific genomic loci or histone residues can be inherited or acquired, and can influence gene expression. The enzymes that regulate epigenetic state can be targeted via CRISPR genome editing or order to generate genomewide perturbations in epigenetic state. This was seen, for example, after CRISPR-mediated KO of all three active DNA methyltransferases (DNMTs), individually or in combination, in human embryonic stem cells (ESCs), allowing researchers to characterize viable, pluripotent cell lines with distinct effects on the DNA methylation landscape (Liao *et al*., 2015).

Researchers increasingly need methods for introducing epigenetic modifications only at desired genomic loci in order to model diseases and test hypotheses regarding potential therapeutic strategies. For example, specific epigenetic alterations are often necessary or sufficient to drive transformation of normal cells into cancerous cells, and play roles in later steps of carcinogenesis; therefore, the enzymes that regulate epigenetic modifications to DNA or histone proteins are candidate targets for cancer therapy (reviewed by Yao *et al.,* 2015).

CRISPR technology allows a catalytically inactive Cas9 to serve as a precisely targeted DNA-binding domain; when fused to epigenetic enzymes such as DNA methylases, histone acetyltransferases or deacetylases (HATs or HDACs), the complex can alter the epigenetic state in a precise way at a single precise location, or at several specific locations simultaneously. For example, a CRISPR-Cas9-based acetyltransferase consisting of dCas9 fused to the catalytic core of the human acetyltransferase p300 was shown to acetylate histone H3 lysine 27 specifically at its target sites and to robustly activate transcription of target genes (Hilton *et al*., 2015).

Similar to the capabilities of the SAM complex for transcription activation, Cas9 epigenetic effectors (epiCas9s) could also be used for genome-wide screening to discover novel relationships between DNA methylation or chromatin states and phenotypes such as cellular differentiation or disease progression (Hsu *et al*., 2014).

Stem Cell Differentiation

CRISPR technology can be used to guide stem cell differentiation for both basic research and therapeutics. Stem cell differentiation typically requires the robust activation of specific genes – typically transcription factors that control broad programs of downstream target gene expression – in specific combinations and sequences, over the course of several weeks or months. A catalytically inactive Cas9 nuclease that is fused to transactivation domains can be used as a programmable transcription activator to activate genes required for differentiation. For example, targeted activation of the endogenous Myod1 gene locus has been shown to yield stable and sustained reprogramming of mouse embryonic fibroblasts into skeletal myocytes (Chakraborty *et al*., 2014) for the repair of skeletal muscle tissue.

Induced pluripotent stem cells (iPSCs) have also become popular choices for stem cell therapy since they can be derived from patient-specific cells, overcoming ethical issues associated with embryonic stem cells. Similar to embryonic stem cells, iPSCs must be pre-differentiated prior to implantation to avoid teratoma formation; however, differentiation efficiency continues to be a bottleneck. Recent reports indicate that CRISPR may be an essential tool to improve differentiation, and has been used to derive a variety of cell types including muscle cells for the treatment of muscular dystrophy (Loperfido *et al*., 2015) and hematopoietic stem cells for the treatment of sickle cell anemia (Song *et al*., 2015). Recently, there have been multiple studies investigating the use of CRISPR to correct deleterious mutations associated with genetic diseases. For instance, the inherited blood disease β-Thalassemia is caused by deletions to the β-globin (HBB) gene, and by generating iPSCs with this mutation corrected could be a potential treatment option (Xu *et al*., 2015). Together these results demonstrate that CRISPR/Cas can improve the efficiency of not only gene targeting, but also directed differentiation.

Therapeutics

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. CRISPR-based therapeutics are already in development for treating blood cancers by modifying patients' T cells; eliminating disease-causing viruses in patients; and correcting single nucleotide mutations that cause many inherited diseases such as sickle-cell anemia.

CRISPR genome editing is especially promising for diseases that can be tackled by modifying cells that can easily be removed from a patient, genome-edited, screened to ensure no off-target genome modifications, and then infused back into the same patient. Autologous cell therapies that use genome editing to correct a mutation in the patient's own cells could be far safer than current therapies that use transplants from healthy donors. For example, combining CRISPR-mediated genome engineering with autologous T-cell therapies holds great promise for many diseases including cancer, HIV, primary immune deficiencies, and autoimmune diseases. It has already been demonstrated that primary human CD4+ T cells can be genome-edited with high efficiency and specificity using Cas9 protein in complex with guide RNA (Cas9 RNPs) (Schumann *et al*., 2015). Fusing GFP to Cas9 allows FACS-based enrichment of transfected T-cells (Meissner *et al*., 2014), and other improvements to CRISPR-based T-cell therapy protocols are doubtless underway. While there are many examples of *in vitro* or animal studies in which CRISPR-mediated gene knockout corrects a disease phenotype, significant challenges nonetheless remain to translate these into safe, efficacious therapies for human patients.

