## A CRISPR Look at Genome Editing

## New Tools Are Driving Science even Further Ahead with the Ability to Interrogate the Function of any DNA Sequence in almost any Animal Model

[*Andrea Toell, Ph.D.*](http://genengnews.com/Contributor/AndreaToell/6668/)

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| Researchers are now using CRISPR-Cas9 to edit eukaryotic genomes, and the race is on to expand its possible uses. [© Stiggdriver/iStock] |

Genome editing techniques have taken a giant leap forwards since the development of recombinant DNA technology back in the 1970s. The ability to manipulate DNA revolutionized the field of biology, allowing researchers to study genes in hitherto unknown levels of detail, teasing out a greater functional understanding. More recently, new genome editing tools are driving science even further ahead with the ability to interrogate the function of any DNA sequence in almost any animal model, in situ, i.e., directly within the genome. The ability to examine gene function and organization within their endogenous setting offers an immense number of options for furthering our understanding.

### The Game Changer

One of the most prominent tools added to the genome editing toolbox has come in the form of an RNA-guided endonuclease known as Cas9, originally found in the prokaryotic adaptive immune system known as the clustered regularly interspaced short palindromic repeats ([CRISPR](http://genengnews.com/search?q=CRISPR)) system. The CRISPR/Cas9 system as an adaptive defense mechanism in bacteria was [first described in 2007](http://www.ncbi.nlm.nih.gov/pubmed/17379808) and proved itself to be very capable at fighting off foreign invaders like viruses, by using Cas9 to cleave the viral genome at specific locations. Life scientists soon realized that they could employ the innate ability of the CRISPR/Cas9 system to target and cleave specific sequences of DNA to modify genetic regions of interest in their own laboratory model systems. By 2013, researchers had started to use CRISPR-Cas9 [to edit eukaryotic genomes](http://www.ncbi.nlm.nih.gov/pubmed/23907171), and the race was on to expand its possible uses.

The CRISPR/Cas9 system is composed of two main components: the Cas9 endonuclease that is responsible for cleaving the target DNA—typically in the form of double strand breaks (DSBs)—and a short piece of guiding RNA (gRNA) that directs Cas9 to a specific location within the genome by hybridizing with its matching sequence in the genome. The gRNA would—in its normal defensive role—have matched the DNA sequence of a foreign invader, but these can easily be swapped out with a sequence of interest to redirect Cas9. This simple, flexible, and powerful system is opening up numerous avenues of novel research by inserting, deleting, or modifying DNA sequences of interest, benefiting science from blue-skies research to medicine and biotechnology.

### Making Valuable Edits

Genome-editing tools like CRISPR/Cas9 have major benefits associated with investigating how genes relate to a certain function and thanks to the flexibility of the system, it is now being applied to a range of additional areas.

Establishing transgenic model animals has traditionally been a challenging and lengthy process involving injecting a viral or bacterial vector containing the relevant transgene into the germ line. The difficulty here lies in the inherent risk of the injected DNA inserting itself at a critical, nonspecific locus with unexpected detrimental mutations, such as gene silencing or over-expression. The CRISPR/Cas9 system on the other hand is inherently specific, and can [accelerate the time for generating transgenic models](http://www.ncbi.nlm.nih.gov/pubmed/23643243). Transgenic animals (knockout or knockin) can be developed through [the injection of Cas9 and its specific gRNA into a fertilized zygote](http://www.ncbi.nlm.nih.gov/pubmed/23643243) for rapid, inheritable changes, facilitating cost-effective mutagenesis studies in conjunction with [very specific targeting](http://www.ncbi.nlm.nih.gov/pubmed/23992846).

Cell models can also be generated rapidly by [transfecting cells with plasmids containing Cas9](http://www.ncbi.nlm.nih.gov/pubmed/24906146) and the target-specific gRNA, or by using recently designed systems that involve transfecting purified[recombinant Cas9 protein pre-complexed with the gRNA](http://www.ncbi.nlm.nih.gov/pubmed/24696461) directly into the target cells. The latter approach eliminates the need for intracellular Cas9 expression via the plasmid, which places less stress on the target cells and results in the production of more colonies, while also reducing the impact of off-target effects.

