

*Review Article***CRISPR-CAS9: New Hope for Precision Genome Editing**

**Rishit N Sathwara, Jay Prakash Gupta, Jagdish D Chaudhari, Bhavesh M Prajapati,  
Girish A Parmar, Amit Kumar Srivastava and Harshadkumar C Chauhan**

College of Veterinary Science & Animal Husbandry, SDAU, Gujarat, INDIA

\*Corresponding author: [kadiarishitchandra9094@gmail.com](mailto:kadiarishitchandra9094@gmail.com)

|              |                                                                                       |
|--------------|---------------------------------------------------------------------------------------|
| Rec. Date:   | Oct 05, 2017 13:18                                                                    |
| Accept Date: | Jan 18, 2018 01:54                                                                    |
| DOI          | <a href="https://doi.org/10.5455/ijlr.20171005011845">10.5455/ijlr.20171005011845</a> |

**Abstract**

*A clustered regularly interspaced short palindromic repeats (CRISPRs) is genome editing tool with multiplexing ability. Early approaches for genome editing were based on the principle of site-specific recognition of DNA sequences by oligonucleotides, small molecules or self-splicing introns. However, CRISPR is not an artificial construction but an essential part of adaptive immune systems of bacteria and archaea against invading genetic elements, ability to manipulate the genome and facilitating the elucidation of target gene function in biology and diseases. The CRISPR system comprise non specific Cas9 nuclease, a set of programmable sequence-specific CRISPR RNA (crRNA) and trans-activating crRNA to form guide RNA which can guide Cas9 to cleave DNA and generate double-strand breaks at specific target site. Then cellular DNA repair process leads to desired insertions, deletions at target sites via, Non-homologous end-joining (NHEJ) and Homology directed repair (HDR). CRISPR system is used to solve the several complex molecular biology problems.*

**Key words:** Genome Editing, PAM, sgRNA, TALENs, ZFNs

**How to cite:** Sathwara, R., Gupta, J., Chaudhari, J., Prajapati, B., Parmar, G., Srivastava, A., & Chauhan, H. (2018). CRISPR-CAS9: New Hope for Precision Genome Editing. International Journal of Livestock Research, 8(6), 1-13. doi: 10.5455/ijlr.20171005011845

**Introduction**

Animals are exceptionally adaptive and this feature has enabled them to flourish all over the planet. They have been genetically and epigenetically adapted to many different habitats and climatic conditions. These adaptive mutations are carried down to subsequent generations. Technological advances in genomic engineering are the key for engineering genetic adaptations of individuals. The ability to manipulate the genome with precision and at specific target is crucial for understanding the contributions of gene and genetics to biology and disease. To facilitate genome editing, programmable sequence-

specific DNA (Deoxyribonucleic acid) nuclease technologies have enabled targeted modification of endogenous genomic sequences with high efficiency.

Ever since the concept of central dogma established in molecular biology, scientists have endeavored to develop new technologies to modify or manipulate the genome. Genome editing, the introduction of a desired change to the sequence of genomic DNA, is driving a revolution in the biomedical sciences and has the potential to provide future treatment for many human or animal diseases with a genetic component. The ideal genome-editing tool should be able to edit any genomic locus with high efficiency, high DNA sequence specificity and little or no undesired by-products. The genome editing technologies are currently in various laboratory development stages and are limited to modification of genetic material of somatic cells (Lanphier *et al.*, 2015). A new system offered the first alternative to the presently prevalent protein-based targeting (TALEN and Zinc Finger) methods used to specifically target a gene (or other DNA sequence).

Early approaches were based on the principle of site-specific recognition of DNA sequences by oligonucleotides (Faruqi *et al.*, 1996), small molecules or self-splicing introns. Recently, the site-directed Zinc Finger Nucleases (ZFNs) based on eukaryotic transcription factors (Miller *et al.*, 2007) and Transcription Activator Like Effector Nucleases (TALENs) from *Xanthomonas* bacteria (Moore *et al.*, 2014; Gaj *et al.*, 2013) using the principles of DNA protein recognition were developed. However, difficulties of protein design, synthesis and validation remained a barrier to widespread adoption of these engineered nucleases for routine use. Zinc Finger Nuclease was the first programmable genome editing tool. It consists of a custom-designed zinc finger (ZF) DNA-binding domain and non specific nuclease domain from the FokI restriction enzyme (Porteus and Carroll, 2005). ZF DNA binding domains can be engineered to bind to a pre-determined DNA sequence at a genomic locus. Each zinc finger domain recognizes a 3 to 4 bp DNA sequence and tandem domains can potentially bind to an extended nucleotide sequence (typically with a length which is a multiple of 3, usually 9 bp to 18 bp) that is unique within a cell's genome. ZFNs are designed as a pair that recognizes two sequences flanking the site, one on the forward strand while the other on the reverse strand. Upon binding of the ZFNs on either side of the site, the pair of FokI domains dimerize and cleave the DNA at the site, generating a double strand break (DSB) with 5' overhangs (Urnov *et al.*, 2010).

