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Development of CRISPR-Cas systems for genome editing and beyond

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Perspective

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Abstract

The development of clustered regularly interspaced short-palindromic repeat (CRISPR)-Cas systems for genome editing has transformed the way life science research is conducted and holds enormous potential for the treatment of disease as well as for many aspects of biotechnology. Here, I provide a personal perspective on the development of CRISPR-Cas9 for genome editing within the broader context of the field and discuss our work to discover novel Cas effectors and develop them into additional molecular tools. The initial demonstration of Cas9-mediated genome editing launched the development of many other technologies, enabled new lines of biological inquiry, and motivated a deeper examination of natural CRISPR-Cas systems, including the discovery of new types of CRISPR-Cas systems. These new discoveries in turn spurred further technological developments. I review these exciting discoveries and technologies as well as provide an overview of the broad array of applications of these technologies in basic research and in the improvement of human health. It is clear that we are only just beginning to unravel the potential within microbial diversity, and it is quite likely that we will continue to discover other exciting phenomena, some of which it may be possible to repurpose as molecular technologies. The transformation of mysterious natural phenomena to powerful tools, however, takes a collective effort to discover, characterize, and engineer them, and it has been a privilege to join the numerous researchers who have contributed to this transformation of CRISPR-Cas systems.

Introduction

Close observers of the diversity in the natural world generally appreciate why evolution has been likened not to the work of an engineer, but to that of a tinkerer (Jacob, 1977). By repurposing a genetic material under selective pressure, nature has evolved a myriad of 'field-tested' solutions to the challenges organisms face. Evolutionary tinkering is particularly evident in the microbial world, where selective pressure is high, effective population size is large, generation time is short, and genetic information can be exchanged widely and relatively quickly. As biologists delve ever deeper into the molecular and genetic mechanisms underlying the observed phenotypic diversity, we continue to learn more about fundamental biological processes and uncover new natural systems and phenomena. In addition to providing insight into the molecular underpinnings of life, some of these novel systems have been developed into various molecular technologies. For example, heat-stable polymerases discovered in thermophilic bacteria enabled the development of polymerase chain reaction (PCR), and restriction enzymes discovered by studying host responses to phages enabled recombinant deoxyribonucleic acid (DNA) technologies.

One of the latest examples of how nature's solutions have been successfully adapted into a molecular technology is the development of clustered regularly interspaced short-palindromic repeat (CRISPR)-Cas (CRISPR-associated) systems for eukaryotic genome editing. CRISPR-Cas-mediated genome editing is a robust, easy-to-use method to precisely alter DNA sequences within the genome of living organisms. Because of the simplicity and efficiency of the system, it has been widely adopted and further developed, leading to an extraordinarily powerful molecular toolbox. Once microbiological curiosity, CRISPR has become a part of the common language of molecular biology, with its reach extending into nearly every corner of the life sciences and its impact going far beyond the confines of the laboratory. The story of CRISPR is one with two-intertwined aspects (Fig. 1): biological investigation to better understand these elegant systems and engineering of these systems into powerful molecular technologies. As the impact of these technologies spreads, it spurs further work into the biology, which continues to provide additional technological opportunities. Thus, the early part of the CRISPR revolution involved engineering Cas9 as genome editing technology, but through the recent discovery and development of additional Cas effectors, particularly the ribonucleic acid (RNA)-targeting Cas13 family, it has continued to expand into new

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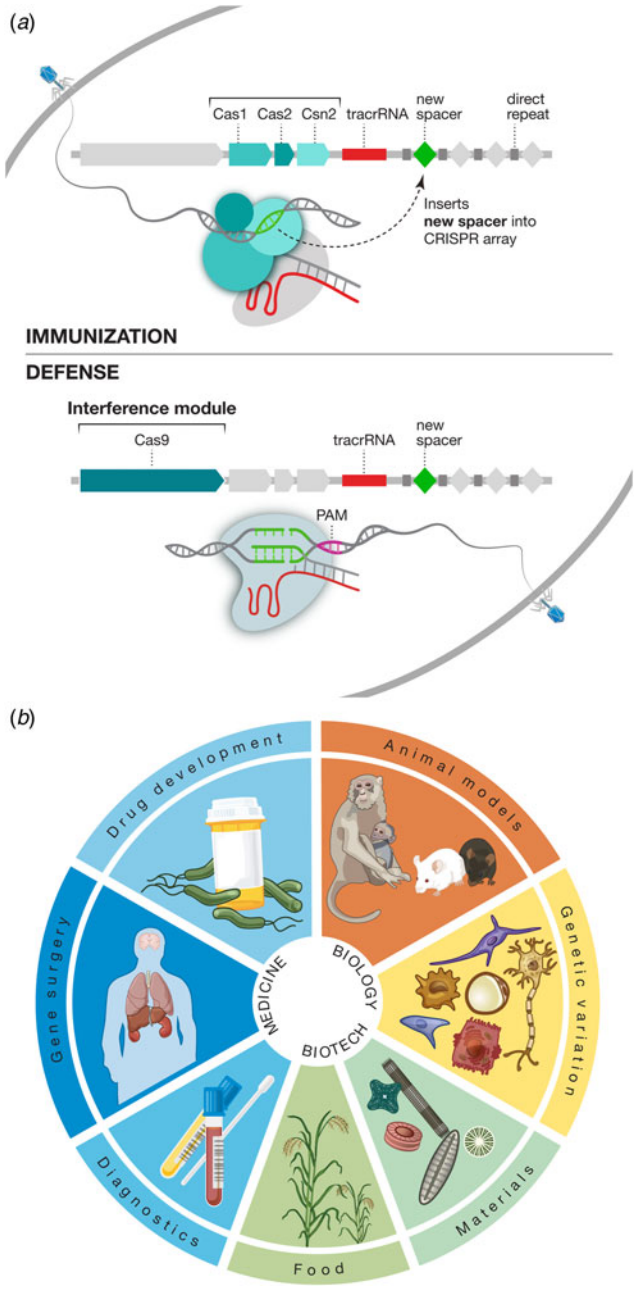


Fig. 1. Two aspects of CRISPR: biology and technology. (a) CRISPR-Cas adaptive immune systems help microbes defend against phages and other foreign genetic materials. During the immunization phase (top), an adaptation module inserts a new spacer, a stretch of DNA derived from the genome of the invader, into the CRISPR array. During the defense phase (bottom), spacers are converted into guide RNAs that direct an interference module to matching target sequences, which are then cleaved. (b) CRISPR technologies have broad applications in the life sciences, medicine, and industrial biotechnology. The CRISPR molecular toolbox allows researchers to carry out precise genome and transcriptome editing in eukaryotic cells to advance our understanding of biology through the generation of useful animal and cellular models and interrogation of genetic variation, to boost biotechnology through engineering and production of novel materials and agricultural products, and to advance human health through detection of pathogens, development of novel therapeutic approaches, and elucidation of disease mechanisms. Image adapted from (Hsu *et al.*, 2014).

areas. CRISPR-based technologies are being employed in diverse ways to improve human health and offer the potential to fundamentally change the way we treat disease.

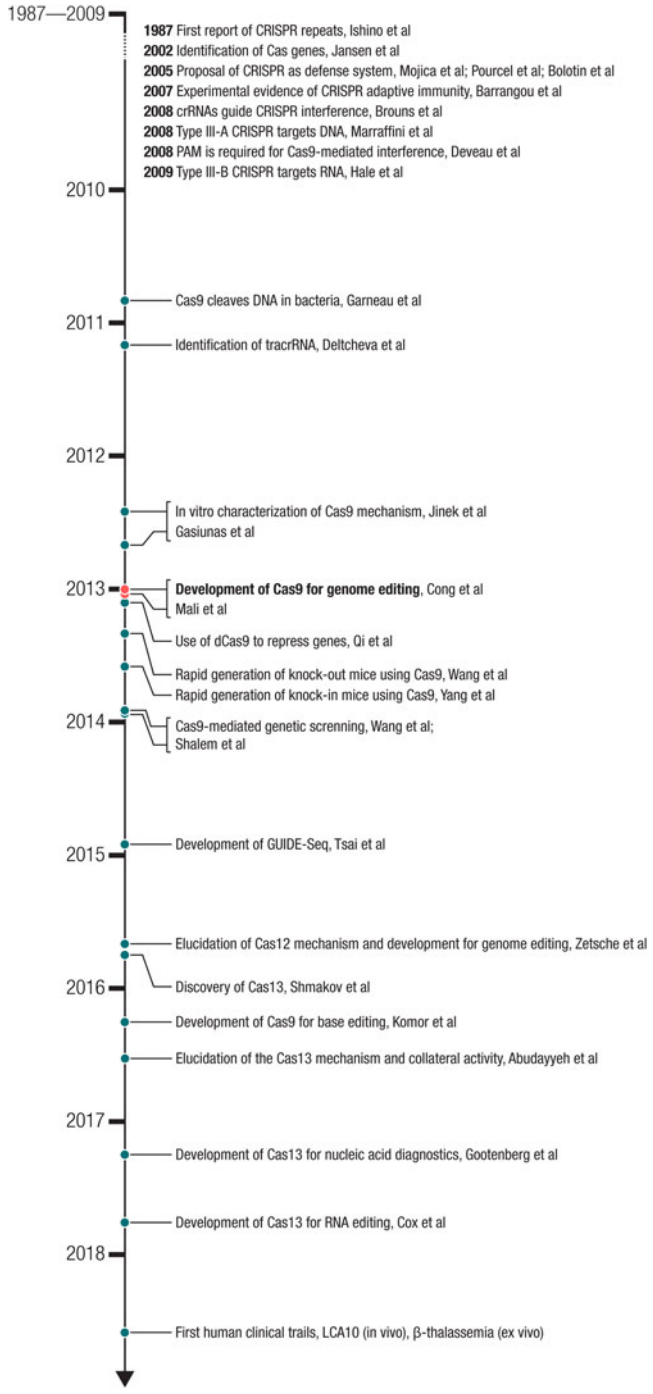


Fig. 2. Milestones in the development of CRISPR-based technologies. The development of Cas9 for genome editing ((Cong *et al.*, 2013) – submitted on October 5, 2012 and (Mali *et al.*, 2013*b*) – submitted on October 26, 2012) built on a number of important biological studies and spurred many powerful applications as well as the discovery of new CRISPR effectors such as the DNA-targeting Cas12 and RNA-targeting Cas13.

Here, I briefly overview the natural function of CRISPR-Cas systems, followed by a personal account and perspective of the time period over which CRISPR-Cas9 was developed for genome editing in eukaryotic cells. I also discuss the continuing study and remarkable biotechnological development of CRISPR-Cas systems beyond Cas9 (Fig. 2). In particular, I highlight some of the exciting applications of this technology and identify areas for future

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improvement. Although I have striven to include many primary studies, I apologize in advance to those whose work might have unintentionally been omitted. In addition to this perspective, there are a number of general reviews covering this topic (Doudna and Charpentier, 2014; Hsu *et al*., 2014; van der Oost *et al*., 2014; Marraffini, 2015; Sontheimer and Barrangou, 2015; Mojica and Rodriguez-Valera, 2016; Barrangou and Horvath, 2017; Koonin and Makarova, 2017; Lemay *et al*., 2017; Ishino *et al*., 2018). I also refer readers to several reviews focused on various aspects related to CRISPR-Cas technologies, including the structure and mechanism of Cas effectors (Jackson and Wiedenheft, 2015; Garcia-Doval and Jinek, 2017; Jiang and Doudna, 2017), classification and evolution of CRISPR-Cas systems (Koonin and Makarova, 2017), and applications of the CRISPR technology in agriculture (Voytas and Gao, 2014; Gao, 2018), animal and cellular modeling (Hotta and Yamanaka, 2015), genetic screening (Shalem *et al*., 2015; Doench, 2017; Jost and Weissman, 2018), genome editing specificity (Tsai and Joung, 2016), base editing (Hess *et al*., 2017; Rees and Liu, 2018), drug discovery and development (Fellmann *et al*., 2017), and therapeutic applications (Cox *et al*., 2015; Porteus, 2015; Xiong *et al*., 2016).

I would also like to take this opportunity to acknowledge all of the members of the CRISPR research community, who have contributed to elucidating the mechanism of CRISPR-Cas systems and developing and applying this extraordinary technology. It has been tremendously inspiring to see the multitude of ways that CRISPR-Cas systems continue to be applied. In addition, I am grateful to all of the collaborators and trainees with whom I have been fortunate to work alongside to uncover novel CRISPR biology and to develop and apply these remarkable technologies.