In order to address safety concerns prior to bringing CRISPR technology in to the clinic, a great deal of attention has already been paid to developing nonviral vectors such as lipid- or polymer-based nanocarriers, and several are already in clinical trials (Li *et al*., 2015). Non-viral CRISPR-mediated gene therapy may bypass some of the risks of prior viral-based gene therapy strategies, including the risk that a viral vector might recombine *in vivo* and become replication-competent; the risk that randomly integrating viruses will induce insertional mutagenesis, inaccurate gene dosage; the risk that genetic modifications could be made at unintended genomic loci or in unintended tissues; or the chance that the gene therapy will simply be ineffective due to immune responses directed against the viral vector. However, even non-viral Cas9 delivery may not completely avoid unwanted immune responses; a study delivering SpCas9 *in vivo* in mouse liver detected Cas9-specific humoral immune responses, highlighting the need for caution in future translational studies, and reinforcing the idea that *ex vivo* genome modification of autologous cells may be a safer route than *in vivo* delivery of Cas9 (Wang *et al*., 2015).

Table 4: Lead Prospects for CRISPR-based Therapeutics

Cancer	CRISPR-mediated knockout of NANOG and NANOGP8 decreases the *in vivo* tumorigenic potential of DU145 prostate cancer cell lines as well as *in vitro* phenotypes associated with malignancy such as sphere formation, anchorage-independent growth, migration capability, and drug resistance, suggesting that CRISPR-mediated gene knockout may be a viable addition to the therapeutic arsenal for prostate cancer patients (Kawamura *et al*., 2015).
Cardiovascular Disease	CRISPR/Cas9-mediated gene therapies could be used to correct inherited or acquired mutations that underlie cardiac disease, or to introduce therapeutic genes such as SERCA2a, S100A1, and adenylate cyclase 6 (Rincon *et al*., 2015)
HIV	HIV has been effectively eliminated in some patients via gene therapy to delete CCR5, which could be accomplished more efficiently in the future using CRISPR technology. In addition, CRISPR could be used in stem cell-based gene therapies to treat chronic HIV infection; hematopoietic stem/progenitor cells have been engineered to express a chimeric antigen receptor (CAR), so that they differentiate into functional cytotoxic T lymphocytes and natural killer cells that are resistant to HIV infection and suppress HIV replication (Zhen *et al.,* 2015).
Viral Diseases	CRISPR genome editing may be used to prevent, control, or cure viral diseases by targeting viral genes essential for replication or virulence. For example, persistent infection with HPV strains that cause genital warts, which have a high rate of recurrence after treatment, could be tackled through CRISPR-mediated inactivation of viral E 7 gene, as has already been demonstrated in transformed keratinocytes *in vitro* (Liu *et al*. 2015). CRISPR could also be used to target human genes that could enhance host immune responses against the virus.
Immunode-ficiences	Immunodeficiencies such as SCID are typically treated by allogeneic hematopoietic stem cell (HSC) transplantation, which carry a significant risk of incompatibility between donor and patient (Ott de Bruin *et al*., 2015).
Genetic Diseases	CRISPR genome editing could enable treatments for a number of genetic diseases, such as Chronic granulomatous disease (CGD) (Flynn *et al*., 2015) and replacing dysfunctional proteins in photoreceptor cells to restore sight in patients with a genetic retinal disease.

Future of CRISPR

CRISPR/Cas has revolutionized genome editing for its ease of use and broad applicability to mammalian cells, microbes, and animal models. Not only does CRISPR have the potential to enhance our ability to analyze and understand gene function, but this new tool can also reform the medical industry. Accessible genome editing techniques can be used to correct genetic mutations that are responsible for inherited disorders or diseases, and also for large-scale production and screening of new drugs (Doudna *et al*., 2014). In addition, the ability of CRISPR/Cas to both activate and repress gene function in both coding and non-coding regions of the genome expands its potential even further.

Considering how recently the CRISPR system has been applied to mammalian and microbial gene editing, there is still room for improvement. As the mechanism for how Cas9 binds to DNA is revealed, more effective Cas9-gRNA constructs can be designed (Sternberg and Doudna, 2015). Along the same vein, delivery of Cas9 into mammalian cells continues to be a bottleneck for some cell types. Designing smaller Cas9 variants that can be transfected into cells more easily will expand its applications and uses.

Regardless of these improvements, the significant role that CRISPR/Cas plays in the biological sciences is apparent. CRISPR/Cas gene editing remains the easiest and most exciting technology in genome engineering. There is no doubt that this is just the beginning of a revolutionary technology that can be used by generations of scientists to come.

Want to keep up with advances in CRISPR technology?

Visit www.genscript.com/crispr.html to learn about the most recent CRISPR-based tools and research findings

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The references cited in this handbook are not intended to be exhaustive. We apologize for omitting, due to space constraints, many important contributions from other researchers.

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