TALENs are naturally produced by certain microbes such as *Xanthomonas*, gram-negative bacteria. The naturally occurring TALE repeats comprise tandem arrays with 10 to 30 repeats that bind and recognize extended DNA sequences (Bogdanove and Voytas, 2011). Each repeat is 33 to 35 amino acids in length, with two polymorphisms at positions 12 and 13 within the module, which are called the repeat variable di-residue (RVD). One RVD binds specifically to one nucleotide of genomic DNA (Moscou and Bogdanove, 2009; Boch *et al.*, 2009). RVDs with different specificities can be assembled into arrays in

order to target DNA sequences that are pre-designed by the worker. TALENs can be successfully used to target endogenous genes and efficiently cleave DNA. The TALENs and CRISPR-Cas9 (CRISPR associated protein 9) methods are relatively similar. However, the TALENs method is not as efficient as the CRISPR-Cas9 method because later uses RNA (Ribonucleic acid) guide for precise DNA cutting, causing it to be more precise and safe. It is also able to be used with multiple cells at once.

### CRISPR-Cas9 System

Clustered regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated proteins (Cas) are an RNA-mediated adaptive immune system of bacteria and archaea that protect them from phages and plasmids. CRISPR is latest genome-editing system. It is not an artificial construction but bacteria use it as a primitive form of adaptive antiviral immunity. Ishino *et al.* (1987) first discovered the CRISPRs in *Escherichia coli*. It is the manifestation of memory of the system; which is the repository of short, directly repeating nucleotide sequences that alternate with small unique DNA fragments (Ishino *et al.*, 1987) acquired from previous infections (Bolotin *et al.*, 2005). Following infection with a pathogen, bacteria armed with CRISPR-Cas system remember the signature of the pathogen in their chromosomal DNA. The cell then transcribes those sequences called CRISPRs. Cas proteins are the actual effectors (Haft *et al.*, 2005). They are able to process CRISPR sequences into small RNAs (Haurwitz *et al.*, 2010) and to cleave the infectious DNA molecules that match the CRISPR-derived RNA (Marraffini and Sontheimer, 2008; Garneau *et al.*, 2010; Gasiunas *et al.*, 2012). A second transcript called a tracrRNA (trans-activating crRNA) along with the CRISPR-derived RNA guides the Cas9 nuclease to its target.

The simplicity of the CRISPR nuclease system is that it acts with only three components (Cas9, crRNA and tracrRNA) and that makes this system attractive for laboratory use. A complex prokaryotic system is converted into a simple genome editing tool by fusing the crRNA and the tracrRNA into an artificial, small guide RNA (sgRNA) of a hairpin RNA structure resembling the tracrRNA linked to a 20 bp sequence homologous to the target DNA (Jinek *et al.*, 2012).

Cas9 is the final effector among all the other Cas proteins DNA (Chylinski *et al.*, 2013) which is able to complex (Nishimasu *et al.*, 2014; Jinek *et al.*, 2014) and cleave both strands of a DNA molecule after detecting a typical homologous base pair match with the sgRNA. If we want to begin with the CRISPR-Cas genome editing system, we only need to identify a 20 bp sequence from the target DNA, followed by the protospacer adjacent motif (PAM) NGG (any nucleotide followed by G and G) and clone it into the sgRNA expression vector appropriate for the organism of interest (Ran *et al.*, 2013a, b). Genome editing with CRISPR-Cas depends mainly on the cell processes triggered by the DNA double strand break (DSB) at the targeted locus. In the absence of template DNA, the non-homologous end-joining (NHEJ) pathway repairs DNA damage more efficiently than homology directed repair (HDR). These results in stochastic

insertions and deletions (indels) are introduced through NHEJ-mediated repair, rendering the genetic modification unpredictable (Barnes, 2001). When a DNA donor template is provided, DSB can also be repaired through homology-driven or HDR; DSB facilitate the frequency of homologous recombination (Liang *et al.*, 1996). This HDR strategy enhanced the efficiency of the desired genome-editing event by two to three times, resulting in targeted incorporation typically being much more efficient than incorporation at random sites in the genome (Chouluka *et al.*, 1995; Jasin, 1996).

### Mechanism of the CRISPR-Cas System

It is now clear that the CRISPR-Cas system requires a CRISPR locus, consisting the hypervariable spacers, which are being acquired from phages or plasmids and is located in the host genome. It further requires different types of Cas genes that are located in the nearby CRISPR locus and encodes various Cas proteins for the multistep defence against foreign DNA (Sapranauskas *et al.*, 2011). The whole process of CRISPR-Cas defense can be divided in three-step sequential process; acquisition, RNA processing and finishing with interference.

### Acquisition Phase

The foreign DNA is first recognized, captured and subsequently integrated as spacers between the two contiguous repeat sequences located in the CRISPR locus. Spacers are derived from phage or plasmid and are also known as protospacer (Deveau *et al.*, 2008). Small nucleotides present near the protospacer are referred to as the protospacer adjacent motif (PAM), which is particularly important during the acquisition of DNA (Deveau *et al.*, 2010).

