Genome editing using CRISPR/Cas9 literatures

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Abstract: Genome editing, or genome engineering is a type of genetic engineering in which DNA is inserted, deleted, modified or replaced in the genome of a living organism. In 2018, the common methods for such editing use engineered nucleases, or "molecular scissors". These nucleases create site-specific double-strand breaks (DSBs) at desired locations in the genome. The induced double-strand breaks are repaired through nonhomologous end-joining (NHEJ) or homologous recombination (HR), resulting in targeted mutations. As of 2015 four families of engineered nucleases were used: meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector-based nucleases (TALEN), and the clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) system. Nine genome editors were available as of 2017. CRISPR is a family of DNA sequences in bacteria. The sequences contain snippets of DNA from viruses that have attacked the bacterium. These snippets are used by the bacterium to detect and destroy DNA from similar viruses during subsequent attacks. These sequences play a key role in a bacterial defense system, and form the basis of a technology known as CRISPR/Cas9 that effectively and specifically changes genes within organisms. The CRISPR/Cas system is a prokaryotic immune system that confers resistance to foreign genetic elements such as those present within plasmids and phages that provides a form of acquired immunity. RNA harboring the spacer sequence helps Cas (CRISPR-associated) proteins recognize and cut exogenous DNA. Other RNA-guided Cas proteins cut foreign RNA. CRISPRs are found in approximately 40% of sequenced bacterial genomes and 90% of sequenced archaea.
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Key words: DNA; eternal; life; genome editing; CRISPR/Cas9

1. Introduction

Genome editing, or genome engineering is a type of genetic engineering in which DNA is inserted, deleted, modified or replaced in the genome of a living organism. In 2018, the common methods for such editing use engineered nucleases, or "molecular scissors". These nucleases create site-specific double-strand breaks (DSBs) at desired locations in the genome. The induced double-strand breaks are repaired through nonhomologous end-joining (NHEJ) or homologous recombination (HR), resulting in targeted mutations. As of 2015 four families of engineered nucleases were used: meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector-based nucleases (TALEN), and the clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) system. Nine genome editors were available as of 2017.

CRISPR is a family of DNA sequences in bacteria. The sequences contain snippets of DNA from viruses that have attacked the bacterium. These snippets are used by the bacterium to detect and destroy DNA from similar viruses during subsequent attacks. These sequences play a key role in a bacterial defense system, and form the basis of a technology known as CRISPR/Cas9 that effectively and specifically changes genes within organisms. The CRISPR/Cas system is a prokaryotic immune system that confers resistance to foreign genetic elements such as those present within plasmids and phages that provides a form of acquired immunity. RNA harboring the spacer sequence helps Cas (CRISPR-associated) proteins recognize and cut exogenous DNA. Other RNA-guided Cas proteins cut foreign RNA. CRISPRs are found in approximately 40% of sequenced bacterial genomes and 90% of sequenced archaea.

Literatures

The following gives some recent reference papers on genome editing using CRISPR/Cas9.

Abbasi, F., et al. (2018). "Revolutionizing male fertility factor research in mice by using the genome editing tool CRISPR/Cas9." Reprod Med Biol **17**(1): 3-10.

Background: Reproductive research is quintessential in understanding not only the cause of infertility, but also for creating family planning tools. The knockout (KO) system approach is conducive to discovering genes that are essential for fertility in mice. However, in vivo research has been limited due to its high cost and length of time needed to establish KO mice. Methods: The mechanisms behind the CRISPR/Cas9 system and its application in investigating male fertility in mice are described by using original and review articles. Results: The

CRISPR/CAS9 SYSTEM has enabled researchers to rapidly, efficiently, and inexpensively produce genetically modified mice to study male fertility. Several genes have been highlighted that were found to be indispensable for male fertility by using the CRISPR/Cas9 system, as well as more complicated gene manipulation techniques, such as point mutations, tag insertions, and double knockouts, which have become easier with this new technology. Conclusion: In order to increase efficiency and usage, new methods of CRISPR/Cas9 integration are being developed, such as electroporation and applying the system to embryonic stem cells. The hidden mysteries of male fertility will be unraveled with the help of this new technology.

Ahn, J., et al. (2017). "Targeted genome editing in a quail cell line using a customized CRISPR/Cas9 system." Poult Sci **96**(5): 1445-1450.

Soon after RNA-guided Cas9 (CRISPR-associated protein 9) endonuclease opened a new era of targeted genome editing, the CRISPR/Cas9 platform began to be extensively used to modify genes in various types of cells and organisms. However, successful CRISPR/Cas9-mediated insertion/deletion (indel) mutation remains to be demonstrated in avian cell lines. The objective of this study was to design a poultry-specific CRISPR/Cas9 system to efficiently introduce targeted deletion mutation in chromosomes of the quail muscle clone 7 (QM7) cell line using a customized quail CRISPR vector. In this study, two avian-specific promoters, quail 7SK (q7SK) promoter and CBh promoter, the hybrid form of cytomegalovirus and chicken beta-actin promoters, were cloned into a CRISPR vector for the expression of guide RNA and Cas9 protein, respectively. Then, guide RNA, which was designed to target 20-base pair (bp) nucleotides in the quail melanophilin (MLPH) locus, was ligated to the modified CRISPR vector and transfected to QM7 cells. Our results showed multiple indel mutations in the quail MLPH locus in nearly half of the alleles being tested, suggesting the high efficiency of the system for targeted gene modification. The new CRISPR vector developed from this study has the potential application to generate knockout avian cell lines and knockout poultry.

Alsaiari, S. K., et al. (2018). "Endosomal Escape and Delivery of CRISPR/Cas9 Genome Editing Machinery Enabled by Nanoscale Zeolitic Imidazolate Framework." J Am Chem Soc **140**(1): 143-146.

CRISPR/Cas9 is a combined protein (Cas9) and an engineered single guide RNA (sgRNA) genome editing platform that offers revolutionary solutions to genetic diseases. It has, however, a double delivery problem owning to the large protein size and the highly charged RNA component. In this work, we report the first example of CRISPR/Cas9 encapsulated by nanoscale zeolitic imidazole frameworks (ZIFs) with a loading efficiency of 17% and enhanced endosomal escape promoted by the protonated imidazole moieties. The gene editing potential of CRISPR/Cas9 encapsulated by ZIF-8 (CC-ZIFs) is further verified by knocking down the gene expression of green fluorescent protein by 37% over 4 days. The nanoscale CC-ZIFs are biocompatible and easily scaled-up offering excellent loading capacity and controlled codelivery of intact Cas9 protein and sgRNA.

Altenbuchner, J. (2016). "Editing of the Bacillus subtilis Genome by the CRISPR-Cas9 System." Appl Environ Microbiol **82**(17): 5421-5427.

UNLABELLED: The clustered regularly interspaced short palindromic repeat (CRISPR)-associated (Cas) systems are adaptive immune systems of bacteria. A type II CRISPR-Cas9 system from Streptococcus pyogenes has recently been developed into a genome engineering tool for prokaryotes and eukaryotes. Here, we present a single-plasmid system which allows efficient genome editing of Bacillus subtilis The plasmid pJOE8999 is a shuttle vector that has a pUC minimal origin of replication for Escherichia coli, the temperature-sensitive replication origin of plasmid pE194(ts) for B. subtilis, and a kanamycin resistance gene working in both organisms. For genome editing, it carries the cas9 gene under the control of the B. subtilis mannose-inducible promoter PmanP and a single guide RNA (sgRNA)-encoding sequence transcribed via a strong promoter. This sgRNA guides the Cas9 nuclease to its target. The 20-nucleotide spacer sequence at the 5' end of the sgRNA sequence, responsible for target specificity, is located between BsaI sites. Thus, the target specificity is altered by changing the spacer sequences via oligonucleotides fitted between the BsaI sites. Cas9 in complex with the sgRNA induces double-strand breaks (DSBs) at its target site. Repair of the DSBs and the required modification of the genome are achieved by adding homology templates, usually two PCR fragments obtained from both sides of the target sequence. Two adjacent SfiI sites enable the ordered integration of these homology templates into the vector. The function of the CRISPR-Cas9 vector was demonstrated by introducing two large deletions in the B. subtilis chromosome and by repair of the trpC2 mutation of B. subtilis 168. IMPORTANCE: In prokaryotes, most methods used for scarless genome engineering are based on selection-counterselection systems. The disadvantages are often the lack of a suitable counterselection marker, the toxicity of the compounds needed for counterselection, and the

requirement of certain mutations in the target strain. CRISPR-Cas systems were recently developed as important tools for genome editing. The single-plasmid system constructed for the genome editing of B. subtilis overcomes the problems of counterselection methods. It allows deletions and introduction of point mutations. It is easy to handle and very efficient, and it may be adapted for use in other firmicutes.

Andrey, G. and M. Spielmann (2017). "CRISPR/Cas9 Genome Editing in Embryonic Stem Cells." Methods Mol Biol **1468**: 221-234.

Targeted mutagenesis is required to evaluate the function of DNA segments across the genome. In recent years the CRISPR/Cas9 technology has been widely used for functional genome studies and is partially replacing classical homologous recombination methods in different aspects. CRISPR/Cas9-derived tools indeed allow the production of a wide-range of engineered mutations: from point mutations to large chromosomal rearrangements such as deletions, duplications and inversions. Here we present a protocol to engineer Embryonic Stem Cells (ESC) with desired mutations using transfection of custom-made CRISPR/Cas9 vectors. These methods allow the in vivo modeling of congenital mutations and the functional interrogation of DNA sequences.

Annunziato, S., et al. (2016). "Modeling invasive lobular breast carcinoma by CRISPR/Cas9-mediated somatic genome editing of the mammary gland." Genes Dev **30**(12): 1470-1480.

Large-scale sequencing studies are rapidly identifying putative oncogenic mutations in human tumors. However, discrimination between passenger and driver events in tumorigenesis remains challenging and requires in vivo validation studies in reliable animal models of human cancer. In this study, we describe a novel strategy for in vivo validation of candidate tumor suppressors implicated in invasive lobular breast carcinoma (ILC), which is hallmarked by loss of the cell-cell adhesion molecule E-cadherin. We describe an approach to model ILC by intraductal injection of lentiviral vectors encoding Cre recombinase, the CRISPR/Cas9 system, or both in female mice carrying conditional alleles of the Cdh1 gene, encoding for E-cadherin. Using this approach, we were able to target ILC-initiating cells and induce specific gene disruption of Pten by CRISPR/Cas9-mediated somatic gene editing. Whereas intraductal injection of Cas9-encoding lentiviruses induced Cas9-specific immune responses and development of tumors that did not resemble ILC, lentiviral delivery of a Pten targeting single-guide RNA (sgRNA) in mice with mammary gland-specific loss of E-cadherin and expression of Cas9 efficiently induced ILC development. This versatile platform can be used for rapid in vivo testing of putative tumor suppressor genes implicated in ILC, providing new opportunities for modeling invasive lobular breast carcinoma in mice.

Bai, Y., et al. (2016). "Efficient Genome Editing in Chicken DF-1 Cells Using the CRISPR/Cas9 System." G3 (Bethesda) **6**(4): 917-923.

In recent years, genome engineering technology has provided unprecedented opportunities for site-specific modification of biological genomes. Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) 9 is one such means that can target a specific genome locus. It has been applied in human cells and many other organisms. Meanwhile, to efficiently enrich targeted cells, several surrogate systems have also been developed. However, very limited information exists on the application of CRISPR/Cas9 in chickens. In this study, we employed the CRISPR/Cas9 system to induce mutations in the peroxisome proliferator-activated receptor-gamma (PPAR-gamma), ATP synthase epsilon subunit (ATP5E), and ovalbumin (OVA) genes in chicken DF-1 cells. The results of T7E1 assays showed that the mutation rate at the three different loci was 0.75%, 0.5%, and 3.0%, respectively. In order to improve the mutation efficiency, we used the Puro (R) gene for efficient enrichment of genetically modified cells with the surrogate reporter system. The mutation rate, as assessed via the T7E1 assay, increased to 60.7%, 61.3%, and 47.3%, and subsequent sequence analysis showed that the mutation efficiency increased to 94.7%, 95%, and 95%, respectively. In addition, there were no detectable off-target mutations in three potential off-target sites using the T7E1 assay. As noted above, the CRISPR/Cas9 system is a robust tool for chicken genome editing.

Bak, R. O., et al. (2018). "CRISPR/Cas9 genome editing in human hematopoietic stem cells." Nat Protoc **13**(2): 358-376.

Genome editing via homologous recombination (HR) (gene targeting) in human hematopoietic stem cells (HSCs) has the power to reveal gene-function relationships and potentially transform curative hematological gene and cell therapies. However, there are no comprehensive and reproducible protocols for targeting HSCs for HR. Herein, we provide a detailed protocol for the production, enrichment, and in vitro and in vivo analyses of HR-targeted HSCs by combining CRISPR/Cas9 technology with the use of rAAV6 and flow cytometry. Using this protocol, researchers can introduce single-nucleotide changes

into the genome or longer gene cassettes with the precision of genome editing. Along with our troubleshooting and optimization guidelines, researchers can use this protocol to streamline HSC genome editing at any locus of interest. The in vitro HSC-targeting protocol and analyses can be completed in 3 weeks, and the long-term in vivo HSC engraftment analyses in immunodeficient mice can be achieved in 16 weeks. This protocol enables manipulation of genes for investigation of gene functions during hematopoiesis, as well as for the correction of genetic mutations in HSC transplantation-based therapies for diseases such as sickle cell disease, beta-thalassemia, and primary immunodeficiencies.

Bassett, A. R. (2017). "Editing the genome of hiPSC with CRISPR/Cas9: disease models." Mamm Genome **28**(7-8): 348-364.

The advent of human-induced pluripotent stem cell (hiPSC) technology has provided a unique opportunity to establish cellular models of disease from individual patients, and to study the effects of the underlying genetic aberrations upon multiple different cell types, many of which would not normally be accessible. Combining this with recent advances in genome editing techniques such as the clustered regularly interspaced short palindromic repeat (CRISPR) system has provided an ability to repair putative causative alleles in patient lines, or introduce disease alleles into a healthy "WT" cell line. This has enabled analysis of isogenic cell pairs that differ in a single genetic change, which allows a thorough assessment of the molecular and cellular phenotypes that result from this abnormality. Importantly, this establishes the true causative lesion, which is often impossible to ascertain from human genetic studies alone. These isogenic cell lines can be used not only to understand the cellular consequences of disease mutations, but also to perform high throughput genetic and pharmacological screens to both understand the underlying pathological mechanisms and to develop novel therapeutic agents to prevent or treat such diseases. In the future, optimising and developing such genetic manipulation technologies may facilitate the provision of cellular or molecular gene therapies, to intervene and ultimately cure many debilitating genetic disorders.

Bassett, A. R. and J. L. Liu (2014). "CRISPR/Cas9 and genome editing in Drosophila." J Genet Genomics **41**(1): 7-19.

Recent advances in our ability to design DNA binding factors with specificity for desired sequences have resulted in a revolution in genetic engineering, enabling directed changes to the genome to be made relatively easily. Traditional techniques for generating genetic mutations in most organisms have relied on selection from large pools of randomly induced mutations for those of particular interest, or time-consuming gene targeting by homologous recombination. Drosophila melanogaster has always been at the forefront of genetic analysis, and application of these new genome editing techniques to this organism will revolutionise our approach to performing analysis of gene function in the future. We discuss the recent techniques that apply the CRISPR/Cas9 system to Drosophila, highlight potential uses for this technology and speculate upon the future of genome engineering in this model organism.

Basu, S., et al. (2017). "A novel tool for monitoring endogenous alpha-synuclein transcription by NanoLuciferase tag insertion at the 3'end using CRISPR-Cas9 genome editing technique." Sci Rep **8**: 45883.

alpha-synuclein (alpha-SYN) is a major pathologic contributor to Parkinson's disease (PD). Multiplication of alpha-SYN encoding gene (SNCA) is correlated with early onset of the disease underlining the significance of its transcriptional regulation. Thus, monitoring endogenous transcription of SNCA is of utmost importance to understand PD pathology. We developed a stable cell line expressing alpha-SYN endogenously tagged with NanoLuc luciferase reporter using CRISPR/Cas9-mediated genome editing. This allows efficient measurement of transcriptional activity of alpha-SYN in its native epigenetic landscape which is not achievable using exogenous transfection-based luciferase reporter assays. The NanoLuc activity faithfully monitored the transcriptional regulation of SNCA following treatment with different drugs known to regulate alpha-SYN expression; while exogenous promoter-reporter assays failed to reproduce the similar outcomes. To our knowledge, this is the first report showing endogenous monitoring of alpha-SYN transcription, thus making it an efficient drug screening tool that can be used for therapeutic intervention in PD.

Batzir, N. A., et al. (2017). "Therapeutic Genome Editing and its Potential Enhancement through CRISPR Guide RNA and Cas9 Modifications." Pediatr Endocrinol Rev **14**(4): 353-363.

Genome editing with engineered nucleases is a rapidly growing field thanks to transformative technologies that allow researchers to precisely alter genomes for numerous applications including basic research, biotechnology, and human gene therapy. The genome editing process relies on creating a site-

specific DNA double-strand break (DSB) by engineered nucleases and then allowing the cell's repair machinery to repair the break such that precise changes are made to the DNA sequence. The recent development of CRISPR-Cas systems as easily accessible and programmable tools for genome editing accelerates the progress towards using genome editing as a new approach to human therapeutics. Here we review how genome editing using engineered nucleases works and how using different genome editing outcomes can be used as a tool set for treating human diseases. We then review the major challenges of therapeutic genome editing and we discuss how its potential enhancement through CRISPR guide RNA and Cas9 protein modifications could resolve some of these challenges.

Beagan, J. A. and J. E. Phillips-Cremins (2016). "CRISPR/Cas9 genome editing throws descriptive 3-D genome folding studies for a loop." Wiley Interdiscip Rev Syst Biol Med **8**(4): 286-299.

CRISPR/Cas9 genome editing studies have recently shed new light into the causal link between the linear DNA sequence and 3-D chromatin architecture. Here we describe current models for the folding of genomes into a nested hierarchy of higher-order structures and discuss new insights into the organizing principles governing genome folding at each length scale. WIREs Syst Biol Med 2016, 8:286-299. doi: 10.1002/wsbm.1338 For further resources related to this article, please visit the WIREs website.

Bellec, J., et al. (2015). "CFTR inactivation by lentiviral vector-mediated RNA interference and CRISPR-Cas9 genome editing in human airway epithelial cells." Curr Gene Ther **15**(5): 447-459.

BACKGROUND: Polarized airway epithelial cell cultures modelling Cystic Fibrosis Transmembrane conductance Regulator (CFTR) defect are crucial for CF and biomedical research. RNA interference has proven its value to generate knockdown models for various pathologies. More recently, genome editing using CRISPR-Cas9 artificial endonuclease was a valuable addition to the toolbox of gene inactivation. METHODS: Calu-3 cells and primary HAECs were transduced with HIV-1-derived lentiviral vectors (LVV) encoding small hairpin RNA (shRNA) sequence or CRISPR-Cas9 components targeting CFTR alongside GFP. After sorting of GFP-positive cells, CFTR expression was measured by RT-qPCR and Western blot in polarized or differentiated cells. CFTR channel function was assessed in Ussing chambers. Il-8 secretion, proliferation and cell migration were also studied in transduced cells. RESULTS: shRNA interference and CRISPRCas9 strategies efficiently decreased CFTR expression in Calu-3 cells. Strong CFTR knockdown was confirmed at the functional level in CRISPR-Cas9-modified cells. CFTR-specific shRNA sequences did not reduce gene expression in primary HAECs, whereas CRISPR-Cas9-mediated gene modification activity was correlated with a reduction of transepithelial secretion and response to a CFTR inhibitor. CFTR inactivation in the CRISPR-Cas9-modified Calu-3 cells did not affect migration and proliferation but slightly increased basal interleukin-8 secretion. CONCLUSION: We generated CFTR inactivated cell lines and demonstrated that CRISPR-Cas9 vectorised in a single LVV efficiently promotes CFTR inactivation in primary HAECs. These results provide a new protocol to engineer CF primary epithelia with their isogenic controls and pave the way for manipulation of CFTR expression in these cultures.

Benakanakere, M. R., et al. (2016). "Investigation of the functional role of human Interleukin-8 gene haplotypes by CRISPR/Cas9 mediated genome editing." Sci Rep **6**: 31180.

Interleukin-8 (IL-8) gene polymorphisms have been considered as susceptibility factors in periodontal disease. However, the functional roles of IL-8 gene haplotypes have not been investigated. Here, we demonstrate for the first time the use of the CRISPR/Cas9 system to engineer the IL-8 gene, and tested the functionality of different haplotypes. Two sgRNAs vectors targeting the IL-8 gene and the naked homologous repair DNA carrying different haplotypes were used to successfully generate HEK293T cells carrying the AT genotype at the first SNP - rs4073 (alias -251), TT genotype at the second SNP - rs2227307 (alias +396), TC or CC genotypes at the third SNP - rs2227306 (alias +781) at the IL-8 locus. When stimulated with Poly I:C, ATC/TTC haplotype, cells significantly up-regulated the IL-8 at both transcriptional and translational levels. To test whether ATC/TTC haplotype is functional, we used a trans-well assay to measure the transmigration of primary neutrophils incubated with supernatants from the Poly I:C stimulation experiment. ATC/TTC haplotype cells significantly increased transmigration of neutrophils confirming the functional role for this IL-8 haplotype. Taken together, our data provides evidence that carriage of the ATC/TTC haplotype in itself may increase the influx of neutrophils in inflammatory lesions and influence disease susceptibility.

Bence, M., et al. (2017). "Combining the auxin-inducible degradation system with CRISPR/Cas9-based genome editing for the conditional depletion of endogenous Drosophila melanogaster proteins." FEBS J **284**(7): 1056-1069.

Inducible protein degradation techniques have considerable advantages over classical genetic approaches, which generate loss-of-function phenotypes at the gene or mRNA level. The plant-derived auxin-inducible degradation system (AID) is a promising technique which enables the degradation of target proteins tagged with the AID motif in nonplant cells. Here, we present a detailed characterization of this method employed during the adult oogenesis of Drosophila. Furthermore, with the help of CRISPR/Cas9-based genome editing, we improve the utility of the AID system in the conditional elimination of endogenously expressed proteins. We demonstrate that the AID system induces efficient and reversible protein depletion of maternally provided proteins both in the ovary and the early embryo. Moreover, the AID system provides a fine spatiotemporal control of protein degradation and allows for the generation of different levels of protein knockdown in a well-regulated manner. These features of the AID system enable the unraveling of the discrete phenotypes of genes with highly complex functions. We utilized this system to generate a conditional loss-of-function allele which allows for the specific degradation of the Vasa protein without affecting its alternative splice variant (solo) and the vasa intronic gene (vig). With the help of this special allele, we demonstrate that dramatic decrease of Vasa protein in the vitellarium does not influence the completion of oogenesis as well as the establishment of proper anteroposterior and dorsoventral polarity in the developing oocyte. Our study suggests that both the localization and the translation of gurken mRNA in the vitellarium is independent from Vasa.

Beneke, T., et al. (2017). "A CRISPR Cas9 high-throughput genome editing toolkit for kinetoplastids." R Soc Open Sci **4**(5): 170095.

Clustered regularly interspaced short palindromic repeats (CRISPR), CRISPR-associated gene 9 (Cas9) genome editing is set to revolutionize genetic manipulation of pathogens, including kinetoplastids. CRISPR technology provides the opportunity to develop scalable methods for high-throughput production of mutant phenotypes. Here, we report development of a CRISPR-Cas9 toolkit that allows rapid tagging and gene knockout in diverse kinetoplastid species without requiring the user to perform any DNA cloning. We developed a new protocol for single-guide RNA (sgRNA) delivery using PCR-generated DNA templates which are transcribed in vivo by T7 RNA polymerase and an online resource (LeishGEdit.net) for automated primer design. We produced a set of plasmids that allows easy and scalable generation of DNA constructs for transfections in just a few hours. We show how these tools allow knock-in of fluorescent protein tags, modified biotin ligase BirA*, luciferase, HaloTag and small epitope tags, which can be fused to proteins at the N- or C-terminus, for functional studies of proteins and localization screening. These tools enabled generation of null mutants in a single round of transfection in promastigote form Leishmania major, Leishmania mexicana and bloodstream form Trypanosoma brucei; deleted genes were undetectable in non-clonal populations, enabling for the first time rapid and large-scale knockout screens.

Bono, J. M., et al. (2015). "Connecting genotypes, phenotypes and fitness: harnessing the power of CRISPR/Cas9 genome editing." Mol Ecol **24**(15): 3810-3822.

One of the fundamental goals in evolution and ecology is to identify the genetic basis of adaptive phenotypes. Unfortunately, progress towards this goal has been hampered by a lack of genetic tools available for nonmodel organisms. The exciting new development of the CRISPR (clustered regularly interspaced short palindromic repeat)/Cas9 (CRISPR-associated nuclease 9) genome-editing system now promises to transform the field of molecular ecology by providing a versatile toolkit for manipulating the genome of a wide variety of organisms. Here, we review the numerous applications of this groundbreaking technology and provide a practical guide to the creation of genetic knockouts, transgenics and other related forms of gene manipulation in nonmodel organisms. We also specifically discuss the potential uses of the CRISPR/Cas9 system in ecological and evolutionary studies, which will further advance the field towards the long-standing goal of connecting genotypes, phenotypes and fitness.

Bortesi, L. and R. Fischer (2015). "The CRISPR/Cas9 system for plant genome editing and beyond." Biotechnol Adv **33**(1): 41-52.

Targeted genome editing using artificial nucleases has the potential to accelerate basic research as well as plant breeding by providing the means to modify genomes rapidly in a precise and predictable manner. Here we describe the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) system, a recently developed tool for the introduction of site-specific double-stranded DNA breaks. We highlight the strengths and weaknesses of this technology compared with two well-established genome editing platforms: zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). We summarize recent results obtained in plants using CRISPR/Cas9 technology,

discuss possible applications in plant breeding and consider potential future developments.

Bruder, M. R., et al. (2016). "Extending CRISPR-Cas9 Technology from Genome Editing to Transcriptional Engineering in the Genus Clostridium." Appl Environ Microbiol **82**(20): 6109-6119.

The discovery and exploitation of the prokaryotic adaptive immunity system based on clustered regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated (Cas) proteins have revolutionized genetic engineering. CRISPR-Cas tools have enabled extensive genome editing as well as efficient modulation of the transcriptional program in a multitude of organisms. Progress in the development of genetic engineering tools for the genus Clostridium has lagged behind that of many other prokaryotes, presenting the CRISPR-Cas technology an opportunity to resolve a long-existing issue. Here, we applied the Streptococcus pyogenes type II CRISPR-Cas9 (SpCRISPR-Cas9) system for genome editing in Clostridium acetobutylicum DSM792. We further explored the utility of the SpCRISPR-Cas9 machinery for gene-specific transcriptional repression. For proof-of-concept demonstration, a plasmid-encoded fluorescent protein gene was used for transcriptional repression in C. acetobutylicum Subsequently, we targeted the carbon catabolite repression (CCR) system of C. acetobutylicum through transcriptional repression of the hprK gene encoding HPr kinase/phosphorylase, leading to the coutilization of glucose and xylose, which are two abundant carbon sources from lignocellulosic feedstocks. Similar approaches based on SpCRISPR-Cas9 for genome editing and transcriptional repression were also demonstrated in Clostridium pasteurianum ATCC 6013. As such, this work lays a foundation for the derivation of clostridial strains for industrial purposes. IMPORTANCE: After recognizing the industrial potential of Clostridium for decades, methods for the genetic manipulation of these anaerobic bacteria are still underdeveloped. This study reports the implementation of CRISPR-Cas technology for genome editing and transcriptional regulation in Clostridium acetobutylicum, which is arguably the most common industrial clostridial strain. The developed genetic tools enable simpler, more reliable, and more extensive derivation of C. acetobutylicum mutant strains for industrial purposes. Similar approaches were also demonstrated in Clostridium pasteurianum, another clostridial strain that is capable of utilizing glycerol as the carbon source for butanol fermentation, and therefore can be arguably applied in other clostridial strains.

Bryant, J. M., et al. (2017). "CRISPR/Cas9 Genome Editing Reveals That the Intron Is Not Essential for var2csa Gene Activation or Silencing in Plasmodium falciparum." MBio **8**(4).

Plasmodium falciparum relies on monoallelic expression of 1 of 60 var virulence genes for antigenic variation and host immune evasion. Each var gene contains a conserved intron which has been implicated in previous studies in both activation and repression of transcription via several epigenetic mechanisms, including interaction with the var promoter, production of long noncoding RNAs (lncRNAs), and localization to repressive perinuclear sites. However, functional studies have relied primarily on artificial expression constructs. Using the recently developed P. falciparum clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system, we directly deleted the var2csa P. falciparum 3D7_1200600 (Pf3D7_1200600) endogenous intron, resulting in an intronless var gene in a natural, marker-free chromosomal context. Deletion of the var2csa intron resulted in an upregulation of transcription of the var2csa gene in ring-stage parasites and subsequent expression of the PfEMP1 protein in late-stage parasites. Intron deletion did not affect the normal temporal regulation and subsequent transcriptional silencing of the var gene in trophozoites but did result in increased rates of var gene switching in some mutant clones. Transcriptional repression of the intronless var2csa gene could be achieved via long-term culture or panning with the CD36 receptor, after which reactivation was possible with chondroitin sulfate A (CSA) panning. These data suggest that the var2csa intron is not required for silencing or activation in ring-stage parasites but point to a subtle role in regulation of switching within the var gene family. IMPORTANCE Plasmodium falciparum is the most virulent species of malaria parasite, causing high rates of morbidity and mortality in those infected. Chronic infection depends on an immune evasion mechanism termed antigenic variation, which in turn relies on monoallelic expression of 1 of ~60 var genes. Understanding antigenic variation and the transcriptional regulation of monoallelic expression is important for developing drugs and/or vaccines. The var gene family encodes the antigenic surface proteins that decorate infected erythrocytes. Until recently, studying the underlying genetic elements that regulate monoallelic expression in P. falciparum was difficult, and most studies relied on artificial systems such as episomal reporter genes. Our study was the first to use CRISPR/Cas9 genome editing for the functional study of an important, conserved genetic element of var genes-the intron-in an endogenous, episome-free manner. Our findings shed light on the role of the var

gene intron in transcriptional regulation of monoallelic expression.

Burby, P. E. and L. A. Simmons (2017). "CRISPR/Cas9 Editing of the Bacillus subtilis Genome." Bio Protoc **7**(8).

A fundamental procedure for most modern biologists is the genetic manipulation of the organism under study. Although many different methods for editing bacterial genomes have been used in laboratories for decades, the adaptation of CRISPR/Cas9 technology to bacterial genetics has allowed researchers to manipulate bacterial genomes with unparalleled facility. CRISPR/Cas9 has allowed for genome edits to be more precise, while also increasing the efficiency of transferring mutations into a variety of genetic backgrounds. As a result, the advantages are realized in tractable organisms and organisms that have been refractory to genetic manipulation. Here, we describe our method for editing the genome of the bacterium Bacillus subtilis. Our method is highly efficient, resulting in precise, markerless mutations. Further, after generating the editing plasmid, the mutation can be quickly introduced into several genetic backgrounds, greatly increasing the speed with which genetic analyses may be performed.

Butt, H., et al. (2017). "Efficient CRISPR/Cas9-Mediated Genome Editing Using a Chimeric Single-Guide RNA Molecule." Front Plant Sci **8**: 1441.

The CRISPR/Cas9 system has been applied in diverse eukaryotic organisms for targeted mutagenesis. However, targeted gene editing is inefficient and requires the simultaneous delivery of a DNA template for homology-directed repair (HDR). Here, we used CRISPR/Cas9 to generate targeted double-strand breaks and to deliver an RNA repair template for HDR in rice (Oryza sativa). We used chimeric single-guide RNA (cgRNA) molecules carrying both sequences for target site specificity (to generate the double-strand breaks) and repair template sequences (to direct HDR), flanked by regions of homology to the target. Gene editing was more efficient in rice protoplasts using repair templates complementary to the non-target DNA strand, rather than the target strand. We applied this cgRNA repair method to generate herbicide resistance in rice, which showed that this cgRNA repair method can be used for targeted gene editing in plants. Our findings will facilitate applications in functional genomics and targeted improvement of crop traits.

Cai, Y., et al. (2015). "CRISPR/Cas9-Mediated Genome Editing in Soybean Hairy Roots." PLoS One **10**(8): e0136064.

As a new technology for gene editing, the CRISPR (clustered regularly interspaced short palindromic repeat)/Cas (CRISPR-associated) system has been rapidly and widely used for genome engineering in various organisms. In the present study, we successfully applied type II CRISPR/Cas9 system to generate and estimate genome editing in the desired target genes in soybean (Glycine max (L.) Merrill.). The single-guide RNA (sgRNA) and Cas9 cassettes were assembled on one vector to improve transformation efficiency, and we designed a sgRNA that targeted a transgene (bar) and six sgRNAs that targeted different sites of two endogenous soybean genes (GmFEI2 and GmSHR). The targeted DNA mutations were detected in soybean hairy roots. The results demonstrated that this customized CRISPR/Cas9 system shared the same efficiency for both endogenous and exogenous genes in soybean hairy roots. We also performed experiments to detect the potential of CRISPR/Cas9 system to simultaneously edit two endogenous soybean genes using only one customized sgRNA. Overall, generating and detecting the CRISPR/Cas9-mediated genome modifications in target genes of soybean hairy roots could rapidly assess the efficiency of each target loci. The target sites with higher efficiencies can be used for regular soybean transformation. Furthermore, this method provides a powerful tool for root-specific functional genomics studies in soybean.

Canny, M. D., et al. (2018). "Inhibition of 53BP1 favors homology-dependent DNA repair and increases CRISPR-Cas9 genome-editing efficiency." Nat Biotechnol **36**(1): 95-102.

Programmable nucleases, such as Cas9, are used for precise genome editing by homology-dependent repair (HDR). However, HDR efficiency is constrained by competition from other double-strand break (DSB) repair pathways, including non-homologous end-joining (NHEJ). We report the discovery of a genetically encoded inhibitor of 53BP1 that increases the efficiency of HDR-dependent genome editing in human and mouse cells. 53BP1 is a key regulator of DSB repair pathway choice in eukaryotic cells and functions to favor NHEJ over HDR by suppressing end resection, which is the rate-limiting step in the initiation of HDR. We screened an existing combinatorial library of engineered ubiquitin variants for inhibitors of 53BP1. Expression of one variant, named i53 (inhibitor of 53BP1), in human and mouse cells, blocked accumulation of 53BP1 at sites of DNA damage and improved gene targeting and chromosomal gene conversion with either double-stranded DNA or single-stranded oligonucleotide donors by up to 5.6-fold. Inhibition of 53BP1 is a

robust method to increase efficiency of HDR-based precise genome editing.

Ceasar, S. A., et al. (2016). "Insert, remove or replace: A highly advanced genome editing system using CRISPR/Cas9." Biochim Biophys Acta **1863**(9): 2333-2344.

The clustered, regularly interspaced, short palindromic repeat (CRISPR) and CRISPR associated protein 9 (Cas9) system discovered as an adaptive immunity mechanism in prokaryotes has emerged as the most popular tool for the precise alterations of the genomes of diverse species. CRISPR/Cas9 system has taken the world of genome editing by storm in recent years. Its popularity as a tool for altering genomes is due to the ability of Cas9 protein to cause double-stranded breaks in DNA after binding with short guide RNA molecules, which can be produced with dramatically less effort and expense than required for production of transcription-activator like effector nucleases (TALEN) and zinc-finger nucleases (ZFN). This system has been exploited in many species from prokaryotes to higher animals including human cells as evidenced by the literature showing increasing sophistication and ease of CRISPR/Cas9 as well as increasing species variety where it is applicable. This technology is poised to solve several complex molecular biology problems faced in life science research including cancer research. In this review, we highlight the recent advancements in CRISPR/Cas9 system in editing genomes of prokaryotes, fungi, plants and animals and provide details on software tools available for convenient design of CRISPR/Cas9 targeting plasmids. We also discuss the future prospects of this advanced molecular technology.

Chadwick, A. C. and K. Musunuru (2017). "Treatment of Dyslipidemia Using CRISPR/Cas9 Genome Editing." Curr Atheroscler Rep **19**(7): 32.

PURPOSE OF REVIEW: Clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated 9 (Cas9) has recently emerged as a top genome editing technology and has afforded investigators the ability to more easily study a number of diseases. This review discusses CRISPR/Cas9's advantages and limitations and highlights a few recent reports on genome editing applications for alleviating dyslipidemia through disruption of proprotein convertase subtilisin/kexin type 9 (PCSK9). RECENT FINDINGS: Targeting of mouse Pcsk9 using CRISPR/Cas9 technology has yielded promising results for lowering total cholesterol levels, and several recent findings are highlighted in this review. Reported on-target mutagenesis efficiency is as high as 90% with a subsequent 40% reduction of blood cholesterol levels in mice, highlighting the potential for use as a therapeutic in human patients. The ability to characterize and treat diseases is becoming easier with the recent advances in genome editing technologies. In this review, we discuss how genome editing strategies can be of use for potential therapeutic applications.

Chadwick, A. C. and K. Musunuru (2018). "CRISPR-Cas9 Genome Editing for Treatment of Atherogenic Dyslipidemia." Arterioscler Thromb Vasc Biol **38**(1): 12-18.

Although human genetics has resulted in the identification of novel lipid-related genes that can be targeted for the prevention of atherosclerotic vascular disease, medications targeting these genes or their protein products have short-term effects and require frequent administration during the course of the lifetime for maximal benefit. Genome-editing technologies, such as CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats-CRISPR-associated 9) have the potential to permanently alter genes in the body and produce long-term and even lifelong protection against atherosclerosis. In this review, we discuss recent advances in genome-editing technologies and early proof-of-concept studies of somatic in vivo genome editing in mice that highlight the potential of genome editing to target disease-related genes in patients, which would establish a novel therapeutic paradigm for atherosclerosis.

Chen, J., et al. (2017). "CRISPR/Cas9-mediated efficient genome editing via blastospore-based transformation in entomopathogenic fungus Beauveria bassiana." Sci Rep **8**: 45763.

Beauveria bassiana is an environmentally friendly alternative to chemical insecticides against various agricultural insect pests and vectors of human diseases. However, its application has been limited due to slow kill and sensitivity to abiotic stresses. Understanding of the molecular pathogenesis and physiological characteristics would facilitate improvement of the fungal performance. Loss-of-function mutagenesis is the most powerful tool to characterize gene functions, but it is hampered by the low rate of homologous recombination and the limited availability of selectable markers. Here, by combining the use of uridine auxotrophy as recipient and donor DNAs harboring auxotrophic complementation gene ura5 as a selectable marker with the blastospore-based transformation system, we established a highly efficient, low false-positive background and cost-effective CRISPR/Cas9-mediated gene editing system in B. bassiana. This system has been demonstrated as a simple and powerful tool for targeted gene knock-out and/or knock-in in B. bassiana in a single gene

disruption. We further demonstrated that our system allows simultaneous disruption of multiple genes via homology-directed repair in a single transformation. This technology will allow us to study functionally redundant genes and holds significant potential to greatly accelerate functional genomics studies of B. bassiana.

Chen, S., et al. (2016). "CRISPR-Cas9: from Genome Editing to Cancer Research." Int J Biol Sci **12**(12): 1427-1436.

Cancer development is a multistep process triggered by innate and acquired mutations, which cause the functional abnormality and determine the initiation and progression of tumorigenesis. Gene editing is a widely used engineering tool for generating mutations that enhance tumorigenesis. The recent developed clustered regularly interspaced short palindromic repeats-CRISPR-associated 9 (CRISPR-Cas9) system renews the genome editing approach into a more convenient and efficient way. By rapidly introducing genetic modifications in cell lines, organs and animals, CRISPR-Cas9 system extends the gene editing into whole genome screening, both in loss-of-function and gain-of-function manners. Meanwhile, the system accelerates the establishment of animal cancer models, promoting in vivo studies for cancer research. Furthermore, CRISPR-Cas9 system is modified into diverse innovative tools for observing the dynamic bioprocesses in cancer studies, such as image tracing for targeted DNA, regulation of transcription activation or repression. Here, we view recent technical advances in the application of CRISPR-Cas9 system in cancer genetics, large-scale cancer driver gene hunting, animal cancer modeling and functional studies.

Chen, W., et al. (2017). "Rapid and Efficient Genome Editing in Staphylococcus aureus by Using an Engineered CRISPR/Cas9 System." J Am Chem Soc **139**(10): 3790-3795.

Staphylococcus aureus, a major human pathogen, has been the cause of serious infectious diseases with a high mortality rate. Although genetics is a key means to study S. aureus physiology, such as drug resistance and pathogenesis, genetic manipulation in S. aureus is always time-consuming and labor-intensive. Here we report a CRISPR/Cas9 system (pCasSA) for rapid and efficient genome editing, including gene deletion, insertion, and single-base substitution mutation in S. aureus. The designed pCasSA system is amenable to the assembly of spacers and repair arms by Golden Gate assembly and Gibson assembly, respectively, enabling rapid construction of the plasmids for editing. We further engineered the pCasSA system to be an efficient transcription inhibition system for gene knockdown and possible genome-wide screening. The development of the CRISPR/Cas9-mediated genome editing and transcription inhibition tools will dramatically accelerate drug-target exploration and drug development.

Chiang, T. W., et al. (2016). "CRISPR-Cas9(D10A) nickase-based genotypic and phenotypic screening to enhance genome editing." Sci Rep **6**: 24356.

The RNA-guided Cas9 nuclease is being widely employed to engineer the genomes of various cells and organisms. Despite the efficient mutagenesis induced by Cas9, off-target effects have raised concerns over the system's specificity. Recently a "double-nicking" strategy using catalytic mutant Cas9(D10A) nickase has been developed to minimise off-target effects. Here, we describe a Cas9(D10A)-based screening approach that combines an All-in-One Cas9(D10A) nickase vector with fluorescence-activated cell sorting enrichment followed by high-throughput genotypic and phenotypic clonal screening strategies to generate isogenic knockouts and knock-ins highly efficiently, with minimal off-target effects. We validated this approach by targeting genes for the DNA-damage response (DDR) proteins MDC1, 53BP1, RIF1 and P53, plus the nuclear architecture proteins Lamin A/C, in three different human cell lines. We also efficiently obtained biallelic knock-in clones, using single-stranded oligodeoxynucleotides as homologous templates, for insertion of an EcoRI recognition site at the RIF1 locus and introduction of a point mutation at the histone H2AFX locus to abolish assembly of DDR factors at sites of DNA double-strand breaks. This versatile screening approach should facilitate research aimed at defining gene functions, modelling of cancers and other diseases underpinned by genetic factors, and exploring new therapeutic opportunities.

Chiou, S. H., et al. (2015). "Pancreatic cancer modeling using retrograde viral vector delivery and in vivo CRISPR/Cas9-mediated somatic genome editing." Genes Dev **29**(14): 1576-1585.

Pancreatic ductal adenocarcinoma (PDAC) is a genomically diverse, prevalent, and almost invariably fatal malignancy. Although conventional genetically engineered mouse models of human PDAC have been instrumental in understanding pancreatic cancer development, these models are much too labor-intensive, expensive, and slow to perform the extensive molecular analyses needed to adequately understand this disease. Here we demonstrate that retrograde pancreatic ductal injection of either adenoviral-Cre or lentiviral-Cre vectors allows titratable initiation of pancreatic neoplasias that progress into invasive and metastatic PDAC. To

enable in vivo CRISPR/Cas9-mediated gene inactivation in the pancreas, we generated a Cre-regulated Cas9 allele and lentiviral vectors that express Cre and a single-guide RNA. CRISPR-mediated targeting of Lkb1 in combination with oncogenic Kras expression led to selection for inactivating genomic alterations, absence of Lkb1 protein, and rapid tumor growth that phenocopied Cre-mediated genetic deletion of Lkb1. This method will transform our ability to rapidly interrogate gene function during the development of this recalcitrant cancer.

Chira, S., et al. (2017). "CRISPR/Cas9: Transcending the Reality of Genome Editing." Mol Ther Nucleic Acids **7**: 211-222.

With the expansion of the microbiology field of research, a new genome editing tool arises from the biology of bacteria that holds the promise of achieving precise modifications in the genome with a simplicity and versatility that surpasses previous genome editing methods. This new technique, commonly named CRISPR/Cas9, led to a rapid expansion of the biomedical field; more specifically, cancer characterization and modeling have benefitted greatly from the genome editing capabilities of CRISPR/Cas9. In this paper, we briefly summarize recent improvements in CRISPR/Cas9 design meant to overcome the limitations that have arisen from the nuclease activity of Cas9 and the influence of this technology in cancer research. In addition, we present challenges that might impede the clinical applicability of CRISPR/Cas9 for cancer therapy and highlight future directions for designing CRISPR/Cas9 delivery systems that might prove useful for cancer therapeutics.