This system also has great potential for studying polygenic diseases such as diabetes, autism, or schizophrenia in animal models due to the ability to encode multiple guide sequences into the CRISPR/Cas9 system, allowing for the [simultaneous editing of several genomics sites](http://www.ncbi.nlm.nih.gov/pubmed/23287718) (commonly referred to as multiplexing). Targeted genome editing would be invaluable in unraveling the contributions of several genes responsible for a disease phenotype, and has already been used to eliminate [two genes in monkeys](http://www.ncbi.nlm.nih.gov/pubmed/24486104) and the simultaneous disruption [of five genes in mice](http://www.ncbi.nlm.nih.gov/pubmed/23643243).

Transgenic animals have also been created that [naturally express Cas9](http://www.ncbi.nlm.nih.gov/pubmed/25263330), meaning only the gRNA needs to be injected in order to alter almost any region of the mouse genome. The so-called “Cas9 mouse” was used to model the competition between gain- and loss-of-function mutations in lung cancer. There are now a number of companies providing knockin animals (primarily rodents) generated with CRISPR/Cas9 that have had human transgenes inserted into their genome. In these so-called “humanized” animals, the human protein encoded is expressed within the animal, providing a model for more accurately assessing the effects of candidate drug molecules or other compounds of interest. Humanized models provide a much more representative look at how a compound might behave in humans, potentially mitigating the high rates of attrition seen in early drug testing phases.

Beyond animal models of disease, research is being directed toward [organ-on-chip models](http://www.ncbi.nlm.nih.gov/pubmed/25093883). These are continually-perfused chambers filled with cells in microfluidic channels that mimic tissues and organs, [enabling high resolution, real-time analysis](http://www.ncbi.nlm.nih.gov/pubmed/25093883). In such in vitro models of human organs, investigators are given the chance to examine an array of human diseases with a genetic basis through the manipulation of key genes of interest, lending valuable insight into disease pathogenesis and potential avenues of treatment. A [heart-on-chip model](http://www.ncbi.nlm.nih.gov/pubmed/24813252) has recently been developed to help understand mitochondrial cardiomyopathy seen in Barth syndrome using CRISPR/Cas9-mediated genome editing in order to isolate a mutation of interest and to establish its sufficiency to cause the disease phenotype.

Other far-reaching applications of this system include more efficient genomic screens via the ability of gRNA (either with Cas9 or in Cas9-expressing cells/animals) to perturb thousands of genomic elements at once, facilitating[genome-wide loss-of-function screens](http://www.ncbi.nlm.nih.gov/pubmed/24336571); live imaging of the genome through [fluorescently tagging Cas9](http://www.ncbi.nlm.nih.gov/pubmed/24360272), as an alternative to DNA-fluorescent in situ hybridization (FISH) that does not require samples to be fixed; or even the possibility of directly treating genetic diseases through correction of the responsible faulty gene—this could be especially effective in treating monogenic disorders resulting from loss-of-function diseases such as cystic fibrosis.

Clearly, genome editing through the CRISPR/Cas9 system is already making waves in the research community. However, this is still a relatively new tool, and as such, there are a number of limitations that many are working hard to overcome.

### Challenges and Solutions

CRISPR-Cas9 is valued because the method tends to make is [easy to use, cost-effective and highly customizable](http://www.biocompare.com/Editorial-Articles/144186-Genome-Editing-with-CRISPRs-TALENs-and-ZFNs/) when compared to TALEN (Transcriptional Activator-Like Effector Nucleases) and ZFN (Zinc Finger Nucleases), while also offering the opportunity to [target multiple genes within a single experiment](http://www.ncbi.nlm.nih.gov/pubmed/23643243). However, while each technique can be [prone to off-target effects](http://www.ncbi.nlm.nih.gov/pubmed/23873081), CRISPR/Cas9 appears to be especially at risk according to some studies.

When Cas9 induces DSBs, there is the possibility that the nuclease could cause mutations in sequences that are similar to the desired target sequence(s). This means, that CRISPR/Cas9 could perturb normal function in important genes such as those responsible for tumor suppression. A great deal of work has been devoted to reducing off-target effects by [modifying the gRNA](http://www.ncbi.nlm.nih.gov/pubmed/24463574), the [Cas9 protein](http://www.ncbi.nlm.nih.gov/pubmed/24770324)itself and even the [delivery system](http://www.ncbi.nlm.nih.gov/pubmed/24696461). However, while these approaches have successfully reduced the frequency of off-target mutations (often substantially so), it has remained unknown whether the whole genome is free of off-target effects. This has caused rising controversy amongst the research community around the safety and effectiveness of using CRISPR/Cas9.