### RNA Processing

The Cas1 and Cas2 are the universal genes present in the genome and they process foreign DNA to generate a functional CRISPR-Cas system (Bhaya *et al.*, 2011). The RNA polymerase transcribes CRISPR locus and produces a pre CRISPR RNA (pre-crRNA) while endonucleases cleave the pre-crRNAs into active CRISPR RNAs (crRNAs).

### Interference

crRNAs form a crRNA-Protein complex which can recognize the regions of incoming foreign DNA (or RNA), through the base pairing rules with great specificity. This complex degrades the foreign DNA and maintains phage immunity (Brouns *et al.*, 2008). On the other hand, if the base pairing is not homologous or the PAM sequence is absent then the bacterial host does not have resistance against the phage, leading to infection and subsequent host cell lysis (Bhaya *et al.*, 2011). There are three types of CRISPR system viz. Type I, Type II and Type III identified across a wide range of bacterial and archaeal hosts (Sinkunas

*et al.*, 2011; Heler *et al.*, 2015; Spilman *et al.*, 2013), where in each system comprised of a cluster of CRISPR-associated (Cas) genes, noncoding RNAs and a distinctive array of repetitive elements (direct repeats).

### Application of CRISPR-Cas9

Ever since scientists understood that DNA carried heritable information, they have tried or desired to modify this rule as per their will. Manipulating the fundamental rule for life would empower the scientists with ability to correct defects and permanently cure genetic disorders. But the tools that were in vogue, although were working but these were similar as of trying to perform surgery while wearing woollen gloves. CRISPRs were used for applied purposes before their functions in host defense were known, particularly by taking advantage of the heterogeneity of CRISPRs among isolates that are otherwise isogenic.

### CRISPR-Cas9 in Treatment of Genetic Diseases

Numerous animal or human genetic diseases are caused by gene mutations like cataract, Duchenne muscular dystrophy (DMD) etc. Diseases such as DMD results from frame shift mutations and the correct reading frame of the gene can be restored through targeted NHEJ induced indels (Ousterout *et al.*, 2013). The corrected cells may ultimately generate many healthy muscle fibres and this strategy, some day in future may allow correction of disease-causing mutations in the muscle tissue of patients with DMD (Long *et al.*, 2014). CRISPR-Cas9 system have the potential to knockout the accurate region of the mutant genes, and this characteristic can be used to repair DNA and correct diseases with ssDNA donors *via* HDR or NHEJ. The targeted mutation can be used for the purpose of gene knockout of mutant allele as in the case of Huntington's disease (Aronin and DiFiglia, 2014).

In addition to this, CRISPR-Cas9 technology can also be used to target regulators or enhancers of pathogenic genes to alleviate diseases. A dominant cataract-causing mutation in the *Crygc* gene in mice was corrected using CRISPR-Cas9 (Wu *et al.*, 2013). The zygotes from mating B6D2F1 females with homozygous cataract males were harvested and then co-microinjected with a sgRNA to specifically target the mutant *Crygc* gene, the Cas9 to cleave double-strand DNA and a ssDNA donor to repair the defect through HDR.

### CRISPR-Cas9 in Generation of Disease Models

By stimulating genetic mutations from the patients, CRISPR-Cas9 system has been utilized to generate specific disease models in cells or animals. Cellular models can be generated easily by introducing Cas9 into the target cells using transient transfection of plasmids carrying Cas9 and the appropriately designed sgRNA.

Besides this, owing to multiplexing capabilities, Cas9 offers a great promise for study of common diseases which are supposed to be polygenic in inheritance, like diabetes, heart disease, schizophrenia, autism etc. Many haplotypes are being identified which have indicated strong association with disease risk. However, due to linkage disequilibrium it is often difficult to determine which of several genetic variants are responsible for a particular phenotype. Using Cas9, one could study the effect of each individual variant and the effect of manipulating each individual variation on an isogenic background by editing stem cells and differentiating them into cell types of interest (Schwank *et al.*, 2013).

Transgenic animal models can be generated by injecting Cas9 protein and transcribed sgRNA, directly into fertilized zygotes to achieve heritable gene modification at one or multiple alleles in animal models such as rodents and monkeys (Yang *et al.*, 2013; Niu *et al.*, 2014). Further, bypassing the typical ES cell targeting stage in generating transgenic lines, the generation time for mutant mice and rats can be reduced from more than a year to only several weeks. Such advances will facilitate cost effective and large-scale *in vivo* mutagenesis studies in rodent models and can be combined with highly specific editing (Fu *et al.*, 2014) to avoid confounding off target mutagenesis.