Chou, Y. Y., et al. (2016). "Inhibition of JCPyV infection mediated by targeted viral genome editing using CRISPR/Cas9." Sci Rep **6**: 36921.

Progressive multifocal leukoencephalopathy (PML) is a debilitating disease resulting from infection of oligodendrocytes by the JC polyomavirus (JCPyV). Currently, there is no anti-viral therapeutic available against JCPyV infection. The clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) system (CRISPR/Cas9) is a genome editing tool capable of introducing sequence specific breaks in double stranded DNA. Here we show that the CRISPR/Cas9 system can restrict the JCPyV life cycle in cultured cells. We utilized CRISPR/Cas9 to target the noncoding control region and the late gene open reading frame of the JCPyV genome. We found significant inhibition of virus replication and viral protein expression in cells recipient of Cas9 together with JCPyV-specific single-guide RNA delivered prior to or after JCPyV infection.

Chung, W. Y., et al. (2016). "Generation of DeltaF508-CFTR T84 cell lines by CRISPR/Cas9-mediated genome editing." Biotechnol Lett **38**(12): 2023-2034.

OBJECTIVES: To provide a simple method to make a stable DeltaF508-CFTR-expressing T84 cell line that can be used as an efficient screening model system for DeltaF508-CFTR rescue. RESULTS: CFTR knockout cell lines were generated by Cas9 with a single-guide RNA (sgRNA) targeting exon 1 of the CFTR genome, which produced indels that abolished CFTR protein expressions. Next, stable DeltaF508-CFTR expression was achieved by genome integration of DeltaF508-CFTR via the lentivirus infection system. Finally, we showed functional rescue of DeltaF508-CFTR not only by growing the cells at a low temperature, but also incubating with VX-809, a DeltaF508-CFTR corrector, in the established T84 cells expressing DeltaF508-CFTR. CONCLUSIONS: This cell system provides an appropriate screening platform for rescue of DeltaF508-CFTR, especially related to protein folding, escaped from endoplasmic-reticulum-associated protein degradation, and membrane transport.

Cottle, R. N., et al. (2015). "Controlled delivery of beta-globin-targeting TALENs and CRISPR/Cas9 into mammalian cells for genome editing using microinjection." Sci Rep **5**: 16031.

Tal-effector nucleases (TALEN) and clustered regularly interspaced short palindromic repeats (CRISPR) with CRISPR-associated (Cas) proteins are genome editing tools with unprecedented potential. However, the ability to deliver optimal amounts of these nucleases into mammalian cells with minimal toxicity poses a major challenge. Common delivery approaches are transfection- and viral-based methods; each associated with significant drawbacks. An alternative method for directly delivering genome-editing reagents into single living cells with high efficiency and controlled volume is microinjection. Here, we characterize a glass microcapillary-based injection system and demonstrate controlled co-injection of TALENs or CRISPR/Cas9 together with donor template into single K562 cells for targeting the human beta-globin gene. We quantified nuclease induced insertions and deletions (indels) and found that, with beta-globin-targeting TALENs, similar levels of on- and off-target activity in cells could be achieved by microinjection compared with nucleofection. Furthermore, we observed 11% and 2% homology directed repair in single K562 cells co-injected with a donor template along with

CRISPR/Cas9 and TALENs respectively. These results demonstrate that a high level of targeted gene modification can be achieved in human cells using glass-needle microinjection of genome editing reagents.

Cuculis, L. and C. M. Schroeder (2017). "A Single-Molecule View of Genome Editing Proteins: Biophysical Mechanisms for TALEs and CRISPR/Cas9." Annu Rev Chem Biomol Eng **8**: 577-597.

Exciting new advances in genome engineering have unlocked the potential to radically alter the treatment of human disease. In this review, we discuss the application of single-molecule techniques to uncover the mechanisms behind two premier classes of genome editing proteins: transcription activator-like effector nucleases (TALENs) and the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated system (Cas). These technologies have facilitated a striking number of gene editing applications in a variety of organisms; however, we are only beginning to understand the molecular mechanisms governing the DNA editing properties of these systems. Here, we discuss the DNA search and recognition process for TALEs and Cas9 that have been revealed by recent single-molecule experiments.

D'Agostino, Y. and S. D'Aniello (2017). "Molecular basis, applications and challenges of CRISPR/Cas9: a continuously evolving tool for genome editing." Brief Funct Genomics **16**(4): 211-216.

The clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) system is a recently discovered tool for genome editing that has quickly revolutionized the ability to generate site-specific mutations in a wide range of animal models, including nonhuman primates. Indeed, a significant number of scientific reports describing single or multiplex guide RNA microinjection, double-nicking strategies, site-specific knock-in and conditional knock-out have been published in less than three years. However, despite the great potential of this new technology, there are some limitations because of the presence of off-target genomic sites, which must be taken into consideration. To address this issue, various research teams have tried to improve the efficiency of the system through enzymatic modifications of the Cas9 protein or by the introduction of alternative strategies. Although several review articles are available that singly describe the molecular mechanism (s), applications and challenges of each of these strategies, a concise compilation of approaches is lacking. In the current review, we describe and evaluate most CRISPR/Cas9 approaches available at present, describing both mechanism of action, in addition to advantages or disadvantages. The primary goal of this work is to serve as a guide for not skilled researchers, facilitating the selection of the best strategy to target their gene of interest and allowing optimization of particular applications to the specific aims of the study. The present article also offers a unique perspective, focusing on the fact that CRISPR technology is opening a new genomic era, providing the means to manipulate specific genes in a targeted manner in all animal models, an endeavor previously considered to be difficult.

Dehler, C. E., et al. (2016). "Development of an Efficient Genome Editing Method by CRISPR/Cas9 in a Fish Cell Line." Mar Biotechnol (NY) **18**(4): 449-452.

CRISPR/Cas9 system has been used widely in animals and plants to direct mutagenesis. To date, no such method exists for fish somatic cell lines. We describe an efficient procedure for genome editing in the Chinook salmon Oncorhynchus tshawytscha CHSE. This cell line was genetically modified to firstly overexpress a monomeric form of EGFP (cell line CHSE-E Geneticin resistant) and additionally to overexpress nCas9n, a nuclear version of Cas9 (cell line CHSE-EC, Hygromycin and Geneticin resistant). A pre-validated sgRNA was produced in vitro and used to transfect CHSE-EC cells. The EGFP gene was disrupted in 34.6 % of cells, as estimated by FACS and microscopy. The targeted locus was characterised by PCR amplification, cloning and sequencing of PCR products; inactivation of the EGFP gene by deletions in the expected site was validated in 25 % of clones. This method opens perspectives for functional genomic studies compatible with high-throughput screening.

Demirci, Y., et al. (2018). "CRISPR/Cas9: An RNA-guided highly precise synthetic tool for plant genome editing." J Cell Physiol **233**(3): 1844-1859.

CRISPR/Cas9 is a newly developed and naturally occurred genome editing tool, which is originally used by bacteria for immune defence. In the past years, it has been quickly employed and modified to precisely edit genome sequences in both plants and animals. Compared with the well-developed zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), CRISPR/Cas9 has lots of advantages, including easier to design and implement, higher targeting efficiency, and less expensive. Thus, it is becoming one of the most powerful tools for knockout of an individual gene as well as insertion of one gene and/or control of gene transcription. Studies have shown that CRISPR/Cas9

is a great tool to edit many genes in a variety of plant species, including the model plant species as well as agriculturally important crops, such as cotton, maize, wheat, and rice. CRISPR/Cas9-based genome editing can be used for plant functional studies and plant improvement to yield, quality, and tolerance to environmental stress.

Ding, Q., et al. (2014). "Permanent alteration of PCSK9 with in vivo CRISPR-Cas9 genome editing." Circ Res **115**(5): 488-492.

RATIONALE: Individuals with naturally occurring loss-of-function proprotein convertase subtilisin/kexin type 9 (PCSK9) mutations experience reduced low-density lipoprotein cholesterol levels and protection against cardiovascular disease. OBJECTIVE: The goal of this study was to assess whether genome editing using a clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated system can efficiently introduce loss-of-function mutations into the endogenous PCSK9 gene in vivo. METHODS AND RESULTS: We used adenovirus to express CRISPR-associated 9 and a CRISPR guide RNA targeting Pcsk9 in mouse liver, where the gene is specifically expressed. We found that <3 to 4 days of administration of the virus, the mutagenesis rate of Pcsk9 in the liver was as high as >50%. This resulted in decreased plasma PCSK9 levels, increased hepatic low-density lipoprotein receptor levels, and decreased plasma cholesterol levels (by 35-40%). No off-target mutagenesis was detected in 10 selected sites. CONCLUSIONS: Genome editing with the CRISPR-CRISPR-associated 9 system disrupts the Pcsk9 gene in vivo with high efficiency and reduces blood cholesterol levels in mice. This approach may have therapeutic potential for the prevention of cardiovascular disease in humans.

Ding, Y., et al. (2016). "Recent Advances in Genome Editing Using CRISPR/Cas9." Front Plant Sci **7**: 703.

The CRISPR (clustered regularly interspaced short palindromic repeat)-Cas9 (CRISPR-associated nuclease 9) system is a versatile tool for genome engineering that uses a guide RNA (gRNA) to target Cas9 to a specific sequence. This simple RNA-guided genome-editing technology has become a revolutionary tool in biology and has many innovative applications in different fields. In this review, we briefly introduce the Cas9-mediated genome-editing method, summarize the recent advances in CRISPR/Cas9 technology, and discuss their implications for plant research. To date, targeted gene knockout using the Cas9/gRNA system has been established in many plant species, and the targeting efficiency and capacity of Cas9 has been improved by optimizing its expression and that of its gRNA. The CRISPR/Cas9 system can also be used for sequence-specific mutagenesis/integration and transcriptional control of target genes. We also discuss off-target effects and the constraint that the protospacer-adjacent motif (PAM) puts on CRISPR/Cas9 genome engineering. To address these problems, a number of bioinformatic tools are available to help design specific gRNAs, and new Cas9 variants and orthologs with high fidelity and alternative PAM specificities have been engineered. Owing to these recent efforts, the CRISPR/Cas9 system is becoming a revolutionary and flexible tool for genome engineering. Adoption of the CRISPR/Cas9 technology in plant research would enable the investigation of plant biology at an unprecedented depth and create innovative applications in precise crop breeding.

Dominguez, A. A., et al. (2016). "Beyond editing: repurposing CRISPR-Cas9 for precision genome regulation and interrogation." Nat Rev Mol Cell Biol **17**(1): 5-15.

The bacterial CRISPR-Cas9 system has emerged as a multifunctional platform for sequence-specific regulation of gene expression. This Review describes the development of technologies based on nuclease-deactivated Cas9, termed dCas9, for RNA-guided genomic transcription regulation, both by repression through CRISPR interference (CRISPRi) and by activation through CRISPR activation (CRISPRa). We highlight different uses in diverse organisms, including bacterial and eukaryotic cells, and summarize current applications of harnessing CRISPR-dCas9 for multiplexed, inducible gene regulation, genome-wide screens and cell fate engineering. We also provide a perspective on future developments of the technology and its applications in biomedical research and clinical studies.

Dong, S., et al. (2015). "Heritable CRISPR/Cas9-mediated genome editing in the yellow fever mosquito, Aedes aegypti." PLoS One **10**(3): e0122353.

In vivo targeted gene disruption is a powerful tool to study gene function. Thus far, two tools for genome editing in Aedes aegypti have been applied, zinc-finger nucleases (ZFN) and transcription activator-like effector nucleases (TALEN). As a promising alternative to ZFN and TALEN, which are difficult to produce and validate using standard molecular biological techniques, the clustered regularly interspaced short palindromic repeats/CRISPR-associated sequence 9 (CRISPR/Cas9) system has recently been discovered as a "do-it-yourself" genome editing tool. Here, we

describe the use of CRISPR/Cas9 in the mosquito vector, Aedes aegypti. In a transgenic mosquito line expressing both Dsred and enhanced cyan fluorescent protein (ECFP) from the eye tissue-specific 3xP3 promoter in separated but tightly linked expression cassettes, we targeted the ECFP nucleotide sequence for disruption. When supplying the Cas9 enzyme and two sgRNAs targeting different regions of the ECFP gene as in vitro transcribed mRNAs for germline transformation, we recovered four different G1 pools (5.5% knockout efficiency) where individuals still expressed DsRed but no longer ECFP. PCR amplification, cloning, and sequencing of PCR amplicons revealed indels in the ECFP target gene ranging from 2-27 nucleotides. These results show for the first time that CRISPR/Cas9 mediated gene editing is achievable in Ae. aegypti, paving the way for further functional genomics related studies in this mosquito species.

Doudna, J. A. and E. Charpentier (2014). "Genome editing. The new frontier of genome engineering with CRISPR-Cas9." Science **346**(6213): 1258096.

The advent of facile genome engineering using the bacterial RNA-guided CRISPR-Cas9 system in animals and plants is transforming biology. We review the history of CRISPR (clustered regularly interspaced palindromic repeat) biology from its initial discovery through the elucidation of the CRISPR-Cas9 enzyme mechanism, which has set the stage for remarkable developments using this technology to modify, regulate, or mark genomic loci in a wide variety of cells and organisms from all three domains of life. These results highlight a new era in which genomic manipulation is no longer a bottleneck to experiments, paving the way toward fundamental discoveries in biology, with applications in all branches of biotechnology, as well as strategies for human therapeutics.

Dow, L. E., et al. (2015). "Inducible in vivo genome editing with CRISPR-Cas9." Nat Biotechnol **33**(4): 390-394.

CRISPR-Cas9-based genome editing enables the rapid genetic manipulation of any genomic locus without the need for gene targeting by homologous recombination. Here we describe a conditional transgenic approach that allows temporal control of CRISPR-Cas9 activity for inducible genome editing in adult mice. We show that doxycycline-regulated Cas9 induction enables widespread gene disruption in multiple tissues and that limiting the duration of Cas9 expression or using a Cas9(D10A) (Cas9n) variant can regulate the frequency and size of target gene modifications, respectively. Further, we show that this inducible CRISPR (iCRISPR) system can be used effectively to create biallelic mutation in multiple target loci and, thus, provides a flexible and fast platform to study loss-of-function phenotypes in vivo.

Duda, K., et al. (2014). "High-efficiency genome editing via 2A-coupled co-expression of fluorescent proteins and zinc finger nucleases or CRISPR/Cas9 nickase pairs." Nucleic Acids Res **42**(10): e84.

Targeted endonucleases including zinc finger nucleases (ZFNs) and clustered regularly interspaced short palindromic repeats (CRISPRs)/Cas9 are increasingly being used for genome editing in higher species. We therefore devised a broadly applicable and versatile method for increasing editing efficiencies by these tools. Briefly, 2A peptide-coupled co-expression of fluorescent protein and nuclease was combined with fluorescence-activated cell sorting (FACS) to allow for efficient isolation of cell populations with increasingly higher nuclease expression levels, which translated into increasingly higher genome editing rates. For ZFNs, this approach, combined with delivery of donors as single-stranded oligodeoxynucleotides and nucleases as messenger ribonucleic acid, enabled high knockin efficiencies in demanding applications, including biallelic codon conversion frequencies reaching 30-70% at high transfection efficiencies and approximately 2% at low transfection efficiencies, simultaneous homozygous knockin mutation of two genes with approximately 1.5% efficiency as well as generation of cell pools with almost complete codon conversion via three consecutive targeting and FACS events. Observed off-target effects were minimal, and when occurring, our data suggest that they may be counteracted by selecting intermediate nuclease levels where off-target mutagenesis is low, but on-target mutagenesis remains relatively high. The method was also applicable to the CRISPR/Cas9 system, including CRISPR/Cas9 mutant nickase pairs, which exhibit low off-target mutagenesis compared to wild-type Cas9.

Eid, A. and M. M. Mahfouz (2016). "Genome editing: the road of CRISPR/Cas9 from bench to clinic." Exp Mol Med **48**(10): e265.

Molecular scissors engineered for site-specific modification of the genome hold great promise for effective functional analyses of genes, genomes and epigenomes and could improve our understanding of the molecular underpinnings of disease states and facilitate novel therapeutic applications. Several platforms for molecular scissors that enable targeted genome engineering have been developed, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and, most recently, clustered regularly interspaced palindromic repeats (CRISPR)/CRISPR-associated-9 (Cas9). The

CRISPR/Cas9 system's simplicity, facile engineering and amenability to multiplexing make it the system of choice for many applications. CRISPR/Cas9 has been used to generate disease models to study genetic diseases. Improvements are urgently needed for various aspects of the CRISPR/Cas9 system, including the system's precision, delivery and control over the outcome of the repair process. Here, we discuss the current status of genome engineering and its implications for the future of biological research and gene therapy.

El-Sayed, A. S. A., et al. (2017). "Genome editing approaches: manipulating of lovastatin and taxol synthesis of filamentous fungi by CRISPR/Cas9 system." Appl Microbiol Biotechnol **101**(10): 3953-3976.

Filamentous fungi are prolific repertoire of structurally diverse secondary metabolites of remarkable biological activities such as lovastatin and paclitaxel that have been approved by FDA as drugs for hypercholesterolemia and cancer treatment. The clusters of genes encoding lovastatin and paclitaxel are cryptic at standard laboratory cultural conditions (Kennedy et al. Science 284:1368-1372, 1999; Bergmann et al. Nature Chem Biol 3:213-217, 2007). The expression of these genes might be triggered in response to nutritional and physical conditions; nevertheless, the overall yield of these metabolites does not match the global need. Consequently, overexpression of the downstream limiting enzymes and/or blocking the competing metabolic pathways of these metabolites could be the most successful technologies to enhance their yield. This is the first review summarizing the different strategies implemented for fungal genome editing, molecular regulatory mechanisms, and prospective of clustered regulatory interspaced short palindromic repeat/Cas9 system in metabolic engineering of fungi to improve their yield of lovastatin and taxol to industrial scale. Thus, elucidating the putative metabolic pathways in fungi for overproduction of lovastatin and taxol was the ultimate objective of this review.

Fang, Y., et al. (2017). "Efficient Genome Editing in the Oomycete Phytophthora sojae Using CRISPR/Cas9." Curr Protoc Microbiol **44**: 21A 21 21-21A 21 26.

Phytophthora is a filamentous fungus-like microorganism, but belongs to the oomycetes, in the kingdom Stramenopila. Phytophthora species are notorious as plant destroyers, causing multibillion-dollar damage to agriculture and natural ecosystems worldwide annually. For a long time, genome editing has been unattainable in oomycetes, because of their extremely low rate of homologous recombination. The recent implementation of the CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR-associated) system in the soybean pathogen Phytophthora sojae, an experimental model for oomycetes, has opened up a powerful new research capability for the oomycete community. Here, we describe a detailed protocol for CRISPR/Cas9-mediated genome editing in P. sojae, including single guide RNA (sgRNA) design and construction, efficient gene replacement, and mutant-screening strategies. This protocol should be generally applicable for most culturable oomycetes. We also describe an optimized transformation method that is useful for other Phytophthora spp. including P. capsici and P. parasitica. (c) 2017 by John Wiley & Sons, Inc.

Farasat, I. and H. M. Salis (2016). "A Biophysical Model of CRISPR/Cas9 Activity for Rational Design of Genome Editing and Gene Regulation." PLoS Comput Biol **12**(1): e1004724.

The ability to precisely modify genomes and regulate specific genes will greatly accelerate several medical and engineering applications. The CRISPR/Cas9 (Type II) system binds and cuts DNA using guide RNAs, though the variables that control its on-target and off-target activity remain poorly characterized. Here, we develop and parameterize a system-wide biophysical model of Cas9-based genome editing and gene regulation to predict how changing guide RNA sequences, DNA superhelical densities, Cas9 and crRNA expression levels, organisms and growth conditions, and experimental conditions collectively control the dynamics of dCas9-based binding and Cas9-based cleavage at all DNA sites with both canonical and non-canonical PAMs. We combine statistical thermodynamics and kinetics to model Cas9:crRNA complex formation, diffusion, site selection, reversible R-loop formation, and cleavage, using large amounts of structural, biochemical, expression, and next-generation sequencing data to determine kinetic parameters and develop free energy models. Our results identify DNA supercoiling as a novel mechanism controlling Cas9 binding. Using the model, we predict Cas9 off-target binding frequencies across the lambdaphage and human genomes, and explain why Cas9's off-target activity can be so high. With this improved understanding, we propose several rules for designing experiments for minimizing off-target activity. We also discuss the implications for engineering dCas9-based genetic circuits.

Farboud, B. (2017). "Targeted genome editing in Caenorhabditis elegans using CRISPR/Cas9." Wiley Interdiscip Rev Dev Biol **6**(6).

Utilization of programmable nucleases to generate DNA lesions at precise endogenous

sequences has transformed the ability to edit genomes from microbes to plants and animals. This is especially true in organisms that previously lacked the means to engineer precise genomic changes, like Caenorhabditis elegans. C. elegans is a 1 mm long free-living, nonparasitic, nematode worm, which is easily cultivated in a laboratory. Its detailed genetic map and relatively compact genome (~100 megabases) helped make it the first metazoan to have its entire genome sequenced. With detailed sequence information came development of numerous molecular tools to dissect gene function. Initially absent from this toolbox, however, were methods to make precise edits at chosen endogenous loci. Adapting site-specific nucleases for use in C. elegans, revolutionized studies of C. elegans biology. Zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and then CRISPR-associated protein 9 (Cas9) were used to target specific endogenous DNA sequences to make double-strand DNA breaks (DSBs). Precise changes could be engineered by providing repair templates targeting the DSB in trans. The ease of programming Cas9 to bind and cleave DNA sequences with few limitations has led to its widespread use in C. elegans research and sped the development of strategies to facilitate mutant recovery. Numerous innovative CRISPR/Cas9 methodologies are now primed for use in C. elegans. WIREs Dev Biol 2017, 6:e287. doi: 10.1002/wdev.287 For further resources related to this article, please visit the WIREs website.

Feng, Y., et al. (2016). "Expanding CRISPR/Cas9 Genome Editing Capacity in Zebrafish Using SaCas9." G3 (Bethesda) **6**(8): 2517-2521.

The type II CRISPR/Cas9 system has been used widely for genome editing in zebrafish. However, the requirement for the 5'-NGG-3' protospacer-adjacent motif (PAM) of Cas9 from Streptococcus pyogenes (SpCas9) limits its targeting sequences. Here, we report that a Cas9 ortholog from Staphylococcus aureus (SaCas9), and its KKH variant, successfully induced targeted mutagenesis with high frequency in zebrafish. Confirming previous findings, the SpCas9 variant, VQR, can also induce targeted mutations in zebrafish. Bioinformatics analysis of these new Cas targets suggests that the number of available target sites in the zebrafish genome can be greatly expanded. Collectively, the expanded target repertoire of Cas9 in zebrafish should further facilitate the utility of this organism for genetic studies of vertebrate biology.

Fernandez, R. and J. Berro (2016). "Use of a fluoride channel as a new selection marker for fission yeast plasmids and application to fast genome editing with CRISPR/Cas9." Yeast **33**(10): 549-557.

Fission yeast is a powerful model organism that has provided insights into important cellular processes thanks to the ease of its genome editing by homologous recombination. However, creation of strains with a large number of targeted mutations or containing plasmids has been challenging because only a very small number of selection markers is available in Schizosaccharomyces pombe. In this paper, we identify two fission yeast fluoride exporter channels (Fex1p and Fex2p) and describe the development of a new strategy using Fex1p as a selection marker for transformants in rich media supplemented with fluoride. To our knowledge this is the first positive selection marker identified in S. pombe that does not use auxotrophy or drug resistance and that can be used for plasmids transformation or genomic integration in rich media. We illustrate the application of our new marker by significantly accelerating the protocol for genome edition using CRISPR/Cas9 in S. pombe. Copyright (c) 2016 John Wiley & Sons, Ltd.

Ferreira, R., et al. (2018). "Multiplexed CRISPR/Cas9 Genome Editing and Gene Regulation Using Csy4 in Saccharomyces cerevisiae." ACS Synth Biol **7**(1): 10-15.

Clustered regularly interspaced short palindromic repeats (CRISPR) technology has greatly accelerated the field of strain engineering. However, insufficient efforts have been made toward developing robust multiplexing tools in Saccharomyces cerevisiae. Here, we exploit the RNA processing capacity of the bacterial endoribonuclease Csy4 from Pseudomonas aeruginosa, to generate multiple gRNAs from a single transcript for genome editing and gene interference applications in S. cerevisiae. In regards to genome editing, we performed a quadruple deletion of FAA1, FAA4, POX1 and TES1 reaching 96% efficiency out of 24 colonies tested. Then, we used this system to efficiently transcriptionally regulate the three genes, OLE1, HMG1 and ACS1. Thus, we demonstrate that multiplexed genome editing and gene regulation can be performed in a fast and effective manner using Csy4.

Fujihara, Y. and M. Ikawa (2014). "CRISPR/Cas9-based genome editing in mice by single plasmid injection." Methods Enzymol **546**: 319-336.

CRISPR/Cas-mediated genome modification has opened a new era for elucidating gene function. Gene knockout mice can be generated by injecting humanized Cas9 (hCas9) mRNA and guide RNA (sgRNA) into fertilized eggs. However, delivery of RNA instead of DNA to the fertilized oocyte requires extra preparation and extra care with storage. To

simplify the method of delivery, we injected the circular pX330 plasmids expressing both hCas9 and sgRNA and found that mutant mice were generated as efficiently as with RNA injection. Different from the linearized plasmid, the circular plasmid decreased the chance of integration into the host genome. We also developed the pCAG-EGxxFP reporter plasmid for evaluating the sgRNA activity by observing EGFP fluorescence in HEK293T cells. The combination of these techniques allowed us to develop a rapid, easy, and reproducible strategy for targeted mutagenesis in living mice. This chapter provides an experimental protocol for the design of sgRNAs, the construction of pX330-sgRNA and pCAG-EGxxFP-target plasmids, the validation of cleavage efficiency in vitro, and the generation of targeted gene mutant mice. These mice can be generated within a month.

Gandhi, S., et al. (2017). "Optimization of CRISPR/Cas9 genome editing for loss-of-function in the early chick embryo." Dev Biol **432**(1): 86-97.

The advent of CRISPR/Cas9 has made genome editing possible in virtually any organism, including those not previously amenable to genetic manipulations. Here, we present an optimization of CRISPR/Cas9 for application to early avian embryos with improved efficiency via a three-fold strategy. First, we employed Cas9 protein flanked with two nuclear localization signal sequences for improved nuclear localization. Second, we used a modified guide RNA (gRNA) scaffold that obviates premature termination of transcription and unstable Cas9-gRNA interactions. Third, we used a chick-specific U6 promoter that yields 4-fold higher gRNA expression than the previously utilized human U6. For rapid screening of gRNAs for in vivo applications, we also generated a chicken fibroblast cell line that constitutively expresses Cas9. As proof of principle, we performed electroporation-based loss-of-function studies in the early chick embryo to knock out Pax7 and Sox10, key transcription factors with known functions in neural crest development. The results show that CRISPR/Cas9-mediated deletion causes loss of their respective proteins and transcripts, as well as predicted downstream targets. Taken together, the results reveal the utility of this optimized CRISPR/Cas9 method for targeted gene knockout in chicken embryos in a manner that is reproducible, robust and specific.

Gao, S., et al. (2016). "Multiplex gene editing of the Yarrowia lipolytica genome using the CRISPR-Cas9 system." J Ind Microbiol Biotechnol **43**(8): 1085-1093.

Yarrowia lipolytica is categorized as a generally recognized as safe (GRAS) organism and is a heavily documented, unconventional yeast that has been widely incorporated into multiple industrial fields to produce valuable biochemicals. This study describes the construction of a CRISPR-Cas9 system for genome editing in Y. lipolytica using a single plasmid (pCAS1yl or pCAS2yl) to transport Cas9 and relevant guide RNA expression cassettes, with or without donor DNA, to target genes. Two Cas9 target genes, TRP1 and PEX10, were repaired by non-homologous end-joining (NHEJ) or homologous recombination, with maximal efficiencies in Y. lipolytica of 85.6 % for the wild-type strain and 94.1 % for the ku70/ku80 double-deficient strain, within 4 days. Simultaneous double and triple multigene editing was achieved with pCAS1yl by NHEJ, with efficiencies of 36.7 or 19.3 %, respectively, and the pCASyl system was successfully expanded to different Y. lipolytica breeding strains. This timesaving method will enable and improve synthetic biology, metabolic engineering and functional genomic studies of Y. lipolytica.

Gao, W., et al. (2017). "Genome Editing in Cotton with the CRISPR/Cas9 System." Front Plant Sci **8**: 1364.

Genome editing is an important tool for gene functional studies as well as crop improvement. The recent development of the CRISPR/Cas9 system using single guide RNA molecules (sgRNAs) to direct precise double strand breaks in the genome has the potential to revolutionize agriculture. Unfortunately, not all sgRNAs are equally efficient and it is difficult to predict their efficiency by bioinformatics. In crops such as cotton (Gossypium hirsutum L.), with labor-intensive and lengthy transformation procedures, it is essential to minimize the risk of using an ineffective sgRNA that could result in the production of transgenic plants without the desired CRISPR-induced mutations. In this study, we have developed a fast and efficient method to validate the functionality of sgRNAs in cotton using a transient expression system. We have used this method to validate target sites for three different genes GhPDS, GhCLA1, and GhEF1 and analyzed the nature of the CRISPR/Cas9-induced mutations. In our experiments, the most frequent type of mutations observed in cotton cotyledons were deletions (approximately 64%). We prove that the CRISPR/Cas9 system can effectively produce mutations in homeologous cotton genes, an important requisite in this allotetraploid crop. We also show that multiple gene targeting can be achieved in cotton with the simultaneous expression of several sgRNAs and have generated mutations in GhPDS and GhEF1 at two target sites. Additionally, we have used the CRISPR/Cas9 system to produce targeted gene fragment deletions in the GhPDS locus. Finally, we obtained transgenic cotton plants containing

CRISPR/Cas9-induced gene editing mutations in the GhCLA1 gene. The mutation efficiency was very high, with 80.6% of the transgenic lines containing mutations in the GhCLA1 target site resulting in an intense albino phenotype due to interference with chloroplast biogenesis.

Gao, X., et al. (2016). "An Effective Strategy for Reliably Isolating Heritable and Cas9-Free Arabidopsis Mutants Generated by CRISPR/Cas9-Mediated Genome Editing." Plant Physiol **171**(3): 1794-1800.

Mutations generated by CRISPR/Cas9 in Arabidopsis (Arabidopsis thaliana) are often somatic and are rarely heritable. Isolation of mutations in Cas9-free Arabidopsis plants can ensure the stable transmission of the identified mutations to next generations, but the process is laborious and inefficient. Here, we present a simple visual screen for Cas9-free T2 seeds, allowing us to quickly obtain Cas9-free Arabidopsis mutants in the T2 generation. To demonstrate this in principle, we targeted two sites in the AUXIN-BINDING PROTEIN1 (ABP1) gene, whose function as a membrane-associated auxin receptor has been challenged recently. We obtained many T1 plants with detectable mutations near the target sites, but only a small fraction of T1 plants yielded Cas9-free abp1 mutations in the T2 generation. Moreover, the mutations did not segregate in Mendelian fashion in the T2 generation. However, mutations identified in the Cas9-free T2 plants were stably transmitted to the T3 generation following Mendelian genetics. To further simplify the screening procedure, we simultaneously targeted two sites in ABP1 to generate large deletions, which can be easily identified by PCR. We successfully generated two abp1 alleles that contained 1,141- and 711-bp deletions in the ABP1 gene. All of the Cas9-free abp1 alleles we generated were stable and heritable. The method described here allows for effectively isolating Cas9-free heritable CRISPR mutants in Arabidopsis.

Gasiunas, G. and V. Siksnys (2013). "RNA-dependent DNA endonuclease Cas9 of the CRISPR system: Holy Grail of genome editing?" Trends Microbiol **21**(11): 562-567.

Tailor-made nucleases for precise genome modification, such as zinc finger or TALE nucleases, currently represent the state-of-the-art for genome editing. These nucleases combine a programmable protein module which guides the enzyme to the target site with a nuclease domain which cuts DNA at the addressed site. Reprogramming of these nucleases to cut genomes at specific locations requires major protein engineering efforts. RNA-guided DNA endonuclease Cas9 of the type II (clustered regularly interspaced short palindromic repeat) CRISPR-Cas system uses CRISPR RNA (crRNA) as a guide to locate the DNA target and the Cas9 protein to cut DNA. Easy programmability of the Cas9 endonuclease using customizable RNAs brings unprecedented flexibility and versatility for targeted genome modification. We highlight the potential of the Cas9 RNA-guided DNA endonuclease as a novel tool for genome surgery, and discuss possible constraints and future prospects.

Gee, P., et al. (2017). "Cellular Reprogramming, Genome Editing, and Alternative CRISPR Cas9 Technologies for Precise Gene Therapy of Duchenne Muscular Dystrophy." Stem Cells Int **2017**: 8765154.

In the past decade, the development of two innovative technologies, namely, induced pluripotent stem cells (iPSCs) and the CRISPR Cas9 system, has enabled researchers to model diseases derived from patient cells and precisely edit DNA sequences of interest, respectively. In particular, Duchenne muscular dystrophy (DMD) has been an exemplary monogenic disease model for combining these technologies to demonstrate that genome editing can correct genetic mutations in DMD patient-derived iPSCs. DMD is an X-linked genetic disorder caused by mutations that disrupt the open reading frame of the dystrophin gene, which plays a critical role in stabilizing muscle cells during contraction and relaxation. The CRISPR Cas9 system has been shown to be capable of targeting the dystrophin gene and rescuing its expression in in vitro patient-derived iPSCs and in vivo DMD mouse models. In this review, we highlight recent advances made using the CRISPR Cas9 system to correct genetic mutations and discuss how emerging CRISPR technologies and iPSCs in a combined platform can play a role in bringing a therapy for DMD closer to the clinic.

Giau, V. V., et al. (2018). "Genome-editing applications of CRISPR-Cas9 to promote in vitro studies of Alzheimer's disease." Clin Interv Aging **13**: 221-233.

Genetic variations play an important role in the clinical presentation and progression of Alzheimer's disease (AD), especially early-onset Alzheimer's disease. Hundreds of mutations have been reported with the majority resulting from alterations in beta-amyloid precursor protein (APP), presenilin 1 (PSEN1), or presenilin 2 (PSEN2) genes. The roles of these mutations in the pathogenesis of AD have been classically confirmed or refuted through functional studies, where the mutations are cloned, inserted into cell lines, and monitored for changes in various properties including cell survival, amyloid production, or Abeta42/40 ratio. However, these verification

studies tend to be expensive, time consuming, and inconsistent. Recently, the clustered regularly interspaced short palindromic repeats-CRISPR-associated protein 9 (CRISPR-Cas9) system was developed, which improves sequence-specific gene editing in cell lines, organs, and animals. CRISPR-Cas9 is a promising tool for the generation of models of human genetic diseases and could facilitate the establishment of new animal AD models and the observation of dynamic bioprocesses in AD. Here, we recapitulated the history of CRISPR technology, recent progress, and, especially, its potential applications in AD-related genetic, animal modeling, and functional studies.

Gokcezade, J., et al. (2014). "Efficient CRISPR/Cas9 plasmids for rapid and versatile genome editing in Drosophila." G3 (Bethesda) **4**(11): 2279-2282.

The CRISPR-associated RNA-guided nuclease Cas9 has emerged as a powerful tool for genome engineering in a variety of organisms. To achieve efficient gene targeting rates in Drosophila, current approaches require either injection of in vitro transcribed RNAs or injection into transgenic Cas9-expressing embryos. We report a simple and versatile alternative method for CRISPR-mediated genome editing in Drosophila using bicistronic Cas9/sgRNA expression vectors. Gene targeting with this single-plasmid injection approach is as efficient as in transgenic nanos-Cas9 embryos and allows the isolation of targeted knock-out and knock-in alleles by molecular screening within 2 months. Our strategy is independent of genetic background and does not require prior establishment of transgenic flies.

Gonzalez, F. (2016). "CRISPR/Cas9 genome editing in human pluripotent stem cells: Harnessing human genetics in a dish." Dev Dyn **245**(7): 788-806.

Because of their extraordinary differentiation potential, human pluripotent stem cells (hPSCs) can differentiate into virtually any cell type of the human body, providing a powerful platform not only for generating relevant cell types useful for cell replacement therapies, but also for modeling human development and disease. Expanding this potential, structures resembling human organs, termed organoids, have been recently obtained from hPSCs through tissue engineering. Organoids exhibit multiple cell types self-organizing into structures recapitulating in part the physiology and the cellular interactions observed in the organ in vivo, offering unprecedented opportunities for human disease modeling. To fulfill this promise, tissue engineering in hPSCs needs to be supported by robust and scalable genome editing technologies. With the advent of the CRISPR/Cas9 technology, manipulating the genome of hPSCs has now become an easy task, allowing modifying their genome with superior precision, speed, and throughput. Here we review current and potential applications of the CRISPR/Cas9 technology in hPSCs and how they contribute to establish hPSCs as a model of choice for studying human genetics. Developmental Dynamics 245:788-806, 2016. (c) 2016 Wiley Periodicals, Inc.

Gori, J. L., et al. (2015). "Delivery and Specificity of CRISPR-Cas9 Genome Editing Technologies for Human Gene Therapy." Hum Gene Ther **26**(7): 443-451.

Genome editing using the clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR associated 9 (Cas9) technology is revolutionizing the study of gene function and likely will give rise to an entire new class of therapeutics for a wide range of diseases. Achieving this goal requires not only characterization of the technology for efficacy and specificity but also optimization of its delivery to the target cells for each disease indication. In this review we survey the various methods by which the CRISPR-Cas9 components have been delivered to cells and highlight some of the more clinically relevant approaches. Additionally, we discuss the methods available for assessing the specificity of Cas9 editing; an important safety consideration for development of the technology.

Gratz, S. J., et al. (2015). "Precise Genome Editing of Drosophila with CRISPR RNA-Guided Cas9." Methods Mol Biol **1311**: 335-348.

The readily programmable CRISPR-Cas9 system is transforming genome engineering. We and others have adapted the S. pyogenes CRISPR-Cas9 system to precisely engineer the Drosophila genome and demonstrated that these modifications are efficiently transmitted through the germline. Here we provide a detailed protocol for engineering small indels, defined deletions, and targeted insertion of exogenous DNA sequences within one month using a rapid DNA injection-based approach.

Gratz, S. J., et al. (2015). "CRISPR-Cas9 Genome Editing in Drosophila." Curr Protoc Mol Biol **111**: 31 32 31-20.

The CRISPR-Cas9 system has transformed genome engineering of model organisms from possible to practical. CRISPR-Cas9 can be readily programmed to generate sequence-specific double-strand breaks that disrupt targeted loci when repaired by error-prone non-homologous end joining (NHEJ) or to catalyze precise genome modification through homology-directed repair (HDR). Here we describe a streamlined

approach for rapid and highly efficient engineering of the Drosophila genome via CRISPR-Cas9-mediated HDR. In this approach, transgenic flies expressing Cas9 are injected with plasmids to express guide RNAs (gRNAs) and positively marked donor templates. We detail target-site selection; gRNA plasmid generation; donor template design and construction; and the generation, identification, and molecular confirmation of engineered lines. We also present alternative approaches and highlight key considerations for experimental design. The approach outlined here can be used to rapidly and reliably generate a variety of engineered modifications, including genomic deletions and replacements, precise sequence edits, and incorporation of protein tags.

Grav, L. M., et al. (2017). "Application of CRISPR/Cas9 Genome Editing to Improve Recombinant Protein Production in CHO Cells." Methods Mol Biol **1603**: 101-118.

Genome editing has become an increasingly important aspect of Chinese Hamster Ovary (CHO) cell line engineering for improving production of recombinant protein therapeutics. Currently, the focus is directed toward expanding the product diversity, controlling and improving product quality and yields. In this chapter, we present our protocol on how to use the genome editing tool Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) to knockout engineering target genes in CHO cells. As an example, we refer to the glutamine synthetase (GS)-encoding gene as the knockout target gene, a knockout that increases the selection efficiency of the GS-mediated gene amplification system.

Grenier, F., et al. (2015). "Selection and Validation of Spacer Sequences for CRISPR-Cas9 Genome Editing and Transcription Regulation in Bacteria." Methods Mol Biol **1334**: 233-244.

RNA-guided Cas9 nucleases derived from clustered regularly interspaced short palindromic repeat (CRISPR)-Cas systems have recently been adapted as sequence-programmable tools for various purposes such as genome editing and transcriptional regulation. A critical aspect of the system is the selection and validation of spacer sequences that allow precise targeting of the guide RNA-Cas9 complex. We describe a procedure involving computational and experimental steps to identify and test potentially interesting spacer sequences in bacterial genomes.

Grobarczyk, B., et al. (2015). "Generation of Isogenic Human iPS Cell Line Precisely Corrected by Genome Editing Using the CRISPR/Cas9 System." Stem Cell Rev **11**(5): 774-787.

Genome engineering and human iPS cells are two powerful technologies, which can be combined to highlight phenotypic differences and identify pathological mechanisms of complex diseases by providing isogenic cellular material. However, very few data are available regarding precise gene correction in human iPS cells. Here, we describe an optimized stepwise protocol to deliver CRISPR/Cas9 plasmids in human iPS cells. We highlight technical issues especially those associated to human stem cell culture and to the correction of a point mutation to obtain isogenic iPS cell line, without inserting any resistance cassette. Based on a two-steps clonal isolation protocol (mechanical picking followed by enzymatic dissociation), we succeed to select and expand corrected human iPS cell line with a great efficiency (more than 2% of the sequenced colonies). This protocol can also be used to obtain knock-out cell line from healthy iPS cell line by the NHEJ pathway (with about 15% efficiency) and reproduce disease phenotype. In addition, we also provide protocols for functional validation tests after every critical step.

Guitart, J. R., Jr., et al. (2016). "Research Techniques Made Simple: The Application of CRISPR-Cas9 and Genome Editing in Investigative Dermatology." J Invest Dermatol **136**(9): e87-93.

Designer nucleases have gained widespread attention for their ability to precisely modify genomic DNA in a programmable manner. These genome-editing nucleases make double-stranded breaks at specified loci, and desired changes can be made to modify, ablate, or excise target genes. This technology has been used widely to develop human disease models in laboratory animals and to study gene functions by silencing, activating, or modifying them. Furthermore, the recent discovery of a bacterially derived programmable nuclease termed clustered regularly interspaced palindromic repeats (CRISPR)-associated protein 9 (Cas9) has revolutionized the field because of its versatility and wide applicability. In this article, we discuss various modalities used to achieve genome editing with an emphasis on CRISPR-Cas9. We discuss genome-editing strategies to either repair or ablate target genes, with emphasis on their applications for investigating dermatological diseases. Additionally, we highlight preclinical studies showing the potential of genome editing as a therapy for congenital blistering diseases and as an antimicrobial agent, and we discuss limitations and future directions of this technology.

Gundry, M. C., et al. (2016). "Highly Efficient Genome Editing of Murine and Human Hematopoietic Progenitor Cells by CRISPR/Cas9." Cell Rep **17**(5): 1453-1461.

Our understanding of the mechanisms that regulate hematopoietic stem/progenitor cells (HSPCs) has been advanced by the ability to genetically manipulate mice; however, germline modification is time consuming and expensive. Here, we describe fast, efficient, and cost-effective methods to directly modify the genomes of mouse and human HSPCs using the CRISPR/Cas9 system. Using plasmid and virus-free delivery of guide RNAs alone into Cas9-expressing HSPCs or Cas9-guide RNA ribonucleoprotein (RNP) complexes into wild-type cells, we have achieved extremely efficient gene disruption in primary HSPCs from mouse (>60%) and human (approximately 75%). These techniques enabled rapid evaluation of the functional effects of gene loss of Eed, Suz12, and DNMT3A. We also achieved homology-directed repair in primary human HSPCs (>20%). These methods will significantly expand applications for CRISPR/Cas9 technologies for studying normal and malignant hematopoiesis.

Gurumurthy, C. B., et al. (2016). "GONAD: A Novel CRISPR/Cas9 Genome Editing Method that Does Not Require Ex Vivo Handling of Embryos." Curr Protoc Hum Genet **88**: Unit 15 18.