Biology of CRISPR-Cas-mediated adaptive immunity

Overview and nomenclature of CRISPR-Cas systems

CRISPR-Cas systems are adaptive immune systems found in roughly 50% of bacterial species and nearly all archaeal species sequenced to date (Makarova *et al*., 2015). These systems evolved over billions of years to defend microbes from the invasion of foreign nucleic acids such as bacteriophage genomes and conjugating plasmids by targeting their DNA or RNA. The molecular machinery involved in CRISPR-Cas immunity is encoded by the CRISPR locus as two sets of genetic components that are often located next to each other in microbial genomes: (1) an operon of multiple *cas* genes, and (2) a set of non-coding CRISPR RNAs (crRNAs) including ones encoded by the signature repetitive CRISPR array consisting of spacers sandwiched between short-CRISPR repeats (Fig. 1*a*). Using these components, CRISPR-Cas systems mediate adaptive immunity (immunization and defense) through three general phases: adaptation, crRNA processing, and interference. First, during the adaptation phase, a subset of Cas proteins called the 'adaptation module' obtains and inserts fragments of an invading virus or other foreign genetic material as a 'spacer' sequence into the beginning of the CRISPR array in the host genome along with a newly duplicated CRISPR repeat. The sequence on the virus or plasmid matching the acquired spacer is called a protospacer. Second, the CRISPR array is transcribed and processed into individual crRNAs, each bearing an RNA fragment corresponding to the previously encountered virus or plasmid along with a portion of the CRISPR repeat. Third, during the interference phase, crRNAs guide the 'interference module', encoded either by complex comprising Cas effector subunits or by a single-effector protein, to destroy the invader.

There are many variations on the CRISPR theme, however, and the natural diversity of CRISPR-Cas systems is remarkably extensive, including systems that target DNA, systems that target RNA, and systems that target both DNA and RNA. CRISPR-Cas systems also operate in different ways, recognizing and cleaving their nucleic acid targets through distinct mechanisms mediated by various effector-crRNA complexes. Based on their unique effector proteins, CRISPR-Cas systems are currently classified into six types (I through VI), which are in turn grouped into two-broad classes (Makarova *et al*., 2015; Shmakov *et al*., 2017): class 1 systems (types I, III, and IV) use a multi-protein complex to achieve interference, and class 2 systems (types II, V, and VI) utilize a single-nuclease effector such as Cas9, Cas12, and Cas13 for interference.

Discovery and characterization of CRISPR-Cas systems

In 1987, a series of regularly-interspaced repeats of unknown function was observed in the genome of *E. coli*, documenting the first instance of a CRISPR array (Ishino *et al*., 1987). In early 2002, clues to the function of CRISPR-Cas systems came from two-bioinformatics studies, one of which reported the presence of conserved operons that appeared to encode a novel DNA repair system, which we now know are *cas* genes (Makarova *et al*., 2002), and the other of which reported the association between CRISPR arrays and *cas* genes (Jansen *et al*., 2002). Next, it was observed that spacer sequences in between CRISPR repeats matched sequences in phage genomes, leading to the suggestion that CRISPR arrays could be involved in immunity against the corresponding phages (Mojica *et al*., 2005; Pourcel *et al*., 2005). Third, work focused on *Streptococcus thermophilus* similarly found that more spacers matched phage sequences and identified a large CRISPR-associated protein containing the DNA-cleaving HNH domain, which is now known as Cas9, the hallmark protein in type II systems (Bolotin *et al*., 2005). Despite the linkage between CRISPR-Cas and phage infection, the specific role that CRISPR spacers played in providing immunity remained unclear.

Experimental work with the type II system of *S. thermophilus* showed that the spacers in the CRISPR array are acquired from phages and specify immunity against specific phages carrying matching sequences. Moreover, *cas* genes are required for both immunization and phage interference (Barrangou *et al*., 2007). These exciting results established CRISPR-Cas as a microbial adaptive immune system. Insight into the molecular mechanism of CRISPR-Cas immunity came from work using a type I CRISPR-Cas system, which revealed that the CRISPR array is transcribed and processed into short crRNAs that provide recognition of the invading phages and that the effector module can be directed to multiple targets by changing the crRNA sequences (Brouns *et al*., 2008). Although the prevailing hypothesis at the time was that CRISPR-Cas systems achieved interference using an RNAi-like mechanism (Makarova *et al*., 2006), there was evidence that the target was DNA, rather than RNA (Brouns *et al*., 2008). Another study reported that a type III-A CRISPR-Cas system limits horizontal gene transfer by targeting DNA (Marraffini and Sontheimer, 2008). However, other systems, such as the type III-B CRISPR-Cas system, target RNA instead (Hale *et al*., 2009), highlighting the substantial mechanistic differences between CRISPR-Cas systems.

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As the overall picture of CRISPR-Cas-mediated adaptive immunity began to take shape, studies also started to clarify the natural mechanism of type II CRISPR-Cas systems, which uses the nuclease effector Cas9. In one study, it was shown that a short well-conserved sequence motif at the end of CRISPR targets, called a protospacer adjacent motif (PAM) (Mojica *et al*., 2009), is required for Cas9-mediated interference (Deveau *et al*., 2008). In 2010, it was shown that *S. thermophilus* Cas9 is guided by crRNAs to create blunt double-strand breaks (DSBs) in DNA 3 bp upstream from the PAM at targeted sites in phage genomes and in plasmids and that Cas9 is the only protein required for DNA cleavage (Garneau *et al*., 2010). In 2011, small-RNA sequencing of *Streptococcus pyogenes* revealed the presence of an additional small RNA associated with the CRISPR array. This additional RNA, termed tracrRNA, forms a duplex with direct repeat sequences on the pre-crRNA to produce mature crRNA, and it is required for Cas9-based interference (Deltcheva *et al*., 2011). Another study in 2011 showed that the CRISPR-Cas locus from *S. thermophilus* could be expressed in *E. coli*, where it could mediate interference against plasmid DNA (Sapranauskas *et al*., 2011). These studies collectively established that the nuclease complex of the natural Cas9 system contains three components (Cas9, crRNA, and tracrRNA) and that the DNA target site needs to be flanked by the appropriate PAM.

As the biology of CRISPR-Cas systems became better understood, it began to be adapted for use, first as an aid for bacterial strain typing (Pourcel *et al*., 2005; Horvath *et al*., 2008, 2009), and then in its native context by inoculating *S. thermophilus* with viruses to generate phage-resistant strains that can be deployed in industrial dairy applications, such as yogurt and cheese making (Quiberoni *et al*., 2010). Additional suggestions for its application were also raised, including microbial gene silencing (Sorek *et al*., 2008), combating antibiotic resistance, and targeted DNA destruction (Marraffini and Sontheimer, 2008; Garneau *et al*., 2010).

Development of CRISPR-Cas9 for genome editing

The ability to make precise changes to the genome holds great promise for advancing our understanding of biology and human health as well as providing new approaches to treating grievous diseases. The demonstration in 1987, the same year that CRISPR was first reported, of targeted gene insertion via homologous recombination in mice was a major breakthrough (Doetschman *et al*., 1987; Thomas and Capecchi, 1987), but the efficiency in mammalian cells was extremely low outside of mouse embryonic stem cells. Work in both yeast and mammalian cells demonstrated that the efficiency of gene insertion could be increased through the generation of a DSB at the target site (Rudin *et al*., 1989; Plessis *et al*., 1992; Rouet *et al*., 1994). These observations motivated the development of targetable nucleases such as meganucleases, zinc finger nucleases, and transcription activator-like effector (TALE) nucleases that can be customized to recognize specific DNA sequences and generate DSBs at specific loci to facilitate genome editing (reviewed in (Urnov *et al*., 2010; Joung and Sander, 2013; Kim and Kim, 2014)). However, the targeting capacity of each of these technologies was limited, and it was challenging to reprogram them in practice, ultimately dampening their impact.

As a Junior Fellow at Harvard in 2009, I had experienced firsthand the challenges of working with zinc finger nucleases. After reading studies describing the DNA recognition mechanism of microbial TALE proteins (Boch *et al*., 2009; Moscou and Bogdanove, 2009), I asked Le Cong, a rotation graduate student, to join me to develop TALEs for use in mammalian cells (Zhang *et al*., 2011). In 2010, I accepted a faculty position at MIT and the Broad Institute, planning to build a research program around genome and transcriptome editing. I started to set up my lab in January 2011, and Cong joined as my first graduate student. The very next month, I heard Michael Gilmore speak at the Broad Institute about his studies on *Enterococcus* bacteria, during which he mentioned that *Enterococcus* carried CRISPR-Cas systems, which contained a new class of nucleases. Given my interest in genome editing, I was intrigued by the prospect of a new class of nucleases. After studying the CRISPR-Cas literature, I immediately recognized that CRISPR-Cas would be easier to reprogram than TALEs, and I decided to refocus a significant portion of my genome editing efforts on adapting Cas9 for genome editing in eukaryotic cells.

In early 2011, it was already known that Cas9 could cleave DNA in bacterial cells when directed by a crRNA (Garneau *et al*., 2010). Based on the literature, it was also known that the nuclease complex of the natural Cas9 system contains three components (Cas9, crRNA, and tracrRNA). However, CRISPR-Cas systems had only been studied in bacterial and biochemical systems, and they had not been explored in the context of eukaryotic cells. Thus, the key question that needed to be answered, in my mind, was whether Cas9 could be engineered to achieve genome editing in eukaryotic cells. Bacterial enzymes evolved to function optimally in their native bacterial environment, which has substantially different biochemical properties than that of the intracellular environment of a eukaryotic cell. Indeed, I knew that previous attempts to harness bacterial systems for use in eukaryotic cells had failed, including Group II introns (Mastroianni *et al*., 2008) and ribozymes (Link and Breaker, 2009). From my past experiences developing microbial opsins for use in mammalian neurons for optogenetics (Boyden *et al*., 2005; Zhang *et al*., 2007) and TALEs for use in mammalian cells for genome editing (Zhang *et al*., 2011), I decided to directly answer the question of whether Cas9 could be used as a programmable nuclease in eukaryotic cells by using a human cell culture system. Working with human cells, I developed a three-component CRISPR-Cas9 system – Cas9, crRNA, and tracrRNA – for genome editing.

As a brand new Assistant Professor, I not only designed but also carried out experiments in the laboratory myself, while mentoring trainees, recruiting lab members, and applying for grants. In one of the grants submitted to the National Institutes of Health in January 2012, I described my strategy to use a three-component Cas9 system (Cas9, crRNA, and tracrRNA) for genome editing in mammalian cells, as was later published in our study (Fig. 3) (Cong *et al*., 2013). The strategy for this work was based on the synthesis of the available literature in the CRISPR field, which had established the requirement for these three components for function in bacteria.

During the course of our experiments, a detailed biochemical analysis of the mechanism of Cas9 *in vitro* was published (Jinek *et al*., 2012). First, it showed that the purified *S. pyogenes* Cas9-crRNA-tracrRNA complex cleaves DNA 3 bp upstream of the PAM of the target site, in agreement with previous *in vivo* results with the *S. thermophilus* system (Garneau *et al*., 2010). Second, it showed that tracrRNA and crRNA are both required for target cleavage by the Cas9-crRNA-tracrRNA complex. Furthermore, by truncating the tracrRNA, the study found that a short fragment of the tracrRNA (nucleotides 23 to 48, without

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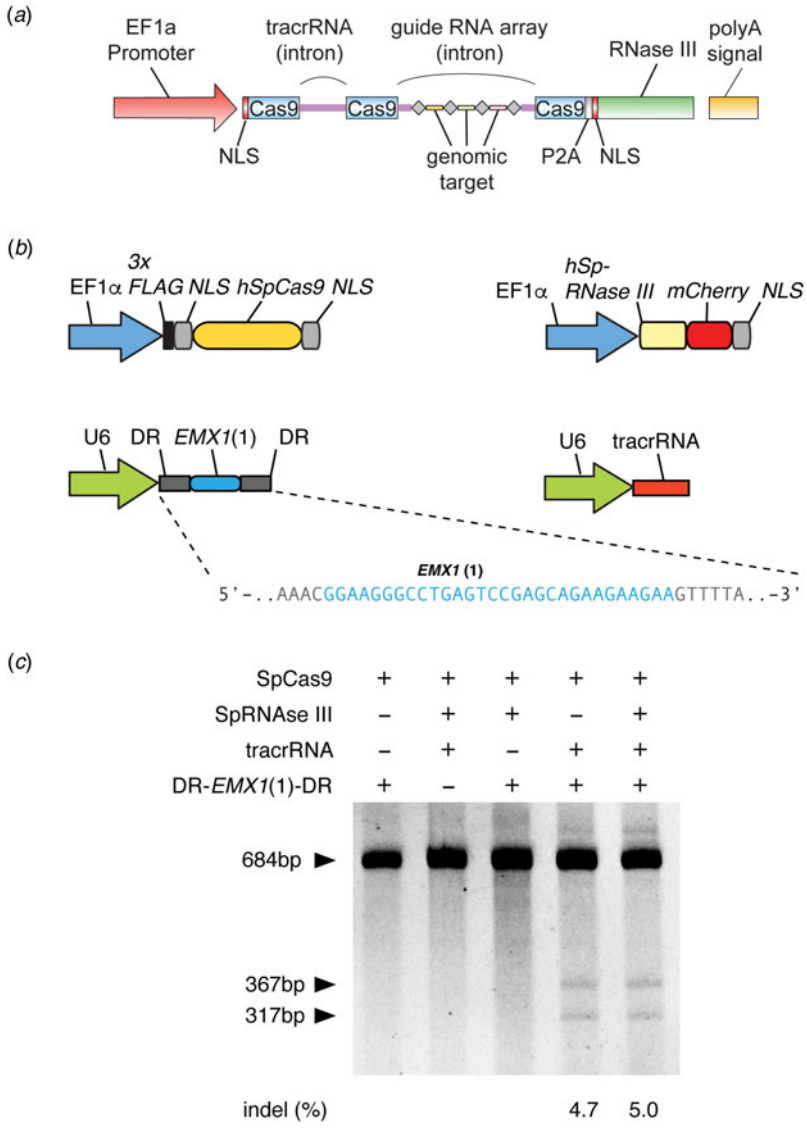