### CRISPR-Cas9 in Somatic Genome Editing

In addition to repairing mutations underlying inherited disorders, Cas9-mediated genome editing might be used to introduce protective mutations in somatic tissues to combat nongenetic or complex diseases. For example, NHEJ-mediated inactivation of the CCR5 receptor in lymphocytes (Lombardo *et al.*, 2007) may be a viable strategy for circumventing HIV (Human Immunodeficiency Virus) infection, whereas deletion of PCSK9 (Cohen *et al.*, 2005) or angiotensin II (Musunuru *et al.*, 2010) may provide therapeutic effects against statin-resistant hypercholesterolemia or hyperlipidemia. Although these targets may be also addressed using siRNA-mediated protein knockdown, a unique advantage of NHEJ-mediated gene inactivation is the ability to achieve permanent therapeutic benefit without the need for continuing treatment. As with all gene therapies, it will of course be important to establish that each proposed therapeutic use has a favorable risk-benefit ratio. Cas9 could be used beyond the direct genome modification of somatic tissue, such as for engineering therapeutic cells. Chimeric antigen receptor (CAR) T-cells can be modified *ex vivo* and reinfused into a patient to specifically target certain cancers (Couzin-Frankel, 2013). The ease of design and testing of Cas9 may also facilitate the treatment of highly rare genetic variants through personalized medicine. To support these tremendous possibilities, there are a number of animal model studies as well as clinical trials using programmable nucleases that had already provided important insights into the future development of Cas9-based therapeutics.

### CRISPR-Cas9 in the Treatment of Infectious Diseases

It is well known fact that the CRISPR-Cas system originally functions as an antiviral adaptive immune system in bacteria, this system could be used for treating infectious diseases i.e. by destroying pathogen genome from infected animals. The CRISPR-Cas9 system can eliminate the HIV-1 genome and prevents new HIV infection (Ebina *et al.*, 2013; Hu *et al.*, 2014). When transfected into HIV-1 provirus-integrated human cells, a sgRNA expression vector targeting the long terminal repeats (LTR) of HIV-1 efficiently cleaves and mutates LTR target sites and suppresses LTR-driven viral gene expression. This system has also been shown to delete viral genes from the host cell chromosome (Hu *et al.*, 2014).

### CRISPR-Cas9 in Genome Editing of Microorganisms

It is always desirable to get the control of infection, virulence and drug resistance, which can be done by manipulating the genome of some of the harmful infectious agents. On the same line manipulation of some of the beneficial microorganism is also desirable as such organisms have been used by mankind since time immemorial for preparation of food and beverages such as curd, cheese, beer, wine etc. To improve the quality of these products, genome editing of these microbes is quintessential, for gene knock-outs, knock-ins and replacement of some of the sequences. Despite this, microbial genome editing remains an unexplored and underrepresented application of CRISPR Cas systems (Selle and Barrangou, 2015). In present scenario, major issue concern to human health is the development of resistance against commonly used antibiotics in certain bacteria. It is quite well understood that more than 200 conserved and essential proteins are present in the bacteria, but only a relatively small number of these have been exploited as antibiotic targets (Bugg *et al.*, 2011). However, the current antibiotics are not specific to selectively kill the desired strains from a complex community (Bikard *et al.*, 2014). Another issue to consider is that most of the antibiotic resistance genes are derived from plasmids (and have multiple copies) which are capable of autonomous transfer into microbial populations (Nordmann *et al.*, 2012). Potentially the build-up of resistance over time may have also occurred due to random mutations in genes and the evolutionary adaptation of strains, the excess and inappropriate use of antibiotics. Current antibiotics have a tendency to be broad spectrum and therefore cannot readily discriminate between the beneficial and harmful strains within a mixed community. In order to specifically target harmful strains, a programmable CRISPR-Cas9 system has been successfully developed for targeting even highly related strains in pure or mixed cultures. This can also offer the development of a new way to control multi-drug resistance (MDR) and to discriminate between the harmful and the beneficial microorganisms within a community (Gomaa *et al.*, 2014).

### CRISPR-Cas9 Applicability in Livestock and Poultry

Tan *et al.* (2013) have injected polled gene into fibroblast obtained from dairy bulls having horns, using TALEN and HDR template containing the polled gene. The successful use of genome editing to introgress desired alteration in bovine  $\beta$ -lactoglobulin gene has been accomplished through co-injection of ZFN/TALEN along with oligonucleotide templates (Wei *et al.*, 2015). Likewise, one-step generation of multiple gene modifications in pigs has been seen through the CRISPR/Cas9 system (Whitworth *et al.*, 2014). When a designed CRISPR/Cas9 system targeting CD163 (resistance to PRRS) and CD1D (biomedical application) was introduced into somatic cells, it was highly efficient in inducing mutations. When these mutated cells were used with somatic cell nuclear transfer, offspring with these modifications were created. This approach has also been used for production of *in vitro* zygotes to generate pigs with specific genetic modifications. Very recently, it has also been demonstrated that the disruption of FGF5 in Cashmere goats using CRISPR/Cas9 resulted in increased secondary hair follicles and longer fibres (Wang *et al.*, 2016).