Transgenic technologies used for creating a desired genomic change in animals involve three critical steps: isolation of fertilized eggs, microinjection of transgenic DNA into them and their subsequent transfer to recipient females. These ex vivo steps have been widely used for over 3 decades and they were also readily adapted for the latest genome editing technologies such as ZFNs, TALENs, and CRISPR/Cas9 systems. We recently developed a method called GONAD (Genome editing via Oviductal Nucleic Acids Delivery) that does not require all the three critical steps of transgenesis and therefore relieves the bottlenecks of widely used animal transgenic technologies. Here we provide protocols for the GONAD system.

Haeussler, M. and J. P. Concordet (2016). "Genome Editing with CRISPR-Cas9: Can It Get Any Better?" J Genet Genomics **43**(5): 239-250.

The CRISPR-Cas revolution is taking place in virtually all fields of life sciences. Harnessing DNA cleavage with the CRISPR-Cas9 system of Streptococcus pyogenes has proven to be extraordinarily simple and efficient, relying only on the design of a synthetic single guide RNA (sgRNA) and its co-expression with Cas9. Here, we review the progress in the design of sgRNA from the original dual RNA guide for S. pyogenes and Staphylococcus aureus Cas9 (SpCas9 and SaCas9). New assays for genome-wide identification of off-targets have provided important insights into the issue of cleavage specificity in vivo. At the same time, the on-target activity of thousands of guides has been determined. These data have led to numerous online tools that facilitate the selection of guide RNAs in target sequences. It appears that for most basic research applications, cleavage activity can be maximized and off-targets minimized by carefully choosing guide RNAs based on computational predictions. Moreover, recent studies of Cas proteins have further improved the flexibility and precision of the CRISPR-Cas toolkit for genome editing. Inspired by the crystal structure of the complex of sgRNA-SpCas9 bound to target DNA, several variants of SpCas9 have recently been engineered, either with novel protospacer adjacent motifs (PAMs) or with drastically reduced off-targets. Novel Cas9 and Cas9-like proteins called Cpf1 have also been characterized from other bacteria and will benefit from the insights obtained from SpCas9. Genome editing with CRISPR-Cas9 may also progress with better understanding and control of cellular DNA repair pathways activated after Cas9-induced DNA cleavage.

Han, Y. and Q. W. Li (2016). "[Application progress of CRISPR/Cas9 genome editing technology in the treatment of HIV-1 infection]." Yi Chuan **38**(1): 9-16.

The goal of gene therapy is to introduce foreign genes into human target cells in a certain way to correct or compensate diseases caused by defective or abnormal genes. Therefore, gene therapy has great practical significance in studying the treatment of persistent or latent HIV-1 infection. At present, the existing methods of gene therapy have some major defects such as limited target site recognition and high frequency of off-targets. The latest research showed that the clustered regularly interspaced short palindromic repeats (CRISPR) /CRISPR-associated nuclease 9 (Cas9) system from bacteria and archaea has been successfully reformed to a targeted genome editing tool. Thus, how to achieve the goal of treating HIV-1 infection by modifying targeted HIV-1 virus genome effectively using the CRISPR/Cas9 system has become a current research focus. Here we review the latest achievements worldwide and briefly introduce applications of the CRISPR/Cas9 genome editing technology in the treatment of HIV-1 infection, including CCR5 gene editing, removal of HIV-1 virus and activation of HIV-1 virus, in order to provide reference for the prevention and treatment of HIV-1 infection.

Han, Y., et al. (2015). "CRISPR-Cas9 genome editing of a single regulatory element nearly abolishes target gene expression in mice--brief report." Arterioscler Thromb Vasc Biol **35**(2): 312-315.

OBJECTIVE: To ascertain the importance of a single regulatory element in the control of Cnn1 expression using CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) genome editing. APPROACH AND RESULTS: The CRISPR/Cas9 system was used to produce 3 of 18 founder mice carrying point mutations in an intronic CArG box of the smooth muscle cell-restricted Cnn1 gene. Each founder was bred for germline transmission of the mutant CArG box and littermate interbreeding to generate homozygous mutant (Cnn1(DeltaCArG/DeltaCArG)) mice. Quantitative reverse transcription polymerase chain reaction, Western blotting, and confocal immunofluorescence microscopy showed dramatic reductions in Cnn1 mRNA and CNN1 protein expression in Cnn1(DeltaCArG/DeltaCArG) mice with no change in other smooth muscle cell-restricted genes and little evidence of off-target edits elsewhere in the genome. In vivo chromatin immunoprecipitation assay revealed a sharp decrease in binding of serum response factor to the mutant CArG box. Loss of CNN1 expression was coincident with an increase in Ki-67 positive cells in the normal vessel wall. CONCLUSIONS: CRISPR/Cas9 genome editing of a single CArG box nearly abolishes Cnn1 expression in vivo and evokes increases in smooth muscle cell DNA synthesis. This facile genome editing system paves the way for a new generation of studies designed to test the importance of individual regulatory elements in living animals, including regulatory variants in conserved sequence blocks linked to human disease.

Hashimoto, M. and T. Takemoto (2015). "Electroporation enables the efficient mRNA delivery into the mouse zygotes and facilitates CRISPR/Cas9-based genome editing." Sci Rep **5**: 11315.

Recent use of the CRISPR/Cas9 system has dramatically reduced the time required to produce mutant mice, but the involvement of a time-consuming microinjection step still hampers its application for high-throughput genetic analysis. Here we developed a simple, highly efficient, and large-scale genome editing method, in which the RNAs for the CRISPR/Cas9 system are electroporated into zygotes rather than microinjected. We used this method to perform single-stranded oligodeoxynucleotide (ssODN)-mediated knock-in in mouse embryos. This method facilitates large-scale genetic analysis in the mouse.

He, Z., et al. (2016). "Comparison of CRISPR/Cas9 and TALENs on editing an integrated EGFP gene in the genome of HEK293FT cells." Springerplus **5**(1): 814.

BACKGROUND: Genome editors such as CRISPR/Cas9 and TALENs are at the forefront of research into methodologies for targeted modification of the mammalian genome. To date few comparative studies have been carried out to investigate the difference of genome editing characteristics between CRISPR/Cas9 and TALENs. While the CRISPR/Cas9 system has overtaken TALENs as the tool of choice for most research groups working in this field, we hypothesized that there could be certain applications whereby the application of TALENs would have specific benefits. Here we compare CRISPR/Cas9 and TALEN as tools for introducing site-specific editing events at an integrated EGFP gene in the genome of HEK293FT cells. RESULTS: Guide RNAs and TALEN pairs were designed to target two loci within the EGFP gene. We found that paired Cas9 nucleases induced targeted genomic deletion more efficiently and precisely than two TALEN pairs. However, when concurrently supplied with a plasmid template spanning the two DNA double-strand breaks (DSBs) within EGFP, TALENs stimulated homology directed repair (HDR) more efficiently than CRISPR/Cas9 and caused fewer targeted genomic deletions. CONCLUSIONS: Our data suggest that the choice of genome editing tool should be determined by the desired genome editing outcome. Such a rational approach is likely to benefit research outputs for groups working in fields as diverse as modification of cell lines, to animal models for disease studies, or gene therapy strategies.

Henao-Mejia, J., et al. (2016). "Generation of Genetically Modified Mice Using the CRISPR-Cas9 Genome-Editing System." Cold Spring Harb Protoc **2016**(2): pdb prot090704.

Genetically modified mice are extremely valuable tools for studying gene function and human diseases. Although the generation of mice with specific genetic modifications through traditional methods using homologous recombination in embryonic stem cells has been invaluable in the last two decades, it is an extremely costly, time-consuming, and, in some cases, uncertain technology. The recently described CRISPR-Cas9 genome-editing technology significantly reduces the time and the cost that are required to generate genetically engineered mice, allowing scientists to test more precise and bold hypotheses in vivo. Using this revolutionary methodology we have generated more than 100 novel genetically engineered mouse strains. In the current protocol, we describe in detail the optimal conditions to generate mice carrying point mutations, chromosomal deletions, conditional alleles, fusion tags, or endogenous reporters.

Heo, M. J., et al. (2017). "Controlling Citrate Synthase Expression by CRISPR/Cas9 Genome Editing for n-Butanol Production in Escherichia coli." ACS Synth Biol **6**(2): 182-189.

Genome editing using CRISPR/Cas9 was successfully demonstrated in Esherichia coli to effectively produce n-butanol in a defined medium under microaerobic condition. The butanol synthetic pathway genes including those encoding oxygen-tolerant alcohol dehydrogenase were overexpressed in metabolically engineered E. coli, resulting in 0.82 g/L butanol production. To increase butanol production, carbon flux from acetyl-CoA to citric acid cycle should be redirected to acetoacetyl-CoA. For this purpose, the 5'-untranslated region sequence of gltA encoding citrate synthase was designed using an expression prediction program, UTR designer, and modified using the CRISPR/Cas9 genome editing method to reduce its expression level. E. coli strains with decreased citrate synthase expression produced more butanol and the citrate synthase activity was correlated with butanol production. These results demonstrate that redistributing carbon flux using genome editing is an efficient engineering tool for metabolite overproduction.

Hindriksen, S., et al. (2017). "Baculoviral delivery of CRISPR/Cas9 facilitates efficient genome editing in human cells." PLoS One **12**(6): e0179514.

The CRISPR/Cas9 system is a highly effective tool for genome editing. Key to robust genome editing is the efficient delivery of the CRISPR/Cas9 machinery. Viral delivery systems are efficient vehicles for the transduction of foreign genes but commonly used viral vectors suffer from a limited capacity in the genetic information they can carry. Baculovirus however is capable of carrying large exogenous DNA fragments. Here we investigate the use of baculoviral vectors as a delivery vehicle for CRISPR/Cas9 based genome-editing tools. We demonstrate transduction of a panel of cell lines with Cas9 and an sgRNA sequence, which results in efficient knockout of all four targeted subunits of the chromosomal passenger complex (CPC). We further show that introduction of a homology directed repair template into the same CRISPR/Cas9 baculovirus facilitates introduction of specific point mutations and endogenous gene tags. Tagging of the CPC recruitment factor Haspin with the fluorescent reporter YFP allowed us to study its native localization as well as recruitment to the cohesin subunit Pds5B.

Hirosawa, M., et al. (2017). "Cell-type-specific genome editing with a microRNA-responsive CRISPR-Cas9 switch." Nucleic Acids Res **45**(13): e118.

The CRISPR-Cas9 system is a powerful genome-editing tool useful in a variety of biotechnology and biomedical applications. Here we developed a synthetic RNA-based, microRNA (miRNA)-responsive CRISPR-Cas9 system (miR-Cas9 switch) in which the genome editing activity of Cas9 can be modulated through endogenous miRNA signatures in mammalian cells. We created miR-Cas9 switches by using a miRNA-complementary sequence in the 5-UTR of mRNA encoding Streptococcus pyogenes Cas9. The miR-21-Cas9 or miR-302-Cas9 switches selectively and efficiently responded to miR-21-5p in HeLa cells or miR-302a-5p in human induced pluripotent stem cells, and post-transcriptionally attenuated the Cas9 activity only in the target cells. Moreover, the miR-Cas9 switches could differentially control the genome editing by sensing endogenous miRNA activities within a heterogeneous cell population. Our miR-Cas9 switch system provides a promising framework for cell-type selective genome editing and cell engineering based on intracellular miRNA information.

Hirose, M., et al. (2017). "CRISPR/Cas9-mediated genome editing in wild-derived mice: generation of tamed wild-derived strains by mutation of the a (nonagouti) gene." Sci Rep **7**: 42476.

Wild-derived mice have contributed to experimental mouse genetics by virtue of their genetic diversity, which may help increase the chance of identifying novel modifier genes responsible for specific phenotypes and diseases. However, gene targeting using wild-derived mice has been unsuccessful because of the unavailability of stable embryonic stem cells. Here, we report that CRISPR/Cas9-mediated gene targeting can be applied to the Japanese wild-derived MSM/Ms strain (Mus musculus molossinus). We targeted the nonagouti (a) gene encoding the agouti protein that is localized in hair and the brain. We obtained three homozygous knockout mice as founders, all showing black coat colour. While homozygous knockout offspring were physiologically indistinguishable from wild-type litter-mates, they showed specific domesticated behaviours: hypoactivity in the dark phase and a decline in the avoidance of a human hand. These phenotypes were consistent over subsequent generations. Our findings support the empirical hypothesis that nonagouti is a domestication-linked gene, the loss of which might repress aggressive behaviour.

Hruscha, A., et al. (2013). "Efficient CRISPR/Cas9 genome editing with low off-target effects in zebrafish." Development **140**(24): 4982-4987.

Gene modifications in animal models have been greatly facilitated through the application of targeted genome editing tools. The prokaryotic CRISPR/Cas9 type II genome editing system has recently been applied in cell lines and vertebrates. However, we still have very limited information about the efficiency of mutagenesis, germline transmission rates and off-target effects in genomes of model organisms. We now demonstrate that CRISPR/Cas9 mutagenesis in zebrafish is highly efficient, reaching up to 86.0%, and is heritable. The efficiency of the CRISPR/Cas9 system further facilitated the targeted knock-in of a protein tag provided by a donor oligonucleotide with knock-in efficiencies of 3.5-15.6%. Mutation rates at potential off-target sites are only 1.1-2.5%, demonstrating the specificity of the CRISPR/Cas9 system. The ease and efficiency of the CRISPR/Cas9 system with limited off-target effects make it a powerful genome engineering tool for in vivo studies.

Hruscha, A. and B. Schmid (2015). "Generation of zebrafish models by CRISPR /Cas9 genome editing." Methods Mol Biol **1254**: 341-350.

The CRISPR /Cas system identified in archaea has been adopted and optimized for genome editing purposes in zebrafish. In vitro transcribed guide RNA and Cas9 mRNA are microinjected into fertilized zebrafish embryos to edit the zebrafish genome. Here, we describe how to design a gRNA, a fast method for in vitro transcription of gRNA from oligonucleotides, microinjection into fertilized zebrafish embryos, and a PCR -based restriction fragment length assay to identify mutations at the gRNA target site.

Ikeda, M., et al. (2017). "Correction of a Disease Mutation using CRISPR/Cas9-assisted Genome Editing in Japanese Black Cattle." Sci Rep **7**(1): 17827.

Isoleucyl-tRNA synthetase (IARS) syndrome is a recessive disease of Japanese Black cattle caused by a single nucleotide substitution. To repair the mutated IARS gene, we designed clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) to create a double-strand break near the mutation site. CRISPR/Cas9 and donor DNA that contained a synonymous codon for the correct amino acid and an Aequorea coerulescens Green Fluorescent Protein (AcGFP) cassette with a piggyBac transposase recognition site at both ends were introduced into bovine fetal fibroblast (BFF) cells isolated from a homozygous mutant calf. Recombinant cells were enriched on the basis of expression of AcGFP, and two cell lines that contained the repaired allele were subcloned. We generated somatic cell nuclear transfer (SCNT) embryos from the repaired cells and transferred 22 blastocysts to recipient cows. In total, five viable fetuses were retrieved at Days 34 and 36. PiggyBac transposase mRNA was introduced into BFF cells isolated from cloned foetuses and AcGFP-negative cells were used for second round of cloning. We transferred nine SCNT embryos to recipient cows and retrieved two fetuses at Day 34. Fetal genomic DNA analysis showed correct repair of the IARS mutation without any additional DNA footprint.

Ikmi, A., et al. (2014). "TALEN and CRISPR/Cas9-mediated genome editing in the early-branching metazoan Nematostella vectensis." Nat Commun **5**: 5486.

Non-bilaterian phyla represent key lineages for exploring the evolutionary history of early animals. However, despite an increasing number of sequenced genomes from early-branching metazoans, efficient and reproducible methodologies for analysis of gene function remain a major challenge. Here we report the utilization of the TALEN and CRISPR/Cas9 systems to induce targeted mutations and homologous recombination-mediated transgenesis in the sea anemone Nematostella vectensis. We also present a new method to isolate genetically modified animals using engineered selection cassettes introduced by homologous recombination. Taken together, these methods will permit sophisticated gain- and loss-of-function analyses in Nematostella and perhaps other early metazoan species that allow for zygotic injection.

Ishizu, H., et al. (2017). "Use of the CRISPR-Cas9 system for genome editing in cultured Drosophila ovarian somatic cells." Methods **126**: 186-192.

The CRISPR-Cas9 system can be used for genome engineering in many organisms. PIWI-interacting RNAs (piRNAs) play a crucial role in repressing transposons to maintain genome integrity in Drosophila ovaries, and cultured ovarian somatic cells (OSCs) are widely used to elucidate the molecular mechanisms underlying the piRNA pathway. However, the germline-specific piRNA amplification system known as the ping-pong machinery does not occur in OSCs, making them unsuitable for elucidating the underlying mechanisms. Mutations in the lethal (3) malignant brain tumor gene (l (3)mbt) have been shown to cause ectopic expression of germline genes, including ping-pong factors. We therefore performed genome editing of Drosophila OSCs using the CRISPR-Cas9 system to achieve l (3)mbt knockout, resulting in successful induction of the piRNA amplification machinery. Here, we describe the detailed procedures for generating knockout and knockin OSC cells.

Jiang, Y., et al. (2015). "Multigene editing in the Escherichia coli genome via the CRISPR-Cas9 system." Appl Environ Microbiol **81**(7): 2506-2514.

An efficient genome-scale editing tool is required for construction of industrially useful microbes. We describe a targeted, continual multigene editing strategy that was applied to the Escherichia coli genome by using the Streptococcus pyogenes type II CRISPR-Cas9 system to realize a variety of precise genome modifications, including gene deletion and insertion, with a highest efficiency of 100%, which was able to achieve simultaneous multigene editing of up to three targets. The system also demonstrated successful targeted chromosomal deletions in Tatumella citrea, another species of the Enterobacteriaceae, with highest efficiency of 100%.

Jing, W., et al. (2015). "CRISPR/CAS9-Mediated Genome Editing of miRNA-155 Inhibits Proinflammatory Cytokine Production by RAW264.7 Cells." Biomed Res Int **2015**: 326042.

MicroRNA 155 (miR-155) is a key proinflammatory regulator in clinical and experimental rheumatoid arthritis (RA). Here we generated a miR-155 genome knockout (GKO) RAW264.7 macrophage cell line using the clustered regulatory interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (CAS9) technology. While upregulating the Src homology-2 domain-containing inositol 5-phosphatase 1 (SHIP1), the miR-155 GKO line is severely impaired in producing proinflammatory cytokines but slightly increased in osteoclastogenesis upon treatment with receptor activator of nuclear factor-kappaB ligand (RANKL). Taken together, our results suggest that genome editing of miR-155 holds the potential as a therapeutic strategy in RA.

Katayama, T., et al. (2016). "Development of a genome editing technique using the CRISPR/Cas9 system in the industrial filamentous fungus Aspergillus oryzae." Biotechnol Lett **38**(4): 637-642.

OBJECTIVES: To develop a genome editing method using the CRISPR/Cas9 system in Aspergillus oryzae, the industrial filamentous fungus used in Japanese traditional fermentation and for the production of enzymes and heterologous proteins. RESULTS: To develop the CRISPR/Cas9 system as a genome editing technique for A. oryzae, we constructed plasmids expressing the gene encoding Cas9 nuclease and single guide RNAs for the mutagenesis of target genes. We introduced these into an A. oryzae strain and obtained transformants containing mutations within each target gene that exhibited expected phenotypes. The mutational rates ranged from 10 to 20 %, and 1 bp deletions or insertions were the most commonly induced mutations. CONCLUSIONS: We developed a functional and versatile genome editing method using the CRISPR/Cas9 system in A. oryzae. This technique will contribute to the use of efficient targeted mutagenesis in many A. oryzae industrial strains.

Katic, I., et al. (2015). "CRISPR/Cas9 Genome Editing in Caenorhabditis elegans: Evaluation of Templates for Homology-Mediated Repair and Knock-Ins by Homology-Independent DNA Repair." G3 (Bethesda) **5**(8): 1649-1656.

Precise genome editing by the Cas9 nuclease depends on exogenously provided templates for homologous recombination. Here, we compare oligonucleotides with short homology and circular DNA molecules with extensive homology to genomic targets as templates for homology-based repair of CRISPR/Cas9 induced double-strand breaks. We find oligonucleotides to be templates of choice for introducing small sequence changes into the genome based on editing efficiency and ease of use. We show that polarity of oligonucleotide templates greatly affects repair efficiency: oligonucleotides in the sense orientation with respect to the target gene are better templates. In addition, combining a gene loss-of-function phenotype screen with detection of integrated fluorescent markers, we demonstrate that targeted knock-ins in Caenorhabditis elegans also can be achieved by homology-independent repair.

Kelley, M. L., et al. (2016). "Versatility of chemically synthesized guide RNAs for CRISPR-Cas9 genome editing." J Biotechnol **233**: 74-83.

The CRISPR-Cas9 system has become the most popular and efficient method for genome engineering in mammalian cells. The Streptococcus pyogenes Cas9 nuclease can function with two types of guide RNAs: the native dual crRNA and tracrRNA (crRNA:tracrRNA) or a chimeric single guide RNA (sgRNA). Although sgRNAs expressed from a DNA vector are predominant in the literature, guide RNAs can be rapidly generated by chemical synthesis and provide equivalent functionality in gene editing experiments. This review highlights the attributes and advantages of chemically synthesized guide RNAs including the incorporation of chemical modifications to enhance gene editing efficiencies in certain applications. The use of synthetic guide RNAs is also uniquely suited to genome-scale high throughput arrayed screening, particularly when using complex phenotypic assays for functional genomics studies. Finally, the use of synthetic guide RNAs along with DNA-free sources of Cas9 (mRNA or protein) allows for transient CRISPR-Cas9 presence in the cell,

thereby resulting in a decreased probability of off-target events.

Khlestkina, E. K. and V. K. Shumny (2016). "[Prospects for application of breakthrough technologies in breeding: The CRISPR/Cas9 system for plant genome editing]." Genetika **52**(7): 774-787.

Integration of the methods of contemporary genetics and biotechnology into the breeding process is assessed, and the potential role and efficacy of genome editing as a novel approach is discussed. Use of molecular (DNA) markers for breeding was proposed more than 30 years ago. Nowadays, they are widely used as an accessory tool in order to select plants by mono- and olygogenic traits. Presently, the genomic approaches are actively introduced into the breeding processes owing to automatization of DNA polymorphism analyses and development of comparatively cheap methods of DNA sequencing. These approaches provide effective selection by complex quantitative traits, and are based on the full-genome genotyping of the breeding material. Moreover, biotechnological tools, such as doubled haploids production, which provides fast obtainment of homozygotes, are widely used in plant breeding. Use of genomic and biotechnological approaches makes the development of varieties less time consuming. It also decreases the cultivated areas and financial expenditures required for accomplishment of the breeding process. However, the capacities of modern breeding are not limited to only these advantages. Experiments carried out on plants about 10 years ago provided the first data on genome editing. In the last two years, we have observed a sharp increase in the number of publications that report about successful experiments aimed at plant genome editing owing to the use of the relatively simple and convenient CRISPR/Cas9 system. The goal of some of these experiments was to modify agriculturally valuable genes of cultivated plants, such as potato, cabbage, tomato, maize, rice, wheat, barley, soybean and sorghum. These studies show that it is possible to obtain nontransgenic plants carrying stably inherited, specifically determined mutations using the CRISPR/Cas9 system. This possibility offers the challenge to obtain varieties with predetermined mono- and olygogenic traits.

Khurshid, H., et al. (2018). "An Era of CRISPR/ Cas9 Mediated Plant Genome Editing." Curr Issues Mol Biol **26**: 47-54.

Recently the engineered nucleases have revolutionized genome editing to perturb gene expression at specific sites in complex eukaryotic genomes. Three important classes of these genome editing tools are Moreover, the more recent type II Clustered Regularly Inter-spaced Short Palindromic Repeats/Crispr associated protein (CRISPR/Cas9) system has become the most favorite plant genome editing tool for its precision and RNA based specificity unlike its counterparts which rely on protein based specificity. Plasmid-mediated co-delivery of multiple sgRNAs and Cas9 to the Plant cell can simultaneously alter more than one target loci which enable multiplex genome editing. In this review, we discuss recent advancements in the CRISPR/ Cas9 technology mechanism, theory and its applications in plants and agriculture. We also suggest that the CRISPR/ Cas9 as an effective genome editing tool, has vast potential for crop improvement and studying gene regulation mechanism and chromatin remodeling.

Klann, T. S., et al. (2017). "CRISPR-Cas9 epigenome editing enables high-throughput screening for functional regulatory elements in the human genome." Nat Biotechnol **35**(6): 561-568.

Large genome-mapping consortia and thousands of genome-wide association studies have identified non-protein-coding elements in the genome as having a central role in various biological processes. However, decoding the functions of the millions of putative regulatory elements discovered in these studies remains challenging. CRISPR-Cas9-based epigenome editing technologies have enabled precise perturbation of the activity of specific regulatory elements. Here we describe CRISPR-Cas9-based epigenomic regulatory element screening (CERES) for improved high-throughput screening of regulatory element activity in the native genomic context. Using dCas9(KRAB) repressor and dCas9(p300) activator constructs and lentiviral single guide RNA libraries to target DNase I hypersensitive sites surrounding a gene of interest, we carried out both loss- and gain-of-function screens to identify regulatory elements for the beta-globin and HER2 loci in human cells. CERES readily identified known and previously unidentified regulatory elements, some of which were dependent on cell type or direction of perturbation. This technology allows the high-throughput functional annotation of putative regulatory elements in their native chromosomal context.

Klimek-Chodacka, M., et al. (2018). "Efficient CRISPR/Cas9-based genome editing in carrot cells." Plant Cell Rep.

KEY MESSAGE: The first report presenting successful and efficient carrot genome editing using CRISPR/Cas9 system. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas9) is a powerful genome editing tool that has been widely adopted in model organisms recently, but has not been used in

carrot-a model species for in vitro culture studies and an important health-promoting crop grown worldwide. In this study, for the first time, we report application of the CRISPR/Cas9 system for efficient targeted mutagenesis of the carrot genome. Multiplexing CRISPR/Cas9 vectors expressing two single-guide RNA (gRNAs) targeting the carrot flavanone-3-hydroxylase (F3H) gene were tested for blockage of the anthocyanin biosynthesis in a model purple-colored callus using Agrobacterium-mediated genetic transformation. This approach allowed fast and visual comparison of three codon-optimized Cas9 genes and revealed that the most efficient one in generating F3H mutants was the Arabidopsis codon-optimized AteCas9 gene with up to 90% efficiency. Knockout of F3H gene resulted in the discoloration of calli, validating the functional role of this gene in the anthocyanin biosynthesis in carrot as well as providing a visual marker for screening successfully edited events. Most resulting mutations were small Indels, but long chromosome fragment deletions of 116-119 nt were also generated with simultaneous cleavage mediated by two gRNAs. The results demonstrate successful site-directed mutagenesis in carrot with CRISPR/Cas9 and the usefulness of a model callus culture to validate genome editing systems. Given that the carrot genome has been sequenced recently, our timely study sheds light on the promising application of genome editing tools for boosting basic and translational research in this important vegetable crop.

Korge, S., et al. (2015). "Highly Efficient Genome Editing via CRISPR/Cas9 to Create Clock Gene Knockout Cells." J Biol Rhythms **30**(5): 389-395.

Targeted genome editing using CRISPR/Cas9 is a relatively new, revolutionary technology allowing for efficient and directed alterations of the genome. It has been widely used for loss-of-function studies in animals and cell lines but has not yet been used to study circadian rhythms. Here, we describe the application of CRISPR/Cas9 genome editing for the generation of an F-box and leucine-rich repeat protein 3 (Fbxl3) knockout in a human cell line. Genomic alterations at the Fbxl3 locus occurred with very high efficiency (70%-100%) and specificity at both alleles, resulting in insertions and deletions that led to premature stop codons and hence FBXL3 knockout. Fbxl3 knockout cells displayed low amplitude and long period oscillations of Bmal1-luciferase reporter activity as well as increased CRY1 protein stability in line with previously published phenotypes for Fbxl3 knockout in mice. Thus, CRISPR/Cas9 genome editing should be highly valuable for studying circadian rhythms not only in human cells but also in classic model systems as well as nonmodel organisms.

Koutroumpa, F. A., et al. (2016). "Heritable genome editing with CRISPR/Cas9 induces anosmia in a crop pest moth." Sci Rep **6**: 29620.

Lepidoptera suffer critical lack of genetic tools and heritable genome edition has been achieved only in a few model species. Here we demonstrate that the CRISPR/Cas9 system is highly efficient for genome editing in a non-model crop pest Lepidoptera, the noctuid moth Spodoptera littoralis. We knocked-out the olfactory receptor co-receptor Orco gene to investigate its function in Lepidoptera olfaction. We find that 89.6% of the injected individuals carried Orco mutations, 70% of which transmitted them to the next generation. CRISPR/Cas9-mediated Orco knockout caused defects in plant odor and sex pheromone olfactory detection in homozygous individuals. Our work genetically defines Orco as an essential OR partner for both host and mate detection in Lepidoptera, and demonstrates that CRISPR/Cas9 is a simple and highly efficient genome editing technique in noctuid pests opening new routes for gene function analysis and the development of novel pest control strategies.

Lander, N., et al. (2016). "Genome Editing by CRISPR/Cas9: A Game Change in the Genetic Manipulation of Protists." J Eukaryot Microbiol **63**(5): 679-690.

Genome editing by CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated gene 9) system has been transformative in biology. Originally discovered as an adaptive prokaryotic immune system, CRISPR/Cas9 has been repurposed for genome editing in a broad range of model organisms, from yeast to mammalian cells. Protist parasites are unicellular organisms producing important human diseases that affect millions of people around the world. For many of these diseases, such as malaria, Chagas disease, leishmaniasis and cryptosporidiosis, there are no effective treatments or vaccines available. The recent adaptation of the CRISPR/Cas9 technology to several protist models will be playing a key role in the functional study of their proteins, in the characterization of their metabolic pathways, and in the understanding of their biology, and will facilitate the search for new chemotherapeutic targets. In this work we review recent studies where the CRISPR/Cas9 system was adapted to protist parasites, particularly to Apicomplexans and trypanosomatids, emphasizing the different molecular strategies used for genome editing of each organism, as well as their advantages. We also discuss the potential usefulness of this technology in the green alga Chlamydomonas reinhardtii.

Laughery, M. F., et al. (2015). "New vectors for simple and streamlined CRISPR-Cas9 genome editing in Saccharomyces cerevisiae." Yeast **32**(12): 711-720.

Clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 technology is an important tool for genome editing because the Cas9 endonuclease can induce targeted DNA double-strand breaks. Targeting of the DNA break is typically controlled by a single-guide RNA (sgRNA), a chimeric RNA containing a structural segment important for Cas9 binding and a 20mer guide sequence that hybridizes to the genomic DNA target. Previous studies have demonstrated that CRISPR-Cas9 technology can be used for efficient, marker-free genome editing in Saccharomyces cerevisiae. However, introducing the 20mer guide sequence into yeast sgRNA expression vectors often requires cloning procedures that are complex, time-consuming and/or expensive. To simplify this process, we have developed a new sgRNA expression cassette with internal restriction enzyme sites that permit rapid, directional cloning of 20mer guide sequences. Here we describe a flexible set of vectors based on this design for cloning and expressing sgRNAs (and Cas9) in yeast using different selectable markers. We anticipate that the Cas9-sgRNA expression vector with the URA3 selectable marker (pML104) will be particularly useful for genome editing in yeast, since the Cas9 machinery can be easily removed by counter-selection using 5-fluoro-orotic acid (5-FOA) following successful genome editing. The availability of new vectors that simplify and streamline the technical steps required for guide sequence cloning should help accelerate the use of CRISPR-Cas9 technology in yeast genome editing.

Lee, C. M., et al. (2016). "The Neisseria meningitidis CRISPR-Cas9 System Enables Specific Genome Editing in Mammalian Cells." Mol Ther **24**(3): 645-654.

The clustered regularly-interspaced short palindromic repeats (CRISPR)-CRISPR-associated (Cas) system from Streptococcus pyogenes (Spy) has been successfully adapted for RNA-guided genome editing in a wide range of organisms. However, numerous reports have indicated that Spy CRISPR-Cas9 systems may have significant off-target cleavage of genomic DNA sequences differing from the intended on-target site. Here, we report the performance of the Neisseria meningitidis (Nme) CRISPR-Cas9 system that requires a longer protospacer-adjacent motif for site-specific cleavage, and present a comparison between the Spy and Nme CRISPR-Cas9 systems targeting the same protospacer sequence. The results with the native crRNA and tracrRNA as well as a chimeric single guide RNA for the Nme CRISPR-Cas9 system were also compared. Our results suggest that, compared with the Spy system, the Nme CRISPR-Cas9 system has similar or lower on-target cleavage activity but a reduced overall off-target effect on a genomic level when sites containing three or fewer mismatches are considered. Thus, the Nme CRISPR-Cas9 system may represent a safer alternative for precision genome engineering applications.

Li, G., et al. (2017). "Small molecules enhance CRISPR/Cas9-mediated homology-directed genome editing in primary cells." Sci Rep **7**(1): 8943.

CRISPR/Cas9 is an efficient customizable nuclease to generate double-strand breaks (DSBs) in the genome. This process results in knockout of the targeted gene or knock-in of a specific DNA fragment at the targeted locus in the genome of various species. However, efficiency of knock-in mediated by homology-directed repair (HDR) pathway is substantially lower compared with the efficiency of knockout mediated by the nonhomologous end-joining (NHEJ) pathway. Suppressing NHEJ pathway or enhancing HDR pathway has been proven to enhance the nuclease-mediated knock-in efficiency in cultured cells and model organisms. We here investigated the effect of small molecules, Scr7, L755507 and resveratrol, on promoting HDR efficiency in porcine fetal fibroblasts. Results from eGFP reporter assay showed that these small molecules could increase the HDR efficiency by 2-3-fold in porcine fetal fibroblasts. When transfecting with the homologous template DNA and CRISPR/Cas9 plasmid and treating with small molecules, the rate of knock-in porcine fetal fibroblast cell lines with large DNA fragment integration could reach more than 50% of the screened cell colonies, compared with 26.1% knock-in cell lines in the DMSO-treated group. The application of small molecules offers a beneficial approach to improve the frequency of precise genetic modifications in primary somatic cells.

Li, H. H. and G. Liu (2017). "The application of CRISPR/Cas9 in genome editing of filamentous fungi." Yi Chuan **39**(5): 355-367.

Filamentous fungi usually refer to the eukaryotic microorganisms with developed mycelia and without large fruiting bodies. Filamentous fungi not only play an important role in material cycle in nature, but also are closely related to human health, industry and agriculture. However, the difficulties in genetic manipulation impede the molecular studies on filamentous fungi. Clustered regulatory interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) is a conservative immune defense mechanism found in bacteria and archaea. Recently, it

has been developed as a convenient and flexible technique for genome editing. At present, CRISPR/Cas9 has been applied in different species for genetic operation. This review summarizes the application of CRISPR/Cas9 in genome editing of filamentous fungi and aims to provide a reference for the research in this field.

Li, J. F., et al. (2015). "Targeted plant genome editing via the CRISPR/Cas9 technology." Methods Mol Biol **1284**: 239-255.

Targeted modification of plant genome is key for elucidating and manipulating gene functions in basic and applied plant research. The CRISPR (clustered regularly interspaced short palindromic repeats)/CRISPR-associated protein (Cas) technology is emerging as a powerful genome editing tool in diverse organisms. This technology utilizes an easily reprogrammable guide RNA (gRNA) to guide Streptococcus pyogenes Cas9 endonuclease to generate a DNA double-strand break (DSB) within an intended genomic sequence and subsequently stimulate chromosomal mutagenesis or homologous recombination near the DSB site through cellular DNA repair machineries. In this chapter, we describe the detailed procedure to design, construct, and evaluate dual gRNAs for plant codon-optimized Cas9 (pcoCas9)-mediated genome editing using Arabidopsis thaliana and Nicotiana benthamiana protoplasts as model cellular systems. We also discuss strategies to apply the CRISPR/Cas9 system to generating targeted genome modifications in whole plants.

Li, K., et al. (2018). "Development of an Efficient Genome Editing Tool in Bacillus licheniformis Using CRISPR-Cas9 Nickase." Appl Environ Microbiol.

Bacillus strains are important industrial bacteria that can produce various biochemical products. However, low transformation efficiencies and a lack of effective genome editing tools have hindered its widespread application. Recently, clustered regularly interspaced short palindromic repeat (CRISPR)/cas9 techniques have been utilized in many organisms as genome editing tools because of their high efficiency and easy manipulation. In this study, an efficient genome editing method was developed for Bacillus licheniformis using a CRISPR-Cas9 nickase integrated into the genome of B. licheniformis DW2 with overexpression driven by the P43 promoter. The yvmC gene was deleted using the CRISPR-Cas9n technique with homology arms of 1.0 kb as a representative example and an efficiency of 100% was achieved. In addition, two genes were simultaneously disrupted with an efficiency of 11.6% and the large DNA fragment bacABC (42.7 kb) was deleted with an efficiency of 79.0%. Furthermore, the heterologous reporter gene aprN, which encodes for nattokinase in Bacillus subtilis, was inserted into the chromosome of B. licheniformis with an efficiency of 76.5%. The activity of nattokinase in the DWc9nDelta7/pP43SNT-SsacC strain reached 59.7 FU/mL, which was 25.7% higher than that of DWc9n/pP43SNT-SsacC Finally, the engineered strain DWc9nDelta7(DeltaeprDeltawprADeltamprDeltaaprE DeltavprDeltabprADeltabacABC) with multiple disrupted genes was construced using the CRISPR-Cas9n technique. Taken together, we have developed an efficient genome editing tool based on CRISPR/Cas9n in B. licheniformis This tool could be applied to strain improvement for future research.Importance As important industrial bacteria, Bacillus strains have attracted significant attention due to their production of biological products. However, genetic manipulation of these bacteria is difficult. The clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 system has been applied to genome editing in some bacteria, and CRISPR/Cas9n was proven to be an efficient and precise tool in the previous reports. The significance of our research is to develop an efficient, more precise, and systematic genome editing method for single gene deletion, multiple gene disruption, large DNA fragment deletion, and single gene integration in Bacillus licheniformis via Cas9 nickase. We also applied this method to the genetic engineering of the host strain for protein expression.

Li, L., et al. (2017). "Artificial Virus Delivers CRISPR-Cas9 System for Genome Editing of Cells in Mice." ACS Nano **11**(1): 95-111.

CRISPR-Cas9 has emerged as a versatile genome-editing platform. However, due to the large size of the commonly used CRISPR-Cas9 system, its effective delivery has been a challenge and limits its utility for basic research and therapeutic applications. Herein, a multifunctional nucleus-targeting "core-shell" artificial virus (RRPHC) was constructed for the delivery of CRISPR-Cas9 system. The artificial virus could efficiently load with the CRISPR-Cas9 system, accelerate the endosomal escape, and promote the penetration into the nucleus without additional nuclear-localization signal, thus enabling targeted gene disruption. Notably, the artificial virus is more efficient than SuperFect, Lipofectamine 2000, and Lipofectamine 3000. When loaded with a CRISPR-Cas9 plasmid, it induced higher targeted gene disruption efficacy than that of Lipofectamine 3000. Furthermore, the artificial virus effectively targets the ovarian cancer via dual-receptor-mediated endocytosis and had minimum side effects. When loaded with the Cas9-hMTH1 system targeting MTH1 gene, RRPHC

showed effective disruption of MTH1 in vivo. This strategy could be adapted for delivering CRISPR-Cas9 plasmid or other functional nucleic acids in vivo.

Li, W. and G. Ou (2016). "The application of somatic CRISPR-Cas9 to conditional genome editing in Caenorhabditis elegans." Genesis **54**(4): 170-181.

Forward and reverse genetic approaches have been well developed in the nematode Caenorhabditis elegans; however, efficient genetic tools to generate conditional gene mutations are still in high demand. Recently, the Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9 (CRISPR-Cas9) system for genome modification has provided an additional tool for C. elegans researchers to achieve simple and efficient conditional targeted mutagenesis. Here, we review recent advances in the somatic expression of Cas9 endonuclease for conditional gene editing. We present some practical considerations for improving the efficiency and reducing the off-target effects of somatic CRISPR-Cas9 and highlight a strategy to analyze somatic mutation at single-cell resolution. Finally, we outline future applications and consider challenges for this emerging genome editing platform that will need to be addressed in the future.

Li, X., et al. (2015). "Outbred genome sequencing and CRISPR/Cas9 gene editing in butterflies." Nat Commun **6**: 8212.

Butterflies are exceptionally diverse but their potential as an experimental system has been limited by the difficulty of deciphering heterozygous genomes and a lack of genetic manipulation technology. Here we use a hybrid assembly approach to construct high-quality reference genomes for Papilio xuthus (contig and scaffold N50: 492 kb, 3.4 Mb) and Papilio machaon (contig and scaffold N50: 81 kb, 1.15 Mb), highly heterozygous species that differ in host plant affiliations, and adult and larval colour patterns. Integrating comparative genomics and analyses of gene expression yields multiple insights into butterfly evolution, including potential roles of specific genes in recent diversification. To functionally test gene function, we develop an efficient (up to 92.5%) CRISPR/Cas9 gene editing method that yields obvious phenotypes with three genes, Abdominal-B, ebony and frizzled. Our results provide valuable genomic and technological resources for butterflies and unlock their potential as a genetic model system.

Liang, G., et al. (2016). "Selection of highly efficient sgRNAs for CRISPR/Cas9-based plant genome editing." Sci Rep **6**: 21451.

The CRISPR/Cas9-sgRNA system has been developed to mediate genome editing and become a powerful tool for biological research. Employing the CRISPR/Cas9-sgRNA system for genome editing and manipulation has accelerated research and expanded researchers' ability to generate genetic models. However, the method evaluating the efficiency of sgRNAs is lacking in plants. Based on the nucleotide compositions and secondary structures of sgRNAs which have been experimentally validated in plants, we instituted criteria to design efficient sgRNAs. To facilitate the assembly of multiple sgRNA cassettes, we also developed a new strategy to rapidly construct CRISPR/Cas9-sgRNA system for multiplex editing in plants. In theory, up to ten single guide RNA (sgRNA) cassettes can be simultaneously assembled into the final binary vectors. As a proof of concept, 21 sgRNAs complying with the criteria were designed and the corresponding Cas9/sgRNAs expression vectors were constructed. Sequencing analysis of transgenic rice plants suggested that 82% of the desired target sites were edited with deletion, insertion, substitution, and inversion, displaying high editing efficiency. This work provides a convenient approach to select efficient sgRNAs for target editing.

Liang, X., et al. (2017). "Enhanced CRISPR/Cas9-mediated precise genome editing by improved design and delivery of gRNA, Cas9 nuclease, and donor DNA." J Biotechnol **241**: 136-146.

While CRISPR-based gene knock out in mammalian cells has proven to be very efficient, precise insertion of genetic elements via the cellular homology directed repair (HDR) pathway remains a rate-limiting step to seamless genome editing. Under the conditions described here, we achieved up to 56% targeted integration efficiency with up to a six-nucleotide insertion in HEK293 cells. In induced pluripotent stem cells (iPSCs), we achieved precise genome editing rates of up to 45% by co-delivering the Cas9 RNP and donor DNA. In addition, the use of a short double stranded DNA oligonucleotide with 3' overhangs allowed integration of a longer FLAG epitope tag along with a restriction site at rates of up to 50%. We propose a model that favors the design of donor DNAs with the change as close to the cleavage site as possible. For small changes such as SNPs or short insertions, asymmetric single stranded donor molecules with 30 base homology arms 3' to the insertion/repair cassette and greater than 40 bases of homology on the 5' end seems to be favored. For larger insertions such as an epitope tag, a dsDNA donor with protruding 3' homology arms of 30 bases is favored. In both cases, protecting the ends of the donor DNA with phosphorothioate modifications improves the editing efficiency.

Lin, C., et al. (2016). "Increasing the Efficiency of CRISPR/Cas9-mediated Precise Genome Editing of HSV-1 Virus in Human Cells." Sci Rep **6**: 34531.

Genetically modified HSV-1 viruses serve as promising vectors for tumour therapy and vaccine development. The CRISPR/Cas9 system is one of the most powerful tools for precise gene editing of the genomes of organisms. However, whether the CRISPR/Cas9 system can precisely and efficiently make gene replacements in the genome of HSV-1 remains essentially unknown. Here, we reported CRISPR/Cas9-mediated editing of the HSV-1 genome in human cells, including the knockout and replacement of large genes. In established cells stably expressing CRISPR/Cas9, gRNA in coordination with Cas9 could direct a precise cleavage within a pre-defined target region, and foreign genes were successfully used to replace the target gene seamlessly by HDR-mediated gene replacement. Introducing the NHEJ inhibitor SCR7 to the CRISPR/Cas9 system greatly facilitated HDR-mediated gene replacement in the HSV-1 genome. We provided the first genetic evidence that two copies of the ICP0 gene in different locations on the same HSV-1 genome could be simultaneously modified with high efficiency and with no off-target modifications. We also developed a revolutionized isolation platform for desired recombinant viruses using single-cell sorting. Together, our work provides a significantly improved method for targeted editing of DNA viruses, which will facilitate the development of anti-cancer oncolytic viruses and vaccines.