Fig. 3. Development of CRISPR-Cas9 for genome editing. (a) The design of a three-component system (Cas9, crRNA, and tracrRNA) for Cas9-mediated genome editing in eukaryotic cells, which was included in a grant submitted in January 2012 to the National Institutes of Health. In this design, the EF1a promoter drives expression of Cas9 (with a NLS) and the tracrRNA and guide RNA array with four genomic targets. In addition, RNase III is expressed to aid the processing of crRNA, although we later determined it is not necessary (see panel c). Image adapted from NIH Grant 5R01DK097768. (b) Design of the three-component system used in human cells to demonstrate editing of the human genome. *S. pyogenes* Cas9, CRISPR array (DR-EMX(1)-DR), and tracrRNA are individually expressed. The guide sequence in the CRISPR array targets the human *EMX1* gene. Image adapted from Cong *et al.* (2013). (c) Polyacrylamide gel showing successful editing of the *EMX1* target in the human genome. The SURVEYOR reaction is used to detect the presence of Cas9-induced indels at the *EMX1* locus. Transfection of Cas9, CRISPR array, and tracrRNA alone mediated successful genome editing (RNase III is not required). Image adapted from Cong *et al.* (2013).

the 3′ stem loops) was sufficient for supporting robust dual-RNA-guided cleavage of DNA by the Cas9-crRNA-tracrRNA complex *in vitro*. Third, it showed that the HNH domain is responsible for cleaving the target DNA strand and the RuvC domain cleaves the non-target DNA strand. Inactivation of either domain turns the Cas9-crRNA-tracrRNA complex into a DNA nickase. Fourth, it showed that single-base mutations in the PAM and in the 3′ region of the guide sequence abolished DNA cleavage by the Cas9-crRNA-tracrRNA complex, whereas single-base mismatches closer to the 5′ region of the guide RNA did not. Fifth, it showed that the crRNA and the 23-48-nt tracrRNA can be fused into a single-guide RNA (sgRNA) and this Cas9-sgRNA two-component system can mediate cleavage of plasmid DNA under biochemical conditions.

Three months later, a biochemical analysis, using a purified complex containing the *S. thermophilus* Cas9 and crRNA, reported similar findings (Gasiunas *et al.*, 2012). First, the study showed that the Cas9-crRNA complex cleaves target DNA 3 bp upstream of the PAM of the target site, also in agreement with previous *in vivo* results with the *S. thermophilus* system (Garneau *et al.*, 2010). Second, it showed that the Cas9-crRNA complex binds to dsDNA containing both the binding site as well as PAM. Third, the study showed that the HNH domain cleaves the target DNA strand and the RuvC domain cleaves the non-target strand. Inactivation of either domain turned the Cas9-crRNA complex into a DNA nickase. However, this study purified the Cas9-crRNA complex from bacteria without analyzing the components of the complex. As a result, the paper provided an incomplete picture of the Cas9 molecular mechanism for *in vitro* cleavage and failed to identify the requirement for tracrRNA for Cas9 function.

While the *in vitro* two-component system highlighted the potential of exploiting Cas9 for genome editing, as this work was conducted entirely *in vitro*, it did not identify the critical components for achieving robust genome editing in cells and did not demonstrate that Cas9 could be used for genome editing. Thus, although the biochemical demonstration of RNA-guided DNA cleavage is often equated with Cas9-mediated genome editing, even within the scientific community there were concerns as to whether Cas9 could be made to function in eukaryotic cells (Carroll, 2012).

At the time, we had already established that Cas9 could function in human cells. Working alongside my first trainees, including Le Cong and Ann Ran, who are co-first authors of our

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publication (Cong *et al.*, 2013), we focused on two orthologs of Cas9 that had been previously studied using bacterial genetics and had complementary advantages: *S. thermophilus* Cas9 (StCas9), which was small enough to be packaged into an adeno-associated viral vector (AAV) for *in vivo* delivery, and *S. pyogenes* Cas9 (SpCas9), which had a less restrictive PAM sequence (SpCas9, PAM 5′-NGG, can target on average every 12·7 bp in the human genome, whereas StCas9, PAM 5′-NNAGAAW, can target on average every 106·6 bp in the human genome), and thus broader targeting potential. First, we found that both SpCas9 and StCas9 can be engineered to mediate genome editing in human and mouse cells. However, Cas9 aggregated in the nucleolus, pointing to the obstacle of correct subcellular localization when moving a bacterial system into eukaryotic cells. After experimenting with a number of nuclear localization signals (NLSs), we found that the combination of a monopartite and a bipartite NLS allowed Cas9 to localize efficiently into the human cell nucleus without any aggregation in the nucleolus. Second, we found that although the natural bacteria expressed multiple isoforms of the tracrRNA, all of which can provide CRISPR immunity in bacteria (Deltcheva *et al.*, 2011), only the 89-nt isoform was stably expressed in human cells and was important for achieving robust genome editing. Third, we found that across 16 target sites in human and mouse cells, the three-component system for SpCas9 and StCas9 can mediate robust editing of the genome. Fourth, we found that a CRISPR array encoding multiple spacers can be processed by human cells into individual guide RNAs to target multiple genes in the genome. Fifth, we showed that DSBs introduced by Cas9 can stimulate homologous recombination, leading to targeted gene insertion, and that Cas9 nickase activity can also stimulate homologous recombination in cells, while avoiding the formation of DSB-induced indels. Sixth, we also explored a two-component design. We found that the additional 3′ stem loops on the tracrRNA are important for gene editing, as the three-component system achieved significantly more robust genome editing in human cells than the two-component design employed, which failed to edit at a number of genomic sites. Together, these results established a foundation for the molecular mechanism by which CRISPR-Cas9 can mediate robust genome editing and further underscores that the ability of CRISPR-Cas9 to function in eukaryotic cells cannot be predicted from *in vitro* studies (Cong *et al.*, 2013).

If Cas9 could function in eukaryotic cells, however, it would unlock the potential for a range of sought after applications in research, biotechnology, and medicine. It was therefore not surprising that, in addition to our efforts to develop Cas9 for genome editing, other groups were inspired by the biochemical characterization of Cas9 (Jinek *et al.*, 2012) to explore applications of Cas9 as well. Concurrent with our study, a second report of gene editing using Cas9 was published (Mali *et al.*, 2013*b*). Shortly thereafter, additional studies also reported the use of Cas9 in human and animal cells (Cho *et al.*, 2013; Hwang *et al.*, 2013; Jinek *et al.*, 2013) and the use of a catalytically inactivated variant of Cas9 to achieve targeted gene repression (Qi *et al.*, 2013).

Initial impact of Cas9-mediated genome editing

Following the demonstration of Cas9-mediated genome editing in eukaryotic cells, many outstanding scientists contributed to the advancement and application of the technology, pushing the field ahead at a remarkable rate. We continued to develop the technology by focusing on three major areas: (1) further understanding the biology of Cas9 so as to improve and extend its utility; (2) developing applications of Cas9, including genome-wide screening, a Cas9 knock-in mouse, and conversion of Cas9 to a catalytically inactive programmable DNA-binding scaffold; and (3) exploring the natural diversity of CRISPR-Cas systems to identify other Cas effectors with unique properties that may be advantageous for technological development. Through these endeavors we had the opportunity to collaborate with a number of talented researchers from diverse backgrounds, further amplifying the impact of CRISPR-based technologies.

One way the immediate impact of Cas9 can be seen is in its rapid adoption for other organisms, which highlights the broad utility of this tool as well as the robustness and ease-of-use of the system. Catalyzed by the success of Cas9-mediated genome editing in human cells, within a year, groups from around the world reported the successful application of Cas9 in a number of eukaryotic model organisms, including yeast (DiCarlo *et al.*, 2013), mice (Wang *et al.*, 2013), *Drosophila* (Gratz *et al.*, 2013), *C. elegans* (Friedland *et al.*, 2013), *Arabidopsis* (Li *et al.*, 2013), *Xenopus* (Nakayama *et al.*, 2013), and non-human primates (Niu *et al.*, 2014). Cas9 was also successfully deployed in a number of agriculturally important species in that first year, such as rice and wheat (Shan *et al.*, 2013), sorghum (Jiang *et al.*, 2013), and maize (Liang *et al.*, 2014). Before the year's end, the first reports were published on the use of Cas9 to correct a cataract-causing mutation in a mouse, leading to reversal of the disease phenotype (Wu *et al.*, 2013). In parallel, a number of improvements and extensions of the technology were reported in quick succession.

The impact of the CRISPR-based technologies is due in no small part to the open sharing culture of the CRISPR field, which has enabled applications and further development of CRISPR-based technologies to flourish. This has been facilitated through on-line resources, such as the creation of numerous web-based tools for guide design (Hsu *et al.*, 2013; Bae *et al.*, 2014; Schmid-Burgk *et al.*, 2014; Labun *et al.*, 2016; Pinello *et al.*, 2016; Concordet and Haeussler, 2018; Listgarten *et al.*, 2018), and through the annual CRISPR meetings. CRISPR reagents have also been shared widely and openly. To date, more than 350 laboratories from around the world have made their CRISPR-based reagents accessible through the non-profit molecular reagent sharing organization Addgene. For my own group, we have made it a priority to help researchers benefit from the CRISPR technological advances we made by disseminating reagents as well as know-how for CRISPR-based technologies. Through a combination of direct mailing as well as distribution through Addgene, we have been able to share more than 52 000 CRISPR reagents to researchers at more than 2300 institutions spanning 62 countries.

From Cas9 to beyond: Cas12 and Cas13

The development of other molecular technologies, such as restriction enzymes (Loenen *et al.*, 2014) and green fluorescent proteins (Rodriguez *et al.*, 2017), has benefitted significantly from explorations of natural diversity. Similarly, my own experience with the development of optogenetics has taught me the power of exploring the diversity of microbial opsins. Therefore, we turned to the natural diversity of CRISPR-Cas systems to identify other Cas effectors with the potential to expand the capabilities of CRISPR-based technologies. By mining the microbial diversity

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for signatures of CRISPR-Cas systems (e.g., conserved genes and CRISPR-like repeat sequences), we discovered and elucidated the functions of two new types of CRISPR-Cas systems and developed them to significantly expand the CRISPR toolbox (Shmakov *et al.*, 2015, 2017; Zetsche *et al.*, 2015*a*; Smargon *et al.*, 2017) (Fig. 4). These discoveries prompted other investigations of microbial diversity, revealing additional subtypes of CRISPR-Cas systems (Burstein *et al.*, 2017; Harrington *et al.*, 2018; Konermann *et al.*, 2018; Shmakov *et al.*, 2018; Yan *et al.*, 2018*b*) and providing insight into the origin, evolution, and function of these elegant systems.

SaCas9

Beyond its immediate utility in the lab, there was enormous interest in using Cas9-mediated genome editing as a therapeutic that could theoretically treat thousands of genetic diseases. One limitation to the therapeutic use of SpCas9, however, was its relatively large size, which made delivering it challenging. We therefore sought to identify smaller Cas9 orthologs that worked efficiently in mammalian cells while maintaining a broad targeting range. We characterized a number of CRISPR-Cas9 systems and profiled their mammalian genome editing activity. One Cas9 ortholog from *Staphylococcus aureus* (SaCas9, PAM 5′-NNGRRT) showed the highest levels of activity in human cells (Ran *et al.*, 2015). SaCas9 is more than 1 kb shorter than SpCas9, which allowed us to deliver it, along with a guide RNA, on a single-AAV vector for *in vivo* use (Ran *et al.*, 2015). SaCas9 is now being developed as the first *in vivo* genome editing medicine for humans (see below) (Allergan, 2019). SpCas9 is also being advanced for therapeutic applications. However, due to its large size, clinical trials employing SpCas9 are focused on electroporation of patient cells *ex vivo* (Vertex, 2018*a*, 2018*b*).