### Limitations

The CRISPR-Cas9 technology is an effective, cheap and quick method for genome editing, which accelerates our pace to realize the mechanisms of many diseases (Yang *et al.*, 2015). It is widely used to edit genes in all kinds of animals, but still there are certain limitations mentioned below-

#### Target Specificity

A guide RNA is specific for the genes they are supposed to target; it is perplexing that how they are so specific? It has been demonstrated that non-target DNA resembling the guide RNA may be cut, activated or deactivated (Pennisi, 2013). Slaymaker *et al.* (2016) increased the specificity of Cas9 by mutating some individual amino acid site and the modified Cas9 variants could retain on-target effect and decrease off target efficiency.

#### PAM Dependence

The another major limitation of the CRISPR/Cas9 system is its relatively low targeting specificity, because it is determined by a 20nt recognition site and the requirement for the neighboring PAM sequence (NGG) (Wei *et al.*, 2013), which is determined by several mismatches within the target sequence.

#### sgRNA Production / Design

The efficiency of cleavage varies considerably at different target sites. This might be due to various reasons such as secondary structures within the sgRNA, thermodynamic stability of the sgRNA-DNA duplex or accessibility of the target sequence within the context of chromatin.

### Mismatches between crRNA and Targeted Sequences

Many of the studies demonstrated that the mismatches between crRNA and targeted sequences can be tolerated by the Cas9 endonuclease (Lin *et al.*, 2014; Mali *et al.*, 2013). Although there are no definitive rules for Cas9 specificity, the number and location of mismatches play a critical role. As an approximation, DNA-RNA duplex mismatches in close proximity to the PAM sequence impair Cas9 activity, whereas, mismatches distal to the PAM sequence are tolerated. While a single nucleotide mismatch does not affect Cas9 activity, two or more mismatches, depending on their location with respect to the PAM sequence, decrease Cas9 activity.

To increase the gene-editing specificity of CRISPR-Cas9 for potential therapeutic applications, more strategies need to be developed to reduce NHEJ alterations and increase HDR. These two competing repair pathways can lead to either gene correction or gene inactivation and must be tightly controlled. Despite of above all limitations, craze of CRISPR is increasing leap and bound and people have already formed companies to harness the technology for treating genetic diseases. “The only limitation today is people’s ability to think of creative ways to harness CRISPR”.

### Future Prospects

CRISPR-Cas9 has various rousing prospects in genome editing of diverse organisms. For example, CRISPR-Cas9 has a great impact on mosquito genome editing in order to control the spread of such dreaded diseases in the nation like India. Alterations in the wild mosquito population could result in extinction of the target mosquito species within a short period. Ledford (2015) had predicted certain undesirable consequences of CRISPR-Cas9-based genome editing. He proposed that the spread of genome edited strains through wild populations, may be extremely difficult to detect and would be challenging to bio-security measures to control the spread of mutated mosquitoes in case they cause serious problems to ecosystem (Ledford, 2015).

It is understood that with all the wisdom CRISPR remains an excellent tool and one of the best that we currently have for genome engineering. The current acceptance of CRISPR-Cas9 technology, in general, has led to many organisations involved in research are using it, employing it directly as well as adapting it to their needs and developing it beyond its current scope. In summary, CRISPR-Cas9 is a key technology for targeted genome editing in a wide range of organisms and cell types. It is a simple, cost-effective, efficient and has the potential to be further expanded towards even greater biomedical, therapeutic, industrial and biotechnological applications.

### Conclusion

The story of how a mysterious prokaryotic viral defense system became one of the most powerful and versatile platforms for engineering biology highlights the importance of basic science research. Just as

recombinant DNA technology benefited from basic investigation of the restriction enzymes that are central to warfare between phage and bacteria, the latest generation of Cas9-based genome engineering tools are also based on components from the microbial anti-phage defense system. The CRISPR system is an elegant, effective and fluid mechanism of defense against foreign genetic elements. It is rightly described as an adaptive immune system, which evolved long before its popularization. Interestingly, CRISPR's ability to acquire a resistance phenotype and pass it to progeny could be construed an example of a soft or Lamarckian, mode of inheritance. Once these technologies will be further refined with more efficient and less off-target effects, genome editing is likely to emerge as a revolutionary technology for rapid advancement of biotechnology, medicine and livestock improvement worldwide.