Lin, C. Y. and Y. H. Su (2016). "Genome editing in sea urchin embryos by using a CRISPR/Cas9 system." Dev Biol **409**(2): 420-428.

Sea urchin embryos are a useful model system for investigating early developmental processes and the underlying gene regulatory networks. Most functional studies using sea urchin embryos rely on antisense morpholino oligonucleotides to knockdown gene functions. However, major concerns related to this technique include off-target effects, variations in morpholino efficiency, and potential morpholino toxicity; furthermore, such problems are difficult to discern. Recent advances in genome editing technologies have introduced the prospect of not only generating sequence-specific knockouts, but also providing genome-engineering applications. Two genome editing tools, zinc-finger nuclease (ZFN) and transcription activator-like effector nucleases (TALENs), have been utilized in sea urchin embryos, but the resulting efficiencies are far from satisfactory. The CRISPR (clustered regularly interspaced short palindromic repeat)-Cas9 (CRISPR-associated nuclease 9) system serves as an easy and efficient method with which to edit the genomes of several established and emerging model organisms in the field of developmental biology. Here, we apply the CRISPR/Cas9 system to the sea urchin embryo. We designed six guide RNAs (gRNAs) against the well-studied nodal gene and discovered that five of the gRNAs induced the expected phenotype in 60-80% of the injected embryos. In addition, we developed a simple method for isolating genomic DNA from individual embryos, enabling phenotype to be precisely linked to genotype, and revealed that the mutation rates were 67-100% among the sequenced clones. Of the two potential off-target sites we examined, no off-target effects were observed. The detailed procedures described herein promise to accelerate the usage of CRISPR/Cas9 system for genome editing in sea urchin embryos.

Liu, G. G., et al. (2015). "[Efficient genome editing in human pluripotent stem cells through CRISPR/Cas9]." Yi Chuan **37**(11): 1167-1173.

The RNA-guided CRISPR (clustered regularly interspaced short palindromic repeat)-associated Cas9 nuclease has offered a new platform for genome editing with high efficiency. Here, we report the use of CRISPR/Cas9 technology to target a specific genomic region in human pluripotent stem cells. We show that CRISPR/Cas9 can be used to disrupt a gene by introducing frameshift mutations to gene coding region; to knock in specific sequences (e.g. FLAG tag DNA sequence) to targeted genomic locus via homology directed repair; to induce large genomic deletion through dual-guide multiplex. Our results demonstrate the versatile application of CRISPR/Cas9 in stem cell genome editing, which can be widely utilized for functional studies of genes or genome loci in human pluripotent stem cells.

Liu, H., et al. (2016). "Genome Editing of Wnt-1, a Gene Associated with Segmentation, via CRISPR/Cas9 in the Pine Caterpillar Moth, Dendrolimus punctatus." Front Physiol **7**: 666.

The pine caterpillar moth, Dendrolimus punctatus, is a devastating forest pest. Genetic manipulation of this insect pest is limited due to the lack of genomic and functional genomic toolsets. Recently, CRISPR/Cas9 technology has been demonstrated to be a promising approach to modify the genome. To investigate gene functions during the embryogenesis, we introduced CRISPR/Cas9 system in D. punctatus to precisely and effectively manipulate gene expressions inmutant embryos. Compared to controls, knocking out of DpWnt-1, a gene well known for its role in the early body planning, led to high embryonic mortality. Among these mutants, 32.9% of the embryos and larvae showed an abnormal

development. DpWnt-1 mutants predominantly exhibited abnormal posterior segments. In addition, multiple phenotypes were observed, including the loss of limbs and the head deformation, suggesting that DpWnt-1 signaling pathway is necessary for anterior segmentation and appendage development. Overall, our results demonstrate that CRISPR/Cas9 system is feasible and efficient in inducing mutations at a specific locus in D. punctatus. This study not only lays the foundation for characterizing gene functions in a non-model species, but also facilitates the future development of pest control alternatives for a major defoliator.

Liu, J., et al. (2017). "Development of a CRISPR/Cas9 genome editing toolbox for Corynebacterium glutamicum." Microb Cell Fact **16**(1): 205.

BACKGROUND: Corynebacterium glutamicum is an important industrial workhorse and advanced genetic engineering tools are urgently demanded. Recently, the clustered regularly interspaced short palindromic repeats (CRISPR) and their CRISPR-associated proteins (Cas) have revolutionized the field of genome engineering. The CRISPR/Cas9 system that utilizes NGG as protospacer adjacent motif (PAM) and has good targeting specificity can be developed into a powerful tool for efficient and precise genome editing of C. glutamicum. RESULTS: Herein, we developed a versatile CRISPR/Cas9 genome editing toolbox for C. glutamicum. Cas9 and gRNA expression cassettes were reconstituted to combat Cas9 toxicity and facilitate effective termination of gRNA transcription. Co-transformation of Cas9 and gRNA expression plasmids was exploited to overcome high-frequency mutation of cas9, allowing not only highly efficient gene deletion and insertion with plasmid-borne editing templates (efficiencies up to 60.0 and 62.5%, respectively) but also simple and time-saving operation. Furthermore, CRISPR/Cas9-mediated ssDNA recombineering was developed to precisely introduce small modifications and single-nucleotide changes into the genome of C. glutamicum with efficiencies over 80.0%. Notably, double-locus editing was also achieved in C. glutamicum. This toolbox works well in several C. glutamicum strains including the widely-used strains ATCC 13032 and ATCC 13869. CONCLUSIONS: In this study, we developed a CRISPR/Cas9 toolbox that could facilitate markerless gene deletion, gene insertion, precise base editing, and double-locus editing in C. glutamicum. The CRISPR/Cas9 toolbox holds promise for accelerating the engineering of C. glutamicum and advancing its application in the production of biochemicals and biofuels.

Liu, K. I., et al. (2016). "A chemical-inducible CRISPR-Cas9 system for rapid control of genome editing." Nat Chem Biol **12**(11): 980-987.

CRISPR-Cas9 has emerged as a powerful technology that enables ready modification of the mammalian genome. The ability to modulate Cas9 activity can reduce off-target cleavage and facilitate precise genome engineering. Here we report the development of a Cas9 variant whose activity can be switched on and off in human cells with 4-hydroxytamoxifen (4-HT) by fusing the Cas9 enzyme with the hormone-binding domain of the estrogen receptor (ERT2). The final optimized variant, termed iCas, showed low endonuclease activity without 4-HT but high editing efficiency at multiple loci with the chemical. We also tuned the duration and concentration of 4-HT treatment to reduce off-target genome modification. Additionally, we benchmarked iCas against other chemical-inducible methods and found that it had the fastest on rate and that its activity could be toggled on and off repeatedly. Collectively, these results highlight the utility of iCas for rapid and reversible control of genome-editing function.

Liu, Q., et al. (2017). "Development of a genome-editing CRISPR/Cas9 system in thermophilic fungal Myceliophthora species and its application to hyper-cellulase production strain engineering." Biotechnol Biofuels **10**: 1.

BACKGROUND: Over the past 3 years, the CRISPR/Cas9 system has revolutionized the field of genome engineering. However, its application has not yet been validated in thermophilic fungi. Myceliophthora thermophila, an important thermophilic biomass-degrading fungus, has attracted industrial interest for the production of efficient thermostable enzymes. Genetic manipulation of Myceliophthora is crucial for metabolic engineering and to unravel the mechanism of lignocellulose deconstruction. The lack of a powerful, versatile genome-editing tool has impeded the broader exploitation of M. thermophila in biotechnology. RESULTS: In this study, a CRISPR/Cas9 system for efficient multiplexed genome engineering was successfully developed in the thermophilic species M. thermophila and M. heterothallica. This CRISPR/Cas9 system could efficiently mutate the imported amdS gene in the genome via NHEJ-mediated events. As a proof of principle, the genes of the cellulase production pathway, including cre-1, res-1, gh1-1, and alp-1, were chosen as editing targets. Simultaneous multigene disruptions of up to four of these different loci were accomplished with neomycin selection marker integration via a single transformation using the CRISPR/Cas9 system. Using this genome-engineering tool, multiple strains exhibiting

pronounced hyper-cellulase production were generated, in which the extracellular secreted protein and lignocellulase activities were significantly increased (up to 5- and 13-fold, respectively) compared with the parental strain. CONCLUSIONS: A genome-wide engineering system for thermophilic fungi was established based on CRISPR/Cas9. Successful expansion of this system without modification to M. heterothallica indicates it has wide adaptability and flexibility for use in other Myceliophthora species. This system could greatly accelerate strain engineering of thermophilic fungi for production of industrial enzymes, such as cellulases as shown in this study and possibly bio-based fuels and chemicals in the future.

Liu, Q., et al. (2017). "CRISPR/Cas9-based efficient genome editing in Staphylococcus aureus." Acta Biochim Biophys Sin (Shanghai) **49**(9): 764-770.

Staphylococcus aureus is an important pathogenic bacterium prevalent in nosocomial infections and associated with high morbidity and mortality rates, which arise from the significant pathogenicity and multi-drug resistance. However, the typical genetic manipulation tools used to explore the relevant molecular mechanisms of S. aureus have multiple limitations: leaving a scar in the genome, comparatively low gene-editing efficiency, and prolonged experimental period. Here, we present a single-plasmid based on the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) system which allows rapid and efficient chromosomal manipulation in S. aureus. The plasmid carries the cas9 gene under the control of the constitutive promoter Pxyl/tet, a single guide RNA-encoding sequence transcribed via a strong promoter Pspac, and donor DNA used to repair the double strand breaks. The function of the CRISPR/Cas9 vector was demonstrated by deleting the tgt gene and the rocA gene, and by inserting the erm R cassette in S. aureus. This research establishes a CRISPR/Cas9 genome editing tool in S. aureus, which enables marker-free, scarless and rapid genetic manipulation, thus accelerating the study of gene function in S. aureus.

Liu, T., et al. (2016). "Development and potential applications of CRISPR-Cas9 genome editing technology in sarcoma." Cancer Lett **373**(1): 109-118.

Sarcomas include some of the most aggressive tumors and typically respond poorly to chemotherapy. In recent years, specific gene fusion/mutations and gene over-expression/activation have been shown to drive sarcoma pathogenesis and development. These emerging genomic alterations may provide targets for novel therapeutic strategies and have the potential to transform sarcoma patient care. The RNA-guided nuclease CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated protein-9 nuclease) is a convenient and versatile platform for site-specific genome editing and epigenome targeted modulation. Given that sarcoma is believed to develop as a result of genetic alterations in mesenchymal progenitor/stem cells, CRISPR-Cas9 genome editing technologies hold extensive application potentials in sarcoma models and therapies. We review the development and mechanisms of the CRISPR-Cas9 system in genome editing and introduce its application in sarcoma research and potential therapy in clinic. Additionally, we propose future directions and discuss the challenges faced with these applications, providing concise and enlightening information for readers interested in this area.

Liu, X., et al. (2017). "CRISPR/Cas9-mediated genome editing in plants." Methods **121-122**: 94-102.

The increasing burden of the world's population on agriculture necessitates the development of more robust crops. As the amount of information from sequenced crop genomes increases, technology can be used to investigate the function of genes in detail and to design improved crops at the molecular level. Recently, an RNA-programmed genome-editing system composed of a clustered regularly interspaced short palindromic repeats (CRISPR)-encoded guide RNA and the nuclease Cas9 has provided a powerful platform to achieve these goals. By combining versatile tools to study and modify plants at different molecular levels, the CRISPR/Cas9 system is paving the way towards a new horizon for basic research and crop development. In this review, the accomplishments, problems and improvements of this technology in plants, including target sequence cleavage, knock-in/gene replacement, transcriptional regulation, epigenetic modification, off-target effects, delivery system and potential applications, will be highlighted.

Liu, Z., et al. (2017). "Genome editing of the HIV co-receptors CCR5 and CXCR4 by CRISPR-Cas9 protects CD4(+) T cells from HIV-1 infection." Cell Biosci **7**: 47.

BACKGROUND: The main approach to treat HIV-1 infection is combination antiretroviral therapy (cART). Although cART is effective in reducing HIV-1 viral load and controlling disease progression, it has many side effects, and is expensive for HIV-1 infected patients who must remain on lifetime treatment. HIV-1 gene therapy has drawn much attention as studies of genome editing tools have progressed. For example, zinc finger nucleases (ZFN), transcription activator

like effector nucleases (TALEN) and clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 have been utilized to successfully disrupt the HIV-1 co-receptors CCR5 or CXCR4, thereby restricting HIV-1 infection. However, the effects of simultaneous genome editing of CXCR4 and CCR5 by CRISPR-Cas9 in blocking HIV-1 infection in primary CD4(+) T cells has been rarely reported. Furthermore, combination of different target sites of CXCR4 and CCR5 for disruption also need investigation. RESULTS: In this report, we designed two different gRNA combinations targeting both CXCR4 and CCR5, in a single vector. The CRISPR-sgRNAs-Cas9 could successfully induce editing of CXCR4 and CCR5 genes in various cell lines and primary CD4(+) T cells. Using HIV-1 challenge assays, we demonstrated that CXCR4-tropic or CCR5-tropic HIV-1 infections were significantly reduced in CXCR4- and CCR5-modified cells, and the modified cells exhibited a selective advantage over unmodified cells during HIV-1 infection. The off-target analysis showed that no non-specific editing was identified in all predicted sites. In addition, apoptosis assays indicated that simultaneous disruption of CXCR4 and CCR5 in primary CD4(+) T cells by CRISPR-Cas9 had no obvious cytotoxic effects on cell viability. CONCLUSIONS: Our results suggest that simultaneous genome editing of CXCR4 and CCR5 by CRISPR-Cas9 can potentially provide an effective and safe strategy towards a functional cure for HIV-1 infection.

Liu, Z., et al. (2016). "Efficient genome editing of genes involved in neural crest development using the CRISPR/Cas9 system in Xenopus embryos." Cell Biosci **6**: 22.

BACKGROUND: The RNA guided CRISPR/Cas9 nucleases have been proven to be effective for gene disruption in various animal models including Xenopus tropicalis. The neural crest (NC) is a transient cell population during embryonic development and contributes to a large variety of tissues. Currently, loss-of-function studies on NC development in X. tropicalis are largely based on morpholino antisense oligonucleotide. It is worthwhile establishing targeted gene knockout X. tropicails line using CRISPR/Cas9 system to study NC development. METHODS: We utilized CRISPR/Cas9 to disrupt genes that are involved in NC formation in X. tropicalis embryos. A single sgRNA and Cas9 mRNA synthesized in vitro, were co-injected into X. tropicalis embryos at one-cell stage to induce single gene disruption. We also induced duplex mutations, large segmental deletions and inversions in X. tropicalis by injecting Cas9 and a pair of sgRNAs. The specificity of CRISPR/Cas9 was assessed in X. tropicalis embryos and the Cas9 nickase was used to reduce the off-target cleavages. Finally, we crossed the G0 mosaic frogs with targeted mutations to wild type frogs and obtained the germline transmission. RESULTS: Total 16 target sites in 15 genes were targeted by CRISPR/Cas9 and resulted in successful indel mutations at 14 loci with disruption efficiencies in a range from 9.3 to 57.8 %. Furthermore, we demonstrated the feasibility of generation of duplex mutations, large segmental deletions and inversions by using Cas9 and a pair of sgRNAs. We observed that CRISPR/Cas9 displays obvious off-target effects at some loci in X. tropicalis embryos. Such off-target cleavages was reduced by using the D10A Cas9 nickase. Finally, the Cas9 induced indel mutations were efficiently passed to G1 offspring. CONCLUSION: Our study proved that CRISPR/Cas9 could mediate targeted gene mutation in X. tropicalis with high efficiency. This study expands the application of CRISPR/Cas9 platform in X. tropicalis and set a basis for studying NC development using genetic approach.

Lo, T. W., et al. (2013). "Precise and heritable genome editing in evolutionarily diverse nematodes using TALENs and CRISPR/Cas9 to engineer insertions and deletions." Genetics **195**(2): 331-348.

Exploitation of custom-designed nucleases to induce DNA double-strand breaks (DSBs) at genomic locations of choice has transformed our ability to edit genomes, regardless of their complexity. DSBs can trigger either error-prone repair pathways that induce random mutations at the break sites or precise homology-directed repair pathways that generate specific insertions or deletions guided by exogenously supplied DNA. Prior editing strategies using site-specific nucleases to modify the Caenorhabditis elegans genome achieved only the heritable disruption of endogenous loci through random mutagenesis by error-prone repair. Here we report highly effective strategies using TALE nucleases and RNA-guided CRISPR/Cas9 nucleases to induce error-prone repair and homology-directed repair to create heritable, precise insertion, deletion, or substitution of specific DNA sequences at targeted endogenous loci. Our robust strategies are effective across nematode species diverged by 300 million years, including necromenic nematodes (Pristionchus pacificus), male/female species (Caenorhabditis species 9), and hermaphroditic species (C. elegans). Thus, genome-editing tools now exist to transform nonmodel nematode species into genetically tractable model organisms. We demonstrate the utility of our broadly applicable genome-editing strategies by creating reagents generally useful to the nematode community and reagents specifically designed to explore the

mechanism and evolution of X chromosome dosage compensation. By developing an efficient pipeline involving germline injection of nuclease mRNAs and single-stranded DNA templates, we engineered precise, heritable nucleotide changes both close to and far from DSBs to gain or lose genetic function, to tag proteins made from endogenous genes, and to excise entire loci through targeted FLP-FRT recombination.

Lowder, L., et al. (2017). "Rapid Construction of Multiplexed CRISPR-Cas9 Systems for Plant Genome Editing." Methods Mol Biol **1578**: 291-307.

Multiplex CRISPR-Cas9 nuclease mediated genome editing is an efficient method for disrupting gene function in plants. Use of CRISPR-Cas9 has escalated rapidly in recent years and is expected to become routine practice in molecular biology and related fields of research. Due to the relatively novel and widespread adoption of this technology, first-time users may not have regular access to experienced guidance or technical support from peers or mentors. Here, we offer guidance and technical support in the form of a detailed and tested protocol for simultaneous targeting of three separate loci on the TRANSPARENT TESTA 4 (TT4) gene in Arabidopsis thaliana using multiplex CRISPR-Cas9. Although we target multiple loci on a single gene in Arabidopsis, the same approach can be used to target multiple genes or alleles in other plant species as well. We recommend the use of a molecular toolkit to streamline the process and make recommendations for this type of approach. The protocol starts with an overview of the reagents and covers designing of gRNAs and assembly of components into a final T-DNA delivery molecule through Golden Gate cloning and Multisite Gateway LR recombination.

Lowder, L. G., et al. (2015). "A CRISPR/Cas9 Toolbox for Multiplexed Plant Genome Editing and Transcriptional Regulation." Plant Physiol **169**(2): 971-985.

The relative ease, speed, and biological scope of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated Protein9 (Cas9)-based reagents for genomic manipulations are revolutionizing virtually all areas of molecular biosciences, including functional genomics, genetics, applied biomedical research, and agricultural biotechnology. In plant systems, however, a number of hurdles currently exist that limit this technology from reaching its full potential. For example, significant plant molecular biology expertise and effort is still required to generate functional expression constructs that allow simultaneous editing, and especially transcriptional regulation, of multiple different genomic loci or multiplexing, which is a significant advantage of CRISPR/Cas9 versus other genome-editing systems. To streamline and facilitate rapid and wide-scale use of CRISPR/Cas9-based technologies for plant research, we developed and implemented a comprehensive molecular toolbox for multifaceted CRISPR/Cas9 applications in plants. This toolbox provides researchers with a protocol and reagents to quickly and efficiently assemble functional CRISPR/Cas9 transfer DNA constructs for monocots and dicots using Golden Gate and Gateway cloning methods. It comes with a full suite of capabilities, including multiplexed gene editing and transcriptional activation or repression of plant endogenous genes. We report the functionality and effectiveness of this toolbox in model plants such as tobacco (Nicotiana benthamiana), Arabidopsis (Arabidopsis thaliana), and rice (Oryza sativa), demonstrating its utility for basic and applied plant research.

Lu, J., et al. (2016). "A redesigned CRISPR/Cas9 system for marker-free genome editing in Plasmodium falciparum." Parasit Vectors **9**: 198.

BACKGROUND: A highly efficient CRISPR/Cas9-based marker-free genome editing system has been established in Plasmodium falciparum (Pf). However, with the current methods, two drug-selectable markers are needed for episome retention, which may present hurdles for consecutive genome manipulations due to the limited number of available selectable markers. The loading capacity of donor DNA is also unsatisfactory due to the large size of the Cas9 nuclease and sgRNA co-expression system, which limits the size of knock-in DNA fragments. Because of the inefficient end joining (EJ) DNA repair mechanism of Pf, a suicide-rescue approach could be used to address the challenges. Cas9 nuclease and sgRNA were co-expressed from a single plasmid (suicide vector) with one selectable marker, and the donor DNA was ligated into the other plasmid (rescue vector) containing only the ampicillin-resistance gene (AmpR) and a ColEl replication origin (ori). Nonetheless, whether this approach can mediate even the regular gene editing in Pf remains unknown. This study aimed to demonstrate the basic gene editing function of this Cas9-mediated suicide-rescue system. FINDINGS: The suicide and rescue vectors were constructed and co-transfected into Pf3D7. This system worked as expected when used to disrupt the Pfset2 gene and to insert a green fluorescent protein-renilla luciferase (gfp-ruc) fusion gene cassette of 3334 base pairs (bp) into the Pf47 locus, demonstrating that the suicide vector actually induced double-strand breaks (DSBs) and that the rescue vector functioned without maintenance via drug selection. CONCLUSIONS: The adapted marker-free CRISPR/Cas9 system with only a single episome-

selectable marker performs well as the current systems for general gene editing which lays a solid foundation for further studies including consecutive gene manipulations and large gene knock-ins.

Lu, J., et al. (2017). "Multimode drug inducible CRISPR/Cas9 devices for transcriptional activation and genome editing." Nucleic Acids Res.

Precise investigation and manipulation of dynamic biological processes often requires molecular modulation in a controlled inducible manner. The clustered, regularly interspaced, short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9) has emerged as a versatile tool for targeted gene editing and transcriptional programming. Here, we designed and vigorously optimized a series of Hybrid drug Inducible CRISPR/Cas9 Technologies (HIT) for transcriptional activation by grafting a mutated human estrogen receptor (ERT2) to multiple CRISPR/Cas9 systems, which renders them 4-hydroxytamoxifen (4-OHT) inducible for the access of genome. Further, extra functionality of simultaneous genome editing was achieved with one device we named HIT2. Optimized terminal devices herein delivered advantageous performances in comparison with several existing designs. They exerted selective, titratable, rapid and reversible response to drug induction. In addition, these designs were successfully adapted to an orthogonal Cas9. HIT systems developed in this study can be applied for controlled modulation of potentially any genomic loci in multiple modes.

Luo, X., et al. (2016). "Application of the genome editing tool CRISPR/Cas9 in non-human primates." Dongwuxue Yanjiu **37**(4): 214-219.

In the past three years, RNA-guided Cas9 nuclease from the microbial clustered regularly interspaced short palindromic repeats (CRISPR) adaptive immune system has been used to facilitate efficient genome editing in many model and non-model animals. However, its application in nonhuman primates is still at the early stage, though in view of the similarities in anatomy, physiology, behavior and genetics, closely related nonhuman primates serve as optimal models for human biology and disease studies. In this review, we summarize the current proceedings of gene editing using CRISPR/Cas9 in nonhuman primates.

Ma, S., et al. (2014). "CRISPR/Cas9 mediated multiplex genome editing and heritable mutagenesis of BmKu70 in Bombyx mori." Sci Rep **4**: 4489.

CRISPR/Cas9, a bacterial adaptive immune system derived genome-editing technique, has become to be one of the most compelling topics in biotechnology. Bombyx mori is an economically important insect and a model organism for studying lepidopteran and arthropod biology. Here we reported highly efficient and multiplex genome editing in B. mori cell line and heritable site-directed mutagenesis of Bmku70, which is required for NHEJ pathway and also related to antigen diversity, telomere length maintenance and subtelomeric gene silencing, using CRISPR/Cas9 system. We established a simple and practicable method and obtained several Bmku70 knockout B. mori lines, and showed that the frequency of HR was increased in embryos of the Bmku70 knockout B. mori. The mutant lines obtained in this study could be a candidate genetic resource for efficient knock-in and fundamental research of DNA repair in B. mori. We also provided a strategy and procedure to perform heritable genome editing of target genes with no significant phenotype effect.

Ma, X. and Y. G. Liu (2016). "CRISPR/Cas9-Based Multiplex Genome Editing in Monocot and Dicot Plants." Curr Protoc Mol Biol **115**: 31 36 31-31 36 21.

The clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-mediated genome targeting system has been applied to a variety of organisms, including plants. Compared to other genome-targeting technologies such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), the CRISPR/Cas9 system is easier to use and has much higher editing efficiency. In addition, multiple "single guide RNAs" (sgRNAs) with different target sequences can be designed to direct the Cas9 protein to multiple genomic sites for simultaneous multiplex editing. Here, we present a procedure for highly efficient multiplex genome targeting in monocot and dicot plants using a versatile and robust CRISPR/Cas9 vector system, emphasizing the construction of binary constructs with multiple sgRNA expression cassettes in one round of cloning using Golden Gate ligation. We also describe the genotyping of targeted mutations in transgenic plants by direct Sanger sequencing followed by decoding of superimposed sequencing chromatograms containing biallelic or heterozygous mutations using the Web-based tool DSDecode. (c) 2016 by John Wiley & Sons, Inc.

Ma, X., et al. (2015). "A Robust CRISPR/Cas9 System for Convenient, High-Efficiency Multiplex Genome Editing in Monocot and Dicot Plants." Mol Plant **8**(8): 1274-1284.

CRISPR/Cas9 genome targeting systems have been applied to a variety of species. However, most CRISPR/Cas9 systems reported for plants can only modify one or a few target sites. Here, we report a

robust CRISPR/Cas9 vector system, utilizing a plant codon optimized Cas9 gene, for convenient and high-efficiency multiplex genome editing in monocot and dicot plants. We designed PCR-based procedures to rapidly generate multiple sgRNA expression cassettes, which can be assembled into the binary CRISPR/Cas9 vectors in one round of cloning by Golden Gate ligation or Gibson Assembly. With this system, we edited 46 target sites in rice with an average 85.4% rate of mutation, mostly in biallelic and homozygous status. We reasoned that about 16% of the homozygous mutations in rice were generated through the non-homologous end-joining mechanism followed by homologous recombination-based repair. We also obtained uniform biallelic, heterozygous, homozygous, and chimeric mutations in Arabidopsis T1 plants. The targeted mutations in both rice and Arabidopsis were heritable. We provide examples of loss-of-function gene mutations in T0 rice and T1 Arabidopsis plants by simultaneous targeting of multiple (up to eight) members of a gene family, multiple genes in a biosynthetic pathway, or multiple sites in a single gene. This system has provided a versatile toolbox for studying functions of multiple genes and gene families in plants for basic research and genetic improvement.

Ma, X., et al. (2016). "CRISPR/Cas9 Platforms for Genome Editing in Plants: Developments and Applications." Mol Plant **9**(7): 961-974.

The clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein9 (Cas9) genome editing system (CRISPR/Cas9) is adapted from the prokaryotic type II adaptive immunity system. The CRISPR/Cas9 tool surpasses other programmable nucleases, such as ZFNs and TALENs, for its simplicity and high efficiency. Various plant-specific CRISPR/Cas9 vector systems have been established for adaption of this technology to many plant species. In this review, we present an overview of current advances on applications of this technology in plants, emphasizing general considerations for establishment of CRISPR/Cas9 vector platforms, strategies for multiplex editing, methods for analyzing the induced mutations, factors affecting editing efficiency and specificity, and features of the induced mutations and applications of the CRISPR/Cas9 system in plants. In addition, we provide a perspective on the challenges of CRISPR/Cas9 technology and its significance for basic plant research and crop genetic improvement.

Ma, X. L. and Y. G. Liu (2016). "[CRISPR/Cas9-based genome editing systems and the analysis of targeted genome mutations in plants]." Yi Chuan **38**(2): 118-125.

Targeted genomic editing technologies use programmable DNA nucleases to cleave genomic target sites, thus inducing targeted mutations in the genomes. The newly prevailed clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) system that consists of the Cas9 nuclease and single guide RNA (sgRNA) has the advantages of simplicity and high efficiency as compared to other programmable DNA nuclease systems such as zinc finger nucleases (ZFNs) and transcription activator like effector nucleases (TALENs). Currently, a number of cases have been reported on the application of the CRISPR/Cas9 genomic editing technology in plants. In this review, we summarize the strategies for preparing the Cas9 and sgRNA expression constructs, the transformation method for obtaining targeted mutations, the efficiency and features of the resulting mutations and the methods for detecting or genotyping of the mutation sites. We also discuss the existing problems and perspectives of CRISPR/Cas9-based genomic editing in plants.

Ma, Y., et al. (2016). "Increasing the efficiency of CRISPR/Cas9-mediated precise genome editing in rats by inhibiting NHEJ and using Cas9 protein." RNA Biol **13**(7): 605-612.

Precise modifications such as site mutation, codon replacement, insertion or precise targeted deletion are needed for studies of accurate gene function. The CRISPR/Cas9 system has been proved as a powerful tool to generate gene knockout and knockin animals. But the homologous recombination (HR)-directed precise genetic modification mediated by CRISPR/Cas9 is relatively lower compared with nonhomologous end-joining (NHEJ) pathway and extremely expected to be improved. Here, in this study 2 strategies were used to increase the precise genetic modification in rats. Scr7, a DNA ligase IV inhibitor, first identified as an anti-cancer compound, and considered as a potential NHEJ inhibitor, was used to increase the HR-mediated precise genetic modification. Meanwhile, the Cas9 protein instead of mRNA was used to save the mRNA to protein translation step to improve the precise modification efficiency. The Fabp2 and Dbndd1 loci were selected to knockin Cre and CreER (T2), respectively. Our result showed that both Scr7 and Cas9 protein can increase the precise modification.

Mabashi-Asazuma, H. and D. L. Jarvis (2017). "CRISPR-Cas9 vectors for genome editing and host engineering in the baculovirus-insect cell system." Proc Natl Acad Sci U S A **114**(34): 9068-9073.

The baculovirus-insect cell system (BICS) has been widely used to produce many different

recombinant proteins for basic research and is being used to produce several biologics approved for use in human or veterinary medicine. Early BICS were technically complex and constrained by the relatively primordial nature of insect cell protein glycosylation pathways. Since then, recombination has been used to modify baculovirus vectors-which has simplified the system-and transform insect cells, which has enhanced its protein glycosylation capabilities. Now, CRISPR-Cas9 tools for site-specific genome editing are needed to facilitate further improvements in the BICS. Thus, in this study, we used various insect U6 promoters to construct CRISPR-Cas9 vectors and assessed their utility for site-specific genome editing in two insect cell lines commonly used as hosts in the BICS. We demonstrate the use of CRISPR-Cas9 to edit an endogenous insect cell gene and alter protein glycosylation in the BICS.

Martinez, V., et al. (2017). "CRISPR/Cas9-based genome editing for simultaneous interference with gene expression and protein stability." Nucleic Acids Res **45**(20): e171.

Interference with genes is the foundation of reverse genetics and is key to manipulation of living cells for biomedical and biotechnological applications. However, classical genetic knockout and transcriptional knockdown technologies have different drawbacks and offer no control over existing protein levels. Here, we describe an efficient genome editing approach that affects specific protein abundances by changing the rates of both RNA synthesis and protein degradation, based on the two cross-kingdom control mechanisms CRISPRi and the N-end rule for protein stability. In addition, our approach demonstrates that CRISPRi efficiency is dependent on endogenous gene expression levels. The method has broad applications in e.g. study of essential genes and antibiotics discovery.

Marusugi, K., et al. (2016). "Functional validation of tensin2 SH2-PTB domain by CRISPR/Cas9-mediated genome editing." J Vet Med Sci **78**(9): 1413-1420.

Podocytes are terminally differentiated and highly specialized cells in the glomerulus, and they form a crucial component of the glomerular filtration barrier. The ICGN mouse is a model of glomerular dysfunction that shows gross morphological changes in the podocyte foot process, accompanied by proteinuria. Previously, we demonstrated that proteinuria in ICR-derived glomerulonephritis mouse ICGN mice might be caused by a deletion mutation in the tensin2 (Tns2) gene (designated Tns2(nph)). To test whether this mutation causes the mutant phenotype, we created knockout (KO) mice carrying a Tns2 protein deletion in the C-terminal Src homology and phosphotyrosine binding (SH2-PTB) domains (designated Tns2(DeltaC)) via CRISPR/Cas9-mediated genome editing. Tns2(nph)/Tns2(DeltaC) compound heterozygotes and Tns2(DeltaC)/Tns2(DeltaC) homozygous KO mice displayed podocyte abnormalities and massive proteinuria similar to ICGN mice, indicating that these two mutations are allelic. Further, this result suggests that the SH2-PTB domain of Tns2 is required for podocyte integrity. Tns2 knockdown in a mouse podocyte cell line significantly enhanced actin stress fiber formation and cell migration. Thus, this study provides evidence that alteration of actin remodeling resulting from Tns2 deficiency causes morphological changes in podocytes and subsequent proteinuria.

Meng, X., et al. (2017). "[CRISPR-Cas9 mediated genome editing in Caenorhabditis elegans]." Sheng Wu Gong Cheng Xue Bao **33**(10): 1693-1699.

The development of genome editing techniques based on CRISPR (Clustered regularly interspaced short palindromic repeats)-Cas9 system has revolutionized biomedical researches. It can be utilized to edit genome sequence in almost any organisms including Caenorhabditis elegans, one of the most convenient and classic genetic model animals. The application of CRISPR-Cas9 mediated genome editing in C. elegans promotes the functional analysis of gene and proteins under many physiological conditions. In this mini-review, we summarized the development of CRISPR-Cas9-based genome editing in C. elegans.

Minkenberg, B., et al. (2017). "CRISPR/Cas9-Enabled Multiplex Genome Editing and Its Application." Prog Mol Biol Transl Sci **149**: 111-132.

The CRISPR/Cas9 system is a prevalent and versatile genome-editing tool of choice for basic and applied biological research. An exchange of a 20-bp spacer sequence in the gRNA can easily reprogram Cas9 to target a different DNA site. By expressing or providing multiple gRNAs, the system also enables multiplex genome editing at high efficiencies. Current approaches for providing multiple gRNAs in vivo include the use of multigene cassettes to express several gRNAs, Csy4-based excision, arrays of crRNAs, ribozyme-flanked gRNAs, tRNA-dependent cleavage of gRNAs, and direct introduction of Cas9 proteins preloaded with different gRNAs. By simultaneously targeting multiple DNA sequences, multiplex genome editing can be used to knockout multiple genes or delete chromosomal fragments. Off-target risk can also be reduced by Cas9-dimers that require the simultaneous expression of two gRNAs. With multiple gRNAs, specific gene expression or methylation status can be efficiently controlled by

dCas9 fused to activators, repressors, methyltransferase, demethylase, or other functional domains. As a result, multiplex genome editing is expected to accelerate functional discovery of plant genes as well as genetic improvement of agricultural crops.

Morineau, C., et al. (2017). "Selective gene dosage by CRISPR-Cas9 genome editing in hexaploid Camelina sativa." Plant Biotechnol J **15**(6): 729-739.

In many plant species, gene dosage is an important cause of phenotype variation. Engineering gene dosage, particularly in polyploid genomes, would provide an efficient tool for plant breeding. The hexaploid oilseed crop Camelina sativa, which has three closely related expressed subgenomes, is an ideal species for investigation of the possibility of creating a large collection of combinatorial mutants. Selective, targeted mutagenesis of the three delta-12-desaturase (FAD2) genes was achieved by CRISPR-Cas9 gene editing, leading to reduced levels of polyunsaturated fatty acids and increased accumulation of oleic acid in the oil. Analysis of mutations over four generations demonstrated the presence of a large variety of heritable mutations in the three isologous CsFAD2 genes. The different combinations of single, double and triple mutants in the T3 generation were isolated, and the complete loss-of-function mutants revealed the importance of delta-12-desaturation for Camelina development. Combinatorial association of different alleles for the three FAD2 loci provided a large diversity of Camelina lines with various lipid profiles, ranging from 10% to 62% oleic acid accumulation in the oil. The different allelic combinations allowed an unbiased analysis of gene dosage and function in this hexaploid species, but also provided a unique source of genetic variability for plant breeding.

Mou, H., et al. (2017). "CRISPR/Cas9-mediated genome editing induces exon skipping by alternative splicing or exon deletion." Genome Biol **18**(1): 108.

CRISPR is widely used to disrupt gene function by inducing small insertions and deletions. Here, we show that some single-guide RNAs (sgRNAs) can induce exon skipping or large genomic deletions that delete exons. For example, CRISPR-mediated editing of beta-catenin exon 3, which encodes an autoinhibitory domain, induces partial skipping of the in-frame exon and nuclear accumulation of beta-catenin. A single sgRNA can induce small insertions or deletions that partially alter splicing or unexpected larger deletions that remove exons. Exon skipping adds to the unexpected outcomes that must be accounted for, and perhaps taken advantage of, in CRISPR experiments.

Muller, M., et al. (2016). "Streptococcus thermophilus CRISPR-Cas9 Systems Enable Specific Editing of the Human Genome." Mol Ther **24**(3): 636-644.

RNA-guided nucleases (RGNs) based on the type II CRISPR-Cas9 system of Streptococcus pyogenes (Sp) have been widely used for genome editing in experimental models. However, the nontrivial level of off-target activity reported in several human cells may hamper clinical translation. RGN specificity depends on both the guide RNA (gRNA) and the protospacer adjacent motif (PAM) recognized by the Cas9 protein. We hypothesized that more stringent PAM requirements reduce the occurrence of off-target mutagenesis. To test this postulation, we generated RGNs based on two Streptococcus thermophilus (St) Cas9 proteins, which recognize longer PAMs, and performed a side-by-side comparison of the three RGN systems targeted to matching sites in two endogenous human loci, PRKDC and CARD11. Our results demonstrate that in samples with comparable on-target cleavage activities, significantly lower off-target mutagenesis was detected using St-based RGNs as compared to the standard Sp-RGNs. Moreover, similarly to SpCas9, the StCas9 proteins accepted truncated gRNAs, suggesting that the specificities of St-based RGNs can be further improved. In conclusion, our results show that Cas9 proteins with longer or more restrictive PAM requirements provide a safe alternative to SpCas9-based RGNs and hence a valuable option for future human gene therapy applications.

Nagaraju, S., et al. (2016). "Genome editing of Clostridium autoethanogenum using CRISPR/Cas9." Biotechnol Biofuels **9**: 219.

BACKGROUND: Impactful greenhouse gas emissions abatement can now be achieved through gas fermentation using acetogenic microbes for the production of low-carbon fuels and chemicals. However, compared to traditional hosts like Escherichia coli or yeast, only basic genetic tools exist for gas-fermenting acetogens. To advance the process, a robust genetic engineering platform for acetogens is essential. RESULTS: In this study, we report scarless genome editing of an industrially used model acetogen, Clostridium autoethanogenum, using the CRISPR/Cas9 system. Initial efforts to retrofit the CRISPR/Cas9 system for C. autoethanogenum resulted in poor efficiency likely due to uncontrolled expression of Cas9. To address this, we constructed and screened a small library of tetracycline-inducible promoters that can also be used to fine-tune gene expression. With a new inducible promoter, the efficiency of CRISPR/Cas9-mediated desired gene deletion in C. autoethanogenum was improved to over

50 %, making it a viable tool for engineering C. autoethanogenum. CONCLUSIONS: Addition of both an inducible promoter library and a scarless genome editing tool is an important expansion to the genetic tool box of industrial C. autoethanogenum strain.

Neggers, J. E., et al. (2015). "Identifying drug-target selectivity of small-molecule CRM1/XPO1 inhibitors by CRISPR/Cas9 genome editing." Chem Biol **22**(1): 107-116.

Validation of drug-target interaction is essential in drug discovery and development. The ultimate proof for drug-target validation requires the introduction of mutations that confer resistance in cells, an approach that is not straightforward in mammalian cells. Using CRISPR/Cas9 genome editing, we show that a homozygous genomic C528S mutation in the XPO1 gene confers cells with resistance to selinexor (KPT-330). Selinexor is an orally bioavailable inhibitor of exportin-1 (CRM1/XPO1) with potent anticancer activity and is currently under evaluation in human clinical trials. Mutant cells were resistant to the induction of cytotoxicity, apoptosis, cell cycle arrest, and inhibition of XPO1 function, including direct binding of the drug to XPO1. These results validate XPO1 as the prime target of selinexor in cells and identify the selectivity of this drug toward the cysteine 528 residue of XPO1. Our findings demonstrate that CRISPR/Cas9 genome editing enables drug-target validation and drug-target selectivity studies in cancer cells.

Nguyen, T. H. and I. Anegon (2016). "Successful correction of hemophilia by CRISPR/Cas9 genome editing in vivo: delivery vector and immune responses are the key to success." EMBO Mol Med **8**(5): 439-441.

Nihongaki, Y., et al. (2015). "Photoactivatable CRISPR-Cas9 for optogenetic genome editing." Nat Biotechnol **33**(7): 755-760.

We describe an engineered photoactivatable Cas9 (paCas9) that enables optogenetic control of CRISPR-Cas9 genome editing in human cells. paCas9 consists of split Cas9 fragments and photoinducible dimerization domains named Magnets. In response to blue light irradiation, paCas9 expressed in human embryonic kidney 293T cells induces targeted genome sequence modifications through both nonhomologous end joining and homology-directed repair pathways. Genome editing activity can be switched off simply by extinguishing the light. We also demonstrate activation of paCas9 in spatial patterns determined by the sites of irradiation. Optogenetic control of targeted genome editing should facilitate improved understanding of complex gene networks and could prove useful in biomedical applications.

Nishitani, C., et al. (2016). "Efficient Genome Editing in Apple Using a CRISPR/Cas9 system." Sci Rep **6**: 31481.

Genome editing is a powerful technique for genome modification in molecular research and crop breeding, and has the great advantage of imparting novel desired traits to genetic resources. However, the genome editing of fruit tree plantlets remains to be established. In this study, we describe induction of a targeted gene mutation in the endogenous apple phytoene desaturase (PDS) gene using the CRISPR/Cas9 system. Four guide RNAs (gRNAs) were designed and stably transformed with Cas9 separately in apple. Clear and partial albino phenotypes were observed in 31.8% of regenerated plantlets for one gRNA, and bi-allelic mutations in apple PDS were confirmed by DNA sequencing. In addition, an 18-bp gRNA also induced a targeted mutation. These CRIPSR/Cas9 induced-mutations in the apple genome suggest activation of the NHEJ pathway, but with some involvement also of the HR pathway. Our results demonstrate that genome editing can be practically applied to modify the apple genome.

Noman, A., et al. (2016). "CRISPR-Cas9: Tool for Qualitative and Quantitative Plant Genome Editing." Front Plant Sci **7**: 1740.

Recent developments in genome editing techniques have aroused substantial excitement among agricultural scientists. These techniques offer new opportunities for developing improved plant lines with addition of important traits or removal of undesirable traits. Increased adoption of genome editing has been geared by swiftly developing Clustered regularly interspaced short palindromic repeats (CRISPR). This is appearing as driving force for innovative utilization in diverse branches of plant biology. CRISPR-Cas9 mediated genome editing is being used for rapid, easy and efficient alteration of genes among diverse plant species. With approximate completion of conceptual work about CRISPR-Cas9, plant scientists are applying this genome editing tool for crop attributes enhancement. The capability of this system for performing targeted and efficient modifications in genome sequence as well as gene expression will certainly spur novel developments not only in model plants but in crop and ornamental plants as well. Additionally, due to non-involvement of foreign DNA, this technique may help alleviating regulatory issues associated with genetically modified plants. We expect that prevailing challenges in plant science like genomic region manipulation, crop specific vectors etc. will be addressed along with sustained growth of

this genome editing tool. In this review, recent progress of CRISPR-Cas9 technology in plants has been summarized and discussed. We reviewed significance of CRISPR-Cas9 for specific and non-traditional aspects of plant life. It also covers strengths of this technique in comparison with other genome editing techniques, e.g., Zinc finger nucleases, Transcription activator-like effector nucleases and potential challenges in coming decades have been described.