Cas12

We next went beyond Cas9 orthologs to study other CRISPR-Cas systems, beginning with a putative new type of class 2 CRISPR-Cas system, type V, characterized by the Cas12 family of effector proteins. The first Cas12 enzyme, classified as type V-A and referred to as Cas12a (previously known as Cpf1) was identified in the genomes of *Prevotella* and *Francisella* and contained a large protein of unknown function (Schunder *et al.*, 2013; Vestergaard *et al.*, 2014; Makarova *et al.*, 2015). Cas12a is a distinct enzyme unrelated to Cas9. A number of *Francisella* species contain Cas12a in association with putative CRISPR arrays, including *F. novicida*. Heterologous expression of the *F. novicida* CRISPR-Cas12a locus in *E. coli* led to interference of plasmid DNA transformation, establishing CRISPR-Cas12a as a *bona fide* CRISPR-Cas system and revealing that Cas12a requires a T-rich PAM sequence preceding the DNA target site (Zetsche *et al.*, 2015*a*). In contrast to Cas9, the Cas12a system does not contain a tracrRNA, and its DNA cleavage results in a 5′ overhang instead of a blunt DSB (Zetsche *et al.*, 2015*a*). Also, unlike Cas9, which utilizes host RNase III to process its CRISPR array, Cas12a itself has RNase activity and processes its own pre-crRNA array into individual crRNAs (Fonfara *et al.*, 2016).

A search for Cas12a orthologs identified two-Cas12a enzymes, from *Acidaminococcus* and *Lachnospiraceae,* with strong cleavage activity in human cells, comparable to SpCas9 (Zetsche *et al.*, 2015*a*). Apart from expanding the range of genomic targets that can be edited given that it has a different PAM than Cas9, Cas12a-mediated editing has several advantages over Cas9: it is significantly more specific (Kleinstiver *et al.*, 2016*b*; Kim *et al.*, 2017*b*), which is important for therapeutic applications; it offers a simplified guide design because it does not require tracrRNA; it generates over-hanging ends, rather than the blunt ends created by Cas9, which may be beneficial for the introduction of new sequences (Moreno-Mateos *et al.*, 2017); it has smaller molecular size which is more suitable for viral packaging, and it is ideally suited for multiplex genome editing because multiple guide RNAs can be easily expressed as a single transcript and subsequently processed into individual guide RNAs by Cas12a itself (Zetsche *et al.*, 2016).

Relative to the Cas9 family of Cas effectors, Cas12 is a much more diverse family. Indeed a number of subtypes of Cas12 systems have recently been reported (denoted type V-A – V-I). The Cas12b effectors (previously known as C2c1) target DNA, but in contrast to Cas12a, they are dual-RNA guided, requiring a tracrRNA (Shmakov *et al.*, 2015). Although initial characterization of Cas12b revealed thermophilic nuclease activity, which prevented application in mammalian cells, subsequent exploration of the Cas12b diversity and protein engineering made possible the development of two-Cas12b systems with robust genome editing activity in human cells (Teng *et al.*, 2018; Strecker *et al.*, 2019). Comparison of Cas12b with SpCas9 showed that Cas12b has substantially reduced off-target activity, indicating it is inherently more specific than wild-type SpCas9 when targeting the human genome (Teng *et al.*, 2018; Strecker *et al.*, 2019). Additional Cas12 effectors have also been identified from bacterial genomic databases, including Cas12c (Shmakov *et al.*, 2015), Cas12d (CasY) and Cas12e (CasX), both of which were found in metagenomic samples (Burstein *et al.*, 2017), and three subtypes of Cas12f (Cas14) (Harrington *et al.*, 2018). Two-Cas12e orthologs, DpbCasX and PlmCasX, have recently been shown to achieve targeted gene knockout in human cells (Liu *et al.*, 2019). A recent effort to holistically identify CRISPR-Cas systems from more than 10 terabytes of genomic and metagenomic data led to the identification of a number of new type V subtype loci, including both DNA- and RNA-targeting Cas12 systems (Yan *et al.,* 2019).

Cas13

The type VI family of CRISPR-Cas systems, signified by the RNA-guided RNA-targeting Cas13 effector, was first found by using the highly conserved adaptation protein Cas1 as the search seed to identify all genomic fragments that contain putative CRISPR-Cas systems. Focusing on conserved proteins of unknown function located within each CRISPR locus, we discovered a family of well-conserved large proteins carrying the higher eukaryotic–prokaryotic nuclease (HEPN) domain, which suggested they are putative RNases (Shmakov *et al.*, 2015). Subsequent expansion of the search to use CRISPR repeats as the search seed led to the identification of additional Cas13 subtypes, including Cas13b, Cas13c, and Cas13d (Shmakov *et al.*, 2017; Smargon *et al.*, 2017; Konermann *et al.*, 2018; Yan *et al.*, 2018*b*).

Using *E. coli* heterologously expressing type VI CRISPR-Cas systems, we showed that CRISPR-Cas13a and Cas13b systems confer resistance to RNA phages, and that they are single-effector RNases guided by crRNAs (Abudayyeh *et al.*, 2016; Smargon *et al.*, 2017). This finding paved the way for an entirely new set of molecular technologies operating at the level of RNA, rather than DNA, and offering a safer therapeutic approach to treating disease (see below). Similar to Cas12a, Cas13 proteins also

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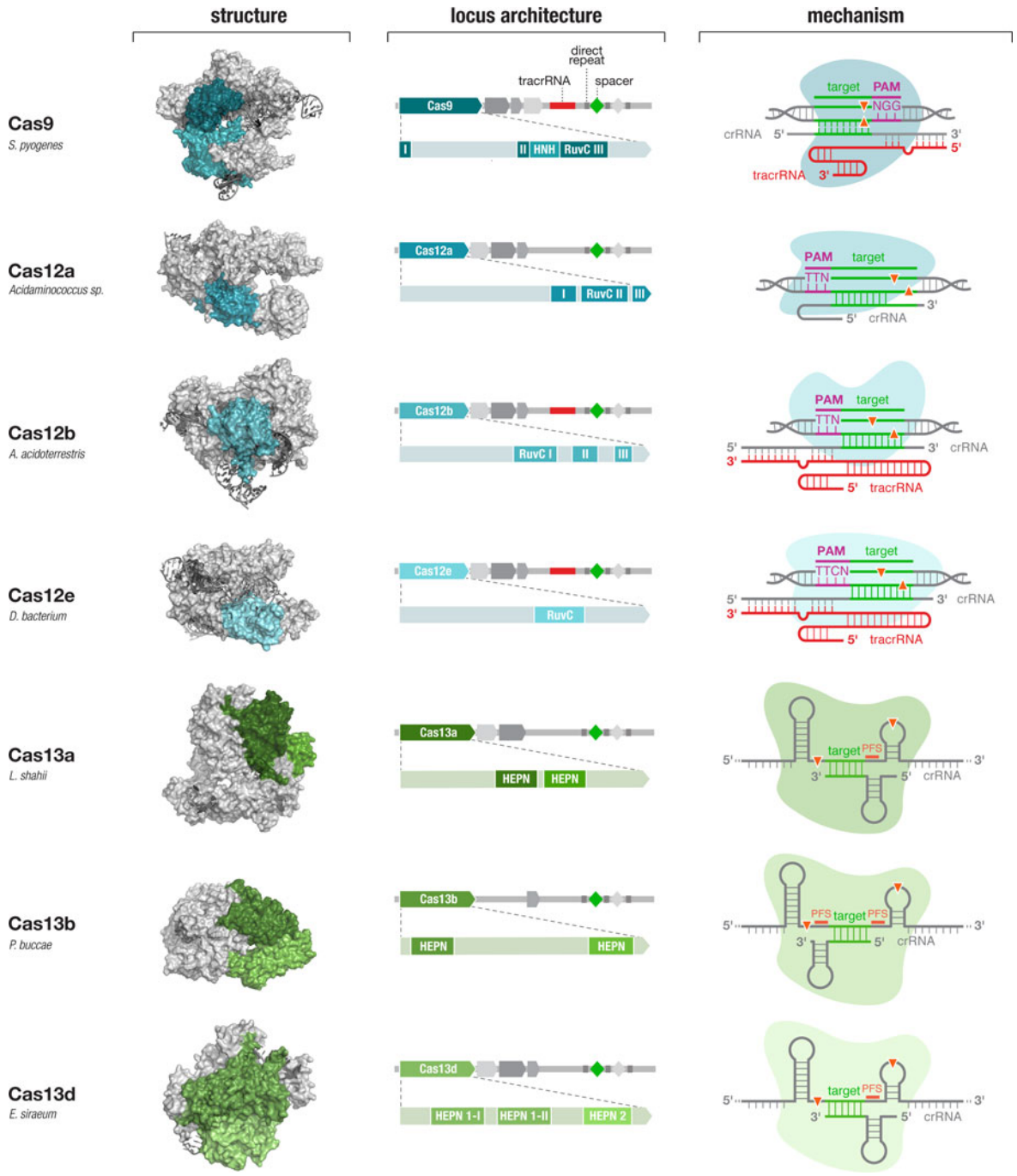


Fig. 4. Diverse class 2 CRISPR effectors have unique molecular features that contribute to an expansive toolbox for genome and transcriptome editing. To date, effectors from seven sub-types of CRISPR-Cas system have been developed for molecular technologies. These effectors differ in their locus architecture, structure, and mechanism, creating many opportunities for engineering CRISPR-based technologies. The locus architecture shows the CRISPR array, tracrRNA (if present), and catalytic domains of the effector protein. The crystal structures for each effector are shown with the catalytic domains colored as in the locus architecture. The mechanism of each effector depicts how it binds its DNA or RNA target, as well as the configuration of the crRNA and tracrRNA (if present). Crystal structures were obtained from the PDB (*S. pyogenes* Cas9, PDB ID: 4OO8; *Acidaminococcus sp.* Cas12a, 5B43; *Alicyclobacillus acidoterrestris* Cas12b, 5U30; *Deltaproteobacteria bacterium* Cas12e, 6NY1; *Leptotrichia shahii* Cas13a, 5WTK; *Prevotella buccae* Cas13b, 6DTD; *Eubacterium siraeum* Cas13d, 6E9E).

contain an RNase processing domain with the ability to cleave their corresponding CRISPR array into individual mature crRNAs (East-Seletsky *et al.*, 2016; Smargon *et al.*, 2017; Konermann *et al.*, 2018). Cas13 cleaves RNA at sites outside of the target region complementary to the crRNA. Analysis of cleavage products of *Leptotrichia shahii* Cas13a (LshCas13a) showed that cut sites do not vary even for crRNAs targeting different positions on the same target, indicating that cut sites are likely dictated by a combination of the target RNA secondary structure and sequence features (Abudayyeh *et al.*, 2017; Smargon *et al.*, 2017). Further exploration of the RNase activity uncovered the 'collateral effect' – recognition of the target RNA by

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the Cas13-crRNA complex leads Cas13 to become a promiscuous RNase, cleaving non-target bystander RNAs at preferred cut sites (Abudayyeh *et al.*, 2016). Collateral activity may play a role in programmed cell death in bacteria, although this remains to be fully explored. This collateral activity has been exploited to expand the applications of Cas effectors into new categories, including the development of sensitive, low-cost, and rapid diagnostics assays for viral and bacterial infections (see below).

Cas13a, b, c, and d have all been adapted for use in mammalian cells to mediate targeted RNA knockdown (Abudayyeh *et al.*, 2017; Cox *et al.*, 2017; Konermann *et al.*, 2018). Interestingly, although in bacteria, each Cas13 ortholog exhibits varying levels of nucleotide preference in sequences flanking the protospacer, referred to as the protospacer flanking site (PFS), the presence of the PFS is not a strict requirement for RNA targeting in mammalian cells (Abudayyeh *et al.*, 2017). Additionally, although collateral activity has been observed *in vitro* and in bacterial cells (Abudayyeh *et al.*, 2016; Meeske and Marraffini, 2018), it has not been detected in mammalian cells (Abudayyeh *et al.*, 2017; Konermann *et al.*, 2018), suggesting that, similarly for Cas9 and Cas12, the differences between biochemical, bacterial, and mammalian environments can substantially affect the behavior of Cas effectors.