## References

1. Aronin N and DiFiglia M. 2014. Huntingtin-lowering strategies in Huntington's disease: antisense oligonucleotides, small RNAs and gene editing. *Movement Disorders*. 29(11): 1455-1461.
2. Barnes DE. 2001. Non-homologous end joining as a mechanism of DNA repair. *Current Biology*. 11(12): 455-457.
3. Bhaya D, Davison M and Barrangou R. 2011. CRISPR-Cas systems in bacteria and archaea: versatile small RNAs for adaptive defense and regulation. *Annual Review of Genetics*. 45: 273-297.
4. Bikard D, Euler CW, Jiang W, Nussenzweig PM, Goldberg GW, Duportet X, Fischetti VA and Marraffini LA. 2014. Exploiting CRISPR-Cas nucleases to produce sequence-specific antimicrobials. *Nature Biotechnology*. 32: 1146-1150.
5. Boch J, Scholze H, Schornack S, Landgraf A, Hahn S, Kay S, Lahaye T, Nickstadt A and Bonas U. 2009. Breaking the code of DNA binding specificity of TAL-type III effectors. *Science*. 326(5959): 1509-1512.
6. Bogdanove AJ and Voytas DF. 2011. TAL effectors: customizable proteins for DNA targeting. *Science*. 333: 1843-1846.
7. Bolotin A, Quinquis B, Sorokin A and Ehrlich SD. 2005. Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. *Microbiology*. 151: 2551-2561.
8. Brouns SJ, Jore MM, Lundgren M, Westra ER, Slijkhuis RJ, Snijders AP, Dickman MJ, Makarova KS, Koonin EV and Van-der-Oost J. 2008. Small CRISPR RNAs guide antiviral defense in prokaryotes. *Science*. 321(5891): 960-964.
9. Bugg TD, Braddick D, Dowson CG and Roper DI. 2011. Bacterial cell wall assembly: still an attractive antibacterial target. *Trends in Biotechnology*. 29(4): 167-173.
10. Chouluka A, Perrin A, Dujon B and Nicolas J. 1995. Induction of homologous recombination in mammalian chromosomes by using the I-SceI system of *Saccharomyces cerevisia*. *Molecular and Cellular Biology*. 15: 1968-1973.
11. Chylinski K, Le-Rhun A and Charpentier E. 2013. The tracrRNA and Cas9 families of type II CRISPR-Cas immunity systems. *RNA Biology*. 10(5): 726-737.
12. Cohen J, Pertsemlidis A, Kotowski IK, Graham R, Garcia CK and Hobbs HH. 2005. Low LDL cholesterol in individuals of African descent resulting from frequent nonsense mutations in PCSK9. *Nature Genetics*. 37: 161-165.
13. Couzin-Frankel J. 2013. Breakthrough of the year 2013. Cancer immunotherapy. *Science*. 342: 1432-1433.

14. Deveau H, Barrangou R, Garneau JE, Labonte J, Fremaux C, Boyaval P, Romero DA, Horvath P and Moineau S. 2008. Phage response to CRISPR-encoded resistance in *Streptococcus thermophilus*. Journal of Bacteriology. 190: 1390-1400.
15. Deveau H, Garneau JE and Moineau S. 2010. CRISPR/Cas system and its role in phage-bacteria interactions. Annual Review of Microbiology. 64: 475-93.
16. Ebina H, Misawa N, Kanemura Y and Koyanagi Y. 2013. Harnessing the CRISPR/Cas9 system to disrupt latent HIV-1 provirus. Scientific Reports. 3: 2510.
17. Faruqi AF, Seidman MM, Segal DJ, Carroll D and Glazer PM. 1996. Recombination induced by triple helix-targeted DNA damage in mammalian cells. Molecular and Cellular Biology. 16: 6820-6828.
18. Fu Y, Sander JD, Reyon D, Cascio VM and Joung JK. 2014. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. Nature Biotechnology. 32(3): 279-284.
19. Gaj T, Gersbach CA and Barbas CF. 2013. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends Biotechnology. 31(7):397-405.
20. Garneau JE, Dupuis ME, Villion M, Romero DA, Barrangou R, Boyaval P, Fremaux C, Horvath P, Magadan AH and Moineau S. 2010. The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. Nature. 468(7320): 67-71.
21. Gasiunas G, Barrangou R, Horvath P and Siksnys V. 2012. Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. Proceeding of the National Academy of Sciences of United States of America. 109(39): 2579-2586.
22. Goma AA, Klumpe HE, Luo ML, Selle K, Barrangou R and Beisel CL. 2014. Programmable removal of bacterial strains by use of genome-targeting CRISPR-Cas systems. The American Society for Microbiology. 5(1): 928-913.
23. Haft DH, Selengut J, Mongodin EM and Nelson KE. 2005. A Guild of 45 CRISPR-associated (Cas) protein families and multiple CRISPR/Cas subtypes exist in prokaryotic genomes. PLoS Computational Biology. 1(6): e60.
24. Haurwitz RE, Jinek M, Wiedenheft B, Zhou K and Doudna JA. 2010. Sequence and structure-specific RNA processing by a CRISPR endonuclease. Science. 329(5997): 1355-1358.
25. Heler R, Samai P, Modell JW, Weiner C, Goldberg GW, Bikard D and Marraffini LA. 2015. Cas9 specifies functional viral targets during CRISPR-Cas adaptation. Nature. 519(7542): 199-202.
26. Hu W, Kaminski R, Yang F, Zhang Y, Cosentino L, Li F, Luo B, Alvarez-Carbonell D, Garcia-Mesa Y, Karn J and Mo X. 2014. RNA-directed gene editing specifically eradicates latent and prevents new HIV-1 infection. Proceeding of National Academy of Sciences of United States of America. 111(31): 11461-11466.
27. Ishino Y, Shinagawa H, Makino K, Amemura M and Nakata A. 1987. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in *Escherichia coli*, and identification of the gene product. Journal of Bacteriology. 169: 5429-5433.
28. Jasin M. 1996. Genetic manipulation of genomes with rare-cutting endonucleases. Trends in Genetics. 12(6): 224-228.
29. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA and Charpentier E. 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. 337(6096): 816-821.
30. Jinek M, Jiang F, Taylor DW, Sternberg SH, Kaya E, Ma E, Anders C, Hauer M, Zhou K, Lin S, Kaplan M, Iavarone AT, Charpentier E, Nogales E and Doudna JA. 2014. Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. Science. 343(6176): 1247997.
31. Lanphier E, Urnov F, Haecker SE, Werner M and Smolenski J. 2015. Don't edit the human germ line. Nature. 519: 410-411.
32. Ledford H. 2015. CRISPR, the disruptor. Nature. 522(7554): 20-24.
33. Liang F, Romanienko PJ, Weavert DT, Jeggo PA and Jasin M. 1996. Chromosomal double-strand break repair in Ku80-deficient cells. Proceeding of the National Academy of Sciences of United States of America. 93: 8929-8933.