Research this year however, has somewhat improved the situation, specifically in human cells. Recent work has made use of whole-genome sequencing (WGS) to [identify off-target effects caused by CRISPR/Cas9](http://www.ncbi.nlm.nih.gov/pubmed/25664545) with a technique termed Digenome-seq to locate both on-target and off-target sequence mutations caused by CRISPR/Cas9 via genome sequencing. Through a modification of the single gRNA, the research team was able to create a highly specific, programmable nuclease that[drastically reduces off-target effects](http://www.ncbi.nlm.nih.gov/pubmed/25664545) on a genome-wide scale in the human cells used. This review of the accuracy of CRISPR/Cas9 is likely to see a great deal of progress in developing new gene or cell therapies.

At the same time, researchers have also been looking to overcome another challenge posed by the CRISPR/Cas9 system: the inefficient repairing of Cas9-mediated DSBs. Once a properly designed gRNA has been used to guide Cas9 to a target, the subsequent DSBs are naturally repaired within the cell by at least one of two competing methods—nonhomologous end joining (NHEJ) or homology-directed repair (HDR), with the latter the preferred outcome as it is more precise. Unfortunately, NHEJ occurs more often than HDR and can introduce unpredictable insertions and deletions of variable size at the second allele of the target gene, which can have an impact on neighboring genes and/or control regions and can [reduce the efficiency of accurately generating the desired mutants](http://www.ncbi.nlm.nih.gov/pubmed/23643243). Therefore, the influence of NHEJ can make precise genome editing a challenge. To solve this problem and to improve the efficiency and precision of CRISPR/Cas9, scientists [used an anticancer agent called Src7](http://www.ncbi.nlm.nih.gov/pubmed/25798939), which also functions as an inhibitor of one of the key enzymes involved in the NHEJ pathway. This method improved efficiency of HDR-mediated genome editing using Cas9 by up to 19-fold.

### Don’t Run before You Can Walk

With scientists around the world working hard to overcome the challenges associated with CRISPR/Cas9 genome editing within cell or animal systems, it is still important to pay close attention to the delivery mechanism. After all, managing off-target effects is of no use if it’s too difficult to first deliver the CRISPR/Cas9 system into a biological system.

Viral vectors, such as adeno-associated virus (AAV) or lentivirus, have long been the vectors of choice for efficient cellular delivery, particularly as they have the potential to enable the in vivo delivery of the CRISPR/Cas9 system into target cells within the body, as would be required for therapeutic applications. However, the Cas9 gene most commonly used (from Streptococcus pyrogens) is relatively large, and close to the top-end capacity for transduction using AAV.

Generating expression plasmids on the other hand can be easier as their construction is [both simple and rapid](http://www.ncbi.nlm.nih.gov/pubmed/24157548), while newly developed methods have suggested it may be more effective to pre-complex the gRNA with a purified form of the Cas9 protein and transfect these components directly into cells,[bypassing the need for a plasmid-based system altogether](http://www.ncbi.nlm.nih.gov/pubmed/24696461). Recent technological innovations have also made [transfecting hard-to-transfect primary and stem cells possible](http://www.ncbi.nlm.nih.gov/pubmed/25517468), such as human hematopoietic stem and progenitor cells (HSPCs), using novel electroporation methods, enabling new avenues of exploration. As such, nonviral approaches for the introduction of CRISPR reagents is an area that is likely to attract a great deal of attention due to the comparative ease and scalability the system offers when compared to viral systems.

### From Humble Beginnings

A relatively unknown innate defense mechanism found within prokaryotes has rapidly become one of the most powerful and flexible tools within the life scientist’s toolbox. With the ability to specifically target both individual and multiple DNA sequences within a genome, CRISPR/Cas9 is providing the scientific community with a powerful tool to unravel much of biology’s intrinsic complexity, lending insight into diseases and how genes can have such pronounced effects on phenotype. While optimization is always an ongoing process, there has already been a great deal of work in order to iron out several teething problems, and with international recognition, CRISPR/Cas9 will continue to be refined, facilitating an exciting new era of biological discovery.