Development of a molecular toolbox based on Cas effectors

DNA and RNA cleavage through the nuclease activities of Cas effectors is only one way CRISPR technology can be applied. The ability to customize the binding specificity of Cas effectors using a short-guide RNA creates many additional opportunities for developing new capabilities for manipulating DNA and RNA. There are two-main categories of molecular tools based on Cas proteins (Fig. 5), with the first category utilizing the intrinsic RNA-guided nuclease activity of each effector, and the second category exploiting nuclease-inactivated Cas proteins (dCas) as RNA-guided nucleic acid binding domains to target effector modules to modulate, monitor, or modify target DNA or RNA. As tools based on Cas effectors rely on the specificity of RNA-guided target recognition, another area of focus has been to assess the specificity of Cas effectors as well as engineering solutions to enhance their specificity. Below is an overview of the broad range of molecular tools that have been developed based on Cas proteins as well as efforts to address the most critical challenges facing CRISPR-based tools.

Leveraging natural and engineered properties of diverse Cas effectors

The opportunities for developing Cas effectors as molecular technologies are further amplified by the natural diversity within each family of class 2 CRISPR-Cas systems. Based on the current publicly accessible bacterial genomic and metagenomic sequencing data, there are over 100 000 Cas9 family members, over 70 000 Cas12 family members, and over 5000 Cas13 family members. Within each family, members can exhibit a number of differences in terms of their size, guide RNA requirement, binding motif (e.g., PAM and PFS), targeting specificity, and suitability for function in eukaryotic cells. In the case of Cas13 family members, they can also exhibit different cleavage motif preferences.

A number of Cas9 orthologs have been discovered (Bolotin *et al.*, 2005; Makarova *et al.*, 2011, 2015; Zhang *et al.*, 2013; Chylinski *et al.*, 2014; Fonfara *et al.*, 2014; Ran *et al.*, 2015; Shmakov *et al.*, 2017), and an increasing number of these have been developed for use as genome editing tools beyond SpCas9 and StCas9 (Esvelt *et al.*, 2013; Hou *et al.*, 2013; Karvelis *et al.*, 2015; Ran *et al.*, 2015; Hirano *et al.*, 2016; Lee *et al.*, 2016; Kim *et al.*, 2017*a*). The natural diversity of these enzymes has allowed expanded applications, for example some smaller Cas9 orthologs, such as *S. aureus* Cas9 (SaCas9), *Neisseria meningitidis* Cas9 (NmeCas9), and *Campylobacter jejunii* Cas9 (CjCas9) have been efficiently delivered *in vivo* using a single-vector strategy (Ran *et al.*, 2015; Kim *et al.*, 2017*a*; Ibraheim *et al.*, 2018).

While exploration of natural Cas diversity provides one avenue for expanding and improving CRISPR-based tools, a complementary approach uses structure-guided engineering to modify and improve Cas effector function. Over the past several years a number of crystal structures have been solved for different members of Cas9 (Anders *et al.*, 2014; Jinek *et al.*, 2014; Nishimasu *et al.*, 2014, 2015; Hirano *et al.*, 2016; Yamada *et al.*, 2017), Cas12 (Dong *et al.*, 2016; Yamano *et al.*, 2016; Yang *et al.*, 2016; Stella *et al.*, 2017; Swarts *et al.*, 2017; Wu *et al.*, 2017; Liu *et al.*, 2019), and Cas13 (Knott *et al.*, 2017; Liu *et al.*, 2017*a*, 2017*b*; Zhang *et al.*, 2018*a*, 2018*b*; Slaymaker *et al.*, 2019) families. These structures include the apo forms with just the effector protein alone, or the effector in complex with its guide RNA alone or guide RNA in complex with target DNA or RNA, providing structural insights into target recognition and cleavage. These structural studies have been complemented by other biochemical and biophysical studies into the target search mechanism of Cas effectors (Sternberg *et al.*, 2014; Knight *et al.*, 2015; Ma *et al.*, 2016*a*).

Expanding the targeting range of DNA-targeting Cas proteins

The DNA targeting range of Cas9 and Cas12 is defined by the PAM sequence, a short-sequence flanking the target sequence is required for DNA targeting. A shorter PAM sequence provides a broader targeting range whereas longer PAM sequences are more restrictive. For example, wild-type SpCas9 (which has an NGG PAM) can target roughly ten times more sites in the human exome than wild-type SaCas9 (which has an NNGRRT PAM) (Scott and Zhang, 2017). In order to increase the flexibility of Cas-mediated DNA targeting, a combination of approaches has been used to expand the number of targetable PAM sequences. First, by exploring phylogenetic diversity, a number of Cas effector orthologs have been identified with distinct PAM requirements. In the case of Cas12a, a survey of more than a dozen orthologs identified one, from *Moraxella bovoculi*, with robust indel activity in human cells and tolerance of a shorter PAM, expanding the available targeting landscape (Zetsche *et al.*, 2017). Ultimately, however, only a handful of Cas effectors have been successfully developed for function in eukaryotic cells (Ran *et al.*, 2015; Zetsche *et al.*, 2015*a*; Abudayyeh *et al.*, 2017; Cox *et al.*, 2017; Kim *et al.*, 2017*a*; Chatterjee *et al.*, 2018; Ibraheim *et al.*, 2018; Konermann *et al.*, 2018; Teng *et al.*, 2018; Liu *et al.*, 2019; Strecker *et al.*, 2019) limiting the extent of this approach.

A second approach for expanding the DNA targeting range of Cas9 and Cas12 is to engineer new variants, either through structure-guided design or directed evolution. Based on the crystal structures of Cas effectors in complex with guide RNA and target DNA (Anders *et al.*, 2014; Nishimasu *et al.*, 2015; Yamano *et al.*, 2016), targeted mutagenesis has been used to generate new protein variants with altered PAM sequences. At the same time, a

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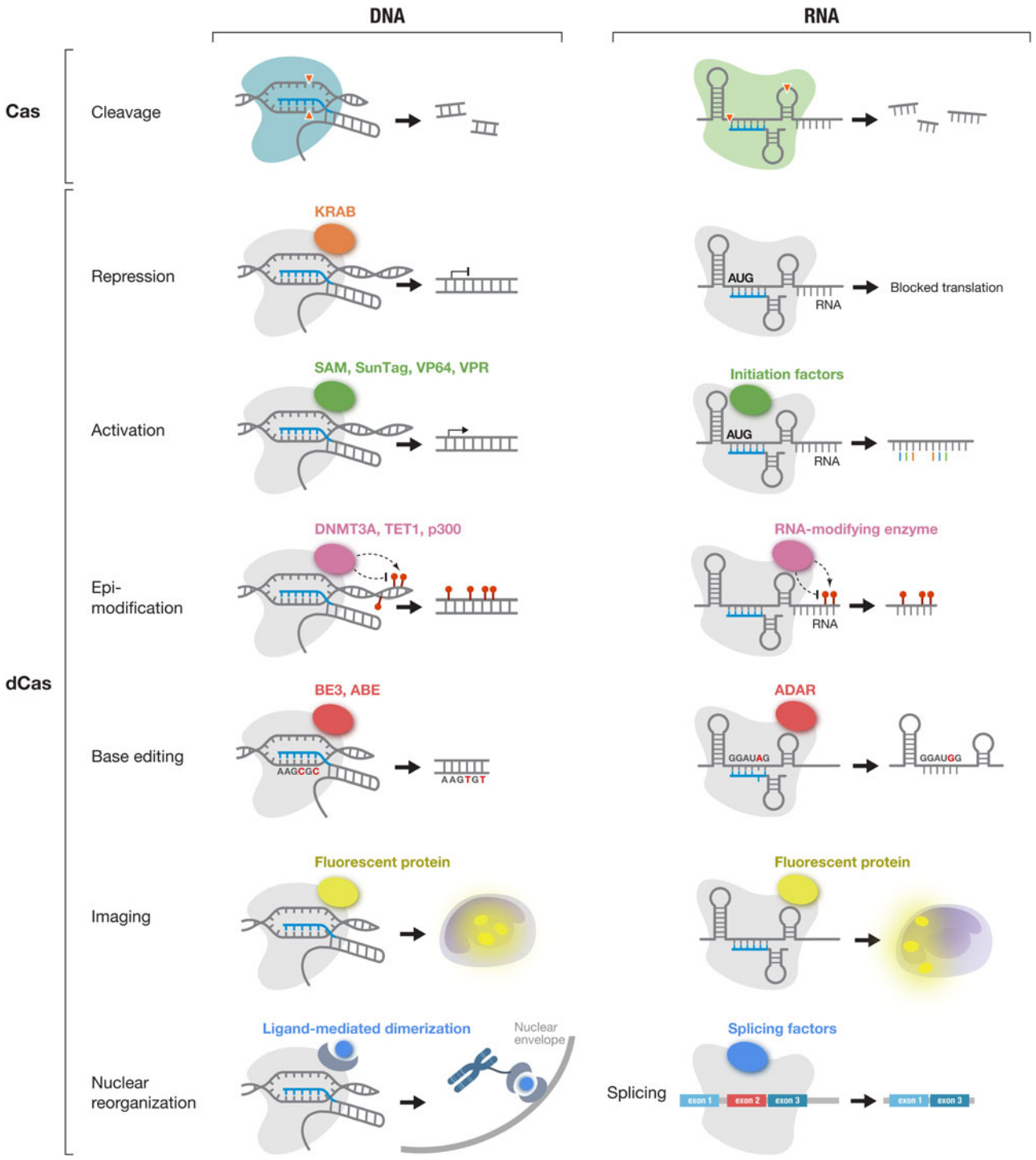


Fig. 5. The CRISPR-Cas toolbox enables a broad range of applications in eukaryotic cells. Applications of DNA-targeting Cas effectors (Cas9 and Cas12a-e) (left column) and applications of RNA-targeting Cas effectors (Cas13a-d) (right column) are shown. Active Cas effectors can be used for nucleic acid cleavage. In addition, Cas effectors can be turned into RNA-guided DNA or RNA binding domains by inactivating their catalytic residues (dCas). dCas can be fused to a variety of functional moieties to achieve targeted repression, activation, epi-modification, base editing, and imaging at either the DNA or RNA level. In addition, dCas13 can be used to modulate splicing through fusion to splicing factors. KRAB, Krüppel associated box; SAM, synergistic activation module; SunTag, SUperNova Tag; DNMT3A, DNA methyltransferase 3 alpha; TET1, ten-eleven translocation 1; p300, E1A-associated protein p300; BE3, base editor 3; ABE, adenine base editor and ADAR, adenosine deaminase acting on RNA;.

number of groups have used directed evolution strategies to evolve new variants of Cas effectors with unique properties, including different PAM preferences. These efforts have led to the development of a number of Cas9 and Cas12a variants with a significantly broadened DNA targeting range (Kleinstiver *et al.*, 2015*a*, 2015*b*; Gao *et al.*, 2017; Hu *et al.*, 2018; Nishimasu *et al.*, 2018). Collectively, these variants enable targeting of virtually any genomic site.

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Assessing the specificity of class 2 Cas effectors

One of the most critical technical requirements for the application of class 2 Cas effectors is their targeting specificity. When applying Cas9 or Cas12 as an active nuclease, minimizing off targets is particularly important because a range of undesirable genomic alterations could arise through the cell's endogenous DNA repair mechanisms, such as translocations between different cleavage sites and large-scale deletions. For nuclease as well as dCas binding applications, it is important that the effector binds selectively to the DNA or RNA targeted by the guide RNA. In the case of active nuclease applications, off-target editing activity due to pseudo-specific interactions between the Cas effector and the genome (arising when there are less than perfect matches between the target and guide RNA) can give rise to additional DSBs that lead to either small insertions and deletions (indels) or larger genomic alterations.