Norton, E. L., et al. (2017). "Development of a CRISPR-Cas9 System for Efficient Genome Editing of Candida lusitaniae." mSphere **2**(3).

Candida lusitaniae is a member of the Candida clade that includes a diverse group of fungal species relevant to both human health and biotechnology. This species exhibits a full sexual cycle to undergo interconversion between haploid and diploid forms. C. lusitaniae is also an emerging opportunistic pathogen that can cause serious bloodstream infections in the clinic and yet has often proven to be refractory to facile genetic manipulations. In this work, we develop a clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated gene 9 (Cas9) system to enable genome editing of C. lusitaniae. We demonstrate that expression of CRISPR-Cas9 components under species-specific promoters is necessary for efficient gene targeting and can be successfully applied to multiple genes in both haploid and diploid isolates. Gene deletion efficiencies with CRISPR-Cas9 were further enhanced in C. lusitaniae strains lacking the established nonhomologous end joining (NHEJ) factors Ku70 and DNA ligase 4. These results indicate that NHEJ plays an important role in directing the repair of DNA double-strand breaks (DSBs) in C. lusitaniae and that removal of this pathway increases integration of gene deletion templates by homologous recombination. The described approaches significantly enhance the ability to perform genetic studies in, and promote understanding of, this emerging human pathogen and model sexual species. IMPORTANCE The ability to perform efficient genome editing is a key development for detailed mechanistic studies of a species. Candida lusitaniae is an important member of the Candida clade and is relevant both as an emerging human pathogen and as a model for understanding mechanisms of sexual reproduction. We highlight the development of a CRISPR-Cas9 system for efficient genome manipulation in C. lusitaniae and demonstrate the importance of species-specific promoters for expression of CRISPR components. We also demonstrate that the NHEJ pathway contributes to non-template-mediated repair of DNA DSBs and that removal of this pathway enhances efficiencies of gene targeting by CRISPR-Cas9. These results therefore establish important genetic tools for further exploration of C. lusitaniae biology.

Numamoto, M., et al. (2017). "Efficient genome editing by CRISPR/Cas9 with a tRNA-sgRNA fusion in the methylotrophic yeast Ogataea polymorpha." J Biosci Bioeng **124**(5): 487-492.

The methylotrophic yeast Ogataea polymorpha (syn. Hansenula polymorpha) is an attractive industrial non-conventional yeast showing high thermo-tolerance (up to 50 degrees C) and xylose assimilation. However, genetic manipulation of O. polymorpha is often laborious and time-consuming because it has lower homologous recombination efficiency relative to Saccharomyces cerevisiae. To overcome this disadvantage, we applied the CRISPR/Cas9 system as a powerful genome editing tool in O. polymorpha. In this system, both single guide RNA (sgRNA) and endonuclease Cas9 were expressed by a single autonomously-replicable plasmid and the sgRNA portion could be easily changed by using PCR and In-Fusion cloning techniques. Because the mutation efficiency of the CRISPR/Cas9 system was relatively low when the sgRNA was expressed under the control of the OpSNR6 promoter, the tRNA (CUG) gene was used for sgRNA expression. The editing efficiency of this system ranged from 17% to 71% of transformants in several target genes tested (ADE12, PHO1, PHO11, and PHO84). These findings indicate that genetic manipulation of O. polymorpha will be more convenient and accelerated by using this CRISPR/Cas9 system.

Odipio, J., et al. (2017). "Efficient CRISPR/Cas9 Genome Editing of Phytoene desaturase in Cassava." Front Plant Sci **8**: 1780.

CRISPR/Cas9 has become a powerful genome-editing tool for introducing genetic changes into crop species. In order to develop capacity for CRISPR/Cas9 technology in the tropical staple cassava (Manihot esculenta), the Phytoene desaturase (MePDS) gene was targeted in two cultivars using constructs carrying gRNAs targeting two sequences within MePDS exon 13. After Agrobacterium-mediated delivery of CRISPR/Cas9 reagents into cassava cells, both constructs induced visible albino phenotypes within cotyledon-stage somatic embryos regenerating on selection medium and the plants regenerated therefrom. A total of 58 (cv. 60444) and 25 (cv. TME 204) plant lines were recovered, of which 38 plant lines (19 from each cultivar) were analyzed for mutagenesis. The frequency of plant lines showing albino phenotype was high, ranging from 90 to 100% in cv. TME 204. Observed albino phenotypes were comprised of full albinos devoid of green tissue and

chimeras containing a mixture of white and green tissues. Sequence analysis revealed that 38/38 (100%) of the plant lines examined carried mutations at the targeted MePDS site, with insertions, deletions, and substitutions recorded. One putatively mono-allelic homozygous line (1/19) was found from cv. 60444, while 1 (1/19) and 4 (4/19) putatively bi-allelic homozygous lines were found in 60444 and TME204, respectively. The remaining plant lines, comprised mostly of the chimeras, were found to be putatively heterozygous. We observed minor (1 bp) nucleotide substitutions and or deletions upstream of the 5' and or downstream of the 3' targeted MePDS region. The data reported demonstrates that CRISPR/Cas9-mediated genome editing of cassava is highly efficient and relatively simple, generating multi-allelic mutations in both cultivars studied. Modification of MePDS described here generates visually detectable mutated events in a relatively short time frame of 6-8 weeks, and does not require sequencing to confirm editing at the target. It therefore provides a valuable platform to facilitate rapid assessment and optimization of CRISPR/Cas9 and other genome-editing technologies in cassava.

Oh-Hashi, K., et al. (2018). "Functional validation of ATF4 and GADD34 in Neuro2a cells by CRISPR/Cas9-mediated genome editing." Mol Cell Biochem **440**(1-2): 65-75.

Activating transcription factor 4 (ATF4), which is ubiquitously expressed, plays a crucial role in regulating various stress-responsive genes under pathophysiological conditions. Further, growth arrest and DNA damage-inducible gene 34 (GADD34), a downstream target of ATF4, has been reported to negatively regulate ATF4 expression. To understand the relationship between intrinsic ATF4 and GADD34 under resting and ER stress conditions, we used a novel gene editing approach, CRISPR/Cas9, to integrate antibiotic-resistant genes into the target genes, ATF4 and GADD34. First, we manipulated the ATF4 gene in the mouse neuroblastoma cell line, Neuro2a, and compared the ER stress responses between parental and ATF4-edited Neuro2a cells. Next, we established Neuro2a cells with edited GADD34 and ATF4/GADD34 genes and found that ATF4 acts as a proapoptotic factor, but GADD34 depletion did not attenuate the expression of cleaved caspase-3 induced by tunicamycin treatment. These findings provide new insights into the ATF4 signaling cascades. Additionally, the rapid establishment of cells lacking multiple genes using this CRISPR/Cas9 system will be a powerful tool for exploring various cellular issues under pathophysiological conditions.

Ohmori, T., et al. (2017). "CRISPR/Cas9-mediated genome editing via postnatal administration of AAV vector cures haemophilia B mice." Sci Rep **7**(1): 4159.

Haemophilia B, a congenital haemorrhagic disease caused by mutations in coagulation factor IX gene (F9), is considered an appropriate target for genome editing technology. Here, we describe treatment strategies for haemophilia B mice using the clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 system. Administration of adeno-associated virus (AAV) 8 vector harbouring Staphylococcus aureus Cas9 (SaCas9) and single guide RNA (sgRNA) to wild-type adult mice induced a double-strand break (DSB) at the target site of F9 in hepatocytes, sufficiently developing haemophilia B. Mutation-specific gene editing by simultaneous induction of homology-directed repair (HDR) sufficiently increased FIX levels to correct the disease phenotype. Insertion of F9 cDNA into the intron more efficiently restored haemostasis via both processes of non-homologous end-joining (NHEJ) and HDR following DSB. Notably, these therapies also cured neonate mice with haemophilia, which cannot be achieved with conventional gene therapy with AAV vector. Ongoing haemophilia therapy targeting the antithrombin gene with antisense oligonucleotide could be replaced by SaCas9/sgRNA-expressing AAV8 vector. Our results suggest that CRISPR/Cas9-mediated genome editing using an AAV8 vector provides a flexible approach to induce DSB at target genes in hepatocytes and could be a good strategy for haemophilia gene therapy.

Oji, A., et al. (2016). "CRISPR/Cas9 mediated genome editing in ES cells and its application for chimeric analysis in mice." Sci Rep **6**: 31666.

Targeted gene disrupted mice can be efficiently generated by expressing a single guide RNA (sgRNA)/CAS9 complex in the zygote. However, the limited success of complicated genome editing, such as large deletions, point mutations, and knockins, remains to be improved. Further, the mosaicism in founder generations complicates the genotypic and phenotypic analyses in these animals. Here we show that large deletions with two sgRNAs as well as dsDNA-mediated point mutations are efficient in mouse embryonic stem cells (ESCs). The dsDNA-mediated gene knockins are also feasible in ESCs. Finally, we generated chimeric mice with biallelic mutant ESCs for a lethal gene, Dnajb13, and analyzed their phenotypes. Not only was the lethal phenotype of hydrocephalus suppressed, but we also found that Dnajb13 is required for sperm cilia formation. The combination of biallelic genome editing in ESCs and

subsequent chimeric analysis provides a useful tool for rapid gene function analysis in the whole organism.

Ou, Z. and X. Sun (2016). "[Application of CRISPR-Cas9 genome editing for constructing animal models of human diseases]." Zhonghua Yi Xue Yi Chuan Xue Za Zhi **33**(4): 559-563.

The CRISPR-Cas9 system is a new targeted nuclease for genome editing, which can directly introduce modifications at the targeted genomic locus. The system utilizes a short single guide RNA (sgRNA) to direct the endonuclease Cas9 in the genome. Upon targeting, Cas9 can generate DNA double-strand breaks (DSBs). As such DSBs are repaired by non-homologous end joining (NHEJ) or homology directed repair (HDR), therefore facilitates introduction of random or specific mutations, repair of endogenous mutations, or insertion of DNA elements. The system has been successfully used to generate gene targeted cell lines including those of human, animals and plants. This article reviews recent advances made in this rapidly evolving technique for the generation of animal models for human diseases.

Ousterout, D. G., et al. (2015). "Multiplex CRISPR/Cas9-based genome editing for correction of dystrophin mutations that cause Duchenne muscular dystrophy." Nat Commun **6**: 6244.

The CRISPR/Cas9 genome-editing platform is a promising technology to correct the genetic basis of hereditary diseases. The versatility, efficiency and multiplexing capabilities of the CRISPR/Cas9 system enable a variety of otherwise challenging gene correction strategies. Here, we use the CRISPR/Cas9 system to restore the expression of the dystrophin gene in cells carrying dystrophin mutations that cause Duchenne muscular dystrophy (DMD). We design single or multiplexed sgRNAs to restore the dystrophin reading frame by targeting the mutational hotspot at exons 45-55 and introducing shifts within exons or deleting one or more exons. Following gene editing in DMD patient myoblasts, dystrophin expression is restored in vitro. Human dystrophin is also detected in vivo after transplantation of genetically corrected patient cells into immunodeficient mice. Importantly, the unique multiplex gene-editing capabilities of the CRISPR/Cas9 system facilitate the generation of a single large deletion that can correct up to 62% of DMD mutations.

Ozaki, A., et al. (2017). "Metabolic engineering of Schizosaccharomyces pombe via CRISPR-Cas9 genome editing for lactic acid production from glucose and cellobiose." Metab Eng Commun **5**: 60-67.

Modification of the Schizosaccharomyces pombe genome is often laborious, time consuming due to the lower efficiency of homologous recombination. Here, we constructed metabolically engineered S. pombe strains using a CRISPR-Cas9 system and also demonstrated D-lactic acid (D-LA) production from glucose and cellobiose. Genes encoding two separate pyruvate decarboxylases (PDCs), an L-lactic acid dehydrogenase (L-LDH), and a minor alcohol dehydrogenase (SPBC337.11) were disrupted, thereby attenuating ethanol production. To increase the cellular supply of acetyl-CoA, an important metabolite for growth, we introduced genes encoding bacterial acetylating acetaldehyde dehydrogenase enzymes (Escherichia coli MhpF and EutE). D-LA production by the resulting strain was achieved by expressing a Lactobacillus plantarum gene encoding D-lactate dehydrogenase. The engineered strain efficiently consumed glucose and produced D-LA at 25.2 g/L from 35.5 g/L of consumed glucose with a yield of 0.71 g D-LA / g glucose. We further modified this strain by expressing beta-glucosidase by cell surface display; the resulting strain produced D-LA at 24.4 g/L from 30 g/L of cellobiose in minimal medium, with a yield of 0.68 g D-LA / g glucose. To our knowledge, this study represents the first report of a S. pombe strain that was metabolically engineered using a CRISPR-Cas9 system, and demonstrates the possibility of engineering S. pombe for the production of value-added chemicals.

Paix, A., et al. (2015). "High Efficiency, Homology-Directed Genome Editing in Caenorhabditis elegans Using CRISPR-Cas9 Ribonucleoprotein Complexes." Genetics **201**(1): 47-54.

Homology-directed repair (HDR) of breaks induced by the RNA-programmed nuclease Cas9 has become a popular method for genome editing in several organisms. Most HDR protocols rely on plasmid-based expression of Cas9 and the gene-specific guide RNAs. Here we report that direct injection of in vitro-assembled Cas9-CRISPR RNA (crRNA) trans-activating crRNA (tracrRNA) ribonucleoprotein complexes into the gonad of Caenorhabditis elegans yields HDR edits at a high frequency. Building on our earlier finding that PCR fragments with 35-base homology are efficient repair templates, we developed an entirely cloning-free protocol for the generation of seamless HDR edits without selection. Combined with the co-CRISPR method, this protocol is sufficiently robust for use with low-efficiency guide RNAs and to generate complex edits, including ORF replacement and simultaneous tagging of two genes with fluorescent proteins.

Paix, A., et al. (2017). "Precision genome editing using CRISPR-Cas9 and linear repair templates in C. elegans." Methods **121-122**: 86-93.

The ability to introduce targeted edits in the genome of model organisms is revolutionizing the field of genetics. State-of-the-art methods for precision genome editing use RNA-guided endonucleases to create double-strand breaks (DSBs) and DNA templates containing the edits to repair the DSBs. Following this strategy, we have developed a protocol to create precise edits in the C. elegans genome. The protocol takes advantage of two innovations to improve editing efficiency: direct injection of CRISPR-Cas9 ribonucleoprotein complexes and use of linear DNAs with short homology arms as repair templates. The protocol requires no cloning or selection, and can be used to generate base and gene-size edits in just 4days. Point mutations, insertions, deletions and gene replacements can all be created using the same experimental pipeline.

Pankowicz, F. P., et al. (2016). "Reprogramming metabolic pathways in vivo with CRISPR/Cas9 genome editing to treat hereditary tyrosinaemia." Nat Commun **7**: 12642.

Many metabolic liver disorders are refractory to drug therapy and require orthotopic liver transplantation. Here we demonstrate a new strategy, which we call metabolic pathway reprogramming, to treat hereditary tyrosinaemia type I in mice; rather than edit the disease-causing gene, we delete a gene in a disease-associated pathway to render the phenotype benign. Using CRISPR/Cas9 in vivo, we convert hepatocytes from tyrosinaemia type I into the benign tyrosinaemia type III by deleting Hpd (hydroxyphenylpyruvate dioxigenase). Edited hepatocytes (Fah (-/-)/Hpd (-/-)) display a growth advantage over non-edited hepatocytes (Fah (-/-)/Hpd (+/+)) and, in some mice, almost completely replace them within 8 weeks. Hpd excision successfully reroutes tyrosine catabolism, leaving treated mice healthy and asymptomatic. Metabolic pathway reprogramming sidesteps potential difficulties associated with editing a critical disease-causing gene and can be explored as an option for treating other diseases.

Park, M. Y., et al. (2017). "Generation of lung cancer cell lines harboring EGFR T790M mutation by CRISPR/Cas9-mediated genome editing." Oncotarget **8**(22): 36331-36338.

Tyrosine kinase inhibitors (TKIs) such as gefitinib and erlotinib are effective against lung adenocarcinomas harboring epidermal growth factor receptor (EGFR) mutations. However, cancer cells can develop resistance to these agents with prolonged exposure; in over 50% of cases, this is attributable to the EGFR T790M mutation. Moreover, additional resistance mutations can arise with the use of new drugs. Cancer cell lines with specific mutations can enable the study of resistance mechanisms. In this study, we introduced the EGFR T790M mutation into the PC9 human lung cancer cell line-which has a deletion in exon 19 of the EGFR gene-by clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas)9-mediated genome editing. EGFR pyrosequencing and peptide nucleic acid clamping revealed that PC9 cells with EGFR T790M generated by CRISPR/Cas 9 had a higher T790M mutation rate than those with the same mutation generated by long-term exposure to gefitinib (PC9-G); moreover, resistance to gefitinib in these clones was higher than that in PC9-G cells. The clones were also highly sensitive to the 3rd-generation EGFR TKI AZD9291, which is cytotoxic to lung cancer cells with EGFR T790M. The CRISPR/Cas9 programmable nuclease system can be used to generate various cancer cell lines with specific mutations that can facilitate studies on resistance mechanisms and drug efficacy.

Paul, J. W., 3rd and Y. Qi (2016). "CRISPR/Cas9 for plant genome editing: accomplishments, problems and prospects." Plant Cell Rep **35**(7): 1417-1427.

The increasing burden of the world population on agriculture requires the development of more robust crops. Dissecting the basic biology that underlies plant development and stress responses will inform the design of better crops. One powerful tool for studying plants at the molecular level is the RNA-programmed genome editing system composed of a clustered regularly interspaced short palindromic repeats (CRISPR)-encoded guide RNA and the nuclease Cas9. Here, some of the recent advances in CRISPR/Cas9 technology that have profound implications for improving the study of plant biology are described. These tools are also paving the way towards new horizons for biotechnologies and crop development.

Pellagatti, A., et al. (2015). "Application of CRISPR/Cas9 genome editing to the study and treatment of disease." Arch Toxicol **89**(7): 1023-1034.

CRISPR/Cas is a microbial adaptive immune system that uses RNA-guided nucleases to cleave foreign genetic elements. The CRISPR/Cas9 method has been engineered from the type II prokaryotic CRISPR system and uses a single-guide RNA to target the Cas9 nuclease to a specific genomic sequence. Cas9 induces double-stranded DNA breaks which are repaired either by imperfect non-homologous end joining to generate insertions or deletions (indels) or, if a repair template is provided, by homology-directed

repair. Due to its specificity, simplicity and versatility, the CRISPR/Cas9 system has recently emerged as a powerful tool for genome engineering in various species. This technology can be used to investigate the function of a gene of interest or to correct gene mutations in cells via genome editing, paving the way for future gene therapy approaches. Improvements to the efficiency of CRISPR repair, in particular to increase the rate of gene correction and to reduce undesired off-target effects, and the development of more effective delivery methods will be required for its broad therapeutic application.

Peng, C., et al. (2015). "CRISPR/Cas9-based tools for targeted genome editing and replication control of HBV." Virol Sin **30**(5): 317-325.

Hepatitis B virus (HBV) infection remains a major global health problem because current therapies rarely eliminate HBV infections to achieve a complete cure. A different treatment paradigm to effectively clear HBV infection and eradicate latent viral reservoirs is urgently required. In recent years, the development of a new RNA-guided gene-editing tool, the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated nuclease 9) system, has greatly facilitated site-specific mutagenesis and represents a very promising potential therapeutic tool for diseases, including for eradication of invasive pathogens such as HBV. Here, we review recent advances in the use of CRISPR/Cas9, which is designed to target HBV specific DNA sequences to inhibit HBV replication and to induce viral genome mutation, in cell lines or animal models. Advantages, limitations and possible solutions, and proposed directions for future research are discussed to highlight the opportunities and challenges of CRISPR/Cas9 as a new, potentially curative therapy for chronic hepatitis B infection.

Peng, R., et al. (2016). "Potential pitfalls of CRISPR/Cas9-mediated genome editing." FEBS J **283**(7): 1218-1231.

Recently, a novel technique named the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas)9 system has been rapidly developed. This genome editing tool has improved our ability tremendously with respect to exploring the pathogenesis of diseases and correcting disease mutations, as well as phenotypes. With a short guide RNA, Cas9 can be precisely directed to target sites, and functions as an endonuclease to efficiently produce breaks in DNA double strands. Over the past 30 years, CRISPR has evolved from the 'curious sequences of unknown biological function' into a promising genome editing tool. As a result of the incessant development in the CRISPR/Cas9 system, Cas9 co-expressed with custom guide RNAs has been successfully used in a variety of cells and organisms. This genome editing technology can also be applied to synthetic biology, functional genomic screening, transcriptional modulation and gene therapy. However, although CRISPR/Cas9 has a broad range of action in science, there are several aspects that affect its efficiency and specificity, including Cas9 activity, target site selection and short guide RNA design, delivery methods, off-target effects and the incidence of homology-directed repair. In the present review, we highlight the factors that affect the utilization of CRISPR/Cas9, as well as possible strategies for handling any problems. Addressing these issues will allow us to take better advantage of this technique. In addition, we also review the history and rapid development of the CRISPR/Cas system from the time of its initial discovery in 2012.

Platt, R. J., et al. (2014). "CRISPR-Cas9 knockin mice for genome editing and cancer modeling." Cell **159**(2): 440-455.

CRISPR-Cas9 is a versatile genome editing technology for studying the functions of genetic elements. To broadly enable the application of Cas9 in vivo, we established a Cre-dependent Cas9 knockin mouse. We demonstrated in vivo as well as ex vivo genome editing using adeno-associated virus (AAV)-, lentivirus-, or particle-mediated delivery of guide RNA in neurons, immune cells, and endothelial cells. Using these mice, we simultaneously modeled the dynamics of KRAS, p53, and LKB1, the top three significantly mutated genes in lung adenocarcinoma. Delivery of a single AAV vector in the lung generated loss-of-function mutations in p53 and Lkb1, as well as homology-directed repair-mediated Kras (G12D) mutations, leading to macroscopic tumors of adenocarcinoma pathology. Together, these results suggest that Cas9 mice empower a wide range of biological and disease modeling applications.

Pohl, C., et al. (2016). "CRISPR/Cas9 Based Genome Editing of Penicillium chrysogenum." ACS Synth Biol **5**(7): 754-764.

CRISPR/Cas9 based systems have emerged as versatile platforms for precision genome editing in a wide range of organisms. Here we have developed powerful CRISPR/Cas9 tools for marker-based and marker-free genome modifications in Penicillium chrysogenum, a model filamentous fungus and industrially relevant cell factory. The developed CRISPR/Cas9 toolbox is highly flexible and allows editing of new targets with minimal cloning efforts. The Cas9 protein and the sgRNA can be either delivered during transformation, as preassembled CRISPR-Cas9 ribonucleoproteins (RNPs) or expressed

from an AMA1 based plasmid within the cell. The direct delivery of the Cas9 protein with in vitro synthesized sgRNA to the cells allows for a transient method for genome engineering that may rapidly be applicable for other filamentous fungi. The expression of Cas9 from an AMA1 based vector was shown to be highly efficient for marker-free gene deletions.

Prykhozhij, S. V., et al. (2016). "A Guide to Computational Tools and Design Strategies for Genome Editing Experiments in Zebrafish Using CRISPR/Cas9." Zebrafish **13**(1): 70-73.

The development of clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology for mainstream biotechnological use based on its discovery as an adaptive immune mechanism in bacteria has dramatically improved the ability of molecular biologists to modify genomes of model organisms. The zebrafish is highly amenable to applications of CRISPR/Cas9 for mutation generation and a variety of DNA insertions. Cas9 protein in complex with a guide RNA molecule recognizes where to cut the homologous DNA based on a short stretch of DNA termed the protospacer-adjacent motif (PAM). Rapid and efficient identification of target sites immediately preceding PAM sites, quantification of genomic occurrences of similar (off target) sites and predictions of cutting efficiency are some of the features where computational tools play critical roles in CRISPR/Cas9 applications. Given the rapid advent and development of this technology, it can be a challenge for researchers to remain up to date with all of the important technological developments in this field. We have contributed to the armamentarium of CRISPR/Cas9 bioinformatics tools and trained other researchers in the use of appropriate computational programs to develop suitable experimental strategies. Here we provide an in-depth guide on how to use CRISPR/Cas9 and other relevant computational tools at each step of a host of genome editing experimental strategies. We also provide detailed conceptual outlines of the steps involved in the design and execution of CRISPR/Cas9-based experimental strategies, such as generation of frameshift mutations, larger chromosomal deletions and inversions, homology-independent insertion of gene cassettes and homology-based knock-in of defined point mutations and larger gene constructs.

Qin, W., et al. (2015). "Efficient CRISPR/Cas9-Mediated Genome Editing in Mice by Zygote Electroporation of Nuclease." Genetics **200**(2): 423-430.

The clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas) system is an adaptive immune system in bacteria and archaea that has recently been exploited for genome engineering. Mutant mice can be generated in one step through direct delivery of the CRISPR/Cas9 components into a mouse zygote. Although the technology is robust, delivery remains a bottleneck, as it involves manual injection of the components into the pronuclei or the cytoplasm of mouse zygotes, which is technically demanding and inherently low throughput. To overcome this limitation, we employed electroporation as a means to deliver the CRISPR/Cas9 components, including Cas9 messenger RNA, single-guide RNA, and donor oligonucleotide, into mouse zygotes and recovered live mice with targeted nonhomologous end joining and homology-directed repair mutations with high efficiency. Our results demonstrate that mice carrying CRISPR/Cas9-mediated targeted mutations can be obtained with high efficiency by zygote electroporation.

Quetier, F. (2016). "The CRISPR-Cas9 technology: Closer to the ultimate toolkit for targeted genome editing." Plant Sci **242**: 65-76.

The first period of plant genome editing was based on Agrobacterium; chemical mutagenesis by EMS (ethyl methanesulfonate) and ionizing radiations; each of these technologies led to randomly distributed genome modifications. The second period is associated with the discoveries of homing and meganuclease enzymes during the 80s and 90s, which were then engineered to provide efficient tools for targeted editing. From 2006 to 2012, a few crop plants were successfully and precisely modified using zinc-finger nucleases. A third wave of improvement in genome editing, which led to a dramatic decrease in off-target events, was achieved in 2009-2011 with the TALEN technology. The latest revolution surfaced in 2013 with the CRISPR-Cas9 system, whose high efficiency and technical ease of use is really impressive; scientists can use in-house kits or commercially available kits; the only two requirements are to carefully choose the location of the DNA double strand breaks to be induced and then to order an oligonucleotide. While this close-to- ultimate toolkit for targeted editing of genomes represents dramatic scientific progress which allows the development of more complex useful agronomic traits through synthetic biology, the social acceptance of genome editing remains regularly questioned by anti-GMO citizens and organizations.

Rahdar, M., et al. (2015). "Synthetic CRISPR RNA-Cas9-guided genome editing in human cells." Proc Natl Acad Sci U S A **112**(51): E7110-7117.

Genome editing with the clustered, regularly interspaced, short palindromic repeats (CRISPR)-Cas9

nuclease system is a powerful technology for manipulating genomes, including introduction of gene disruptions or corrections. Here we develop a chemically modified, 29-nucleotide synthetic CRISPR RNA (scrRNA), which in combination with unmodified transactivating crRNA (tracrRNA) is shown to functionally replace the natural guide RNA in the CRISPR-Cas9 nuclease system and to mediate efficient genome editing in human cells. Incorporation of rational chemical modifications known to protect against nuclease digestion and stabilize RNA-RNA interactions in the tracrRNA hybridization region of CRISPR RNA (crRNA) yields a scrRNA with enhanced activity compared with the unmodified crRNA and comparable gene disruption activity to the previously published single guide RNA. Taken together, these findings provide a platform for therapeutic applications, especially for nervous system disease, using successive application of cell-permeable, synthetic CRISPR RNAs to activate and then silence Cas9 nuclease activity.

Ran, F. A., et al. (2013). "Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity." Cell **154**(6): 1380-1389.

Targeted genome editing technologies have enabled a broad range of research and medical applications. The Cas9 nuclease from the microbial CRISPR-Cas system is targeted to specific genomic loci by a 20 nt guide sequence, which can tolerate certain mismatches to the DNA target and thereby promote undesired off-target mutagenesis. Here, we describe an approach that combines a Cas9 nickase mutant with paired guide RNAs to introduce targeted double-strand breaks. Because individual nicks in the genome are repaired with high fidelity, simultaneous nicking via appropriately offset guide RNAs is required for double-stranded breaks and extends the number of specifically recognized bases for target cleavage. We demonstrate that using paired nicking can reduce off-target activity by 50- to 1,500-fold in cell lines and to facilitate gene knockout in mouse zygotes without sacrificing on-target cleavage efficiency. This versatile strategy enables a wide variety of genome editing applications that require high specificity.

Ren, X., et al. (2017). "Genome editing in Drosophila melanogaster: from basic genome engineering to the multipurpose CRISPR-Cas9 system." Sci China Life Sci **60**(5): 476-489.

Nowadays, genome editing tools are indispensable for studying gene function in order to increase our knowledge of biochemical processes and disease mechanisms. The extensive availability of mutagenesis and transgenesis tools make Drosophila melanogaster an excellent model organism for geneticists. Early mutagenesis tools relied on chemical or physical methods, ethyl methane sulfonate (EMS) and X-rays respectively, to randomly alter DNA at a nucleotide or chromosomal level. Since the discovery of transposable elements and the availability of the complete fly genome, specific genome editing tools, such as P-elements, zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), have undergone rapid development. Currently, one of the leading and most effective contemporary tools is the CRISPR-cas9 system made popular because of its low cost, effectiveness, specificity and simplicity of use. This review briefly addresses the most commonly used mutagenesis and transgenesis tools in Drosophila, followed by an in-depth review of the multipurpose CRISPR-Cas9 system and its current applications.

Renaud, J. B., et al. (2016). "Improved Genome Editing Efficiency and Flexibility Using Modified Oligonucleotides with TALEN and CRISPR-Cas9 Nucleases." Cell Rep **14**(9): 2263-2272.

Genome editing has now been reported in many systems using TALEN and CRISPR-Cas9 nucleases. Precise mutations can be introduced during homology-directed repair with donor DNA carrying the wanted sequence edit, but efficiency is usually lower than for gene knockout and optimal strategies have not been extensively investigated. Here, we show that using phosphorothioate-modified oligonucleotides strongly enhances genome editing efficiency of single-stranded oligonucleotide donors in cultured cells. In addition, it provides better design flexibility, allowing insertions more than 100 bp long. Despite previous reports of phosphorothioate-modified oligonucleotide toxicity, clones of edited cells are readily isolated and targeted sequence insertions are achieved in rats and mice with very high frequency, allowing for homozygous loxP site insertion at the mouse ROSA locus in particular. Finally, when detected, imprecise knockin events exhibit indels that are asymmetrically positioned, consistent with genome editing taking place by two steps of single-strand annealing.

Ringer, K. P., et al. (2018). "Comparative Analysis of Lipid-Mediated CRISPR-Cas9 Genome Editing Techniques." Cell Biol Int.

CRISPR-Cas technology has revolutionized genome engineering. While Cas9 was not the first programmable endonuclease identified, its simplicity of use has driven widespread adoption in a short period of time. While CRISPR-Cas genome editing holds enormous potential for clinical applications, its use in laboratory settings for genotype-phenotype studies and genome-wide screens has led to

breakthroughs in the understanding of many molecular pathways. Numerous protocols have been described for introducing CRISPR-Cas components into cells, and here we sought to simplify and optimize a protocol for genome editing using readily available and inexpensive tools. We compared plasmid, ribonucleoprotein (RNP), and RNA transfection to determine which was method was most optimal for editing cells in a laboratory setting. We limited our comparison to lipofection-mediated introduction because the reagents are widely available. To facilitate optimization, we developed a novel reporter assay to measure gene disruption and the introduction of a variety of exogenous DNA tags. Each method efficiently disrupted endogenous genes and was able to stimulate the introduction of foreign DNA at specific sites, albeit to varying efficiencies. RNP transfection produced the highest level of gene disruption and was the most rapid and efficient method overall. Finally, we show that very short homology arms of 30 base pairs can mediate site-specific editing. The methods described here should broaden the accessibility of RNP-mediated lipofection for laboratory genome-editing experiments.

Rodriguez-Lopez, M., et al. (2016). "A CRISPR/Cas9-based method and primer design tool for seamless genome editing in fission yeast." Wellcome Open Res **1**: 19.

In the fission yeast Schizosaccharomyces pombe the prevailing approach for gene manipulations is based on homologous recombination of a PCR product that contains genomic target sequences and a selectable marker. The CRISPR/Cas9 system has recently been implemented in fission yeast, which allows for seamless genome editing without integration of a selection marker or leaving any other genomic 'scars'. The published method involves manual design of the single guide RNA (sgRNA), and digestion of a large plasmid with a problematic restriction enzyme to clone the sgRNA. To increase the efficiency of this approach, we have established and optimized a PCR-based system to clone the sgRNA without restriction enzymes into a plasmid with a dominant natMX6 (nourseothricin) selection marker. We also provide a web-tool, CRISPR4P, to support the design of the sgRNAs and the primers required for the entire process of seamless DNA deletion. Moreover, we report the preparation of G1-synchronized and cryopreserved S. pombe cells, which greatly increases the efficiency and speed for transformations, and may also facilitate standard gene manipulations. Applying this optimized CRISPR/Cas9-based approach, we have successfully deleted over 80 different non-coding RNA genes, which are generally lowly expressed, and have inserted 7 point mutations in 4 different genomic regions.

Ronda, C., et al. (2014). "Accelerating genome editing in CHO cells using CRISPR Cas9 and CRISPy, a web-based target finding tool." Biotechnol Bioeng **111**(8): 1604-1616.

Chinese hamster ovary (CHO) cells are widely used in the biopharmaceutical industry as a host for the production of complex pharmaceutical proteins. Thus genome engineering of CHO cells for improved product quality and yield is of great interest. Here, we demonstrate for the first time the efficacy of the CRISPR Cas9 technology in CHO cells by generating site-specific gene disruptions in COSMC and FUT8, both of which encode proteins involved in glycosylation. The tested single guide RNAs (sgRNAs) created an indel frequency up to 47.3% in COSMC, while an indel frequency up to 99.7% in FUT8 was achieved by applying lectin selection. All eight sgRNAs examined in this study resulted in relatively high indel frequencies, demonstrating that the Cas9 system is a robust and efficient genome-editing methodology in CHO cells. Deep sequencing revealed that 85% of the indels created by Cas9 resulted in frameshift mutations at the target sites, with a strong preference for single base indels. Finally, we have developed a user-friendly bioinformatics tool, named "CRISPy" for rapid identification of sgRNA target sequences in the CHO-K1 genome. The CRISPy tool identified 1,970,449 CRISPR targets divided into 27,553 genes and lists the number of off-target sites in the genome. In conclusion, the proven functionality of Cas9 to edit CHO genomes combined with our CRISPy database have the potential to accelerate genome editing and synthetic biology efforts in CHO cells.

Roper, J., et al. (2018). "Colonoscopy-based colorectal cancer modeling in mice with CRISPR-Cas9 genome editing and organoid transplantation." Nat Protoc **13**(2): 217-234.

Most genetically engineered mouse models (GEMMs) of colorectal cancer are limited by tumor formation in the small intestine, a high tumor burden that limits metastasis, and the need to generate and cross mutant mice. Cell line or organoid transplantation models generally produce tumors in ectopic locations-such as the subcutaneous space, kidney capsule, or cecal wall-that do not reflect the native stromal environment of the colon mucosa. Here, we describe detailed protocols to rapidly and efficiently induce site-directed tumors in the distal colon of mice that are based on colonoscopy-guided mucosal injection. These techniques can be adapted to deliver viral vectors carrying Cre recombinase,

CRISPR-Cas9 components, CRISPR-engineered mouse tumor organoids, or human cancer organoids to mice to model the adenoma-carcinoma-metastasis sequence of tumor progression. The colonoscopy injection procedure takes approximately 15 min, including preparation. In our experience, anyone with reasonable hand-eye coordination can become proficient with mouse colonoscopy and mucosal injection with a few hours of practice. These approaches are ideal for a wide range of applications, including assessment of gene function in tumorigenesis, examination of tumor-stroma interactions, studies of cancer metastasis, and translational research with patient-derived cancers.

Ruan, G. X., et al. (2017). "CRISPR/Cas9-Mediated Genome Editing as a Therapeutic Approach for Leber Congenital Amaurosis 10." Mol Ther **25**(2): 331-341.

As the most common subtype of Leber congenital amaurosis (LCA), LCA10 is a severe retinal dystrophy caused by mutations in the CEP290 gene. The most frequent mutation found in patients with LCA10 is a deep intronic mutation in CEP290 that generates a cryptic splice donor site. The large size of the CEP290 gene prevents its use in adeno-associated virus (AAV)-mediated gene augmentation therapy. Here, we show that targeted genomic deletion using the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system represents a promising therapeutic approach for the treatment of patients with LCA10 bearing the CEP290 splice mutation. We generated a cellular model of LCA10 by introducing the CEP290 splice mutation into 293FT cells and we showed that guide RNA pairs coupled with SpCas9 were highly efficient at removing the intronic splice mutation and restoring the expression of wild-type CEP290. In addition, we demonstrated that a dual AAV system could effectively delete an intronic fragment of the Cep290 gene in the mouse retina. To minimize the immune response to prolonged expression of SpCas9, we developed a self-limiting CRISPR/Cas9 system that minimizes the duration of SpCas9 expression. These results support further studies to determine the therapeutic potential of CRISPR/Cas9-based strategies for the treatment of patients with LCA10.

Ryder, P., et al. (2017). "Generation of stable nulliplex autopolyploid lines of Arabidopsis thaliana using CRISPR/Cas9 genome editing." Plant Cell Rep **36**(6): 1005-1008.

RNA-guided endonuclease-mediated targeted mutagenesis using the clustered regularly interspersed short palindromic repeats (CRISPR)/Cas9 system has been successful at targeting specific loci for modification in plants. While polyploidy is an evolutionary mechanism enabling plant adaptation, the analysis of gene function in polyploid plants has been limited due to challenges associated with generating polyploid knockout mutants for all gene copies in polyploid plant lines. This study investigated whether CRISPR/Cas9 mediated targeted mutagenesis can generate nulliplex tetraploid mutant lines in Arabidopsis thaliana, while also comparing the relative efficiency of targeted mutagenesis in tetraploid (4x) versus diploid (2x) backgrounds. Using CRISPR/Cas9 genome editing to generate knockout alleles of the TTG1 gene, we demonstrate that homozygous nulliplex mutants can be directly generated in tetraploid Arabidopsis thaliana plants. CRISPR/Cas9 genome editing now provides a route to more efficient generation of polyploid mutants for improving understanding of genome dosage effects in plants.

Salsman, J., et al. (2017). "CRISPR/Cas9 Gene Editing: From Basic Mechanisms to Improved Strategies for Enhanced Genome Engineering In Vivo." Curr Gene Ther **17**(4): 263-274.

INTRODUCTION: Targeted genome editing using the CRISPR/Cas9 technology is becoming a major area of research due to its high potential for the treatment of genetic diseases. Our understanding of this approach has expanded in recent years yet several new challenges have presented themselves as we explore the boundaries of this exciting new technology. Chief among these is improving the efficiency but also the preciseness of genome editing. The efficacy of CRISPR/Cas9 technology relies in part on the use of one of the major DNA repair pathways, Homologous recombination (HR), which is primarily active in S and G2 phases of the cell cycle. Problematically, the HR potential is highly variable from cell type to cell type and most of the cells of interest to be targeted in vivo for precise genome editing are in a quiescent state. CONCLUSION: In this review, we discuss the recent advancements in improving targeted CRISPR/Cas9 based genome editing and the promising ways of delivering this technology in vivo to the cells of interest.

Sato, M., et al. (2018). "Timing of CRISPR/Cas9-related mRNA microinjection after activation as an important factor affecting genome editing efficiency in porcine oocytes." Theriogenology **108**: 29-38.

Recently, successful one-step genome editing by microinjection of CRISPR/Cas9-related mRNA components into the porcine zygote has been described. Given the relatively long gestational period and the high cost of housing swine, the establishment of an effective microinjection-based porcine genome

editing method is urgently required. Previously, we have attempted to disrupt a gene encoding alpha-1,3-galactosyltransferase (GGTA1), which synthesizes the alpha-Gal epitope, by microinjecting CRISPR/Cas9-related nucleic acids and enhanced green fluorescent protein (EGFP) mRNA into porcine oocytes immediately after electrical activation. We found that genome editing was indeed induced, although the resulting blastocysts were mosaic and the frequency of modified cells appeared to be low (50%). To improve genome editing efficiency in porcine oocytes, cytoplasmic injection was performed 6 h after electrical activation, a stage wherein the pronucleus is formed. The developing blastocysts exhibited higher levels of EGFP. Furthermore, the T7 endonuclease 1 assay and subsequent sequencing demonstrated that these embryos exhibited increased genome editing efficiencies (69%), although a high degree of mosaicism for the induced mutation was still observed. Single blastocyst-based cytochemical staining with fluorescently labeled isolectin BS-I-B4 also confirmed this mosaicism. Thus, the development of a technique that avoids or reduces such mosaicism would be a key factor for efficient knock out piglet production via microinjection.

Savic, N. and G. Schwank (2016). "Advances in therapeutic CRISPR/Cas9 genome editing." Transl Res **168**: 15-21.

Targeted nucleases are widely used as tools for genome editing. Two years ago the clustered regularly interspaced short palindromic repeat (CRISPR)-associated Cas9 nuclease was used for the first time, and since then has largely revolutionized the field. The tremendous success of the CRISPR/Cas9 genome editing tool is powered by the ease design principle of the guide RNA that targets Cas9 to the desired DNA locus, and by the high specificity and efficiency of CRISPR/Cas9-generated DNA breaks. Several studies recently used CRISPR/Cas9 to successfully modulate disease-causing alleles in vivo in animal models and ex vivo in somatic and induced pluripotent stem cells, raising hope for therapeutic genome editing in the clinics. In this review, we will summarize and discuss such preclinical CRISPR/Cas9 gene therapy reports.

Schaeffer, S. M. and P. A. Nakata (2015). "CRISPR/Cas9-mediated genome editing and gene replacement in plants: Transitioning from lab to field." Plant Sci **240**: 130-142.

The CRISPR/Cas9 genome engineering system has ignited and swept through the scientific community like wildfire. Owing largely to its efficiency, specificity, and flexibility, the CRISPR/Cas9 system has quickly become the preferred genome-editing tool of plant scientists. In plants, much of the early CRISPR/Cas9 work has been limited to proof of concept and functional studies in model systems. These studies, along with those in other fields of biology, have led to the development of several utilities of CRISPR/Cas9 beyond single gene editing. Such utilities include multiplexing for inducing multiple cleavage events, controlling gene expression, and site specific transgene insertion. With much of the conceptual CRISPR/Cas9 work nearly complete, plant researchers are beginning to apply this gene editing technology for crop trait improvement. Before rational strategies can be designed to implement this technology to engineer a wide array of crops there is a need to expand the availability of crop-specific vectors, genome resources, and transformation protocols. We anticipate that these challenges will be met along with the continued evolution of the CRISPR/Cas9 system particularly in the areas of manipulation of large genomic regions, transgene-free genetic modification, development of breeding resources, discovery of gene function, and improvements upon CRISPR/Cas9 components. The CRISPR/Cas9 editing system appears poised to transform crop trait improvement.

Schwank, G. and H. Clevers (2016). "CRISPR/Cas9-Mediated Genome Editing of Mouse Small Intestinal Organoids." Methods Mol Biol **1422**: 3-11.

The CRISPR/Cas9 system is an RNA-guided genome-editing tool that has been recently developed based on the bacterial CRISPR-Cas immune defense system. Due to its versatility and simplicity, it rapidly became the method of choice for genome editing in various biological systems, including mammalian cells. Here we describe a protocol for CRISPR/Cas9-mediated genome editing in murine small intestinal organoids, a culture system in which somatic stem cells are maintained by self-renewal, while giving rise to all major cell types of the intestinal epithelium. This protocol allows the study of gene function in intestinal epithelial homeostasis and pathophysiology and can be extended to epithelial organoids derived from other internal mouse and human organs.

Schwartz, C. M., et al. (2016). "Synthetic RNA Polymerase III Promoters Facilitate High-Efficiency CRISPR-Cas9-Mediated Genome Editing in Yarrowia lipolytica." ACS Synth Biol **5**(4): 356-359.