34. Lin Y, Cradick TJ, Brown MT, Deshmukh H, Ranjan P, Sarode N, Wile BM, Vertino PM, Stewart FJ and Bao G. 2014. CRISPR/Cas9 systems have off-target activity with insertions or deletions between target DNA and guide RNA sequences. *Nucleic Acids Research*. 42(11): 7473-7485.
35. Lombardo A, Genovese P, Beausejour CM, Colleoni S, Lee YL, Kim KA, Ando D, Urnov FD, Galli C, Gregory PD, Holmes MC and Naldini L. 2007. Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. *Nature Biotechnology*. 25(11): 1298-1306.
36. Long C, McAnally JR, Shelton JM, Mireault AA, Bassel-Duby R and Olson EN. 2014. Prevention of muscular dystrophy in mice by CRISPR/Cas9-mediated editing of germline DNA. *Science*. 345(6201): 1184-1188.
37. Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE and Church GM. 2013. RNA-guided human genome engineering via Cas9. *Science*. 339(6121): 823-826.
38. Marraffini LA and Sontheimer EJ. 2008. CRISPR interference limits horizontal gene transfer in *staphylococci* by targeting DNA. *Science*. 322(5909): 1843-1845.
39. Miller JC, Holmes MC, Wang J, Guschin DY, Lee YL, Rupniewski I, Beausejour CM, Waite AJ, Wang NS and Kim KA. 2007. An improved zinc-finger nuclease architecture for highly specific genome editing. *Nature Biotechnology*. 25: 778-785.
40. Moore R, Chandrasekhar A and Bleris L. 2014. Transcription activator-like effectors: a toolkit for synthetic biology. *ACS Synthetic Biology*. 3(10): 708-716.
41. Moscou MJ and Bogdanove AJ. 2009. A simple cipher governs DNA recognition by TAL effectors. *Science*. 326(5959): 1501.
42. Musunuru K, Pirruccello JP, Do R, Peloso GM, Guiducci C, Sougnez C, Garimella KV, Fisher S, Abreu J, Barry AJ, Fennell T, Banks E, Ambrogio L, Cibulskis K, Kernysky A, Gonzalez E, Rudzicz N, Engert JC, DePristo MA, Daly MJ, Cohen JC, Hobbs HH, Altshuler D, Schonfeld G, Gabriel SB, Yue P and Kathiresan S. 2010. Exome sequencing, ANGPTL3 mutations and familial combined hypolipidemia. *The New England Journal of Medicine*. 363(23): 2220-2227.
43. Nishimasu H, Ran FA, Hsu PD, Konermann S, Shehata SI, Dohmae N, Ishitani R, Zhang F and Nureki O. 2014. Crystal structure of Cas9 in complex with guide RNA and target DNA. *Cell*. 156(5): 935-949.
44. Niu Y, Shen B, Cui Y, Chen Y, Wang J, Wang L, Kang Y, Zhao X, Si W, Li W, Xiang AP, Zhou J, Guo X, Bi Y, Si C, Hu B, Dong G, Wang H, Zhou Z, Li T, Tan T, Pu X, Wang F, Ji S, Zhou Q, Huang X, Ji W and Sha J. 2014. Generation of gene-modified cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. *Cell*. 156(4): 836-843.
45. Nordmann P, Dortet L and Poirel L. 2012. Carbapenem resistance in Enterobacteriaceae: here is the storm. *Trends in Molecular Medicine*. 18(5): 263-272.
46. Ousterout DG, Perez-Pinera P, Thakore PI, Kabadi AM, Brown MT, Qin X, Fedrigo O, Mouly V, Tremblay JP and Gersbach CA. 2013. Reading frame correction by targeted genome editing restores dystrophin expression in cells from duchenne muscular dystrophy patients. *Molecular Therapy*. 21: 1718-1726.
47. Pennisi E. 2013. The CRISPR craze. *Science*. 341(6148): 833-836.
48. Porteus MH and Carroll D. 2005. Gene targeting using zinc finger nucleases. *Nature Biotechnology*. 23(8): 967-973.
49. Ran FA, Hsu PD, Lin CY, Gootenberg JS, Konermann S, Trevino AE, Scott DA, Inoue A, Matoba S, Zhang Y and Zhang F. 2013<sup>a</sup>. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell*. 154: 1380-1389.
50. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA and Zhang F. 2013<sup>b</sup>. Genome engineering using the CRISPR-Cas9 system. *Nature Protocols*. 8(11): 2281-2308.
51. Sapranaukas R, Gasiunas G, Fremaux C, Barrangou R, Horvath P and Siksnys V. 2011. The *Streptococcus thermophilus* CRISPR/Cas system provides immunity in *Escherichia coli*. *Nucleic Acids Research*. 39: 9275-9282.