An initial study to characterize off-target indels used computational analyses to identify loci in the genome that share a high degree of homology to the target site, and then assayed editing events at these computationally predicted off-target loci using deep sequencing (Fu *et al.*, 2013). The study found that SpCas9 can indeed induce off-target edits at genomic loci that carried three or fewer mismatches compared with the guide sequence. Additional studies using different approaches also showed that Cas9 can indeed introduce off-target edits (Hsu *et al.*, 2013; Mali *et al.*, 2013*a*; Pattanayak *et al.*, 2013). As the early approaches covered only a very limited set of off-target sites, subsequent investigations focused on developing genome-wide unbiased approaches including *in vitro* assays like Digenome-seq (Kim *et al.*, 2015), CIRCLE-seq (circularization for *in vitro* reporting of cleavage effects by sequencing) (Tsai *et al.*, 2017), and SITE-seq (selective enrichment and identification of tagged genomic DNA ends by sequencing) (Cameron *et al.*, 2017) and cellular assays like GUIDE-seq (genome-wide, unbiased identification of DSBs enabled by sequencing) (Tsai *et al.*, 2015), BLESS (direct *in situ* breaks labeling, enrichment on streptavidin and next-generation sequencing) and BLISS (breaks labeling *in situ* and sequencing) (Crosetto *et al.*, 2013; Yan *et al.*, 2017), linear amplification-mediated PCR followed by high-throughput genome-wide translocation sequencing (LAM-HTGTS) (Frock *et al.*, 2015), and VIVO (verification of *in vivo* off-targets) (Akcakaya *et al.*, 2018). The use of these assays found that the editing specificity of SpCas9 varied widely depending on the guide RNA. When these unbiased techniques were used to profile the specificity of Cas9 orthologs as well as Cas12 family members, it was found that SaCas9 as well as Cas12a and Cas12b are much more specific than SpCas9, with most guide RNAs exhibiting no detectable off-target editing (Kleinstiver *et al.*, 2016*b*; Yan *et al.*, 2017; Strohkendl *et al.*, 2018; Tycko *et al.*, 2018; Strecker *et al.*, 2019). It is worth noting, however, that the functional impact of off-target edits will vary depending on their location. For example, off-target indels within coding regions, regulatory elements, and non-coding RNAs are likely to have more undesirable effects.

A number of studies have also explored the landscape of larger genomic alterations arising from Cas9 activity. For translocations, the use of LAM-HTGTS showed that the frequency of SpCas9-induced translocation varies considerably with the guide RNA, from undetectable to ~3% (Frock *et al.*, 2015), and the use of a tagmentation strategy found SpCas9-mediated translocation rates of ~2·5% for two-different guides (Giannoukos *et al.*, 2018). For large deletions, the use of PacBio and Sanger sequencing with SpCas9-edited hemizygous embryonic stem cells showed that 10 out of 48 edited alleles represented deletions larger than 250 bp (ranging up to nearly 6 kb) (Kosicki *et al.*, 2018). The same study also identified a number of other events such as transversions, duplications, and other structural rearrangements (Kosicki *et al.*, 2018).

Similar approaches have not been applied to the high-specificity variants of Cas9 or to Cas12a/b, all of which show substantially fewer indel off-targets, and it will be interesting to see if the number of large deletions and structural rearrangements is similarly reduced with these other Cas effectors. These studies, along with empirical testing of guide RNAs, will inform the best choice of the Cas effector for applications requiring particularly high levels of editing specificity. In addition, methods have been developed to quantify the on-target editing outcomes of Cas9 (Miyaoka *et al.*, 2016, 2018).

When assessing the RNA targeting specificity of Cas13, the considerations are slightly different. Although off-target cleavage arising from pseudo-specific binding remains a concern, an additional issue with Cas13 is potential collateral cleavage of bystander transcripts. To assess the likelihood of off-target RNA knockdown, the effect of an increasing number of mismatches between the guide sequence and its RNA target was examined. From these studies it was found that Cas13 can tolerate up to a single mismatch throughout the guide sequence and still cleave the target RNA (Abudayyeh *et al.*, 2017). Additionally, transcriptome-wide sequencing revealed that Cas13 can achieve highly specific knockdown of the target transcript without significant off-target effects. In contrast, short-hairpin RNA (shRNA) knockdown of the same transcript led to downregulation of hundreds of off-targets (Abudayyeh *et al.*, 2017). In addition, biochemical analysis of target RNA binding by Cas13a revealed that perfect matching in a central seed region of the guide sequence is required for binding, but a different guide region is required for the activation of RNase activity (Tambe *et al.*, 2018). Together, these studies provided detailed insight into the potential off-target effects due to pseudo-specific binding as well as suggested that collateral activity is not significant in mammalian cells, which has been supported by additional studies (Konermann *et al.*, 2018).

Improving the targeting specificity of Cas effectors

A number of approaches have been developed to improve the specificity of DNA editing by Cas9, and many of these approaches have also been applied or are relevant to Cas12 as well. The approaches can be divided into strategies that either seek to reduce overall exposure to the nuclease or directly improve specificity through engineering of the system.

For the first category of approaches, we observed early on that the editing specificity can be improved by more than 10-fold by introducing less Cas9 into cells (Hsu *et al.*, 2013). In agreement with this observation, several other groups have demonstrated that using either mRNA to deliver Cas9 or delivering Cas9-sgRNA ribonucleoprotein (RNP) complexes directly into the target cell can significantly increase the editing specificity (Cho *et al.*, 2014; Lin *et al.*, 2014). In addition to use of different delivery methods to limit the dosage of Cas9 in cells, it is also possible to engineer Cas9 so that its nuclease activity becomes drug- or light-inducible (Davis *et al.*, 2015; Nihongaki *et al.*, 2015; Truong *et al.*, 2015; Wright *et al.*, 2015; Zetsche *et al.*, 2015*b*; Liu *et al.*, 2016*a*; Nguyen *et al.*, 2016; Rose *et al.*, 2017). This

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inducible approach has also been applied to Cas12a (Tak *et al.*, 2017).

For the second category of approaches, various strategies have been used, beginning with engineering the system to increase the number of target DNA bases that must be specifically recognized by Cas9 in order to activate nuclease activity. The first strategy doubles the number of DNA bases that need to be recognized to introduce a DSB by utilizing a Cas9 nickase and two-juxtaposed guide RNAs to create two off-set nicks (Mali *et al.*, 2013*a*; Ran *et al.*, 2013). This method was found to reduce off-target edits beyond the detection limit (Ran *et al.*, 2013). Related to this double-nicking approach, a second strategy uses dCas9-FokI fusions and a pair of juxtaposed guide RNAs to facilitate the introduction of a DSB (Guilinger *et al.*, 2014; Tsai *et al.*, 2014). The specificity of Cas9 targeting can also be enhanced by modifying the guide RNA. One study showed that SpCas9 targeting can be significantly improved using truncated-guide RNAs with 17 nt of the targeting sequence (Fu *et al.*, 2014), which decreases the tolerance for mismatches. More recently, it was shown that the use of bridged or locked-nucleic acids can also improve Cas9 specificity by slowing the reaction rate (Cromwell *et al.*, 2018), and that engineering the guide RNA to create a hairpin in the spacer region improves specificity of a number of Cas9 and Cas12 enzymes (Kocak *et al.*, 2019).

In addition to these strategies, rational engineering of Cas9 and Cas12 has been used to create high-specificity variants, offering a simpler solution to the specificity challenge. We developed the first of these variants, eSpCas9, which exhibits substantially reduced off-target activity while maintaining on-target efficiency (Slaymaker *et al.*, 2015). A number of groups have subsequently used structural information or directed evolution to develop additional high-specificity variants of Cas9 (Kleinstiver *et al.*, 2016*a*; Chen *et al.*, 2017; Casini *et al.*, 2018; Hu *et al.*, 2018; Lee *et al.*, 2018; Vakulskas *et al.*, 2018) and Cas12a (Kleinstiver *et al.*, 2019).

The specificity of Cas effectors will continue to be improved through rational engineering and directed evolution, which will be particularly important for the clinical use of Cas effectors. It is important to note that the development of variants of Cas effectors with increased specificity needs to be complemented with higher sensitivity assays, such as the recently developed genome-wide off-target analysis by the two-cell embryo injection (GOTI) method (Zuo *et al.*, 2019), for detecting the presence of off-target activity, particularly large deletions and chromosomal rearrangements.

dCas platforms

In addition to their utility as nucleases, class 2 Cas effectors can also be inactivated to turn the proteins into RNA-guided DNA- or RNA-binding domains (Fig. 5). These inactivated variants can be used for a wide variety of powerful applications by serving as programmable nucleic acid binding scaffolds for the recruitment of a variety of effector functions. To deactivate the nuclease activity of Cas9, alanine substitutions are introduced into the catalytic residues of the HNH and RuvC nuclease domains (Sapranauskas *et al.*, 2011). In early 2013, using this mutant version of Cas9, termed dead Cas9 (dCas9), it was shown that dCas9 could achieve programmable gene repression in bacteria and mammalian cells by simply binding to the genome and blocking transcription (Qi *et al.*, 2013). Since then, many new applications have been developed by using dCas9 to recruit effectors that modulate, modify, or visualize DNA or RNA (for examples see: (Bikard *et al.*, 2013; Chen *et al.*, 2013; Gilbert *et al.*, 2013, 2014; Konermann *et al.*, 2013, 2014; Maeder *et al.*, 2013; Perez-Pinera *et al.*, 2013; Tanenbaum *et al.*, 2014; Hilton *et al.*, 2015; Ma *et al.*, 2015, 2016*b*; Thakore *et al.*, 2015)). Similar to Cas9, the RuvC nuclease domain of Cas12 (Zetsche *et al.*, 2015*a*), and the HEPN nuclease domains of Cas13 (Abudayyeh *et al.*, 2016) can also be inactivated to generate dCas12 and dCas13, respectively.

In the case of Cas9, we showed that it is also possible to use a truncated guide sequence that does not trigger the nuclease activity of Cas9 (Dahlman *et al.*, 2015). Using this strategy, researchers can simultaneously use Cas9 as a nuclease to cleave one set of genomic targets and as a DNA binding domain for a different set of genomic targets simply by using guide RNAs with full length (20-nt) or truncated (12-nt) guide sequences, respectively. This approach is particularly relevant when using transgenic mouse lines expressing the nuclease-active form of Cas9 (Platt *et al.*, 2014). Using this truncated guide RNA strategy, DNA binding experiments can be conducted without creating an additional mouse line expressing dCas9 (Liao *et al.*, 2017).

There are a several ways to recruit effectors to dCas. The simplest method is to directly fuse the effector protein to either the N- or C-terminus of the Cas protein (Gilbert *et al.*, 2013; Konermann *et al.*, 2013; Abudayyeh *et al.*, 2017; Tak *et al.*, 2017). However, in some applications, particularly for the recruitment of fluorescent proteins for imaging, a second strategy has been used where a SunTag is attached to dCas9 to attract effectors that are fused to a single-chain variable fragment antibody fragment with SunTag affinity (Tanenbaum *et al.*, 2014). Yet another approach is to engineer the guide RNA such that exposed hairpins can serve as potential sites for insertion of RNA aptamers. By engineering new guide RNAs carrying the MS2 aptamer inserted into stem loops on the guide RNA, we showed that effector domains can be recruited via MS2 binding (Konermann *et al.*, 2014). Subsequent studies have shown that other aptamers such as PP7 and com can also be inserted into the guide RNA to allow for multiplexing applications (Zalatan *et al.*, 2015; Liu *et al.*, 2016*a*).

The applications of dCas are quite broad. Initial work showed that simply by recruiting dCas9 to target loci, gene expression could be repressed in both bacterial and human cells (Bikard *et al.*, 2013; Qi *et al.*, 2013). Fusions of dCas9 to transcriptional repressors, such as Krüppel-associated box (KRAB), have also been used to programmably repress gene expression (Gilbert *et al.*, 2013) in human cell lines. dCas9-KRAB fusions have been combined with inducible Cas9 systems for fine-tuned regulatory control of gene networks (Mandegar *et al.*, 2016). dCas9 can also be used to facilitate transcriptional activation of target genes (Bikard *et al.*, 2013; Gilbert *et al.*, 2013; Konermann *et al.*, 2013, 2014; Maeder *et al.*, 2013; Perez-Pinera *et al.*, 2013). Additionally, dCas9 has been fused with epigenetic modifiers to achieve targeted histone acetylation (Hilton *et al.*, 2015), histone demethylation (Kearns *et al.*, 2015), and DNA methylation and demethylation (Liu *et al.*, 2016b; Vojta *et al.*, 2016; Xu *et al.*, 2016). A number of groups have used dCas9 for genomic locus and chromosome imaging as well as spatial manipulation of genomic organization (Chen *et al.*, 2013; Morgan *et al.*, 2017; Wang *et al.*, 2018). Through the use of either orthogonal Cas enzymes or aptamers and multiple fluorophores, multiplex locus imaging can be achieved (Chen *et al.*, 2016; Liu *et al.*, 2016*a*). Similarly, RNA can be imaged using dCas effectors, including dCas9 (Nelles *et al.*, 2016) and dCas13a (Abudayyeh *et al.*, 2017). dCas13 has also been fused to hnRNP1, a negative regulator of

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splicing, to achieve targeting exon skipping (Konermann *et al.*, 2018). Through fusion to the engineered peroxidase APEX2, dCas9 can be used to identify proteins associated with a specific genomic locus (Myers *et al.*, 2018). Additional functional platforms have also been developed, such as the fusion of a Cas9 nickase with an error-prone polymerase to create EvolvR, a system for rapid diversification of the DNA sequence within a few hundred base-pair window (Halperin *et al.*, 2018). Another system, CRISPR-X, uses dCas9 and modified-guide RNAs to recruit cytidine deaminase variants to generate localized windows of variation, which may have applications for directed evolution (Hess *et al.*, 2016).