The oleaginous yeast Yarrowia lipolytica is a valuable microbial host for chemical production because it has a high capacity to synthesize, modify, and store intracellular lipids; however, rapid strain development has been hampered by the limited availability of genome engineering tools. We address this limitation by adapting the CRISPR-Cas9 system

from Streptococcus pyogenes for markerless gene disruption and integration in Y. lipolytica. Single gene disruption efficiencies of 92% and higher were achieved when single guide RNAs (sgRNA) were transcribed with synthetic hybrid promoters that combine native RNA polymerase III (Pol III) promoters with tRNA. The Pol III-tRNA hybrid promoters exploit endogenous tRNA processing to produce mature sgRNA for Cas9 targeting. The highest efficiencies were achieved with a SCR1'-tRNA (Gly) promoter and Y. lipolytica codon-optimized Cas9 expressed from a UAS1B8-TEF promoter. Cotransformation of the Cas9 and sgRNA expressing plasmid with a homologous recombination donor plasmid resulted in markerless homologous recombination efficiency of over 64%. Homologous recombination was observed in 100% of transformants when nonhomologous end joining was disrupted. The end result of these studies was the development of pCRISPRyl, a modular tool for markerless gene disruption and integration in Y. lipolytica.

Shao, M., et al. (2016). "The big bang of genome editing technology: development and application of the CRISPR/Cas9 system in disease animal models." Dongwuxue Yanjiu **37**(4): 191-204.

Targeted genome editing technology has been widely used in biomedical studies. The CRISPR-associated RNA-guided endonuclease Cas9 has become a versatile genome editing tool. The CRISPR/Cas9 system is useful for studying gene function through efficient knock-out, knock-in or chromatin modification of the targeted gene loci in various cell types and organisms. It can be applied in a number of fields, such as genetic breeding, disease treatment and gene functional investigation. In this review, we introduce the most recent developments and applications, the challenges, and future directions of Cas9 in generating disease animal model. Derived from the CRISPR adaptive immune system of bacteria, the development trend of Cas9 will inevitably fuel the vital applications from basic research to biotechnology and bio-medicine.

Shen, B., et al. (2017). "Development of CRISPR/Cas9 for Efficient Genome Editing in Toxoplasma gondii." Methods Mol Biol **1498**: 79-103.

Efficient and site-specific alteration of the genome is key to decoding and altering the genomic information of an organism. Over the last couple of years, the RNA-guided Cas9 nucleases derived from the prokaryotic type 2 CRISPR (clustered regularly interspaced short palindromic repeats) systems have drastically improved our ability to engineer the genomes of a variety of organisms including Toxoplasma gondii. In this chapter, we describe detailed protocols for using the CRISPR/Cas9 system adapted from Streptococcus pyogenes to perform efficient genetic manipulations in T. gondii such as gene disruption, gene tagging and genetic complementation. The technical details of the strategy, including CRISPR plasmid construction, target construct generation, parasite transfection and positive clone identification are also provided. These methods are easy to customize to any gene of interest (GOI) and will greatly accelerate studies on this important pathogen.

Shen, Y., et al. (2018). "Generation of PTEN knockout bone marrow mesenchymal stem cell lines by CRISPR/Cas9-mediated genome editing." Cytotechnology.

The tumor suppressor PTEN is involved in the regulation of cell proliferation, lineage determination, motility, adhesion and apoptosis. Loss of PTEN in the bone mesenchymal stem cells (BMSCs) was shown to change their function in the repair tissue. So far, the CRISPR/Cas9 system has been proven extremely simple and flexible. Using this system to manipulate PTEN gene editing could produce the PTEN-Knocking-out (PTEN-KO) strain. We knocked out PTEN in MSCs and validated the expression by PCR and Western blot. To clarify the changes in proliferation, CCK-8 assay was applied. In support, living cell proportion was assessed by Trypan blue staining. For osteogenic and adipogenic induction, cells were cultured in different media for 2 weeks. Oil red staining and alizarin red staining were performed for assessment of osteogenic or adipogenic differentiation. The expression of Id4, Runx2, ALP and PPARgamma was examined by qPCR and immunocytochemistry staining. The PTEN-KO strain was identified by sequencing. The PTEN-KO cells had an increased cell viability and higher survival compared with the wild type. However, decreased expression of Runx2 and PPARgamma was found in the PTEN loss strain after induction, and consistently decreased osteogenic or adipogenic differentiation was observed by alizarin and oil red staining. Together, PTEN-KO strain showed an increased proliferation capability but decreased multi-directional differentiation potential. When BMSCs serve as seed cells for tissue engineering, the PTEN gene may be used as an indicator.

Shi, T. Q., et al. (2017). "CRISPR/Cas9-based genome editing of the filamentous fungi: the state of the art." Appl Microbiol Biotechnol **101**(20): 7435-7443.

In recent years, a variety of genetic tools have been developed and applied to various filamentous fungi, which are widely applied in agriculture and the

food industry. However, the low efficiency of gene targeting has for many years hampered studies on functional genomics in this important group of microorganisms. The emergence of CRISPR/Cas9 genome-editing technology has sparked a revolution in genetic research due to its high efficiency, versatility, and easy operation and opened the door for the discovery and exploitation of many new natural products. Although the application of the CRISPR/Cas9 system in filamentous fungi is still in its infancy compared to its common use in E. coli, yeasts, and mammals, the deep development of this system will certainly drive the exploitation of fungal diversity. In this review, we summarize the research progress on CRISPR/Cas9 systems in filamentous fungi and finally highlight further prospects in this area.

Singh, V., et al. (2017). "Exploring the potential of genome editing CRISPR-Cas9 technology." Gene **599**: 1-18.

CRISPR-Cas9 is an RNA-mediated adaptive immune system that protects bacteria and archaea from viruses or plasmids. Herein we discuss the recent development of CRISPR-Cas9 into a key technology for genome editing, targeting, and regulation in a wide range of organisms and cell types. It requires a custom designed single guide-RNA (sgRNA), a Cas9 endonuclease, and PAM sequences in the target region. The sgRNA-Cas9 complex binds to its target and creates a double-strand break (DSB) that can be repaired by non-homologous end joining (NHEJ) or by the homology-directed repair (HDR) pathway, modifying or permanently replacing the genomic target sequence. Additionally, we highlight recent advances in the repurposing of CRISPR-Cas9 for repression, activation, and loci imaging. In this review, we underline the current progress and the future potential of the CRISPR-Cas9 system towards biomedical, therapeutic, industrial, and biotechnological applications.

Soares Medeiros, L. C., et al. (2017). "Rapid, Selection-Free, High-Efficiency Genome Editing in Protozoan Parasites Using CRISPR-Cas9 Ribonucleoproteins." MBio **8**(6).

Trypanosomatids (order Kinetoplastida), including the human pathogens Trypanosoma cruzi (agent of Chagas disease), Trypanosoma brucei, (African sleeping sickness), and Leishmania (leishmaniasis), affect millions of people and animals globally. T. cruzi is considered one of the least studied and most poorly understood tropical disease-causing parasites, in part because of the relative lack of facile genetic engineering tools. This situation has improved recently through the application of clustered regularly interspaced short palindromic repeats-CRISPR-associated protein 9 (CRISPR-Cas9) technology, but a number of limitations remain, including the toxicity of continuous Cas9 expression and the long drug marker selection times. In this study, we show that the delivery of ribonucleoprotein (RNP) complexes composed of recombinant Cas9 from Staphylococcus aureus (SaCas9), but not from the more routinely used Streptococcus pyogenes Cas9 (SpCas9), and in vitro-transcribed single guide RNAs (sgRNAs) results in rapid gene edits in T. cruzi and other kinetoplastids at frequencies approaching 100%. The highly efficient genome editing via SaCas9/sgRNA RNPs was obtained for both reporter and endogenous genes and observed in multiple parasite life cycle stages in various strains of T. cruzi, as well as in T. brucei and Leishmania major RNP complex delivery was also used to successfully tag proteins at endogenous loci and to assess the biological functions of essential genes. Thus, the use of SaCas9 RNP complexes for gene editing in kinetoplastids provides a simple, rapid, and cloning- and selection-free method to assess gene function in these important human pathogens.IMPORTANCE Protozoan parasites remain some of the highest-impact human and animal pathogens, with very limited treatment and prevention options. The development of improved therapeutics and vaccines depends on a better understanding of the unique biology of these organisms, and understanding their biology, in turn, requires the ability to track and manipulate the products of genes. In this work, we describe new methods that are available to essentially any laboratory and applicable to any parasite isolate for easily and rapidly editing the genomes of kinetoplastid parasites. We demonstrate that these methods provide the means to quickly assess function, including that of the products of essential genes and potential targets of drugs, and to tag gene products at their endogenous loci. This is all achieved without gene cloning or drug selection. We expect this advance to enable investigations, especially in Trypanosoma cruzi and Leishmania spp., that have eluded investigators for decades.

Soda, N., et al. (2017). "CRISPR-Cas9 based plant genome editing: Significance, opportunities and recent advances." Plant Physiol Biochem.

Precise genome editing is a quantum leap in the field of plant sciences. Clustered regularly interspaced short palindromic repeats (CRISPR) and its associated Cas9 protein have emerged as a powerful tool for precise genome editing. CRISPR-Cas9 system introduces small heritable mutations (indels) in the genome of an organism. This system also enables precise gene characterization in plants with complex genomes. Besides, it offers new opportunities of trait stacking, where addition of desirable traits or removal

of undesirable traits can be achieved simultaneously in a single event. With CRISPR-Cas9 RNPs technology, raising transgene free genetically modified plants is within realm of possibility which would be helpful in addressing regulatory concerns of transgenic plants. Several new advancements have been made in this technology which has extended its applications in almost every aspect of plant science. For example, recently developed catalytically inactive dCas9 fused with transcriptional effector domains allows targeted activation or silencing of the gene of interest. Apart from this, dCas9 fused with fluorescent labels is a budding tool in chromatin imaging studies. In this review, we summarize these recent advancements in CRISPR/Cas system and methods for analyzing the induced mutations, and its implementations in crop improvement.

Sollelis, L., et al. (2015). "First efficient CRISPR-Cas9-mediated genome editing in Leishmania parasites." Cell Microbiol **17**(10): 1405-1412.

Protozoan pathogens that cause leishmaniasis in humans are relatively refractory to genetic manipulation. In this work, we implemented the CRISPR-Cas9 system in Leishmania parasites and demonstrated its efficient use for genome editing. The Cas9 endonuclease was expressed under the control of the Dihydrofolate Reductase-Thymidylate Synthase (DHFR-TS) promoter and the single guide RNA was produced under the control of the U6snRNA promoter and terminator. As a proof of concept, we chose to knockout a tandemly repeated gene family, the paraflagellar rod-2 locus. We were able to obtain null mutants in a single round of transfection. In addition, we confirmed the absence of off-target editions by whole genome sequencing of two independent clones. Our work demonstrates that CRISPR-Cas9-mediated gene knockout represents a major improvement in comparison with existing methods. Beyond gene knockout, this genome editing tool opens avenues for a multitude of functional studies to speed up research on leishmaniasis.

Spencer, N. Y., et al. (2016). "Definitive localization of intracellular proteins: Novel approach using CRISPR-Cas9 genome editing, with glucose 6-phosphate dehydrogenase as a model." Anal Biochem **494**: 55-67.

Studies to determine subcellular localization and translocation of proteins are important because subcellular localization of proteins affects every aspect of cellular function. Such studies frequently utilize mutagenesis to alter amino acid sequences hypothesized to constitute subcellular localization signals. These studies often utilize fluorescent protein tags to facilitate live cell imaging. These methods are excellent for studies of monomeric proteins, but for multimeric proteins, they are unable to rule out artifacts from native protein subunits already present in the cells. That is, native monomers might direct the localization of fluorescent proteins with their localization signals obliterated. We have developed a method for ruling out such artifacts, and we use glucose 6-phosphate dehydrogenase (G6PD) as a model to demonstrate the method's utility. Because G6PD is capable of homodimerization, we employed a novel approach to remove interference from native G6PD. We produced a G6PD knockout somatic (hepatic) cell line using CRISPR-Cas9 mediated genome engineering. Transfection of G6PD knockout cells with G6PD fluorescent mutant proteins demonstrated that the major subcellular localization sequences of G6PD are within the N-terminal portion of the protein. This approach sets a new gold standard for similar studies of subcellular localization signals in all homodimerization-capable proteins.

Stolfi, A., et al. (2014). "Tissue-specific genome editing in Ciona embryos by CRISPR/Cas9." Development **141**(21): 4115-4120.

The CRISPR/Cas9 system has ushered in a new era of targeted genetic manipulations. Here, we report the use of CRISPR/Cas9 to induce double-stranded breaks in the genome of the sea squirt Ciona intestinalis. We use electroporation to deliver CRISPR/Cas9 components for tissue-specific disruption of the Ebf (Collier/Olf/EBF) gene in hundreds of synchronized Ciona embryos. Phenotyping of transfected embryos in the 'F0' generation revealed that endogenous Ebf function is required for specification of Islet-expressing motor ganglion neurons and atrial siphon muscles. We demonstrate that CRISPR/Cas9 is sufficiently effective and specific to generate large numbers of embryos carrying mutations in a targeted gene of interest, which should allow for rapid screening of gene function in Ciona.

Sun, W., et al. (2015). "Self-assembled DNA nanoclews for the efficient delivery of CRISPR-Cas9 for genome editing." Angew Chem Int Ed Engl **54**(41): 12029-12033.

CRISPR-Cas9 represents a promising platform for genome editing, yet means for its safe and efficient delivery remain to be fully realized. A novel vehicle that simultaneously delivers the Cas9 protein and single guide RNA (sgRNA) is based on DNA nanoclews, yarn-like DNA nanoparticles that are synthesized by rolling circle amplification. The biologically inspired vehicles were efficiently loaded with Cas9/sgRNA complexes and delivered the

complexes to the nuclei of human cells, thus enabling targeted gene disruption while maintaining cell viability. Editing was most efficient when the DNA nanoclew sequence and the sgRNA guide sequence were partially complementary, offering a design rule for enhancing delivery. Overall, this strategy provides a versatile method that could be adapted for delivering other DNA-binding proteins or functional nucleic acids.

Suzuki, K., et al. (2016). "In vivo genome editing via CRISPR/Cas9 mediated homology-independent targeted integration." Nature **540**(7631): 144-149.

Targeted genome editing via engineered nucleases is an exciting area of biomedical research and holds potential for clinical applications. Despite rapid advances in the field, in vivo targeted transgene integration is still infeasible because current tools are inefficient, especially for non-dividing cells, which compose most adult tissues. This poses a barrier for uncovering fundamental biological principles and developing treatments for a broad range of genetic disorders. Based on clustered regularly interspaced short palindromic repeat/Cas9 (CRISPR/Cas9) technology, here we devise a homology-independent targeted integration (HITI) strategy, which allows for robust DNA knock-in in both dividing and non-dividing cells in vitro and, more importantly, in vivo (for example, in neurons of postnatal mammals). As a proof of concept of its therapeutic potential, we demonstrate the efficacy of HITI in improving visual function using a rat model of the retinal degeneration condition retinitis pigmentosa. The HITI method presented here establishes new avenues for basic research and targeted gene therapies.

Svitashev, S., et al. (2016). "Genome editing in maize directed by CRISPR-Cas9 ribonucleoprotein complexes." Nat Commun **7**: 13274.

Targeted DNA double-strand breaks have been shown to significantly increase the frequency and precision of genome editing. In the past two decades, several double-strand break technologies have been developed. CRISPR-Cas9 has quickly become the technology of choice for genome editing due to its simplicity, efficiency and versatility. Currently, genome editing in plants primarily relies on delivering double-strand break reagents in the form of DNA vectors. Here we report biolistic delivery of pre-assembled Cas9-gRNA ribonucleoproteins into maize embryo cells and regeneration of plants with both mutated and edited alleles. Using this method of delivery, we also demonstrate DNA- and selectable marker-free gene mutagenesis in maize and recovery of plants with mutated alleles at high frequencies. These results open new opportunities to accelerate breeding practices in a wide variety of crop species.

Takayama, K., et al. (2017). "Highly efficient biallelic genome editing of human ES/iPS cells using a CRISPR/Cas9 or TALEN system." Nucleic Acids Res **45**(9): 5198-5207.

Genome editing research of human ES/iPS cells has been accelerated by clustered regularly interspaced short palindromic repeats/CRISPR-associated 9 (CRISPR/Cas9) and transcription activator-like effector nucleases (TALEN) technologies. However, the efficiency of biallelic genetic engineering in transcriptionally inactive genes is still low, unlike that in transcriptionally active genes. To enhance the biallelic homologous recombination efficiency in human ES/iPS cells, we performed screenings of accessorial genes and compounds. We found that RAD51 overexpression and valproic acid treatment enhanced biallelic-targeting efficiency in human ES/iPS cells regardless of the transcriptional activity of the targeted locus. Importantly, RAD51 overexpression and valproic acid treatment synergistically increased the biallelic homologous recombination efficiency. Our findings would facilitate genome editing study using human ES/iPS cells.

Tang, X., et al. (2016). "A Single Transcript CRISPR-Cas9 System for Efficient Genome Editing in Plants." Mol Plant **9**(7): 1088-1091.

Tong, Y., et al. (2018). "CRISPR-Cas9 Toolkit for Actinomycete Genome Editing." Methods Mol Biol **1671**: 163-184.

Bacteria of the order Actinomycetales are one of the most important sources of bioactive natural products, which are the source of many drugs. However, many of them still lack efficient genome editing methods, some strains even cannot be manipulated at all. This restricts systematic metabolic engineering approaches for boosting known and discovering novel natural products. In order to facilitate the genome editing for actinomycetes, we developed a CRISPR-Cas9 toolkit with high efficiency for actinomyces genome editing. This basic toolkit includes a software for spacer (sgRNA) identification, a system for in-frame gene/gene cluster knockout, a system for gene loss-of-function study, a system for generating a random size deletion library, and a system for gene knockdown. For the latter, a uracil-specific excision reagent (USER) cloning technology was adapted to simplify the CRISPR vector construction process. The application of this toolkit was successfully demonstrated by perturbation of genomes of Streptomyces coelicolor A3(2) and Streptomyces

collinus Tu 365. The CRISPR-Cas9 toolkit and related protocol described here can be widely used for metabolic engineering of actinomycetes.

Tothova, Z., et al. (2017). "Multiplex CRISPR/Cas9-Based Genome Editing in Human Hematopoietic Stem Cells Models Clonal Hematopoiesis and Myeloid Neoplasia." Cell Stem Cell **21**(4): 547-555 e548.

Hematologic malignancies are driven by combinations of genetic lesions that have been difficult to model in human cells. We used CRISPR/Cas9 genome engineering of primary adult and umbilical cord blood CD34(+) human hematopoietic stem and progenitor cells (HSPCs), the cells of origin for myeloid pre-malignant and malignant diseases, followed by transplantation into immunodeficient mice to generate genetic models of clonal hematopoiesis and neoplasia. Human hematopoietic cells bearing mutations in combinations of genes, including cohesin complex genes, observed in myeloid malignancies generated immunophenotypically defined neoplastic clones capable of long-term, multi-lineage reconstitution and serial transplantation. Employing these models to investigate therapeutic efficacy, we found that TET2 and cohesin-mutated hematopoietic cells were sensitive to azacitidine treatment. These findings demonstrate the potential for generating genetically defined models of human myeloid diseases, and they are suitable for examining the biological consequences of somatic mutations and the testing of therapeutic agents.

Tycko, J., et al. (2016). "Methods for Optimizing CRISPR-Cas9 Genome Editing Specificity." Mol Cell **63**(3): 355-370.

Advances in the development of delivery, repair, and specificity strategies for the CRISPR-Cas9 genome engineering toolbox are helping researchers understand gene function with unprecedented precision and sensitivity. CRISPR-Cas9 also holds enormous therapeutic potential for the treatment of genetic disorders by directly correcting disease-causing mutations. Although the Cas9 protein has been shown to bind and cleave DNA at off-target sites, the field of Cas9 specificity is rapidly progressing, with marked improvements in guide RNA selection, protein and guide engineering, novel enzymes, and off-target detection methods. We review important challenges and breakthroughs in the field as a comprehensive practical guide to interested users of genome editing technologies, highlighting key tools and strategies for optimizing specificity. The genome editing community should now strive to standardize such methods for measuring and reporting off-target activity, while keeping in mind that the goal for specificity should be continued improvement and vigilance.

van Agtmaal, E. L., et al. (2017). "CRISPR/Cas9-Induced (CTGCAG)n Repeat Instability in the Myotonic Dystrophy Type 1 Locus: Implications for Therapeutic Genome Editing." Mol Ther **25**(1): 24-43.

Myotonic dystrophy type 1 (DM1) is caused by (CTGCAG)n-repeat expansion within the DMPK gene and thought to be mediated by a toxic RNA gain of function. Current attempts to develop therapy for this disease mainly aim at destroying or blocking abnormal properties of mutant DMPK (CUG)n RNA. Here, we explored a DNA-directed strategy and demonstrate that single clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-cleavage in either its 5' or 3' unique flank promotes uncontrollable deletion of large segments from the expanded trinucleotide repeat, rather than formation of short indels usually seen after double-strand break repair. Complete and precise excision of the repeat tract from normal and large expanded DMPK alleles in myoblasts from unaffected individuals, DM1 patients, and a DM1 mouse model could be achieved at high frequency by dual CRISPR/Cas9-cleavage at either side of the (CTGCAG)n sequence. Importantly, removal of the repeat appeared to have no detrimental effects on the expression of genes in the DM1 locus. Moreover, myogenic capacity, nucleocytoplasmic distribution, and abnormal RNP-binding behavior of transcripts from the edited DMPK gene were normalized. Dual sgRNA-guided excision of the (CTGCAG)n tract by CRISPR/Cas9 technology is applicable for developing isogenic cell lines for research and may provide new therapeutic opportunities for patients with DM1.

van Diemen, F. R., et al. (2016). "CRISPR/Cas9-Mediated Genome Editing of Herpesviruses Limits Productive and Latent Infections." PLoS Pathog **12**(6): e1005701.

Herpesviruses infect the majority of the human population and can cause significant morbidity and mortality. Herpes simplex virus (HSV) type 1 causes cold sores and herpes simplex keratitis, whereas HSV-2 is responsible for genital herpes. Human cytomegalovirus (HCMV) is the most common viral cause of congenital defects and is responsible for serious disease in immuno-compromised individuals. Epstein-Barr virus (EBV) is associated with infectious mononucleosis and a broad range of malignancies, including Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease, and post-transplant lymphomas. Herpesviruses persist in their host for life by establishing a latent infection that is interrupted by periodic reactivation events during which replication

occurs. Current antiviral drug treatments target the clinical manifestations of this productive stage, but they are ineffective at eliminating these viruses from the infected host. Here, we set out to combat both productive and latent herpesvirus infections by exploiting the CRISPR/Cas9 system to target viral genetic elements important for virus fitness. We show effective abrogation of HCMV and HSV-1 replication by targeting gRNAs to essential viral genes. Simultaneous targeting of HSV-1 with multiple gRNAs completely abolished the production of infectious particles from human cells. Using the same approach, EBV can be almost completely cleared from latently infected EBV-transformed human tumor cells. Our studies indicate that the CRISPR/Cas9 system can be effectively targeted to herpesvirus genomes as a potent prophylactic and therapeutic anti-viral strategy that may be used to impair viral replication and clear latent virus infection.

Vejnar, C. E., et al. (2016). "Optimization Strategies for the CRISPR-Cas9 Genome-Editing System." Cold Spring Harb Protoc **2016**(10): pdb top090894.

The CRISPR-Cas9 system uncovered in bacteria has emerged as a powerful genome-editing technology in eukaryotic cells. It consists of two components-a single guide RNA (sgRNA) that directs the Cas9 endonuclease to a complementary DNA target site. Efficient targeting of individual genes requires highly active sgRNAs. Recent efforts have made significant progress in understanding the sequence features that increase sgRNA activity. In this introduction, we highlight advancements in the field of CRISPR-Cas9 targeting and discuss our web tool CRISPRscan, which predicts the targeting activity of sgRNAs and improves the efficiency of the CRISPR-Cas9 system for in vivo genome engineering.

Vejnar, C. E., et al. (2016). "Optimized CRISPR-Cas9 System for Genome Editing in Zebrafish." Cold Spring Harb Protoc **2016**(10): pdb prot086850.

This protocol describes how to generate and genotype mutants using an optimized CRISPR-Cas9 genome-editing system in zebrafish (CRISPRscan). Because single guide RNAs (sgRNAs) have variable efficiency when targeting specific loci, our protocol starts by explaining how to use the web tool CRISPRscan to design highly efficient sgRNAs. The CRISPRscan algorithm is based on the results of an integrated analysis of more than 1000 sgRNAs in zebrafish, which uncovered highly predictive factors that influence Cas9 activity. Next, we describe how to easily generate sgRNAs in vitro, which can then be injected in vivo to target specific loci. The use of highly efficient sgRNAs can lead to biallelic mutations in the injected embryos, causing lethality. We explain how targeting Cas9 to the germline increases viability by reducing somatic mutations. Finally, we combine two methods to identify F1 heterozygous fish carrying the desired mutations: (i) Mut-Seq, a method based on high-throughput sequencing to detect F0 founder fish; and (ii) a polymerase chain reaction-based fragment analysis method that identifies F1 heterozygous fish characterized by Mut-Seq. In summary, this protocol includes the steps to generate and characterize mutant zebrafish lines using the CRISPR-Cas9 genome engineering system.

Vigentini, I., et al. (2017). "CRISPR/Cas9 System as a Valuable Genome Editing Tool for Wine Yeasts with Application to Decrease Urea Production." Front Microbiol **8**: 2194.

An extensive repertoire of molecular tools is available for genetic analysis in laboratory strains of S. cerevisiae. Although this has widely contributed to the interpretation of gene functionality within haploid laboratory isolates, the genetics of metabolism in commercially-relevant polyploid yeast strains is still poorly understood. Genetic engineering in industrial yeasts is undergoing major changes due to Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated protein (Cas) engineering approaches. Here we apply the CRISPR/Cas9 system to two commercial "starter" strains of S. cerevisiae (EC1118, AWRI796), eliminating the CAN1 arginine permease pathway to generate strains with reduced urea production (18.5 and 35.5% for EC1118 and AWRI796, respectively). In a wine-model environment based on two grape musts obtained from Chardonnay and Cabernet Sauvignon cultivars, both S. cerevisiae starter strains and CAN1 mutants completed the must fermentation in 8-12 days. However, recombinant strains carrying the can1 mutation failed to produce urea, suggesting that the genetic modification successfully impaired the arginine metabolism. In conclusion, the reduction of urea production in a wine-model environment confirms that the CRISPR/Cas9 system has been successfully established in S. cerevisiae wine yeasts.

Wagner, J. C., et al. (2014). "Efficient CRISPR-Cas9-mediated genome editing in Plasmodium falciparum." Nat Methods **11**(9): 915-918.

Malaria is a major cause of global morbidity and mortality, and new strategies for treating and preventing this disease are needed. Here we show that the Streptococcus pyogenes Cas9 DNA endonuclease and single guide RNAs (sgRNAs) produced using T7 RNA polymerase (T7 RNAP) efficiently edit the Plasmodium falciparum genome. Targeting the genes encoding native knob-associated histidine-rich protein

(kahrp) and erythrocyte binding antigen 175 (eba-175), we achieved high (>/= 50-100%) gene disruption frequencies within the usual time frame for generating transgenic parasites.

Wang, D., et al. (2015). "Adenovirus-Mediated Somatic Genome Editing of Pten by CRISPR/Cas9 in Mouse Liver in Spite of Cas9-Specific Immune Responses." Hum Gene Ther **26**(7): 432-442.

CRISPR/Cas9 derived from the bacterial adaptive immunity pathway is a powerful tool for genome editing, but the safety profiles of in vivo delivered Cas9 (including host immune responses to the bacterial Cas9 protein) have not been comprehensively investigated in model organisms. Nonalcoholic steatohepatitis (NASH) is a prevalent human liver disease characterized by excessive fat accumulation in the liver. In this study, we used adenovirus (Ad) vector to deliver a Streptococcus pyogenes-derived Cas9 system (SpCas9) targeting Pten, a gene involved in NASH and a negative regulator of the PI3K-AKT pathway, in mouse liver. We found that the Ad vector mediated efficient Pten gene editing even in the presence of typical Ad vector-associated immunotoxicity in the liver. Four months after vector infusion, mice receiving the Pten gene-editing Ad vector showed massive hepatomegaly and features of NASH, consistent with the phenotypes following Cre-loxP-induced Pten deficiency in mouse liver. We also detected induction of humoral immunity against SpCas9 and the potential presence of an SpCas9-specific cellular immune response. Our findings provide a strategy to model human liver diseases in mice and highlight the importance considering Cas9-specific immune responses in future translational studies involving in vivo delivery of CRISPR/Cas9.

Wang, D. Y., et al. (2016). "[The application of CRISPR/Cas9 genome editing technology in cancer research]." Yi Chuan **38**(1): 1-8.

The CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein-9 nuclease) genome editing technology has become more and more popular in gene editing because of its simple design and easy operation. Using the CRISPR/Cas9 system, researchers can perform site-directed genome modification at the base level. Moreover, it has been widely used in genome editing in multiple species and related cancer research. In this review, we summarize the application of the CRISPR/Cas9 system in cancer research based on the latest research progresses as well as our understanding of cancer research and genome editing techniques.

Wang, G., et al. (2017). "Efficient, footprint-free human iPSC genome editing by consolidation of Cas9/CRISPR and piggyBac technologies." Nat Protoc **12**(1): 88-103.

Genome editing of human induced pluripotent stem cells (hiPSCs) offers unprecedented opportunities for in vitro disease modeling and personalized cell replacement therapy. The introduction of Cas9-directed genome editing has expanded adoption of this approach. However, marker-free genome editing using standard protocols remains inefficient, yielding desired targeted alleles at a rate of approximately 1-5%. We developed a protocol based on a doxycycline-inducible Cas9 transgene carried on a piggyBac transposon to enable robust and highly efficient Cas9-directed genome editing, so that a parental line can be expeditiously engineered to harbor many separate mutations. Treatment with doxycycline and transfection with guide RNA (gRNA), donor DNA and piggyBac transposase resulted in efficient, targeted genome editing and concurrent scarless transgene excision. Using this approach, in 7 weeks it is possible to efficiently obtain genome-edited clones with minimal off-target mutagenesis and with indel mutation frequencies of 40-50% and homology-directed repair (HDR) frequencies of 10-20%.

Wang, H., et al. (2016). "CRISPR/Cas9 in Genome Editing and Beyond." Annu Rev Biochem **85**: 227-264.

The Cas9 protein (CRISPR-associated protein 9), derived from type II CRISPR (clustered regularly interspaced short palindromic repeats) bacterial immune systems, is emerging as a powerful tool for engineering the genome in diverse organisms. As an RNA-guided DNA endonuclease, Cas9 can be easily programmed to target new sites by altering its guide RNA sequence, and its development as a tool has made sequence-specific gene editing several magnitudes easier. The nuclease-deactivated form of Cas9 further provides a versatile RNA-guided DNA-targeting platform for regulating and imaging the genome, as well as for rewriting the epigenetic status, all in a sequence-specific manner. With all of these advances, we have just begun to explore the possible applications of Cas9 in biomedical research and therapeutics. In this review, we describe the current models of Cas9 function and the structural and biochemical studies that support it. We focus on the applications of Cas9 for genome editing, regulation, and imaging, discuss other possible applications and some technical considerations, and highlight the many advantages that CRISPR/Cas9 technology offers.

Wang, H. X., et al. (2017). "CRISPR/Cas9-Based Genome Editing for Disease Modeling and Therapy:

Challenges and Opportunities for Nonviral Delivery." Chem Rev **117**(15): 9874-9906.

Genome editing offers promising solutions to genetic disorders by editing DNA sequences or modulating gene expression. The clustered regularly interspaced short palindromic repeats (CRISPR)/associated protein 9 (CRISPR/Cas9) technology can be used to edit single or multiple genes in a wide variety of cell types and organisms in vitro and in vivo. Herein, we review the rapidly developing CRISPR/Cas9-based technologies for disease modeling and gene correction and recent progress toward Cas9/guide RNA (gRNA) delivery based on viral and nonviral vectors. We discuss the relative merits of delivering the genome editing elements in the form of DNA, mRNA, or protein, and the opportunities of combining viral delivery of a transgene encoding Cas9 with nonviral delivery of gRNA. We highlight the lessons learned from nonviral gene delivery in the past three decades and consider their applicability for CRISPR/Cas9 delivery. We also include a discussion of bioinformatics tools for gRNA design and chemical modifications of gRNA. Finally, we consider the extracellular and intracellular barriers to nonviral CRISPR/Cas9 delivery and propose strategies that may overcome these barriers to realize the clinical potential of CRISPR/Cas9-based genome editing.

Wang, J., et al. (2017). "CRISPR/Cas9 mediated genome editing of Helicoverpa armigera with mutations of an ABC transporter gene HaABCA2 confers resistance to Bacillus thuringiensis Cry2A toxins." Insect Biochem Mol Biol **87**: 147-153.

High levels of resistance to Bt toxin Cry2Ab have been identified to be genetically linked with loss of function mutations of an ABC transporter gene (ABCA2) in two lepidopteran insects, Helicoverpa armigera and Helicoverpa punctigera. To further confirm the causal relationship between the ABCA2 gene (HaABCA2) and Cry2Ab resistance in H. armigera, two HaABCA2 knockout strains were created from the susceptible SCD strain with the CRISPR/Cas9 genome editing system. One strain (SCD-A2KO1) is homozygous for a 2-bp deletion in exon 2 of HaABCA2 created by non-homologous end joining (NHEJ). The other strain (SCD-A2KO2) is homozygous for a 5-bp deletion in exon 18 of HaABCA2 made by homology-directed repair (HDR), which was produced to mimic the r2 resistance allele of a field-derived Cry2Ab-resistant strain from Australia. Both knockout strains obtained high levels of resistance to both Cry2Aa (>120-fold) and Cry2Ab (>100-fold) compared with the original SCD strain, but no or very limited resistance to Cry1Ac (<4-fold). Resistance to Cry2Ab in both knockouts is recessive, and genetic complementary tests confirmed Cry2Ab resistance alleles are at the same locus (i.e. HaABCA2) for the two strains. Brush border membrane vesicles (BBMVs) of midguts from both knockout strains lost binding with Cry2Ab, but maintained the same binding with Cry1Ac as the SCD strain. In vivo functional evidence from this study demonstrates knockout of HaABCA2 confers high levels of resistance to both Cry2Aa and Cry2Ab, confirming that HaABCA2 plays a key role in mediating toxicity of both Cry2Aa and Cry2Ab against H. armigera.

Wang, L., et al. (2017). "CRISPR-Cas9-mediated genome editing in one blastomere of two-cell embryos reveals a novel Tet3 function in regulating neocortical development." Cell Res **27**(6): 815-829.

Studying the early function of essential genes is an important and challenging problem in developmental biology. Here, we established a method for rapidly inducing CRISPR-Cas9-mediated mutations in one blastomere of two-cell stage embryos, termed 2-cell embryo-CRISPR-Cas9 injection (2CC), to study the in vivo function of essential (or unknown) genes in founder chimeric mice. By injecting both Cre mRNA and CRISPR-Cas9 targeting the gene of interest into fluorescent reporter mice, the 2CC method can trace both wild-type and mutant cells at different developmental stages, offering internal control for phenotypic analyses of mutant cells. Using this method, we identified novel functions of the essential gene Tet3 in regulating excitatory and inhibitory synaptic transmission in the developing mouse cerebral cortex. By generating chimeric mutant mice, the 2CC method allows for the rapid screening of gene function in multiple tissues and cell types in founder chimeric mice, significantly expanding the current armamentarium of genetic tools.

Wang, P., et al. (2018). "High efficient multisites genome editing in allotetraploid cotton (Gossypium hirsutum) using CRISPR/Cas9 system." Plant Biotechnol J **16**(1): 137-150.

Gossypium hirsutum is an allotetraploid with a complex genome. Most genes have multiple copies that belong to At and Dt subgenomes. Sequence similarity is also very high between gene homologues. To efficiently achieve site/gene-specific mutation is quite needed. Due to its high efficiency and robustness, the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 system has exerted broad site-specific genome editing from prokaryotes to eukaryotes. In this study, we utilized a CRISPR/Cas9 system to generate two sgRNAs in a single vector to conduct multiple sites genome editing in allotetraploid cotton. An exogenously transformed

gene Discosoma red fluorescent protein2(DsRed2) and an endogenous gene GhCLA1 were chosen as targets. The DsRed2-edited plants in T0 generation reverted its traits to wild type, with vanished red fluorescence the whole plants. Besides, the mutated phenotype and genotype were inherited to their T1 progenies. For the endogenous gene GhCLA1, 75% of regenerated plants exhibited albino phenotype with obvious nucleotides and DNA fragments deletion. The efficiency of gene editing at each target site is 66.7-100%. The mutation genotype was checked for both genes with Sanger sequencing. Barcode-based high-throughput sequencing, which could be highly efficient for genotyping to a population of mutants, was conducted in GhCLA1-edited T0 plants and it matched well with Sanger sequencing results. No off-target editing was detected at the potential off-target sites. These results prove that the CRISPR/Cas9 system is highly efficient and reliable for allotetraploid cotton genome editing.

Wang, Q., et al. (2016). "Genome editing of model oleaginous microalgae Nannochloropsis spp. by CRISPR/Cas9." Plant J **88**(6): 1071-1081.

Microalgae are promising feedstock for biofuels yet mechanistic probing of their cellular network and industrial strain development have been hindered by lack of genome-editing tools. Nannochloropsis spp. are emerging model microalgae for scalable oil production and carbon sequestration. Here we established a CRISPR/Cas9-based precise genome-editing approach for the industrial oleaginous microalga Nannochloropsis oceanica, using nitrate reductase (NR; g7988) as example. A new screening procedure that compares between restriction enzyme-digested nested PCR (nPCR) products derived from enzyme-digested and not-digested genomic DNA of transformant pools was developed to quickly, yet reliably, detect genome-engineered mutants. Deep sequencing of nPCR products directly amplified from pooled genomic DNA revealed over an 1% proportion of 5-bp deletion mutants and a lower frequency of 12-bp deletion mutants, with both types of editing precisely located at the targeted site. The isolated mutants, in which precise deletion of five bases caused a frameshift in NR translation, grow normally under NH4 Cl but fail to grow under NaNO3, and thus represent a valuable chassis strain for transgenic-strain development. This demonstration of CRISPR/Cas9-based genome editing in industrial microalgae opens many doors for microalgae-based biotechnological applications.

Wang, S., et al. (2017). "Genome Editing in Clostridium saccharoperbutylacetonicum N1-4 with the CRISPR-Cas9 System." Appl Environ Microbiol **83**(10).

Clostridium saccharoperbutylacetonicum N1-4 is well known as a hyper-butanol-producing strain. However, the lack of genetic engineering tools hinders further elucidation of its solvent production mechanism and development of more robust strains. In this study, we set out to develop an efficient genome engineering system for this microorganism based on the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated 9 (CRISPR-Cas9) system. First, the functionality of the CRISPR-Cas9 system previously customized for Clostridium beijerinckii was evaluated in C. saccharoperbutylacetonicum by targeting pta and buk, two essential genes for acetate and butyrate production, respectively. pta and buk single and double deletion mutants were successfully obtained based on this system. However, the genome engineering efficiency was rather low (the mutation rate is <20%). Therefore, the efficiency was further optimized by evaluating various promoters for guide RNA (gRNA) expression. With promoter P J23119, we achieved a mutation rate of 75% for pta deletion without serial subculturing as suggested previously for C. beijerinckii Thus, this developed CRISPR-Cas9 system is highly desirable for efficient genome editing in C. saccharoperbutylacetonicum Batch fermentation results revealed that both the acid and solvent production profiles were altered due to the disruption of acid production pathways; however, neither acetate nor butyrate production was eliminated with the deletion of the corresponding gene. The butanol production, yield, and selectivity were improved in mutants, depending on the fermentation medium. In the pta buk double deletion mutant, the butanol production in P2 medium reached 19.0 g/liter, which is one of the highest levels ever reported from batch fermentations. IMPORTANCE An efficient CRISPR-Cas9 genome engineering system was developed for C. saccharoperbutylacetonicum N1-4. This paves the way for elucidating the solvent production mechanism in this hyper-butanol-producing microorganism and developing strains with desirable butanol-producing features. This tool can be easily adapted for use in closely related microorganisms. As also reported by others, here we demonstrated with solid data that the highly efficient expression of gRNA is the key factor determining the efficiency of CRISPR-Cas9 for genome editing. The protocol developed in this study can provide essential references for other researchers who work in the areas of metabolic engineering and synthetic biology. The developed mutants can be used as excellent starting strains for development of more robust ones for desirable solvent production.

Wang, Y., et al. (2016). "Identification of genomic sites for CRISPR/Cas9-based genome editing in the Vitis vinifera genome." BMC Plant Biol **16**: 96.

BACKGROUND: CRISPR/Cas9 has been recently demonstrated as an effective and popular genome editing tool for modifying genomes of humans, animals, microorganisms, and plants. Success of such genome editing is highly dependent on the availability of suitable target sites in the genomes to be edited. Many specific target sites for CRISPR/Cas9 have been computationally identified for several annual model and crop species, but such sites have not been reported for perennial, woody fruit species. In this study, we identified and characterized five types of CRISPR/Cas9 target sites in the widely cultivated grape species Vitis vinifera and developed a user-friendly database for editing grape genomes in the future. RESULTS: A total of 35,767,960 potential CRISPR/Cas9 target sites were identified from grape genomes in this study. Among them, 22,597,817 target sites were mapped to specific genomic locations and 7,269,788 were found to be highly specific. Protospacers and PAMs were found to distribute uniformly and abundantly in the grape genomes. They were present in all the structural elements of genes with the coding region having the highest abundance. Five PAM types, TGG, AGG, GGG, CGG and NGG, were observed. With the exception of the NGG type, they were abundantly present in the grape genomes. Synteny analysis of similar genes revealed that the synteny of protospacers matched the synteny of homologous genes. A user-friendly database containing protospacers and detailed information of the sites was developed and is available for public use at the Grape-CRISPR website (http://biodb.sdau.edu.cn/gc/index.html). CONCLUSION: Grape genomes harbour millions of potential CRISPR/Cas9 target sites. These sites are widely distributed among and within chromosomes with predominant abundance in the coding regions of genes. We developed a publicly-accessible Grape-CRISPR database for facilitating the use of the CRISPR/Cas9 system as a genome editing tool for functional studies and molecular breeding of grapes. Among other functions, the database allows users to identify and select multi-protospacers for editing similar sequences in grape genomes simultaneously.

Wang, Y., et al. (2016). "Bacterial Genome Editing with CRISPR-Cas9: Deletion, Integration, Single Nucleotide Modification, and Desirable "Clean" Mutant Selection in Clostridium beijerinckii as an Example." ACS Synth Biol **5**(7): 721-732.

CRISPR-Cas9 has been demonstrated as a transformative genome engineering tool for many eukaryotic organisms; however, its utilization in bacteria remains limited and ineffective. Here we explored Streptococcus pyogenes CRISPR-Cas9 for genome editing in Clostridium beijerinckii (industrially significant but notorious for being difficult to metabolically engineer) as a representative attempt to explore CRISPR-Cas9 for genome editing in microorganisms that previously lacked sufficient genetic tools. By combining inducible expression of Cas9 and plasmid-borne editing templates, we successfully achieved gene deletion and integration with high efficiency in single steps. We further achieved single nucleotide modification by applying innovative two-step approaches, which do not rely on availability of Protospacer Adjacent Motif sequences. Severe vector integration events were observed during the genome engineering process, which is likely difficult to avoid but has never been reported by other researchers for the bacterial genome engineering based on homologous recombination with plasmid-borne editing templates. We then further successfully employed CRISPR-Cas9 as an efficient tool for selecting desirable "clean" mutants in this study. The approaches we developed are broadly applicable and will open the way for precise genome editing in diverse microorganisms.

Wasels, F., et al. (2017). "A two-plasmid inducible CRISPR/Cas9 genome editing tool for Clostridium acetobutylicum." J Microbiol Methods **140**: 5-11.

CRISPR/Cas-based genetic engineering has revolutionised molecular biology in both eukaryotes and prokaryotes. Several tools dedicated to the genomic transformation of the Clostridium genus of Gram-positive bacteria have been described in the literature; however, the integration of large DNA fragments still remains relatively limited. In this study, a CRISPR/Cas9 genome editing tool using a two-plasmid strategy was developed for the solventogenic strain Clostridium acetobutylicum ATCC 824. Codon-optimised cas9 from Streptococcus pyogenes was placed under the control of an anhydrotetracycline-inducible promoter on one plasmid, while the gRNA expression cassettes and editing templates were located on a second plasmid. Through the sequential introduction of these vectors into the cell, we achieved highly accurate genome modifications, including nucleotide substitution, gene deletion and cassette insertion up to 3.6kb. To demonstrate its potential, this genome editing tool was used to generate a marker-free mutant of ATCC 824 that produced an isopropanol-butanol-ethanol mixture. Whole-genome sequencing confirmed that no off-target modifications were present in the mutants. Such a tool is a prerequisite for efficient metabolic engineering in this solventogenic strain and provides an alternative

editing strategy that might be applicable to other Clostridium strains.