52. Schwank G, Koo BK, Sasselli V, Dekkers JF, Heo I, Demircan T, Sasaki N, Boymans S, Cuppen E, Van-der-ent CK, Nieuwenhuis EE, Beekman JM and Clevers H. 2013. Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. *Cell Stem Cell*. 13(6): 653-658.
53. Selle K and Barrangou R. 2015. Harnessing CRISPR-Cas systems for bacterial genome editing. *Trends in Microbiology*. 23(4): 225-232.
54. Sinkunas T, Gasiunas G, Fremaux C, Barrangou R, Horvath P and Siksnys V. 2011. Cas3 is a single-stranded DNA nuclease and ATP-dependent helicase in the CRISPR/Cas immune system. *The European Molecular Biology Organization Journal*. 30(7):1335-1342.
55. Slaymaker IM, Gao L, Zetsche B, Scott DA, Yan WX and Zhang F. 2016. Rationally engineered Cas9 nucleases with improved specificity. *Science*. 351(6268): 84-88.
56. Spilman M, Coccozaki A, Hale C, Shao Y, Ramia N, Terns R, Terns M, Li H and Stagg S. 2013. Structure of an RNA silencing complex of the CRISPR-Cas immune system. *Molecular Cell*. 52(1): 146-152.
57. Tan W, Carlson DF, Lancto CA, Garbe JR, Webster DA, Hackett PB and Fahrenkrug SC. 2013. Efficient non meiotic allele introgression in livestock using custom endonucleases. *Proceedings of the National Academy of Science of the United States of America*. 110(41): 16526-16531.
58. Urnov FD, Rebar EJ, Holmes MC, Zhang HS and Gregory PD. 2010. Genome editing with engineered zinc finger nucleases. *Nature Reviews Genetics*. 11(9): 636-646.
59. Wang X, Cai B, Zhou J, Zhu H, Niu Y, Ma B, Yu H, Lei A, Yan H, Shen Q, Shi L, Zhao X, Hua J, Huang X, Qu L and Chen Y. 2016. Disruption of FGF5 in cashmere goats using CRISPR/Cas9 results in more secondary hair follicles and longer fibers. *Public Library of Science one*. 11(10): 164640.
60. Wei C, Liu J, Yu Z, Zhang B, Gao G and Jiao R. 2013. TALEN or Cas9 - rapid, efficient and specific choices for genome modifications. *Journal of Genetics and Genomics*. 40(6): 281-289.
61. Wei L, Nakajima S, Bohm S, Bernstein KA, Shen Z, Tsang M, Levine AS and Lan L. 2015. DNA damage during the G0/G1 phase triggers RNA-templated, Cockayne syndrome B-dependent homologous recombination. *Proceedings of the National Academy of Science of the United States of America*. 112(27): 3495-3504.
62. Whitworth KM, Lee K, Benne JA, Beaton BP, Spate LD, Murphy SL, Samuel MS, Mao J, O'Gorman C, Walters EM, Murphy CN, Driver J, Mileham A, McLaren D, Wells KD and Prather RS. 2014. Use of the CRISPR/Cas9 system to produce genetically engineered pigs from in vitro-derived oocytes and embryos. *Biology of Reproduction*. 91(3): 78.
63. Wu Y, Liang D, Wang Y, Bai M, Tang W, Bao S, Yan Z, Li D and Li J. 2013. Correction of a genetic disease in mouse via use of CRISPR-Cas9. *Cell Stem Cell*. 13(6): 659-662.
64. Yang H, Wang H, Shivalila CS, Cheng AW, Shi L and Jaenisch R. 2013. One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas mediated genome engineering. *Cell*. 154(6): 1370-1379.
65. Yang L, Guell M, Niu D, George H, Lesha E, Grishin D, Aach J, Shrock E, Xu W, Poci J, Cortazio R, Wilkinson RA, Fishman JA and Church G. 2015. Genome-wide inactivation of porcine endogenous retroviruses (PERVs). *Science*. 350(6264): 1101-1104.