Targeted base editing of DNA and RNA

Another exciting application of dCas enzymes has been the development of programmable DNA and RNA base editors, which can achieve the precise chemical change of one base to another (reviewed comprehensively by (Rees and Liu, 2018)). Base editors are particularly promising for the development of therapeutic applications, as more than half of the known pathological variants are point mutations. Furthermore, both DNA and RNA base editing provide the possibility of making targeted changes without relying on homologous recombination, which is inefficient especially in post-mitotic cells such as neurons.

DNA base editors are generated by fusing dCas9 or Cas9 nickase (Komor *et al.*, 2016; Nishida *et al.*, 2016; Gaudelli *et al.*, 2017) or dCas12 (Li *et al.*, 2018*c*) to single-strand DNA deaminases (Fig. 5). The first type of base editor developed used dCas9 or Cas9 nickase to bring a single-stranded DNA cytosine deaminase such as AID or APOBEC to mediate C • G to T • A conversions on target DNA. Binding of DNA by dCas9 or Cas9 nickase forms an R-loop which exposes a short stretch of single-stranded DNA for deamination by the tethered cytosine deaminase (Komor *et al.*, 2016; Nishida *et al.*, 2016). Application of the cytosine base editor in a variety of animal and plant cell types can lead to high levels of targeted base conversion (Rees and Liu, 2018). To expand the types of base changes achievable, a second type of base editor capable of converting A • T to G • C was created by fusing dCas9 or Cas9 nickase to an evolved form of the bacterial tRNA-specific adenine deaminase TadA (Gaudelli *et al.*, 2017). TadA naturally acts on single-stranded RNA, but through an impressive series of directed evolution steps, TadA was converted into a DNA deaminase. DNA base editing has already been applied in animal models of disease, highlighting its potential for therapeutic use (Villiger *et al.*, 2018). This powerful technology is being rapidly optimized to increase specificity, efficacy, and precision (Rees and Liu, 2018).

RNA base editors have been engineered by fusing dCas13 to the adenine deaminase ADAR to achieve a precise, targeted A-to-I conversion (inosine is read out by cells as guanosine) (Fig. 5) (Cox *et al.*, 2017). Because ADAR acts on an RNA duplex formed between the target RNA and the guide RNA at adenosines in A • C mismatch bubbles, a specific adenine can be targeted for deamination by intentional mis-pairing with a cytosine on the guide RNA (Cox *et al.*, 2017). The ability to direct adenine deamination with single-nucleotide precision, which is not currently possible with DNA editing, has inspired efforts to use directed evolution to convert ADAR into a duplex RNA-acting cytosine deaminase, an activity which has not been found in nature, to develop a precise C to U RNA editor. RNA and DNA base editing complement each other to expand the range of applications. In particular, RNA editing does not depend on the presence of DNA-repair machinery for base conversion and therefore can be applied in virtually all cell types. Furthermore, because RNA editing can be potentially temporally restricted when paired with transient delivery systems, it can serve as a reversible editing system, which further expands the therapeutic potential of CRISPR-based technologies.

The specificity of base editors has recently been comprehensively profiled. Although cytosine DNA base editors have been found to generate a large number of off-target edits throughout the genome, the adenine base editor is able to achieve specific editing of the target site (Jin *et al.*, 2019; Kim *et al.*, 2019; Zuo *et al.*, 2019). A study in mice also examined potential off-target effects of adenine base editing and similarly found that A • T to G • C conversion was quite specific (Liu *et al.*, 2018*d*). Additionally, a recent study looking at the specificity of base editors found substantial off-target editing in the transcriptome (Grunewald *et al.*, 2019). Future refinements aimed at reducing non-specific interactions with DNA should significantly increase the specificity of cytosine base editors. In addition to improving the specificity of the deaminase domain, specificity may also be improved through the use of more specific Cas effectors such as dSaCas9 or dCas12a rather than dSpCas9. As DNA base editors do not rely on the introduction of DSBs, the likelihood of large deletions or translocations is significantly lower than with nuclease-based approaches. The specificity of RNA base editors has also been comprehensively profiled using high-coverage transcriptome analysis. Although the initial version of the RNA base editing platform showed broad transcriptome-wide off-target editing, structure-guided engineering of ADAR reduced non-specific interactions and improved the targeting specificity by ~1000 fold (Cox *et al.*, 2017). The promising results with both DNA and RNA base editing are prompting rapid improvements in technology.

Additional applications of CRISPR-based technologies

CRISPR-based technologies have also been coopted for information recording, either about cell fate, activity, or even non-biological data. Many of these approaches rely on the ability of CRISPR-Cas systems to create traceable scars that can be read out through sequencing and then related back to specific events. One of the first such uses was lineage tracing to reconstruct cellular or organismal development (McKenna *et al.*, 2016; Frieda *et al.*, 2017; Kalhor *et al.*, 2017). Trackers have also been built that can record information about the cellular state, such as the presence of small molecules, metabolites, external stimuli, or transcriptional activity (Perli *et al.*, 2016; Schmidt *et al.*, 2018; Tang and Liu, 2018). Cas effectors have also been used to build synthetic gene circuits to advance synthetic biology applications (Nissim *et al.*, 2014; Zalatan *et al.*, 2015; Nakamura *et al.*, 2019). Finally, using the adaptation modules of CRISPR-Cas systems, the Cas1 and Cas2 enzymes (Heler *et al.*, 2015; Nunez *et al.*, 2015; Silas *et al.*, 2016), an approach has been developed for storing non-biological data (Shipman *et al.*, 2016, 2017; Schmidt *et al.*, 2018).

Advancing biological research

CRISPR-based tools have been deployed widely in the life sciences, and due to their accessibility and ease of use, they are contributing to the advancement of biological studies on nearly every

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front. Here, I highlight a few of the ways these tools are being used in high-impact applications, including creation of new animal and cellular models as well as large-scale gene function interrogation.

Accelerating the generation of cellular and animal models

One of the most immediate impacts CRISPR-based technologies have had on the advancement of biological studies is on the generation of plant, animal, and cellular models. First, CRISPR-based technologies have dramatically reduced the time and labor needed to modify the genome of conventional eukaryotic model organisms and cell lines (with the exception of yeast, for which a powerful toolbox for genetic manipulation has existed for decades). A key example of this is the generation of knockout mice. Prior to 2013, knockout mice were created using modified-embryonic stem cells and the entire process took 1–2 years, whereas Cas9-mediated knockout can be achieved in weeks to months (Wang *et al.*, 2013; Yang *et al.*, 2013). Recently, it was suggested that the generation of tailored mouse models could be further accelerated through use of a Cas9-mediated gene drive approach (Grunwald *et al.*, 2019). This approach has also made it substantially more feasible to create non-human primate models (Niu *et al.*, 2014; Chen *et al.*, 2015*b*; Wan *et al.*, 2015). Similarly, the ease of reprogramming CRISPR-based tools has enabled their large-scale application to rapidly create libraries of cell lines. Second, CRISPR technology has rendered a variety of additional organisms genetically tractable, including parasites (Ghorbal *et al.*, 2014; Sollelis *et al.*, 2015; Vinayak *et al.*, 2015; Sidik *et al.*, 2016), microorganisms (Shapiro *et al.*, 2018), and non-model organisms such as crustaceans (Martin *et al.*, 2016), wasps (Li *et al.*, 2017), butterflies (Li *et al.*, 2015), and diatoms (Nymark *et al.*, 2016). This robustness of CRISPR-based technologies across organisms provides opportunities for studying many biological processes in their native context. Third, CRISPR-based technologies are being used to create tailored animal and cellular models that recapitulate genetic variants found in human patients (Birling *et al.*, 2017). These tailored models enable more accurate disease modeling that can be used to understand the molecular pathology of a range of human diseases as well as to develop novel therapeutic strategies to treat these diseases. For example, a pig model of Huntington's disease has been created through Cas9-mediated gene editing, enabling the study of this disease in a more relevant animal model (Yan *et al.*, 2018a). Fourth, Cas9 has also been used *in vivo* to accelerate the modeling of diseases, such as cancer (Maddalo *et al.*, 2014; Sanchez-Rivera *et al.*, 2014). Finally, Cas9 has also been used to successfully edit human embryos in the laboratory for research purposes (the embryos were discarded without being implanted to establish pregnancy) (Liang *et al.*, 2015; Fogarty *et al.*, 2017; Ma *et al.*, 2017). These studies have the potential to advance our understanding of human embryogenesis and reproductive challenges. For one of these studies (Ma *et al.*, 2017), an active dialog is currently underway regarding the interpretation of the editing results (Adikusuma *et al.*, 2018; Egli *et al.*, 2018; Ma *et al.*, 2018).

One particularly useful advance has been the creation of transgenic mice that constitutively or conditionally express Cas9, increasing the ease of gene knockout studies *in vivo* (Platt *et al.*, 2014; Dow *et al.*, 2015). Additionally, a dCas9-EGFP knock-in mouse has been created to enable easy, dynamic tracking of targeted genomic regions (Duan *et al.*, 2018). Using these mouse models, researchers can much more easily target specific cell types, achieve multiplexed gene knockouts, and much more rapidly model diseases. These models have been used to look at a number of biological processes, including cancer (Platt *et al.*, 2014), wound healing (Ge *et al.*, 2017), synaptic transmission (Yamasaki *et al.*, 2017), circadian rhythms (Tso *et al.*, 2017), and T cell differentiation (Zhang *et al.*, 2017), among others.

Genome-wide functional screening

A second way that CRISPR technology is accelerating research is through the development of robust high-throughput screening methods (Koike-Yusa *et al.*, 2014; Shalem *et al.*, 2014; Wang *et al.*, 2014; Zhou *et al.*, 2014) that can systematically assay the impact of genes or regulatory regions on a phenotype of interest (see (Doench, 2017; Guo *et al.*, 2017) for a recent review of CRISPR-based genetic screening) (Fig. 6). Large-guide RNA expression libraries can be computationally designed to target every gene in the genome. Delivery of this library of guides along with Cas9 into cells generates a population of cells, each with a single gene perturbed, collectively knocking out every gene in the genome (Shalem *et al.*, 2014). By phenotypically screening the library of cells, candidate genes involved in a process of interest can be identified through sequencing the guides in selected cells in the perturbed population. Although the approach is similar to methodologies using genome-wide libraries of shRNAs, CRISPR screens are significantly more reliable (Shalem *et al.*, 2014). In addition, as interest in non-coding regions and *cis*-regulatory elements has grown in recent years (nearly 99% of the human genome is non-coding and the overwhelming majority of disease-associated variants identified in genome-wide association studies (GWAS) are in non-coding regions), CRISPR-based screening has also been extended to identify non-coding regulatory elements in the endogenous genome (Canver *et al.*, 2015; Sanjana *et al.*, 2016).

dCas9-based transcription screening systems have also been developed for genome-scale screening. dCas9 alone or tethered to a transcriptional repression domain has been used to mediate genome-scale loss of function screening. Because sgRNAs for dCas9-mediated knockdown are harder to design and are not as potent as Cas9-mediated knockout, transcriptional repression screening typically requires guide RNA libraries with more redundancy per gene (Gilbert *et al.*, 2014). However, for genes that are essential and therefore cannot be permanently knocked-out, transcription repression-based screening may be used to uncover their function. In addition, repression-based approaches circumvent some of the limitations of knockout screening, such as cellular toxicity associated with targeting DSBs to high-copy chromosomal regions (Aguirre *et al.*, 2016; Munoz *et al.*, 2016) and the variability in the outcome of NHEJ repair, which could generate gain-of-function alleles (Donovan *et al.*, 2017; Ipsaro *et al.*, 2017). dCas9-based transcription activators may also be used to carry out gain-of-function genetic screens (Gilbert *et al.*, 2014; Konermann *et al.*, 2014).