Wei, W., et al. (2014). "Heritable genome editing with CRISPR/Cas9 in the silkworm, Bombyx mori." PLoS One **9**(7): e101210.

We report the establishment of an efficient and heritable gene mutagenesis method in the silkworm Bombyx mori using modified type II clustered regularly interspaced short palindromic repeats (CRISPR) with an associated protein (Cas9) system. Using four loci Bm-ok, BmKMO, BmTH, and Bmtan as candidates, we proved that genome alterations at specific sites could be induced by direct microinjection of specific guide RNA and Cas9-mRNA into silkworm embryos. Mutation frequencies of 16.7-35.0% were observed in the injected generation, and DNA fragments deletions were also noted. Bm-ok mosaic mutants were used to test for mutant heritability due to the easily determined translucent epidermal phenotype of Bm-ok-disrupted cells. Two crossing strategies were used. In the first, injected Bm-ok moths were crossed with wild-type moths, and a 28.6% frequency of germline mutation transmission was observed. In the second strategy, two Bm-ok mosaic mutant moths were crossed with each other, and 93.6% of the offsprings appeared mutations in both alleles of Bm-ok gene (compound heterozygous). In summary, the CRISPR/Cas9 system can act as a highly specific and heritable gene-editing tool in Bombyx mori.

Williams, A., et al. (2016). "Editing the Mouse Genome Using the CRISPR-Cas9 System." Cold Spring Harb Protoc **2016**(2): pdb top087536.

The ability to modify the murine genome is perhaps one of the most important developments in modern biology. However, traditional methods of genomic engineering are costly and relatively clumsy in their approach. The use of programmable nucleases such as zinc finger nucleases and transcription activator-like effector nucleases significantly improved the precision of genome-editing technology, but the design and use of these nucleases remains cumbersome and prohibitively expensive. The CRISPR-Cas9 system is the next installment in the line of programmable nucleases; it provides highly efficient and precise genome-editing capabilities using reagents that are simple to design and inexpensive to generate. Furthermore, with the CRISPR-Cas9 system, it is possible to move from a hypothesis to an in vivo mouse model in less than a month. The simplicity, cost effectiveness, and speed of the CRISPR-Cas9 system allows researchers to tackle questions that otherwise would not be technically or financially viable. In this introduction, we discuss practical considerations for the use of Cas9 in genome engineering in mice.

Wolf, T., et al. (2016). "Targeted genome editing in the rare actinomycete Actinoplanes sp. SE50/110 by using the CRISPR/Cas9 System." J Biotechnol **231**: 122-128.

The application of genome editing technologies, like CRISPR/Cas9 for industrially relevant microorganisms, is becoming increasingly important. Compared to other methods of genetic engineering the decisive factor is that CRISPR/Cas9 is relatively easy to apply and thus time and effort can be significantly reduced in organisms, which are otherwise genetically difficult to access. Because of its many advantages and opportunities, we adopted the CRISPR/Cas9 technology for Actinoplanes sp. SE50/110, the producer of the diabetes type II drug acarbose. The functionality of genome editing was successfully shown by the scarless and antibiotic marker-free deletion of the gene encoding the tyrosinase MelC, which catalyzes the formation of the dark pigment eumelanin in the wild type strain. The generated DeltamelC2 mutant of Actinoplanes sp. SE50/110 no longer produces this pigment and therefore the supernatant does not darken. Furthermore, it was shown that the plasmid containing the gene for the Cas9 protein was removed by increasing the temperature due to its temperature-sensitive replication. The precision of the intended mutation was proven and possible off-target effects caused by the genome editing system were ruled out by genome sequencing of several mutants.

Woo, J. W., et al. (2015). "DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins." Nat Biotechnol **33**(11): 1162-1164.

Editing plant genomes without introducing foreign DNA into cells may alleviate regulatory concerns related to genetically modified plants. We transfected preassembled complexes of purified Cas9 protein and guide RNA into plant protoplasts of Arabidopsis thaliana, tobacco, lettuce and rice and achieved targeted mutagenesis in regenerated plants at frequencies of up to 46%. The targeted sites contained germline-transmissible small insertions or deletions that are indistinguishable from naturally occurring genetic variation.

Xi, L., et al. (2015). "A novel two-step genome editing strategy with CRISPR-Cas9 provides new insights into telomerase action and TERT gene expression." Genome Biol **16**: 231.

BACKGROUND: To facilitate indefinite proliferation, stem cells and most cancer cells require the activity of telomerase, which counteracts the successive shortening of telomeres caused by

incomplete DNA replication at the very end of each chromosome. Human telomerase activity is often determined by the expression level of telomerase reverse transcriptase (TERT), the catalytic subunit of the ribonucleoprotein complex. The low expression level of TERT and the lack of adequate antibodies have made it difficult to study telomerase-related processes in human cells. RESULTS: To overcome the low CRISPR-Cas9 editing efficiency at the TERT locus, we develop a two-step "pop-in/pop-out" strategy to enrich cells that underwent homologous recombination (HR). Using this technique, we fuse an N-terminal FLAG-SNAP-tag to TERT, which allows us to reliably detect TERT in western blots, immunopurify it for biochemical analysis, and determine its subcellular localization by fluorescence microscopy. TERT co-localizes detectably with only 5-7 % of the telomeres at a time in S-phase HeLa cells; no nucleolar localization is detected.

Xiang, G., et al. (2017). "Temperature effect on CRISPR-Cas9 mediated genome editing." J Genet Genomics **44**(4): 199-205.

Zinc-finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR-Cas9) are the most commonly used genome editing tools. Previous studies demonstrated that hypothermia treatment increased the mutation rates induced by ZFNs and TALENs in mammalian cells. Here, we characterize the effect of different culture temperatures on CRISPR-Cas9 mediated genome editing and find that the genome editing efficiency of CRISPR-Cas9 is significantly hampered by hypothermia treatment, unlike ZFN and TALEN. In addition, hyperthermia culture condition enhances genome editing by CRISPR-Cas9 in some cell lines, due to the higher enzyme activity and sgRNA expression level at higher temperature. Our study has implications on CRISPR-Cas9 applications in a broad spectrum of species, many of which do not live at 37 degrees C.

Xie, C., et al. (2016). "Genome editing with CRISPR/Cas9 in postnatal mice corrects PRKAG2 cardiac syndrome." Cell Res **26**(10): 1099-1111.

PRKAG2 cardiac syndrome is an autosomal dominant inherited disease resulted from mutations in the PRKAG2 gene that encodes gamma2 regulatory subunit of AMP-activated protein kinase. Affected patients usually develop ventricular tachyarrhythmia and experience progressive heart failure that is refractory to medical treatment and requires cardiac transplantation. In this study, we identify a H530R mutation in PRKAG2 from patients with familial Wolff-Parkinson-White syndrome. By generating H530R PRKAG2 transgenic and knock-in mice, we show that both models recapitulate human symptoms including cardiac hypertrophy and glycogen storage, confirming that the H530R mutation is causally related to PRKAG2 cardiac syndrome. We further combine adeno-associated virus-9 (AAV9) and the CRISPR/Cas9 gene-editing system to disrupt the mutant PRKAG2 allele encoding H530R while leaving the wild-type allele intact. A single systemic injection of AAV9-Cas9/sgRNA at postnatal day 4 or day 42 substantially restores the morphology and function of the heart in H530R PRKAG2 transgenic and knock-in mice. Together, our work suggests that in vivo CRISPR/Cas9 genome editing is an effective tool in the treatment of PRKAG2 cardiac syndrome and other dominant inherited cardiac diseases by selectively disrupting disease-causing mutations.

Xie, D. J., et al. (2016). "Construction of CTCF degradation cell line by CRISPR/Cas9 mediated genome editing." Yi Chuan **38**(7): 651-657.

The CCCTC-binding factor (CTCF) is the main insulator protein described in vertebrates. It plays fundamental roles during diverse cellular processes. CTCF gene knockout mice led to death during embryonic development. To further explore the functions of CTCF, we employed a CRISPR/Cas9-based genome engineering strategy to in-frame insert the mitosis-special degradation domain (MD) of cyclin B into the upstream open reading frame of CTCF gene. Fusion protein is designed to degrade during mitosis leaded by MD. As a control group, mutation of a single arginine (R42A) within the destruction box inactivates the MD leading to constitutive expression of MD (*)-CTCF. The homozygous clones were obtained via the screening by puromycin when coexpressed with puromycin resistence gene. The protein level of CTCF in MD-CTCF cell line was about 10% of wild-type cells throughout cell cycles by the analyses of Western blotting and immunofluorescence.

Xing, H. L., et al. (2014). "A CRISPR/Cas9 toolkit for multiplex genome editing in plants." BMC Plant Biol **14**: 327.

BACKGROUND: To accelerate the application of the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/ CRISPR-associated protein 9) system to a variety of plant species, a toolkit with additional plant selectable markers, more gRNA modules, and easier methods for the assembly of one or more gRNA expression cassettes is required. RESULTS: We developed a CRISPR/Cas9 binary vector set based on the pGreen or pCAMBIA backbone, as well as a gRNA (guide RNA) module vector set, as a toolkit for multiplex genome editing in

plants. This toolkit requires no restriction enzymes besides BsaI to generate final constructs harboring maize-codon optimized Cas9 and one or more gRNAs with high efficiency in as little as one cloning step. The toolkit was validated using maize protoplasts, transgenic maize lines, and transgenic Arabidopsis lines and was shown to exhibit high efficiency and specificity.

Xu, S. (2015). "The application of CRISPR-Cas9 genome editing in Caenorhabditis elegans." J Genet Genomics **42**(8): 413-421.

Genome editing using the Cas9 endonuclease of Streptococcus pyogenes has demonstrated unparalleled efficacy and facility for modifying genomes in a wide variety of organisms. Caenorhabditis elegans is one of the most convenient multicellular organisms for genetic analysis, and the application of this novel genome editing technique to this organism promises to revolutionize analysis of gene function in the future. CRISPR-Cas9 has been successfully used to generate imprecise insertions and deletions via non-homologous end-joining mechanisms and to create precise mutations by homology-directed repair from donor templates. Key variables are the methods used to deliver the Cas9 endonuclease and the efficiency of the single guide RNAs. CRISPR-Cas9-mediated editing appears to be highly specific in C. elegans, with no reported off-target effects.

Xu, T., et al. (2015). "Efficient Genome Editing in Clostridium cellulolyticum via CRISPR-Cas9 Nickase." Appl Environ Microbiol **81**(13): 4423-4431.

The CRISPR-Cas9 system is a powerful and revolutionary genome-editing tool for eukaryotic genomes, but its use in bacterial genomes is very limited. Here, we investigated the use of the Streptococcus pyogenes CRISPR-Cas9 system in editing the genome of Clostridium cellulolyticum, a model microorganism for bioenergy research. Wild-type Cas9-induced double-strand breaks were lethal to C. cellulolyticum due to the minimal expression of nonhomologous end joining (NHEJ) components in this strain. To circumvent this lethality, Cas9 nickase was applied to develop a single-nick-triggered homologous recombination strategy, which allows precise one-step editing at intended genomic loci by transforming a single vector. This strategy has a high editing efficiency (>95%) even using short homologous arms (0.2 kb), is able to deliver foreign genes into the genome in a single step without a marker, enables precise editing even at two very similar target sites differing by two bases preceding the seed region, and has a very high target site density.

Yamanaka, Y. (2016). "CRISPR/Cas9 Genome Editing as a Strategy to Study the Tumor Microenvironment in Transgenic Mice." Methods Mol Biol **1458**: 261-271.

Development of engineered site-specific endonucleases like zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR/Cas9 has been revolutionizing genetic approaches in biomedical research fields. These new tools have opened opportunities to carry out targeted genome editing in mouse zygotes without the need for manipulating embryonic stem cells, which have a higher technical burden and many constraints in strain availability. Specific genetic modifications can be directly generated in working genetic backgrounds. This new approach saves time and costs associated with generation and backcrossing of genetically modified animals and will facilitate their use in various cancer research fields.

Yang, H., et al. (2018). "Break Breast Cancer Addiction by CRISPR/Cas9 Genome Editing." J Cancer **9**(2): 219-231.

Breast cancer is the leading diagnosed cancer for women globally. Evolution of breast cancer in tumorigenesis, metastasis and treatment resistance appears to be driven by the aberrant gene expression and protein degradation encoded by the cancer genomes. The uncontrolled cancer growth relies on these cellular events, thus constituting the cancerous programs and rendering the addiction towards them. These programs are likely the potential anticancer biomarkers for Personalized Medicine of breast cancer. This review intends to delineate the impact of the CRSPR/Cas-mediated genome editing in identification and validation of these anticancer biomarkers. It reviews the progress in three aspects of CRISPR/Cas9-mediated editing of the breast cancer genomes: Somatic genome editing, transcription and protein degradation addictions.

Yang, H., et al. (2017). "CRISPR/Cas9-mediated genome editing efficiently creates specific mutations at multiple loci using one sgRNA in Brassica napus." Sci Rep **7**(1): 7489.

CRISPR/Cas9 is a valuable tool for both basic and applied research that has been widely applied to different plant species. Nonetheless, a systematical assessment of the efficiency of this method is not available for the allotetraploid Brassica napus-an important oilseed crop. In this study, we examined the mutation efficiency of the CRISPR/Cas9 method for 12 genes and also determined the pattern, specificity and heritability of these gene modifications in B. napus. The average mutation frequency for a single-

gene targeted sgRNA in the T0 generation is 65.3%. For paralogous genes located in conserved regions that were targeted by sgRNAs, we observed mutation frequencies that ranged from 27.6% to 96.6%. Homozygotes were readily found in T0 plants. A total of 48.2% of the gene mutations, including homozygotes, bi-alleles, and heterozygotes were stably inherited as classic Mendelian alleles in the next generation (T1) without any new mutations or reversions. Moreover, no mutation was found in the putative off-target sites among the examined T0 plants.

Yang, N., et al. (2017). "Revolutionize Genetic Studies and Crop Improvement with High-Throughput and Genome-Scale CRISPR/Cas9 Gene Editing Technology." Mol Plant **10**(9): 1141-1143.

Yang, Y., et al. (2016). "Targeting ABCB1-mediated tumor multidrug resistance by CRISPR/Cas9-based genome editing." Am J Transl Res **8**(9): 3986-3994.

The RNA-guided clustered regularly interspaced short palindromic (CRISPR) in combination with a CRISPR-associated nuclease 9 (Cas9) nuclease system is a new rapid and precise technology for genome editing. In the present study, we applied the CRISPR/Cas9 system to target ABCB1 (also named MDR1) gene which encodes a 170 kDa transmembrane glycoprotein (P-glycoprotein/P-gp) transporting multiple types of chemotherapeutic drugs including taxanes, epipodophyllotoxins, vinca alkaloids and anthracyclines out of cells to contribute multidrug resistance (MDR) in cancer cells. Our data showed that knockout of ABCB1 by CRISPR/Cas9 system was succesfully archieved with two target sgRNAs in two MDR cancer cells due to the alteration of genome sequences. Knockout of ABCB1 by CRISPR/Cas9 system significantly enhances the sensitivity of ABCB1 substrate chemotherapeutic agents and the intracellular accumulation of rhodamine 123 and doxorubicin in MDR cancer cells. Although now there are lots of limitations to the application of CRISPR/Cas9 for editing cancer genes in human patients, our study provides valuable clues for the use of the CRISPR/Cas9 technology in the investigation and conquest of cancer MDR.

Yao, S., et al. (2015). "CRISPR/Cas9-Mediated Genome Editing of Epigenetic Factors for Cancer Therapy." Hum Gene Ther **26**(7): 463-471.

Advances in engineered recombinant nuclease have provided facile and reliable methods for genome editing. Especially with the development of the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated protein-9 nuclease) system, the discovery of various versions of Cas9 proteins and delivery carriers, it is now practicable to introduce desired mutations into the genome, to correct disease-related mutations, and to activate or suppress genes of interest. Epigenetic regulators are often disturbed in cancer cells and are essential for the transformation of normal to cancerous cells. Tumor-related epigenetic alterations or epigenetic factor mutations play a major part during the various steps of carcinogenesis and affect a variety of cancer-related genes and a wide range of cancerous phenotypes. Therefore, epigenetic regulatory enzymes might be candidate targets for cancer therapy. In this review, we discuss prospects of CRISPR/Cas9-based genome editing in targeting epigenetics for cancer gene therapy.

Ye, L., et al. (2016). "Genome editing using CRISPR-Cas9 to create the HPFH genotype in HSPCs: An approach for treating sickle cell disease and beta-thalassemia." Proc Natl Acad Sci U S A **113**(38): 10661-10665.

Hereditary persistence of fetal hemoglobin (HPFH) is a condition in some individuals who have a high level of fetal hemoglobin throughout life. Individuals with compound heterozygous beta-thalassemia or sickle cell disease (SCD) and HPFH have milder clinical manifestations. Using RNA-guided clustered regularly interspaced short palindromic repeats-associated Cas9 (CRISPR-Cas9) genome-editing technology, we deleted, in normal hematopoietic stem and progenitor cells (HSPCs), 13 kb of the beta-globin locus to mimic the naturally occurring Sicilian HPFH mutation. The efficiency of targeting deletion reached 31% in cells with the delivery of both upstream and downstream breakpoint guide RNA (gRNA)-guided Staphylococcus aureus Cas9 nuclease (SaCas9). The erythroid colonies differentiated from HSPCs with HPFH deletion showed significantly higher gamma-globin gene expression compared with the colonies without deletion. By T7 endonuclease 1 assay, we did not detect any off-target effects in the colonies with deletion. We propose that this strategy of using nonhomologous end joining (NHEJ) to modify the genome may provide an efficient approach toward the development of a safe autologous transplantation for patients with homozygous beta-thalassemia and SCD.

Young, S. A., et al. (2015). "Advantages of using the CRISPR/Cas9 system of genome editing to investigate male reproductive mechanisms using mouse models." Asian J Androl **17**(4): 623-627.

Gene disruption technology has long been beneficial for the study of male reproductive biology. However, because of the time and cost involved, this

technology was not a viable method except in specialist laboratories. The advent of the CRISPR/Cas9 system of gene disruption has ushered in a new era of genetic investigation. Now, it is possible to generate gene-disrupted mouse models in very little time and at very little cost. This Highlight article discusses the application of this technology to study the genetics of male fertility and looks at some of the future uses of this system that could be used to reveal the essential and nonessential genetic components of male reproductive mechanisms.

Yuen, K. S., et al. (2015). "CRISPR/Cas9-mediated genome editing of Epstein-Barr virus in human cells." J Gen Virol **96**(Pt 3): 626-636.

The CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated 9) system is a highly efficient and powerful tool for RNA-guided editing of the cellular genome. Whether CRISPR/Cas9 can also cleave the genome of DNA viruses such as Epstein-Barr virus (EBV), which undergo episomal replication in human cells, remains to be established. Here, we reported on CRISPR/Cas9-mediated editing of the EBV genome in human cells. Two guide RNAs (gRNAs) were used to direct a targeted deletion of 558 bp in the promoter region of BART (BamHI A rightward transcript) which encodes viral microRNAs (miRNAs). Targeted editing was achieved in several human epithelial cell lines latently infected with EBV, including nasopharyngeal carcinoma C666-1 cells. CRISPR/Cas9-mediated editing of the EBV genome was efficient. A recombinant virus with the desired deletion was obtained after puromycin selection of cells expressing Cas9 and gRNAs. No off-target cleavage was found by deep sequencing. The loss of BART miRNA expression and activity was verified, supporting the BART promoter as the major promoter of BART RNA. Although CRISPR/Cas9-mediated editing of the multicopy episome of EBV in infected HEK293 cells was mostly incomplete, viruses could be recovered and introduced into other cells at low m.o.i. Recombinant viruses with an edited genome could be further isolated through single-cell sorting.

Zhang, C., et al. (2014). "Efficient editing of malaria parasite genome using the CRISPR/Cas9 system." MBio **5**(4): e01414-01414.

Malaria parasites are unicellular organisms residing inside the red blood cells, and current methods for editing the parasite genes have been inefficient. The CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats and Cas9 endonuclease-mediated genome editing) system is a new powerful technique for genome editing and has been widely employed to study gene function in various organisms. However, whether this technique can be applied to modify the genomes of malaria parasites has not been determined. In this paper, we demonstrated that Cas9 is able to introduce site-specific DNA double-strand breaks in the Plasmodium yoelii genome that can be repaired through homologous recombination. By supplying engineered homologous repair templates, we generated targeted deletion, reporter knock-in, and nucleotide replacement in multiple parasite genes, achieving up to 100% efficiency in gene deletion and 22 to 45% efficiencies in knock-in and allelic replacement. Our results establish methodologies for introducing desired modifications in the P. yoelii genome with high efficiency and accuracy, which will greatly improve our ability to study gene function of malaria parasites. Importance: Malaria, caused by infection of Plasmodium parasites, remains a world-wide public health burden. Although the genomes of many malaria parasites have been sequenced, we still do not know the functions of approximately half of the genes in the genomes. Studying gene function has become the focus of many studies; however, editing genes in malaria parasite genomes is still inefficient.

Zhang, C., et al. (2018). "CRISPR/Cas9-mediated genome editing reveals the synergistic effects of beta-defensin family members on sperm maturation in rat epididymis." FASEB J: fj201700936R.

The epididymis is a male reproductive organ involved in posttesticular sperm maturation and storage, but the mechanism underlying sperm maturation remains unclear. beta-Defensins (Defbs) belong to a family of small, cysteine-rich, cationic peptides that are antimicrobial and modulate the immune response. A large number of Defb genes are expressed abundantly in the male reproductive tract, especially in the epididymis. We and other groups have shown the involvement of several Defb genes in regulation of sperm function. In this study, we found that Defb23, Defb26, and Defb42 were highly expressed in specific regions of the epididymis. Rats with CRISPR/Cas9-mediated single-gene disruption of Defb23, Defb26, or Defb42 had no obvious fertility phenotypes. Those with the deletion of Defb23/ 26 or Defb23/ 26/ 42 became subfertile, and sperm isolated from the epididymal cauda of multiple-mutant rats were demonstrated decreased motility. Meanwhile, the sperm showed precocious capacitation and increased spontaneous acrosome reaction. Consistent with premature capacitation and acrosome reaction, sperm from multiple-gene-knockout rats had significantly increased intracellular calcium.

Zhang, F., et al. (2014). "CRISPR/Cas9 for genome editing: progress, implications and challenges." Hum Mol Genet **23**(R1): R40-46.

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) protein 9 system provides a robust and multiplexable genome editing tool, enabling researchers to precisely manipulate specific genomic elements, and facilitating the elucidation of target gene function in biology and diseases. CRISPR/Cas9 comprises of a nonspecific Cas9 nuclease and a set of programmable sequence-specific CRISPR RNA (crRNA), which can guide Cas9 to cleave DNA and generate double-strand breaks at target sites. Subsequent cellular DNA repair process leads to desired insertions, deletions or substitutions at target sites. The specificity of CRISPR/Cas9-mediated DNA cleavage requires target sequences matching crRNA and a protospacer adjacent motif locating at downstream of target sequences. Here, we review the molecular mechanism, applications and challenges of CRISPR/Cas9-mediated genome editing and clinical therapeutic potential of CRISPR/Cas9 in future.

Zhang, H., et al. (2017). "A Novel and Efficient Method for Bacteria Genome Editing Employing both CRISPR/Cas9 and an Antibiotic Resistance Cassette." Front Microbiol **8**: 812.

As Cas9-mediated cleavage requires both protospacer and protospacer adjacent motif (PAM) sequences, it is impossible to employ the CRISPR/Cas9 system to directly edit genomic sites without available PAM sequences nearby. Here, we optimized the CRISPR/Cas9 system and developed an innovative two-step strategy for efficient genome editing of any sites, which did not rely on the availability of PAM sequences. An antibiotic resistance cassette was employed as both a positive and a negative selection marker. By integrating the optimized two-plasmid CRISPR/Cas system and donor DNA, we achieved gene insertion and point mutation with high efficiency in Escherichia coli, and importantly, obtained clean mutants with no other unwanted mutations. Moreover, genome editing of essential genes was successfully achieved using this approach with a few modifications. Therefore, our newly developed method is PAM-independent and can be used to edit any genomic loci, and we hope this method can also be used for efficient genome editing in other organisms.

Zhang, J. H., et al. (2016). "Optimization of genome editing through CRISPR-Cas9 engineering." Bioengineered **7**(3): 166-174.

CRISPR (Clustered Regularly-Interspaced Short Palindromic Repeats)-Cas9 (CRISPR associated protein 9) has rapidly become the most promising genome editing tool with great potential to revolutionize medicine. Through guidance of a 20 nucleotide RNA (gRNA), CRISPR-Cas9 finds and cuts target protospacer DNA precisely 3 base pairs upstream of a PAM (Protospacer Adjacent Motif). The broken DNA ends are repaired by either NHEJ (Non-Homologous End Joining) resulting in small indels, or by HDR (Homology Directed Repair) for precise gene or nucleotide replacement. Theoretically, CRISPR-Cas9 could be used to modify any genomic sequences, thereby providing a simple, easy, and cost effective means of genome wide gene editing. However, the off-target activity of CRISPR-Cas9 that cuts DNA sites with imperfect matches with gRNA have been of significant concern because clinical applications require 100% accuracy. Additionally, CRISPR-Cas9 has unpredictable efficiency among different DNA target sites and the PAM requirements greatly restrict its genome editing frequency. A large number of efforts have been made to address these impeding issues, but much more is needed to fully realize the medical potential of CRISPR-Cas9. In this article, we summarize the existing problems and current advances of the CRISPR-Cas9 technology and provide perspectives for the ultimate perfection of Cas9-mediated genome editing.

Zhang, W. W., et al. (2017). "Optimized CRISPR-Cas9 Genome Editing for Leishmania and Its Use To Target a Multigene Family, Induce Chromosomal Translocation, and Study DNA Break Repair Mechanisms." mSphere **2**(1).

CRISPR-Cas9-mediated genome editing has recently been adapted for Leishmania spp. parasites, the causative agents of human leishmaniasis. We have optimized this genome-editing tool by selecting for cells with CRISPR-Cas9 activity through cotargeting the miltefosine transporter gene; mutation of this gene leads to miltefosine resistance. This cotargeting strategy integrated into a triple guide RNA (gRNA) expression vector was used to delete all 11 copies of the A2 multigene family; this was not previously possible with the traditional gene-targeting method. We found that the Leishmania donovani rRNA promoter is more efficient than the U6 promoter in driving gRNA expression, and sequential transfections of the oligonucleotide donor significantly eased the isolation of edited mutants. A gRNA and Cas9 coexpression vector was developed that was functional in all tested Leishmania species, including L. donovani, L. major, and L. mexicana. By simultaneously targeting sites from two different chromosomes, all four types of targeted chromosomal translocations were generated, regardless of the polycistronic transcription direction from the parent

chromosomes. It was possible to use this CRISPR system to create a single conserved amino acid substitution (A189G) mutation for both alleles of RAD51, a DNA recombinase involved in homology-directed repair. We found that RAD51 is essential for L. donovani survival based on direct observation of the death of mutants with both RAD51 alleles disrupted, further confirming that this CRISPR system can reveal gene essentiality. Evidence is also provided that microhomology-mediated end joining (MMEJ) plays a major role in double-strand DNA break repair in L. donovani. IMPORTANCELeishmania parasites cause human leishmaniasis. To accelerate characterization of Leishmania genes for new drug and vaccine development, we optimized and simplified the CRISPR-Cas9 genome-editing tool for Leishmania. We show that co-CRISPR targeting of the miltefosine transporter gene and serial transfections of an oligonucleotide donor significantly eased isolation of edited mutants. This cotargeting strategy was efficiently used to delete all 11 members of the A2 virulence gene family. This technical advancement is valuable, since there are many gene clusters and supernumerary chromosomes in the various Leishmania species and isolates. We simplified this CRISPR system by developing a gRNA and Cas9 coexpression vector which could be used to delete genes in various Leishmania species.

Zhang, W. W. and G. Matlashewski (2015). "CRISPR-Cas9-Mediated Genome Editing in Leishmania donovani." MBio **6**(4): e00861.

UNLABELLED: The prokaryotic CRISPR (clustered regularly interspaced short palindromic repeat)-Cas9, an RNA-guided endonuclease, has been shown to mediate efficient genome editing in a wide variety of organisms. In the present study, the CRISPR-Cas9 system has been adapted to Leishmania donovani, a protozoan parasite that causes fatal human visceral leishmaniasis. We introduced the Cas9 nuclease into L. donovani and generated guide RNA (gRNA) expression vectors by using the L. donovani rRNA promoter and the hepatitis delta virus (HDV) ribozyme. It is demonstrated within that L. donovani mainly used homology-directed repair (HDR) and microhomology-mediated end joining (MMEJ) to repair the Cas9 nuclease-created double-strand DNA break (DSB). The nonhomologous end-joining (NHEJ) pathway appears to be absent in L. donovani. With this CRISPR-Cas9 system, it was possible to generate knockouts without selection by insertion of an oligonucleotide donor with stop codons and 25-nucleotide homology arms into the Cas9 cleavage site. Likewise, we disrupted and precisely tagged endogenous genes by inserting a bleomycin drug selection marker and GFP gene into the Cas9 cleavage site. With the use of Hammerhead and HDV ribozymes, a double-gRNA expression vector that further improved gene-targeting efficiency was developed, and it was used to make precise deletion of the 3-kb miltefosine transporter gene (LdMT). In addition, this study identified a novel single point mutation caused by CRISPR-Cas9 in LdMT (M381T) that led to miltefosine resistance, a concern for the only available oral antileishmanial drug. Together, these results demonstrate that the CRISPR-Cas9 system represents an effective genome engineering tool for L. donovani. IMPORTANCE: Leishmania donovani is the causative agent of fatal visceral leishmaniasis. To understand Leishmania infection and pathogenesis and identify new drug targets for control of leishmaniasis, more-efficient ways to manipulate this parasite genome are required. In this study, we have implemented CRISPR-Cas9 genome-editing technology in L. donovani. Both single- and dual-gRNA expression vectors were developed using a strong RNA polymerase I promoter and ribozymes. With this system, it was possible to generate loss-of-function insertion and deletion mutations and introduce drug selection markers and the GFP sequence precisely into the L. donovani genome.

Zhang, Y., et al. (2017). "Programmable base editing of zebrafish genome using a modified CRISPR-Cas9 system." Nat Commun **8**(1): 118.

Precise genetic modifications in model animals are essential for biomedical research. Here, we report a programmable "base editing" system to induce precise base conversion with high efficiency in zebrafish. Using cytidine deaminase fused to Cas9 nickase, up to 28% of site-specific single-base mutations are achieved in multiple gene loci. In addition, an engineered Cas9-VQR variant with 5'-NGA PAM specificities is used to induce base conversion in zebrafish. This shows that Cas9 variants can be used to expand the utility of this technology. Collectively, the targeted base editing system represents a strategy for precise and effective genome editing in zebrafish.The use of base editing enables precise genetic modifications in model animals. Here the authors show high efficient single-base editing in zebrafish using modified Cas9 and its VQR variant with an altered PAM specificity.

Zhang, Y., et al. (2018). "CRISPR/Cas9 Genome Editing: A Promising Tool For Therapeutic Applications of Induced Pluripotent Stem Cells." Curr Stem Cell Res Ther.

Induced pluripotent stem cells hold tremendous potential for biological and therapeutic applications. The development of efficient technologies for the targeted genome alteration of stem cells in disease

models is a prerequisite for utilizing stem cells to their full potential. The revolutionary technology for genome editing known as the clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein 9 (Cas9) system is recently recognized as a powerful tool for editing DNA at specific loci. The ease of use of the CRISPR-Cas9 technology will allow us to improve our understanding of genomic variation in disease processes via cellular and animal models. More recently, this system was modified to repress (CRISPR interference, CRISPRi) or activate (CRISPR activation, CRISPRa) gene expression without alterations in the DNA, which amplified the scope of applications of CRISPR systems for stem cell biology. Here, we highlight latest advances of CRISPR-associated applications in human pluripotent stem cells. The challenges and future prospects of CRISPR-based systems for human research are also discussed.

Zhang, Z., et al. (2017). "CRISPR/Cas9 Genome-Editing System in Human Stem Cells: Current Status and Future Prospects." Mol Ther Nucleic Acids **9**: 230-241.

Genome-editing involves the insertion, deletion, or replacement of DNA in the genome of a living organism using "molecular scissors." Traditional genome editing with engineered nucleases for human stem cells is limited by its low efficiency, high cost, and poor specificity. The CRISPR system has recently emerged as a powerful gene manipulation technique with advantages of high editing efficiency and low cost. Although this technique offers huge potential for gene manipulation in various organisms ranging from prokaryotes to higher mammals, there remain many challenges in human stem cell research. In this review, we highlight the basic biology and application of the CRISPR/Cas9 system in current human stem cell research, discuss its advantages and challenges, and debate the future prospects for human stem cells in regenerative medicine.

Zhao, D., et al. (2016). "Development of a fast and easy method for Escherichia coli genome editing with CRISPR/Cas9." Microb Cell Fact **15**(1): 205.

BACKGROUND: Microbial genome editing is a powerful tool to modify chromosome in way of deletion, insertion or replacement, which is one of the most important techniques in metabolic engineering research. The emergence of CRISPR/Cas9 technique inspires various genomic editing methods. RESULTS: In this research, the goal of development of a fast and easy method for Escherichia coli genome editing with high efficiency is pursued. For this purpose, we designed modular plasmid assembly strategy, compared effects of different length of homologous arms for recombination, and tested different sets of recombinases. The final technique we developed only requires one plasmid construction and one transformation of practice to edit a genomic locus with 3 days and minimal lab work. In addition, the single temperature sensitive plasmid is easy to eliminate for another round of editing. Especially, process of the modularized editing plasmid construction only takes 4 h.

Zheng, X., et al. (2018). "Heterologous and endogenous U6 snRNA promoters enable CRISPR/Cas9 mediated genome editing in Aspergillus niger." Fungal Biol Biotechnol **5**: 2.

Background: U6 promoters have been used for single guide RNA (sgRNA) transcription in the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein (CRISPR/Cas9) genome editing system. However, no available U6 promoters have been identified in Aspergillus niger, which is an important industrial platform for organic acid and protein production. Two CRISPR/Cas9 systems established in A. niger have recourse to the RNA polymerase II promoter or in vitro transcription for sgRNA synthesis, but these approaches generally increase cloning efforts and genetic manipulation. The validation of functional RNA polymerase II promoters is therefore an urgent need for A. niger. Results: Here, we developed a novel CRISPR/Cas9 system in A. niger for sgRNA expression, based on one endogenous U6 promoter and two heterologous U6 promoters. The three tested U6 promoters enabled sgRNA transcription and the disruption of the polyketide synthase albA gene in A. niger. Furthermore, this system enabled highly efficient gene insertion at the targeted genome loci in A. niger using donor DNAs with homologous arms as short as 40-bp.

Zhou, J., et al. (2017). "CRISPR-Cas9 Based Genome Editing Reveals New Insights into MicroRNA Function and Regulation in Rice." Front Plant Sci **8**: 1598.

MicroRNAs (miRNAs) are small non-coding RNAs that play important roles in plant development and stress responses. Loss-of-function analysis of miRNA genes has been traditionally challenging due to lack of appropriate knockout tools. In this study, single miRNA genes (OsMIR408 and OsMIR528) and miRNA gene families (miR815a/b/c and miR820a/b/c) in rice were targeted by CRISPR-Cas9. We showed single strand conformation polymorphism (SSCP) is a more reliable method than restriction fragment length polymorphism (RFLP) for identifying CRISPR-Cas9 generated mutants. Frequencies of targeted mutagenesis among regenerated T0 lines ranged from 48 to 89% at all tested miRNA target sites. In the case

of miRNA528, three independent guide RNAs (gRNAs) all generated biallelic mutations among confirmed mutant lines. When targeted by two gRNAs, miRNA genes were readily to be deleted at a frequency up to 60% in T0 rice lines. Thus, we demonstrate CRISPR-Cas9 is an effective tool for knocking out plant miRNAs. Single-base pair (bp) insertion/deletion mutations (indels) in mature miRNA regions can lead to the generation of functionally redundant miRNAs. Large deletions at either the mature miRNA or the complementary miRNA (*) were found to readily abolish miRNA function.

Zhu, G. H., et al. (2016). "Functional characterization of SlitPBP3 in Spodoptera litura by CRISPR/Cas9 mediated genome editing." Insect Biochem Mol Biol **75**: 1-9.

Functional gene analysis by using genome editing techniques is limited only in few model insects. Here, we reported an efficient and heritable gene mutagenesis analysis in an important lepidopteran pest, Spodoptera litura, using the CRISPR/Cas9 system. By using this system, we successfully obtained the homozygous S. litura strain by targeting the pheromone binding protein 3 gene (SlitPBP3), which allowed us to elucidate the role of this gene in the olfaction of the female sex pheromones. By co-injection of Cas9 mRNA and sgRNA into S. litura eggs, highly efficient chimera mutation in SlitPBP3 loci was detected both in injected eggs (39.1%) and in the resulting individual moths (87.5%). We used the mutant moths as parents to obtain the G1 offspring and the homozygous mutant strain in G2. The function of SlitPBP3 was explored by Electroantennogram (EAG) recordings with a homozygous mutant strain. The result showed that the EAG responses were significantly decreased in mutant males than in control males when treated with the major sex pheromone component (Z9,E11-14:Ac) and a minor component (Z9-14:Ac) at higher dosages. The results demonstrate that s SlitPBP3 gene plays a minor role in the perception of the female sex pheromones. Furthermore, our study provides a useful methodology with the CRISPR/Cas9 system for gene in vivo functional study, particular for lepidopteran species in which the RNAi approach is not efficient.

Zhu, P., et al. (2017). "CRISPR/Cas9-Mediated Genome Editing Corrects Dystrophin Mutation in Skeletal Muscle Stem Cells in a Mouse Model of Muscle Dystrophy." Mol Ther Nucleic Acids **7**: 31-41.

Muscle stem cells (MuSCs) hold great therapeutic potential for muscle genetic disorders, such as Duchenne muscular dystrophy (DMD). The CRISP/Cas9-based genome editing is a promising technology for correcting genetic alterations in mutant genes. In this study, we used fibrin-gel culture system to selectively expand MuSCs from crude skeletal muscle cells of mdx mice, a mouse model of DMD. By CRISP/Cas9-based genome editing, we corrected the dystrophin mutation in expanded MuSCs and restored the skeletal muscle dystrophin expression upon transplantation in mdx mice. Our studies established a reliable and feasible platform for gene correction in MuSCs by genome editing, thus greatly advancing tissue stem cell-based therapies for DMD and other muscle disorders.

References

1. Abbasi, F., et al. (2018). "Revolutionizing male fertility factor research in mice by using the genome editing tool CRISPR/Cas9." Reprod Med Biol 17(1): 3-10.
2. Ahn, J., et al. (2017). "Targeted genome editing in a quail cell line using a customized CRISPR/Cas9 system." Poult Sci 96(5): 1445-1450.
3. Alsaiari, S. K., et al. (2018). "Endosomal Escape and Delivery of CRISPR/Cas9 Genome Editing Machinery Enabled by Nanoscale Zeolitic Imidazolate Framework." J Am Chem Soc 140(1): 143-146.
4. Altenbuchner, J. (2016). "Editing of the Bacillus subtilis Genome by the CRISPR-Cas9 System." Appl Environ Microbiol 82(17): 5421-5427.
5. Andrey, G. and M. Spielmann (2017). "CRISPR/Cas9 Genome Editing in Embryonic Stem Cells." Methods Mol Biol 1468: 221-234.
6. Annunziato, S., et al. (2016). "Modeling invasive lobular breast carcinoma by CRISPR/Cas9-mediated somatic genome editing of the mammary gland." Genes Dev 30(12): 1470-1480.
7. Bai, Y., et al. (2016). "Efficient Genome Editing in Chicken DF-1 Cells Using the CRISPR/Cas9 System." G3 (Bethesda) 6(4): 917-923.
8. Baidu. http://www.baidu.com. 2017.
9. Bak, R. O., et al. (2018). "CRISPR/Cas9 genome editing in human hematopoietic stem cells." Nat Protoc 13(2): 358-376.
10. Bassett, A. R. (2017). "Editing the genome of hiPSC with CRISPR/Cas9: disease models." Mamm Genome 28(7-8): 348-364.
11. Bassett, A. R. and J. L. Liu (2014). "CRISPR/Cas9 and genome editing in Drosophila." J Genet Genomics 41(1): 7-19.
12. Basu, S., et al. (2017). "A novel tool for monitoring endogenous alpha-synuclein transcription by NanoLuciferase tag insertion at the 3'end using CRISPR-Cas9 genome editing technique." Sci Rep 8: 45883.
13. Batzir, N. A., et al. (2017). "Therapeutic Genome Editing and its Potential Enhancement through CRISPR Guide RNA and Cas9 Modifications." Pediatr Endocrinol Rev 14(4): 353-363.
14. Beagan, J. A. and J. E. Phillips-Cremins (2016). "CRISPR/Cas9 genome editing throws descriptive 3-D genome folding studies for a loop." Wiley Interdiscip Rev Syst Biol Med 8(4): 286-299.
15. Bellec, J., et al. (2015). "CFTR inactivation by lentiviral vector-mediated RNA interference and CRISPR-Cas9 genome editing in human airway epithelial cells." Curr Gene Ther 15(5): 447-459.
16. Benakanakere, M. R., et al. (2016). "Investigation of the functional role of human Interleukin-8 gene haplotypes by CRISPR/Cas9 mediated genome editing." Sci Rep 6: 31180.
17. Bence, M., et al. (2017). "Combining the auxin-inducible degradation system with CRISPR/Cas9-based genome editing for the conditional depletion of endogenous Drosophila melanogaster proteins." FEBS J 284(7): 1056-1069.

18. Beneke, T., et al. (2017). "A CRISPR Cas9 high-throughput genome editing toolkit for kinetoplastids." R Soc Open Sci 4(5): 170095.
19. Bono, J. M., et al. (2015). "Connecting genotypes, phenotypes and fitness: harnessing the power of CRISPR/Cas9 genome editing." Mol Ecol 24(15): 3810-3822.
20. Bortesi, L. and R. Fischer (2015). "The CRISPR/Cas9 system for plant genome editing and beyond." Biotechnol Adv 33(1): 41-52.
21. Bruder, M. R., et al. (2016). "Extending CRISPR-Cas9 Technology from Genome Editing to Transcriptional Engineering in the Genus Clostridium." Appl Environ Microbiol 82(20): 6109-6119.
22. Bryant, J. M., et al. (2017). "CRISPR/Cas9 Genome Editing Reveals That the Intron Is Not Essential for var2csa Gene Activation or Silencing in Plasmodium falciparum." MBio 8(4).
23. Burby, P. E. and L. A. Simmons (2017). "CRISPR/Cas9 Editing of the Bacillus subtilis Genome." Bio Protoc 7(8).
24. Butt, H., et al. (2017). "Efficient CRISPR/Cas9-Mediated Genome Editing Using a Chimeric Single-Guide RNA Molecule." Front Plant Sci 8: 1441.
25. Cai, Y., et al. (2015). "CRISPR/Cas9-Mediated Genome Editing in Soybean Hairy Roots." PLoS One 10(8): e0136064.
26. Canny, M. D., et al. (2018). "Inhibition of 53BP1 favors homology-dependent DNA repair and increases CRISPR-Cas9 genome-editing efficiency." Nat Biotechnol 36(1): 95-102.
27. Ceasar, S. A., et al. (2016). "Insert, remove or replace: A highly advanced genome editing system using CRISPR/Cas9." Biochim Biophys Acta 1863(9): 2333-2344.
28. Chadwick, A. C. and K. Musunuru (2017). "Treatment of Dyslipidemia Using CRISPR/Cas9 Genome Editing." Curr Atheroscler Rep 19(7): 32.
29. Chadwick, A. C. and K. Musunuru (2018). "CRISPR-Cas9 Genome Editing for Treatment of Atherogenic Dyslipidemia." Arterioscler Thromb Vasc Biol 38(1): 12-18.
30. Chen, J., et al. (2017). "CRISPR/Cas9-mediated efficient genome editing via blastospore-based transformation in entomopathogenic fungus Beauveria bassiana." Sci Rep 8: 45763.
31. Chen, S., et al. (2016). "CRISPR-Cas9: from Genome Editing to Cancer Research." Int J Biol Sci 12(12): 1427-1436.
32. Chen, W., et al. (2017). "Rapid and Efficient Genome Editing in Staphylococcus aureus by Using an Engineered CRISPR/Cas9 System." J Am Chem Soc 139(10): 3790-3795.
33. Chiang, T. W., et al. (2016). "CRISPR-Cas9(D10A) nickase-based genotypic and phenotypic screening to enhance genome editing." Sci Rep 6: 24356.
34. Chiou, S. H., et al. (2015). "Pancreatic cancer modeling using retrograde viral vector delivery and in vivo CRISPR/Cas9-mediated somatic genome editing." Genes Dev 29(14): 1576-1585.
35. Chira, S., et al. (2017). "CRISPR/Cas9: Transcending the Reality of Genome Editing." Mol Ther Nucleic Acids 7: 211-222.
36. Chou, Y. Y., et al. (2016). "Inhibition of JCPyV infection mediated by targeted viral genome editing using CRISPR/Cas9." Sci Rep 6: 36921.
37. Chung, W. Y., et al. (2016). "Generation of DeltaF508-CFTR T84 cell lines by CRISPR/Cas9-mediated genome editing." Biotechnol Lett 38(12): 2023-2034.
38. Cottle, R. N., et al. (2015). "Controlled delivery of beta-globin-targeting TALENs and CRISPR/Cas9 into mammalian cells for genome editing using microinjection." Sci Rep 5: 16031.
39. Cuculis, L. and C. M. Schroeder (2017). "A Single-Molecule View of Genome Editing Proteins: Biophysical Mechanisms for TALEs and CRISPR/Cas9." Annu Rev Chem Biomol Eng 8: 577-597.
40. D'Agostino, Y. and S. D'Aniello (2017). "Molecular basis, applications and challenges of CRISPR/Cas9: a continuously evolving tool for genome editing." Brief Funct Genomics 16(4): 211-216.
41. Dehler, C. E., et al. (2016). "Development of an Efficient Genome Editing Method by CRISPR/Cas9 in a Fish Cell Line." Mar Biotechnol (NY) 18(4): 449-452.
42. Demirci, Y., et al. (2018). "CRISPR/Cas9: An RNA-guided highly precise synthetic tool for plant genome editing." J Cell Physiol 233(3): 1844-1859.
43. Ding, Y., et al. (2016). "Recent Advances in Genome Editing Using CRISPR/Cas9." Front Plant Sci 7: 703.
44. Dominguez, A. A., et al. (2016). "Beyond editing: repurposing CRISPR-Cas9 for precision genome regulation and interrogation." Nat Rev Mol Cell Biol 17(1): 5-15.
45. Dong, S., et al. (2015). "Heritable CRISPR/Cas9-mediated genome editing in the yellow fever mosquito, Aedes aegypti." PLoS One 10(3): e0122353.
46. Doudna, J. A. and E. Charpentier (2014). "Genome editing. The new frontier of genome engineering with CRISPR-Cas9." Science 346(6213): 1258096.
47. Dow, L. E., et al. (2015). "Inducible in vivo genome editing with CRISPR-Cas9." Nat Biotechnol 33(4): 390-394.
48. Duda, K., et al. (2014). "High-efficiency genome editing via 2A-coupled co-expression of fluorescent proteins and zinc finger nucleases or CRISPR/Cas9 nickase pairs." Nucleic Acids Res 42(10): e84.
49. Eid, A. and M. M. Mahfouz (2016). "Genome editing: the road of CRISPR/Cas9 from bench to clinic." Exp Mol Med 48(10): e265.
50. El-Sayed, A. S. A., et al. (2017). "Genome editing approaches: manipulating of lovastatin and taxol synthesis of filamentous fungi by CRISPR/Cas9 system." Appl Microbiol Biotechnol 101(10): 3953-3976.
51. Fang, Y., et al. (2017). "Efficient Genome Editing in the Oomycete Phytophthora sojae Using CRISPR/Cas9." Curr Protoc Microbiol 44: 21A 21 21-21A 21 26.
52. Farasat, I. and H. M. Salis (2016). "A Biophysical Model of CRISPR/Cas9 Activity for Rational Design of Genome Editing and Gene Regulation." PLoS Comput Biol 12(1): e1004724.
53. Farboud, B. (2017). "Targeted genome editing in Caenorhabditis elegans using CRISPR/Cas9." Wiley Interdiscip Rev Dev Biol 6(6).
54. Feng, Y., et al. (2016). "Expanding CRISPR/Cas9 Genome Editing Capacity in Zebrafish Using SaCas9." G3 (Bethesda) 6(8): 2517-2521.
55. Fernandez, R. and J. Berro (2016). "Use of a fluoride channel as a new selection marker for fission yeast plasmids and application to fast genome editing with CRISPR/Cas9." Yeast 33(10): 549-557.
56. Ferreira, R., et al. (2018). "Multiplexed CRISPR/Cas9 Genome Editing and Gene Regulation Using Csy4 in Saccharomyces cerevisiae." ACS Synth Biol 7(1): 10-15.
57. Fujihara, Y. and M. Ikawa (2014). "CRISPR/Cas9-based genome editing in mice by single plasmid injection." Methods Enzymol 546: 319-336.
58. Gandhi, S., et al. (2017). "Optimization of CRISPR/Cas9 genome editing for loss-of-function in the early chick embryo." Dev Biol 432(1): 86-97.
59. Gao, S., et al. (2016). "Multiplex gene editing of the Yarrowia lipolytica genome using the CRISPR-Cas9 system." J Ind Microbiol Biotechnol 43(8): 1085-1093.
60. Gao, W., et al. (2017). "Genome Editing in Cotton with the CRISPR/Cas9 System." Front Plant Sci 8: 1364.
61. Gao, X., et al. (2016). "An Effective Strategy for Reliably Isolating Heritable and Cas9-Free Arabidopsis Mutants

Generated by CRISPR/Cas9-Mediated Genome Editing." Plant Physiol 171(3): 1794-1800.
62. Gasiunas, G. and V. Siksnys (2013). "RNA-dependent DNA endonuclease Cas9 of the CRISPR system: Holy Grail of genome editing?" Trends Microbiol 21(11): 562-567.
63. Gee, P., et al. (2017). "Cellular Reprogramming, Genome Editing, and Alternative CRISPR Cas9 Technologies for Precise Gene Therapy of Duchenne Muscular Dystrophy." Stem Cells Int 2017: 8765154.
64. Giau, V. V., et al. (2018). "Genome-editing applications of CRISPR-Cas9 to promote in vitro studies of Alzheimer's disease." Clin Interv Aging 13: 221-233.
65. Gokcezade, J., et al. (2014). "Efficient CRISPR/Cas9 plasmids for rapid and versatile genome editing in Drosophila." G3 (Bethesda) 4(11): 2279-2282.
66. Gonzalez, F. (2016). "CRISPR/Cas9 genome editing in human pluripotent stem cells: Harnessing human genetics in a dish." Dev Dyn 245(7): 788-806.
67. Google. http://www.google.com. 2017.
68. Gori, J. L., et al. (2015). "Delivery and Specificity of CRISPR-Cas9 Genome Editing Technologies for Human Gene Therapy." Hum Gene Ther 26(7): 443-451.
69. Gratz, S. J., et al. (2015). "CRISPR-Cas9 Genome Editing in Drosophila." Curr Protoc Mol Biol 111: 31 32 31-20.
70. Gratz, S. J., et al. (2015). "Precise Genome Editing of Drosophila with CRISPR RNA-Guided Cas9." Methods Mol Biol 1311: 335-348.
71. Grav, L. M., et al. (2017). "Application of CRISPR/Cas9 Genome Editing to Improve Recombinant Protein Production in CHO Cells." Methods Mol Biol 1603: 101-118.
72. Grenier, F., et al. (2015). "Selection and Validation of Spacer Sequences for CRISPR-Cas9 Genome Editing and Transcription Regulation in Bacteria." Methods Mol Biol 1334: 233-244.
73. Grobarczyk, B., et al. (2015). "Generation of Isogenic Human iPS Cell Line Precisely Corrected by Genome Editing Using the CRISPR/Cas9 System." Stem Cell Rev 11(5): 774-787.
74. Guitart, J. R., Jr., et al. (2016). "Research Techniques Made Simple: The Application of CRISPR-Cas9 and Genome Editing in Investigative Dermatology." J Invest Dermatol 136(9): e87-93.
75. Gundry, M. C., et al. (2016). "Highly Efficient Genome Editing of Murine and Human Hematopoietic Progenitor Cells by CRISPR/Cas9." Cell Rep 17(5): 1453-1461.
76. Gurumurthy, C. B., et al. (2016). "GONAD: A Novel CRISPR/Cas9 Genome Editing Method that Does Not Require Ex Vivo Handling of Embryos." Curr Protoc Hum Genet 88: Unit 15 18.
77. Haeussler, M. and J. P. Concordet (2016). "Genome Editing with CRISPR-Cas9: Can It Get Any Better?" J Genet Genomics 43(5): 239-250.
78. Han, Y. and Q. W. Li (2016). "[Application progress of CRISPR/Cas9 genome editing technology in the treatment of HIV-1 infection]." Yi Chuan 38(1): 9-16.
79. Han, Y., et al. (2015). "CRISPR-Cas9 genome editing of a single regulatory element nearly abolishes target gene expression in mice--brief report." Arterioscler Thromb Vasc Biol 35(2): 312-315.
80. Hashimoto, M. and T. Takemoto (2015). "Electroporation enables the efficient mRNA delivery into the mouse zygotes and facilitates CRISPR/Cas9-based genome editing." Sci Rep 5: 11315.
81. He, Z., et al. (2016). "Comparison of CRISPR/Cas9 and TALENs on editing an integrated EGFP gene in the genome of HEK293FT cells." Springerplus 5(1): 814.
82. Henao-Mejia, J., et al. (2016). "Generation of Genetically Modified Mice Using the CRISPR-Cas9 Genome-Editing System." Cold Spring Harb Protoc 2016(2): pdb prot090704.
83. Heo, M. J., et al. (2017). "Controlling Citrate Synthase Expression by CRISPR/Cas9 Genome Editing for n-Butanol Production in Escherichia coli." ACS Synth Biol 6(2): 182-189.
84. Hindriksen, S., et al. (2017). "Baculoviral delivery of CRISPR/Cas9 facilitates efficient genome editing in human cells." PLoS One 12(6): e0179514.
85. Hirosawa, M., et al. (2017). "Cell-type-specific genome editing with a microRNA-responsive CRISPR-Cas9 switch." Nucleic Acids Res 45(13): e118.
86. Hirose, M., et al. (2017). "CRISPR/Cas9-mediated genome editing in wild-derived mice: generation of tamed wild-derived strains by mutation of the a (nonagouti) gene." Sci Rep 7: 42476.
87. Hruscha, A. and B. Schmid (2015). "Generation of zebrafish models by CRISPR /Cas9 genome editing." Methods Mol Biol 1254: 341-350.
88. Hruscha, A., et al. (2013). "Efficient CRISPR/Cas9 genome editing with low off-target effects in zebrafish." Development 140(24): 4982-4987.
89. https://en.wikipedia.org/wiki/CRISPR
90. https://en.wikipedia.org/wiki/Genome_editing
91. Ikeda, M., et al. (2017). "Correction of a Disease Mutation using CRISPR/Cas9-assisted Genome Editing in Japanese Black Cattle." Sci Rep 7(1): 17827.
92. Ikmi, A., et al. (2014). "TALEN and CRISPR/Cas9-mediated genome editing in the early-branching metazoan Nematostella vectensis." Nat Commun 5: 5486.
93. Ishizu, H., et al. (2017). "Use of the CRISPR-Cas9 system for genome editing in cultured Drosophila ovarian somatic cells." Methods 126: 186-192.
94. Jiang, Y., et al. (2015). "Multigene editing in the Escherichia coli genome via the CRISPR-Cas9 system." Appl Environ Microbiol 81(7): 2506-2514.
95. Jing, W., et al. (2015). "CRISPR/CAS9-Mediated Genome Editing of miRNA-155 Inhibits Proinflammatory Cytokine Production by RAW264.7 Cells." Biomed Res Int 2015: 326042.
96. Katayama, T., et al. (2016). "Development of a genome editing technique using the CRISPR/Cas9 system in the industrial filamentous fungus Aspergillus oryzae." Biotechnol Lett 38(4): 637-642.
97. Katic, I., et al. (2015). "CRISPR/Cas9 Genome Editing in Caenorhabditis elegans: Evaluation of Templates for Homology-Mediated Repair and Knock-Ins by Homology-Independent DNA Repair." G3 (Bethesda) 5(8): 1649-1656.
98. Kelley, M. L., et al. (2016). "Versatility of chemically synthesized guide RNAs for CRISPR-Cas9 genome editing." J Biotechnol 233: 74-83.
99. Khlestkina, E. K. and V. K. Shumny (2016). "[Prospects for application of breakthrough technologies in breeding: The CRISPR/Cas9 system for plant genome editing]." Genetika 52(7): 774-787.
100. Khurshid, H., et al. (2018). "An Era of CRISPR/ Cas9 Mediated Plant Genome Editing." Curr Issues Mol Biol 26: 47-54.
101. Klann, T. S., et al. (2017). "CRISPR-Cas9 epigenome editing enables high-throughput screening for functional regulatory elements in the human genome." Nat Biotechnol 35(6): 561-568.
102. Klimek-Chodacka, M., et al. (2018). "Efficient CRISPR/Cas9-based genome editing in carrot cells." Plant Cell Rep.
103. Korge, S., et al. (2015). "Highly Efficient Genome Editing via CRISPR/Cas9 to Create Clock Gene Knockout Cells." J Biol Rhythms 30(5): 389-395.
104. Koutroumpa, F. A., et al. (2016). "Heritable genome editing with CRISPR/Cas9 induces anosmia in a crop pest moth." Sci Rep 6: 29620.
105. Lander, N., et al. (2016). "Genome Editing by CRISPR/Cas9: A Game Change in the Genetic Manipulation of Protists." J Eukaryot Microbiol 63(5): 679-690.

106. Laughery, M. F., et al. (2015). "New vectors for simple and streamlined CRISPR-Cas9 genome editing in Saccharomyces cerevisiae." Yeast 32(12): 711-720.
107. Lee, C. M., et al. (2016). "The Neisseria meningitidis CRISPR-Cas9 System Enables Specific Genome Editing in Mammalian Cells." Mol Ther 24(3): 645-654.
108. Lepidoptera suffer critical lack of genetic tools and heritable genome edition has been achieved only in a few model species. Here we demonstrate that the CRISPR/Cas9 system is highly efficient for genome editing in a non-model crop pest Lepidoptera, the noctuid moth Spodoptera littoralis. We knocked-out the olfactory receptor co-receptor Orco gene to investigate its function in Lepidoptera olfaction. We find that 89.6% of the injected individuals carried Orco mutations, 70% of which transmitted them to the next generation. CRISPR/Cas9-mediated Orco knockout caused defects in plant odor and sex pheromone olfactory detection in homozygous individuals. Our work genetically defines Orco as an essential OR partner for both host and mate detection in Lepidoptera, and demonstrates that CRISPR/Cas9 is a simple and highly efficient genome editing technique in noctuid pests opening new routes for gene function analysis and the development of novel pest control strategies.
109. Li, G., et al. (2017). "Small molecules enhance CRISPR/Cas9-mediated homology-directed genome editing in primary cells." Sci Rep 7(1): 8943.
110. Li, H. H. and G. Liu (2017). "The application of CRISPR/Cas9 in genome editing of filamentous fungi." Yi Chuan 39(5): 355-367.
111. Li, J. F., et al. (2015). "Targeted plant genome editing via the CRISPR/Cas9 technology." Methods Mol Biol 1284: 239-255.
112. Li, K., et al. (2018). "Development of an Efficient Genome Editing Tool in Bacillus licheniformis Using CRISPR-Cas9 Nickase." Appl Environ Microbiol.
113. Li, L., et al. (2017). "Artificial Virus Delivers CRISPR-Cas9 System for Genome Editing of Cells in Mice." ACS Nano 11(1): 95-111.
114. Li, W. and G. Ou (2016). "The application of somatic CRISPR-Cas9 to conditional genome editing in Caenorhabditis elegans." Genesis 54(4): 170-181.
115. Li, X., et al. (2015). "Outbred genome sequencing and CRISPR/Cas9 gene editing in butterflies." Nat Commun 6: 8212.
116. Liang, G., et al. (2016). "Selection of highly efficient sgRNAs for CRISPR/Cas9-based plant genome editing." Sci Rep 6: 21451.
117. Liang, X., et al. (2017). "Enhanced CRISPR/Cas9-mediated precise genome editing by improved design and delivery of gRNA, Cas9 nuclease, and donor DNA." J Biotechnol 241: 136-146.
118. Lin, C. Y. and Y. H. Su (2016). "Genome editing in sea urchin embryos by using a CRISPR/Cas9 system." Dev Biol 409(2): 420-428.
119. Lin, C., et al. (2016). "Increasing the Efficiency of CRISPR/Cas9-mediated Precise Genome Editing of HSV-1 Virus in Human Cells." Sci Rep 6: 34531.
120. Liu, G. G., et al. (2015). "[Efficient genome editing in human pluripotent stem cells through CRISPR/Cas9]." Yi Chuan 37(11): 1167-1173.
121. Liu, H., et al. (2016). "Genome Editing of Wnt-1, a Gene Associated with Segmentation, via CRISPR/Cas9 in the Pine Caterpillar Moth, Dendrolimus punctatus." Front Physiol 7: 666.
122. Liu, J., et al. (2017). "Development of a CRISPR/Cas9 genome editing toolbox for Corynebacterium glutamicum." Microb Cell Fact 16(1): 205.
123. Liu, K. I., et al. (2016). "A chemical-inducible CRISPR-Cas9 system for rapid control of genome editing." Nat Chem Biol 12(11): 980-987.
124. Liu, Q., et al. (2017). "CRISPR/Cas9-based efficient genome editing in Staphylococcus aureus." Acta Biochim Biophys Sin (Shanghai) 49(9): 764-770.
125. Liu, Q., et al. (2017). "Development of a genome-editing CRISPR/Cas9 system in thermophilic fungal Myceliophthora species and its application to hyper-cellulase production strain engineering." Biotechnol Biofuels 10: 1.
126. Liu, T., et al. (2016). "Development and potential applications of CRISPR-Cas9 genome editing technology in sarcoma." Cancer Lett 373(1): 109-118.
127. Liu, X., et al. (2017). "CRISPR/Cas9-mediated genome editing in plants." Methods 121-122: 94-102.
128. Liu, Z., et al. (2016). "Efficient genome editing of genes involved in neural crest development using the CRISPR/Cas9 system in Xenopus embryos." Cell Biosci 6: 22.
129. Liu, Z., et al. (2017). "Genome editing of the HIV co-receptors CCR5 and CXCR4 by CRISPR-Cas9 protects CD4(+) T cells from HIV-1 infection." Cell Biosci 7: 47.
130. Lo, T. W., et al. (2013). "Precise and heritable genome editing in evolutionarily diverse nematodes using TALENs and CRISPR/Cas9 to engineer insertions and deletions." Genetics 195(2): 331-348.
131. Lowder, L. G., et al. (2015). "A CRISPR/Cas9 Toolbox for Multiplexed Plant Genome Editing and Transcriptional Regulation." Plant Physiol 169(2): 971-985.
132. Lowder, L., et al. (2017). "Rapid Construction of Multiplexed CRISPR-Cas9 Systems for Plant Genome Editing." Methods Mol Biol 1578: 291-307.
133. Lu, J., et al. (2016). "A redesigned CRISPR/Cas9 system for marker-free genome editing in Plasmodium falciparum." Parasit Vectors 9: 198.
134. Lu, J., et al. (2017). "Multimode drug inducible CRISPR/Cas9 devices for transcriptional activation and genome editing." Nucleic Acids Res.
135. Luo, X., et al. (2016). "Application of the genome editing tool CRISPR/Cas9 in non-human primates." Dongwuxue Yanjiu 37(4): 214-219.
136. Ma H, Chen G. Stem cell. The Journal of American Science 2005;1(2):90-92.
137. Ma H, Cherng S. Eternal Life and Stem Cell. Nature and Science. 2007;5(1):81-96.
138. Ma H, Cherng S. Nature of Life. Life Science Journal 2005;2(1):7-15.
139. Ma H, Yang Y. Turritopsis nutricula. Nature and Science 2010;8(2):15-20. http://www.sciencepub.net/nature/ns0802/03_1279_hongbao_turritopsis_ns0802_15_20.pdf.
140. Ma H. The Nature of Time and Space. Nature and science 2003;1(1):1-11. Nature and science 2007;5(1):81-96.
141. Ma, S., et al. (2014). "CRISPR/Cas9 mediated multiplex genome editing and heritable mutagenesis of BmKu70 in Bombyx mori." Sci Rep 4: 4489.
142. Ma, X. and Y. G. Liu (2016). "CRISPR/Cas9-Based Multiplex Genome Editing in Monocot and Dicot Plants." Curr Protoc Mol Biol 115: 31 36 31-31 36 21.
143. Ma, X. L. and Y. G. Liu (2016). "[CRISPR/Cas9-based genome editing systems and the analysis of targeted genome mutations in plants]." Yi Chuan 38(2): 118-125.
144. Ma, X., et al. (2015). "A Robust CRISPR/Cas9 System for Convenient, High-Efficiency Multiplex Genome Editing in Monocot and Dicot Plants." Mol Plant 8(8): 1274-1284.
145. Ma, X., et al. (2016). "CRISPR/Cas9 Platforms for Genome Editing in Plants: Developments and Applications." Mol Plant 9(7): 961-974.
146. Ma, Y., et al. (2016). "Increasing the efficiency of CRISPR/Cas9-mediated precise genome editing in rats by inhibiting NHEJ and using Cas9 protein." RNA Biol 13(7): 605-612.
147. Mabashi-Asazuma, H. and D. L. Jarvis (2017). "CRISPR-Cas9 vectors for genome editing and host engineering in the

baculovirus-insect cell system." Proc Natl Acad Sci U S A 114(34): 9068-9073.
148. Marsland Press. http://www.sciencepub.net. 2017.
149. Martinez, V., et al. (2017). "CRISPR/Cas9-based genome editing for simultaneous interference with gene expression and protein stability." Nucleic Acids Res 45(20): e171.
150. Marusugi, K., et al. (2016). "Functional validation of tensin2 SH2-PTB domain by CRISPR/Cas9-mediated genome editing." J Vet Med Sci 78(9): 1413-1420.
151. Meng, X., et al. (2017). "[CRISPR-Cas9 mediated genome editing in Caenorhabditis elegans]." Sheng Wu Gong Cheng Xue Bao 33(10): 1693-1699.
152. Minkenberg, B., et al. (2017). "CRISPR/Cas9-Enabled Multiplex Genome Editing and Its Application." Prog Mol Biol Transl Sci 149: 111-132.
153. Morineau, C., et al. (2017). "Selective gene dosage by CRISPR-Cas9 genome editing in hexaploid Camelina sativa." Plant Biotechnol J 15(6): 729-739.
154. Mou, H., et al. (2017). "CRISPR/Cas9-mediated genome editing induces exon skipping by alternative splicing or exon deletion." Genome Biol 18(1): 108.
155. Muller, M., et al. (2016). "Streptococcus thermophilus CRISPR-Cas9 Systems Enable Specific Editing of the Human Genome." Mol Ther 24(3): 636-644.
156. Nagaraju, S., et al. (2016). "Genome editing of Clostridium autoethanogenum using CRISPR/Cas9." Biotechnol Biofuels 9: 219.
157. National Center for Biotechnology Information, U.S. National Library of Medicine. http://www.ncbi.nlm.nih.gov/pubmed. 2017.
158. Neggers, J. E., et al. (2015). "Identifying drug-target selectivity of small-molecule CRM1/XPO1 inhibitors by CRISPR/Cas9 genome editing." Chem Biol 22(1): 107-116.
159. Nguyen, T. H. and I. Anegon (2016). "Successful correction of hemophilia by CRISPR/Cas9 genome editing in vivo: delivery vector and immune responses are the key to success." EMBO Mol Med 8(5): 439-441.
160. Nihongaki, Y., et al. (2015). "Photoactivatable CRISPR-Cas9 for optogenetic genome editing." Nat Biotechnol 33(7): 755-760.
161. Nishitani, C., et al. (2016). "Efficient Genome Editing in Apple Using a CRISPR/Cas9 system." Sci Rep 6: 31481.
162. Noman, A., et al. (2016). "CRISPR-Cas9: Tool for Qualitative and Quantitative Plant Genome Editing." Front Plant Sci 7: 1740.
163. Norton, E. L., et al. (2017). "Development of a CRISPR-Cas9 System for Efficient Genome Editing of Candida lusitaniae." mSphere 2(3).
164. Numamoto, M., et al. (2017). "Efficient genome editing by CRISPR/Cas9 with a tRNA-sgRNA fusion in the methylotrophic yeast Ogataea polymorpha." J Biosci Bioeng 124(5): 487-492.
165. Odipio, J., et al. (2017). "Efficient CRISPR/Cas9 Genome Editing of Phytoene desaturase in Cassava." Front Plant Sci 8: 1780.
166. Oh-Hashi, K., et al. (2018). "Functional validation of ATF4 and GADD34 in Neuro2a cells by CRISPR/Cas9-mediated genome editing." Mol Cell Biochem 440(1-2): 65-75.
167. Ohmori, T., et al. (2017). "CRISPR/Cas9-mediated genome editing via postnatal administration of AAV vector cures haemophilia B mice." Sci Rep 7(1): 4159.
168. Oji, A., et al. (2016). "CRISPR/Cas9 mediated genome editing in ES cells and its application for chimeric analysis in mice." Sci Rep 6: 31666.
169. Ou, Z. and X. Sun (2016). "[Application of CRISPR-Cas9 genome editing for constructing animal models of human diseases]." Zhonghua Yi Xue Yi Chuan Xue Za Zhi 33(4): 559-563.
170. Ousterout, D. G., et al. (2015). "Multiplex CRISPR/Cas9-based genome editing for correction of dystrophin mutations that cause Duchenne muscular dystrophy." Nat Commun 6: 6244.
171. Ozaki, A., et al. (2017). "Metabolic engineering of Schizosaccharomyces pombe via CRISPR-Cas9 genome editing for lactic acid production from glucose and cellobiose." Metab Eng Commun 5: 60-67.
172. Paix, A., et al. (2015). "High Efficiency, Homology-Directed Genome Editing in Caenorhabditis elegans Using CRISPR-Cas9 Ribonucleoprotein Complexes." Genetics 201(1): 47-54.
173. Paix, A., et al. (2017). "Precision genome editing using CRISPR-Cas9 and linear repair templates in C. elegans." Methods 121-122: 86-93.
174. Pankowicz, F. P., et al. (2016). "Reprogramming metabolic pathways in vivo with CRISPR/Cas9 genome editing to treat hereditary tyrosinaemia." Nat Commun 7: 12642.
175. Park, M. Y., et al. (2017). "Generation of lung cancer cell lines harboring EGFR T790M mutation by CRISPR/Cas9-mediated genome editing." Oncotarget 8(22): 36331-36338.
176. Paul, J. W., 3rd and Y. Qi (2016). "CRISPR/Cas9 for plant genome editing: accomplishments, problems and prospects." Plant Cell Rep 35(7): 1417-1427.
177. Pellagatti, A., et al. (2015). "Application of CRISPR/Cas9 genome editing to the study and treatment of disease." Arch Toxicol 89(7): 1023-1034.
178. Peng, C., et al. (2015). "CRISPR/Cas9-based tools for targeted genome editing and replication control of HBV." Virol Sin 30(5): 317-325.
179. Peng, R., et al. (2016). "Potential pitfalls of CRISPR/Cas9-mediated genome editing." FEBS J 283(7): 1218-1231.
180. Platt, R. J., et al. (2014). "CRISPR-Cas9 knockin mice for genome editing and cancer modeling." Cell 159(2): 440-455.
181. Pohl, C., et al. (2016). "CRISPR/Cas9 Based Genome Editing of Penicillium chrysogenum." ACS Synth Biol 5(7): 754-764.
182. Qin, W., et al. (2015). "Efficient CRISPR/Cas9-Mediated Genome Editing in Mice by Zygote Electroporation of Nuclease." Genetics 200(2): 423-430.
183. Quetier, F. (2016). "The CRISPR-Cas9 technology: Closer to the ultimate toolkit for targeted genome editing." Plant Sci 242: 65-76.
184. Rahdar, M., et al. (2015). "Synthetic CRISPR RNA-Cas9-guided genome editing in human cells." Proc Natl Acad Sci U S A 112(51): E7110-7117.
185. Ran, F. A., et al. (2013). "Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity." Cell 154(6): 1380-1389.
186. Ren, X., et al. (2017). "Genome editing in Drosophila melanogaster: from basic genome engineering to the multipurpose CRISPR-Cas9 system." Sci China Life Sci 60(5): 476-489.
187. Renaud, J. B., et al. (2016). "Improved Genome Editing Efficiency and Flexibility Using Modified Oligonucleotides with TALEN and CRISPR-Cas9 Nucleases." Cell Rep 14(9): 2263-2272.
188. Ringer, K. P., et al. (2018). "Comparative Analysis of Lipid-Mediated CRISPR-Cas9 Genome Editing Techniques." Cell Biol Int.
189. Rodriguez-Lopez, M., et al. (2016). "A CRISPR/Cas9-based method and primer design tool for seamless genome editing in fission yeast." Wellcome Open Res 1: 19.
190. Ronda, C., et al. (2014). "Accelerating genome editing in CHO cells using CRISPR Cas9 and CRISPy, a web-based target finding tool." Biotechnol Bioeng 111(8): 1604-1616.
191. Roper, J., et al. (2018). "Colonoscopy-based colorectal cancer modeling in mice with CRISPR-Cas9 genome editing and organoid transplantation." Nat Protoc 13(2): 217-234.
192. Ruan, G. X., et al. (2017). "CRISPR/Cas9-Mediated Genome Editing as a Therapeutic Approach for Leber Congenital Amaurosis 10." Mol Ther 25(2): 331-341.
193. Ryder, P., et al. (2017). "Generation of stable nulliplex autopolyploid lines of Arabidopsis thaliana using

CRISPR/Cas9 genome editing." Plant Cell Rep 36(6): 1005-1008.
194. Salsman, J., et al. (2017). "CRISPR/Cas9 Gene Editing: From Basic Mechanisms to Improved Strategies for Enhanced Genome Engineering In Vivo." Curr Gene Ther 17(4): 263-274.
195. Sato, M., et al. (2018). "Timing of CRISPR/Cas9-related mRNA microinjection after activation as an important factor affecting genome editing efficiency in porcine oocytes." Theriogenology 108: 29-38.
196. Savic, N. and G. Schwank (2016). "Advances in therapeutic CRISPR/Cas9 genome editing." Transl Res 168: 15-21.
197. Schaeffer, S. M. and P. A. Nakata (2015). "CRISPR/Cas9-mediated genome editing and gene replacement in plants: Transitioning from lab to field." Plant Sci 240: 130-142.
198. Schwank, G. and H. Clevers (2016). "CRISPR/Cas9-Mediated Genome Editing of Mouse Small Intestinal Organoids." Methods Mol Biol 1422: 3-11.
199. Schwartz, C. M., et al. (2016). "Synthetic RNA Polymerase III Promoters Facilitate High-Efficiency CRISPR-Cas9-Mediated Genome Editing in Yarrowia lipolytica." ACS Synth Biol 5(4): 356-359.
200. Shao, M., et al. (2016). "The big bang of genome editing technology: development and application of the CRISPR/Cas9 system in disease animal models." Dongwuxue Yanjiu 37(4): 191-204.
201. Shen, B., et al. (2017). "Development of CRISPR/Cas9 for Efficient Genome Editing in Toxoplasma gondii." Methods Mol Biol 1498: 79-103.
202. Shen, Y., et al. (2018). "Generation of PTEN knockout bone marrow mesenchymal stem cell lines by CRISPR/Cas9-mediated genome editing." Cytotechnology.
203. Shi, T. Q., et al. (2017). "CRISPR/Cas9-based genome editing of the filamentous fungi: the state of the art." Appl Microbiol Biotechnol 101(20): 7435-7443.
204. Singh, V., et al. (2017). "Exploring the potential of genome editing CRISPR-Cas9 technology." Gene 599: 1-18.
205. Soares Medeiros, L. C., et al. (2017). "Rapid, Selection-Free, High-Efficiency Genome Editing in Protozoan Parasites Using CRISPR-Cas9 Ribonucleoproteins." MBio 8(6).
206. Soda, N., et al. (2017). "CRISPR-Cas9 based plant genome editing: Significance, opportunities and recent advances." Plant Physiol Biochem.
207. Sollelis, L., et al. (2015). "First efficient CRISPR-Cas9-mediated genome editing in Leishmania parasites." Cell Microbiol 17(10): 1405-1412.
208. Stolfi, A., et al. (2014). "Tissue-specific genome editing in Ciona embryos by CRISPR/Cas9." Development 141(21): 4115-4120.
209. Sun, W., et al. (2015). "Self-assembled DNA nanoclews for the efficient delivery of CRISPR-Cas9 for genome editing." Angew Chem Int Ed Engl 54(41): 12029-12033.
210. Suzuki, K., et al. (2016). "In vivo genome editing via CRISPR/Cas9 mediated homology-independent targeted integration." Nature 540(7631): 144-149.
211. Svitashev, S., et al. (2016). "Genome editing in maize directed by CRISPR-Cas9 ribonucleoprotein complexes." Nat Commun 7: 13274.
212. Takayama, K., et al. (2017). "Highly efficient biallelic genome editing of human ES/iPS cells using a CRISPR/Cas9 or TALEN system." Nucleic Acids Res 45(9): 5198-5207.
213. Tang, X., et al. (2016). "A Single Transcript CRISPR-Cas9 System for Efficient Genome Editing in Plants." Mol Plant 9(7): 1088-1091.
214. Tong, Y., et al. (2018). "CRISPR-Cas9 Toolkit for Actinomycete Genome Editing." Methods Mol Biol 1671: 163-184.
215. Tothova, Z., et al. (2017). "Multiplex CRISPR/Cas9-Based Genome Editing in Human Hematopoietic Stem Cells Models Clonal Hematopoiesis and Myeloid Neoplasia." Cell Stem Cell 21(4): 547-555 e548.
216. van Agtmaal, E. L., et al. (2017). "CRISPR/Cas9-Induced (CTGCAG)n Repeat Instability in the Myotonic Dystrophy Type 1 Locus: Implications for Therapeutic Genome Editing." Mol Ther 25(1): 24-43.
217. van Diemen, F. R., et al. (2016). "CRISPR/Cas9-Mediated Genome Editing of Herpesviruses Limits Productive and Latent Infections." PLoS Pathog 12(6): e1005701.
218. Vejnar, C. E., et al. (2016). "Optimization Strategies for the CRISPR-Cas9 Genome-Editing System." Cold Spring Harb Protoc 2016(10): pdb top090894.
219. Vejnar, C. E., et al. (2016). "Optimized CRISPR-Cas9 System for Genome Editing in Zebrafish." Cold Spring Harb Protoc 2016(10): pdb prot086850.
220. Vigentini, I., et al. (2017). "CRISPR/Cas9 System as a Valuable Genome Editing Tool for Wine Yeasts with Application to Decrease Urea Production." Front Microbiol 8: 2194.
221. Wagner, J. C., et al. (2014). "Efficient CRISPR-Cas9-mediated genome editing in Plasmodium falciparum." Nat Methods 11(9): 915-918.
222. Wang, D. Y., et al. (2016). "[The application of CRISPR/Cas9 genome editing technology in cancer research]." Yi Chuan 38(1): 1-8.
223. Wang, D., et al. (2015). "Adenovirus-Mediated Somatic Genome Editing of Pten by CRISPR/Cas9 in Mouse Liver in Spite of Cas9-Specific Immune Responses." Hum Gene Ther 26(7): 432-442.
224. Wang, G., et al. (2017). "Efficient, footprint-free human iPSC genome editing by consolidation of Cas9/CRISPR and piggyBac technologies." Nat Protoc 12(1): 88-103.
225. Wang, H. X., et al. (2017). "CRISPR/Cas9-Based Genome Editing for Disease Modeling and Therapy: Challenges and Opportunities for Nonviral Delivery." Chem Rev 117(15): 9874-9906.
226. Wang, H., et al. (2016). "CRISPR/Cas9 in Genome Editing and Beyond." Annu Rev Biochem 85: 227-264.
227. Wang, J., et al. (2017). "CRISPR/Cas9 mediated genome editing of Helicoverpa armigera with mutations of an ABC transporter gene HaABCA2 confers resistance to Bacillus thuringiensis Cry2A toxins." Insect Biochem Mol Biol 87: 147-153.
228. Wang, L., et al. (2017). "CRISPR-Cas9-mediated genome editing in one blastomere of two-cell embryos reveals a novel Tet3 function in regulating neocortical development." Cell Res 27(6): 815-829.
229. Wang, P., et al. (2018). "High efficient multisites genome editing in allotetraploid cotton (Gossypium hirsutum) using CRISPR/Cas9 system." Plant Biotechnol J 16(1): 137-150.
230. Wang, Q., et al. (2016). "Genome editing of model oleaginous microalgae Nannochloropsis spp. by CRISPR/Cas9." Plant J 88(6): 1071-1081.
231. Wang, S., et al. (2017). "Genome Editing in Clostridium saccharoperbutylacetonicum N1-4 with the CRISPR-Cas9 System." Appl Environ Microbiol 83(10).
232. Wang, Y., et al. (2016). "Bacterial Genome Editing with CRISPR-Cas9: Deletion, Integration, Single Nucleotide Modification, and Desirable "Clean" Mutant Selection in Clostridium beijerinckii as an Example." ACS Synth Biol 5(7): 721-732.
233. Wang, Y., et al. (2016). "Identification of genomic sites for CRISPR/Cas9-based genome editing in the Vitis vinifera genome." BMC Plant Biol 16: 96.
234. Wasels, F., et al. (2017). "A two-plasmid inducible CRISPR/Cas9 genome editing tool for Clostridium acetobutylicum." J Microbiol Methods 140: 5-11.
235. Wei, W., et al. (2014). "Heritable genome editing with CRISPR/Cas9 in the silkworm, Bombyx mori." PLoS One 9(7): e101210.

236. Wikipedia. The free encyclopedia. http://en.wikipedia.org. 2017.
237. Williams, A., et al. (2016). "Editing the Mouse Genome Using the CRISPR-Cas9 System." Cold Spring Harb Protoc 2016(2): pdb top087536.
238. Wolf, T., et al. (2016). "Targeted genome editing in the rare actinomycete Actinoplanes sp. SE50/110 by using the CRISPR/Cas9 System." J Biotechnol 231: 122-128.
239. Woo, J. W., et al. (2015). "DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins." Nat Biotechnol 33(11): 1162-1164.
240. Xi, L., et al. (2015). "A novel two-step genome editing strategy with CRISPR-Cas9 provides new insights into telomerase action and TERT gene expression." Genome Biol 16: 231.
241. Xiang, G., et al. (2017). "Temperature effect on CRISPR-Cas9 mediated genome editing." J Genet Genomics 44(4): 199-205.
242. Xie, C., et al. (2016). "Genome editing with CRISPR/Cas9 in postnatal mice corrects PRKAG2 cardiac syndrome." Cell Res 26(10): 1099-1111.
243. Xie, D. J., et al. (2016). "Construction of CTCF degradation cell line by CRISPR/Cas9 mediated genome editing." Yi Chuan 38(7): 651-657.
244. Xing, H. L., et al. (2014). "A CRISPR/Cas9 toolkit for multiplex genome editing in plants." BMC Plant Biol 14: 327.
245. Xu, S. (2015). "The application of CRISPR-Cas9 genome editing in Caenorhabditis elegans." J Genet Genomics 42(8): 413-421.
246. Xu, T., et al. (2015). "Efficient Genome Editing in Clostridium cellulolyticum via CRISPR-Cas9 Nickase." Appl Environ Microbiol 81(13): 4423-4431.
247. Yamanaka, Y. (2016). "CRISPR/Cas9 Genome Editing as a Strategy to Study the Tumor Microenvironment in Transgenic Mice." Methods Mol Biol 1458: 261-271.
248. Yang, H., et al. (2017). "CRISPR/Cas9-mediated genome editing efficiently creates specific mutations at multiple loci using one sgRNA in Brassica napus." Sci Rep 7(1): 7489.
249. Yang, H., et al. (2018). "Break Breast Cancer Addiction by CRISPR/Cas9 Genome Editing." J Cancer 9(2): 219-231.
250. Yang, N., et al. (2017). "Revolutionize Genetic Studies and Crop Improvement with High-Throughput and Genome-Scale CRISPR/Cas9 Gene Editing Technology." Mol Plant 10(9): 1141-1143.
251. Yang, Y., et al. (2016). "Targeting ABCB1-mediated tumor multidrug resistance by CRISPR/Cas9-based genome editing." Am J Transl Res 8(9): 3986-3994.
252. Yao, S., et al. (2015). "CRISPR/Cas9-Mediated Genome Editing of Epigenetic Factors for Cancer Therapy." Hum Gene Ther 26(7): 463-471.
253. Ye, L., et al. (2016). "Genome editing using CRISPR-Cas9 to create the HPFH genotype in HSPCs: An approach for treating sickle cell disease and beta-thalassemia." Proc Natl Acad Sci U S A 113(38): 10661-10665.
254. Young, S. A., et al. (2015). "Advantages of using the CRISPR/Cas9 system of genome editing to investigate male reproductive mechanisms using mouse models." Asian J Androl 17(4): 623-627.
255. Yuen, K. S., et al. (2015). "CRISPR/Cas9-mediated genome editing of Epstein-Barr virus in human cells." J Gen Virol 96(Pt 3): 626-636.
256. Zhang, C., et al. (2014). "Efficient editing of malaria parasite genome using the CRISPR/Cas9 system." MBio 5(4): e01414-01414.
257. Zhang, C., et al. (2018). "CRISPR/Cas9-mediated genome editing reveals the synergistic effects of beta-defensin family members on sperm maturation in rat epididymis." FASEB J: fj201700936R.
258. Zhang, F., et al. (2014). "CRISPR/Cas9 for genome editing: progress, implications and challenges." Hum Mol Genet 23(R1): R40-46.
259. Zhang, H., et al. (2017). "A Novel and Efficient Method for Bacteria Genome Editing Employing both CRISPR/Cas9 and an Antibiotic Resistance Cassette." Front Microbiol 8: 812.
260. Zhang, J. H., et al. (2016). "Optimization of genome editing through CRISPR-Cas9 engineering." Bioengineered 7(3): 166-174.
261. Zhang, W. W. and G. Matlashewski (2015). "CRISPR-Cas9-Mediated Genome Editing in Leishmania donovani." MBio 6(4): e00861.
262. Zhang, W. W., et al. (2017). "Optimized CRISPR-Cas9 Genome Editing for Leishmania and Its Use To Target a Multigene Family, Induce Chromosomal Translocation, and Study DNA Break Repair Mechanisms." mSphere 2(1).
263. Zhang, Y., et al. (2017). "Programmable base editing of zebrafish genome using a modified CRISPR-Cas9 system." Nat Commun 8(1): 118.
264. Zhang, Y., et al. (2018). "CRISPR/Cas9 Genome Editing: A Promising Tool For Therapeutic Applications of Induced Pluripotent Stem Cells." Curr Stem Cell Res Ther.
265. Zhang, Z., et al. (2017). "CRISPR/Cas9 Genome-Editing System in Human Stem Cells: Current Status and Future Prospects." Mol Ther Nucleic Acids 9: 230-241.
266. Zhao, D., et al. (2016). "Development of a fast and easy method for Escherichia coli genome editing with CRISPR/Cas9." Microb Cell Fact 15(1): 205.
267. Zheng, X., et al. (2018). "Heterologous and endogenous U6 snRNA promoters enable CRISPR/Cas9 mediated genome editing in Aspergillus niger." Fungal Biol Biotechnol 5: 2.
268. Zhou, J., et al. (2017). "CRISPR-Cas9 Based Genome Editing Reveals New Insights into MicroRNA Function and Regulation in Rice." Front Plant Sci 8: 1598.
269. Zhu, G. H., et al. (2016). "Functional characterization of SlitPBP3 in Spodoptera litura by CRISPR/Cas9 mediated genome editing." Insect Biochem Mol Biol 75: 1-9.
270. Zhu, P., et al. (2017). "CRISPR/Cas9-Mediated Genome Editing Corrects Dystrophin Mutation in Skeletal Muscle Stem Cells in a Mouse Model of Muscle Dystrophy." Mol Ther Nucleic Acids 7: 31-41.

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