Additional modes of CRISPR-based screening include the use of paired guides to create libraries of large deletions, which has enabled further interrogation of lncRNAs (Zhu *et al.*, 2016), and dCas9 fused to epigenomic modifiers to screen for functional regulatory elements (Klann *et al.*, 2017). The basic screening approach has also been adapted to achieve multiplex perturbations to identify gene sets involved in specific drug responses (Wong *et al.*, 2016) and to systematically map genetic interactions at an unprecedented scale (Horlbeck *et al.*, 2018). Pooled CRISPR screens have been combined with single-cell RNA-sequencing,

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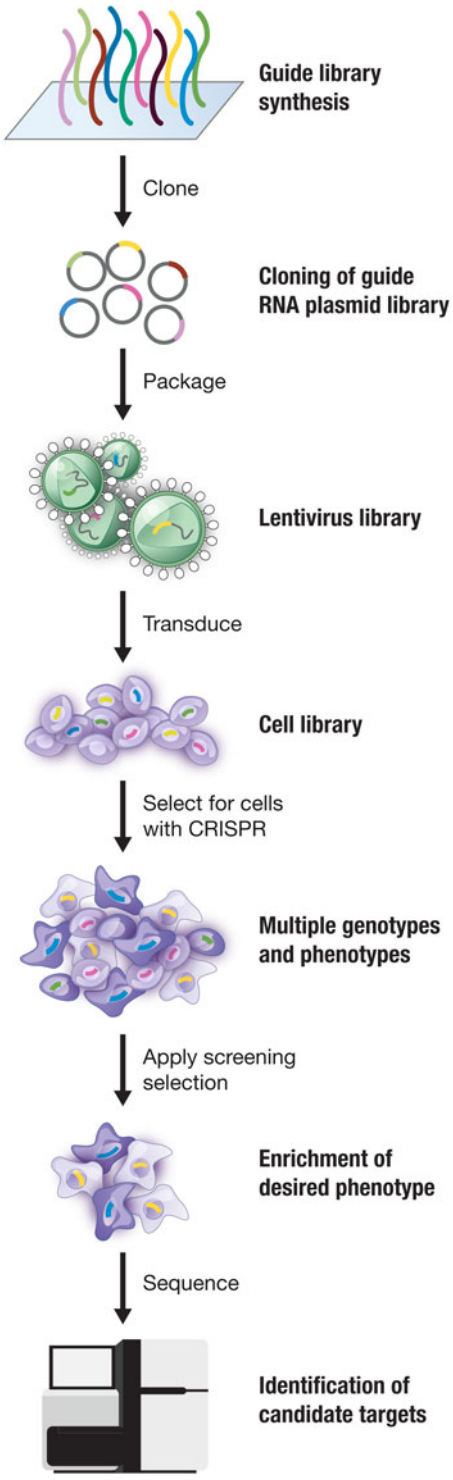


Fig. 6. CRISPR-mediated genome-wide screening allows large-scale interrogation of gene function. Cas9 can be combined with libraries of guide RNAs to facilitate gain- or loss-of-function genetic screening. First, guide RNAs designed to target coding or noncoding elements of the genome are synthesized and cloned into a Cas9 guide RNA plasmid library. This library is then packaged into lentivirus, which is transduced into cells of interest. After selecting for cells with Cas9 and then applying a selective pressure, cells with the desired phenotype are enriched. Sequencing of the guide RNAs in this enriched cell population identifies candidate loci involved in the phenotype. Image adapted from Hsu *et al.* (2014).

enabling dissection of complex phenotypes at the transcriptional level (Adamson *et al.*, 2016; Dixit *et al.*, 2016; Jaitin *et al.*, 2016) including eQTLs (Gasperini *et al.*, 2019). Finally, CRISPR-based screens can be adapted for *in vivo* large-scale gene interrogation of unique phenotypes, such as metastasis to distal organs, that cannot be captured *in vitro* (Chen *et al.*, 2015*a*; Manguso *et al.*, 2017).

Over the past five years, CRISPR-based screens have been applied to a large number of biological questions, including a range of aspects of cancer biology (Chen *et al.*, 2015*a*; Hart *et al.*, 2015; Shi *et al.*, 2015; Toledo *et al.*, 2015; Han *et al.*, 2017, 2018; Manguso *et al.*, 2017; Patel *et al.*, 2017), mitochondrial disease (Jain *et al.*, 2016), host-pathogen interactions (Marceau *et al.*, 2016; Zhang *et al.*, 2016; Park *et al.*, 2017), the immune system (Parnas *et al.*, 2015), gene essentiality (Wang *et al.*, 2015), cell fate specification (Liu *et al.*, 2018*c*), mechanisms of DNA repair (Noordermeer *et al.*, 2018), regulatory sequences in enhancers (Canver *et al.*, 2015; Fulco *et al.*, 2016; Sanjana *et al.*, 2016; Liu *et al.*, 2018*a*), and the role of long non-coding RNAs (Zhu *et al.*, 2016; Joung *et al.*, 2017; Liu *et al.*, 2017*c*), to name just a few.

To extend the utility of CRISPR-based screens and make them even more robust, a number of groups have also contributed to refining and optimizing screening approaches as well as developing a host of computational tools to aid in the design of large-scale CRISPR-mediated screens, including CRISPR inhibition (CRISPRi) and activation (CRISPRa) approaches (Doench *et al.*, 2014, 2016; Hart *et al.*, 2015, 2017; Heigwer *et al.*, 2016; Horlbeck *et al.*, 2016; Meier *et al.*, 2017; Morgens *et al.*, 2017; Sanson *et al.*, 2018). In addition, software has been developed for the analysis of CRISPR-mediated screens (Li *et al.*, 2014; Hart and Moffat, 2016; Winter *et al.*, 2016; Wang *et al.*, 2019). To date, a number of CRISPR knockout screening libraries, some with improved efficacy and others targeting themed gene collections (e.g., kinome or transcription factors) have been developed. Together, these tools have substantially reduced the barrier to forward genetic approaches in mammalian cells, uncovering exciting new biology and revealing new potential therapeutic targets.

Providing new opportunities for plant and agricultural science

Another area of the life sciences that CRISPR technology has deeply impacted is plant biology. In particular, it has revolutionized plant breeding by dramatically reducing the time to generate new genotypes. In some plant species, homozygous knockout mutants can now be produced in a single generation (Feng *et al.*, 2013; Mao *et al.*, 2013; Brooks *et al.*, 2014; Xu *et al.*, 2014; Zhang *et al.*, 2014). The natural ability of Cas enzymes for multiplex editing is particularly helpful for editing polyploid genomes, such as wheat, where traditional genetic manipulation strategies are difficult as well as altering complex agronomic traits. To date, CRISPR-mediated gene knockout has been applied in a number of agricultural crops, including rice, barley, soy, maize, wheat, tomato, potato, lettuce, citrus trees, mushroom, cucumber, grape, watermelon and others, and there is a substantial effort to engineer these and other plants to achieve a range of traits such as drought resistance, increased yield, pathogen resistance, and

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decreased time to ripening (Schindele *et al.*, 2018). Cas-based approaches have also succeeded in plants that have traditionally been inaccessible to targeted gene changes, such as woody plants (Bewg *et al.*, 2018). Methods are also being developed to achieve transgene-free gene editing in plants that rely on transforming plants with Cas9-guide RNA RNP complexes to avoid introducing foreign DNA (Woo *et al.*, 2015; Liang *et al.*, 2017)

Similar to the extension of Cas enzymes for many purposes in other systems, there is a growing toolbox of CRISPR-based technologies tailored to plant biology. For example, Cas12a has proven to be particularly effective in plants (Zaidi *et al.*, 2017). Base editing approaches have also been applied successfully in a number of plant species including rice, maze, tomato, and wheat (Shimatani *et al.*, 2017; Zong *et al.*, 2017; Kang *et al.*, 2018; Li *et al.*, 2018*a*; Hua *et al.*, 2019). Cas13a has been used for interference against RNA viruses in tobacco, providing a new strategy for conferring immunity (Aman *et al.*, 2018). Together, this toolbox is advancing basic plant biology studies and holds substantial potential to contribute to global food security without relying on transgenes. To date, numerous crop strains have been generated through CRISPR-mediated genome editing, including tomatoes with higher yield (Soyk *et al.*, 2017), reduced-gluten wheat, virus-resistant cacao, caffeine-free coffee, and mushrooms that were engineered to resist browning, which have received USDA approval (https://www.nature.com/news/gene-edited-crispr-mushroom-escapes-us-regulation-1·19754).

Advancing human health

The ability to precisely manipulate the genome, and in particular our ability to edit DNA and RNA, offers enormous potential for improving human health by offering a platform that can be tailored to any of thousands of genetic disorders (reviewed in (Cox *et al.*, 2015)). Achieving this potential, however, will require a suite of highly specific and efficient Cas enzymes and a toolbox of delivery modalities that can be seamlessly combined to address the specific challenges of individual diseases. To date, the field has produced a spectacular diversity of editing tools, some of which are now entering clinical trials. There are still outstanding challenges in the development of CRISPR-based therapeutics, however, notably delivery and potential immunogenicity. Below I discuss some of the ways CRISPR-based technologies are advancing human health and highlight areas where additional research is needed.

Applications for elimination of bacterial and viral pathogens

CRISPR-Cas systems have been applied to improve human health in a range of ways, including the generation of new antibacterial agents. Several groups reported that CRISPR-Cas systems could be packaged in phages and used to selectively treat targeted bacteria, generating programmable, sequence-specific antimicrobial agents (Bikard *et al.*, 2014; Citorik *et al.*, 2014). There have also been multiple studies that leverage the natural function of CRISPR-Cas systems to treat viral infections. For example, it was shown that in a cellular model of HIV, Cas9 could be programmed to target integrated copies of the HIV virome as well as prevent HIV infection (Hu *et al.*, 2014). With many viral infections, the persistence of latent virus in the body represents a major therapeutic challenge. Using a mouse model of Hepatitis B virus (HBV) chronic infection, it was demonstrated that Cas9 targeting the genome of HBV can reduce viral load (Ramanan *et al.*, 2015). Finally, CRISPR-based technologies are being used to eliminate endogenous retroviruses in pigs to generate animals that may be suitable sources of organs for transplantation into human patients (Yang *et al.*, 2015; Niu *et al.*, 2017).

Applications for detection of bacterial and viral pathogens

Another way that CRISPR-Cas systems are advancing human health is through the development of CRISPR-based diagnostics. Our discovery of the collateral RNase activity of Cas13 – upon binding to the target RNA, Cas13a becomes activated as a non-specific RNase and can cleave the target RNA as well as other RNA molecules in the vicinity that do not have complementarity with the guide RNA target sequence – has made possible the development of a new approach for nucleic acid detection (Abudayyeh *et al.*, 2016). With programmable target recognition triggering non-specific collateral cleavage of reporter molecules, these systems can be used to detect target molecules of interest at very low levels (Fig. 7). This technology has a range of applications, notably in the detection and monitoring of infectious diseases in the field, such as Zika and Ebola, and in highly sensitive genotyping, such as the detection of cancer-associated alleles in circulating DNA. Moreover, CRISPR diagnostics can be used in agricultural and industrial settings to ensure food safety and prevent the spread of contaminating agents.

A number of modalities can be integrated with collateral-cleavage of reporter RNA to provide diagnostic readout. Using gel electrophoresis for visualization, diagnostic reporting can be achieved by assessing cleavage of a fluorescently tagged reporter RNA by Cas13 upon crRNA-guided recognition of target RNA (Abudayyeh *et al.*, 2016). Using a fluorimeter for readout, detection of target nucleic acid triggers Cas13 collateral activity and unlocks fluorescent reporters such as the commercially available RNaseAlert by unleashing the fluorophore from its quencher to yield fluorescence. Direct application of Cas13 collateral RNase activity with RNaseAlert achieved detection sensitivity in the picomolar range (~ 1 000 000 molecules per test) (East-Seletsky *et al.*, 2016). By integrating Cas13 collateral activity with isothermal amplification, we developed a technique called SHERLOCK, which allowed for significantly increased detection sensitivity to the attomolar range (Gootenberg *et al.*, 2017), enabling clinical applications. Finally, using colorimetric paper test strips, Cas13-based diagnostics can be applied in low-resource settings or in the field. To achieve this low-cost modality, we developed a lateral flow paper-based test, similar to a commonly-used pregnancy test strip, by exploiting Cas13 collateral activity to cleave and unlock two-different affinity molecules so that the presence of the target can be read out as two-stained lines on the paper strip (Gootenberg *et al.*, 2018). Cas13-based diagnostics tests can also be lyophilized and easily reconstituted through the addition of water, which makes it possible for SHERLOCK to be distributed and stored without requiring refrigeration (Gootenberg *et al.*, 2017), which is especially important for application in low-resource settings in the developing world.

CRISPR-based detection technology is rapidly expanding. Recently, Cas12 was found to have natural ssDNA collateral activity that is triggered by binding DNA (Chen *et al.*, 2018; Li *et al.*, 2018*b*). Cas12 can be similarly combined with isothermal amplification in a technique called DETECTOR or integrated into the SHERLOCK platform for multiplex detection (Chen *et al.*, 2018; Gootenberg *et al.*, 2018; Li *et al.*, 2018*b*). Different types of Cas proteins with collateral activity can be combined to achieve

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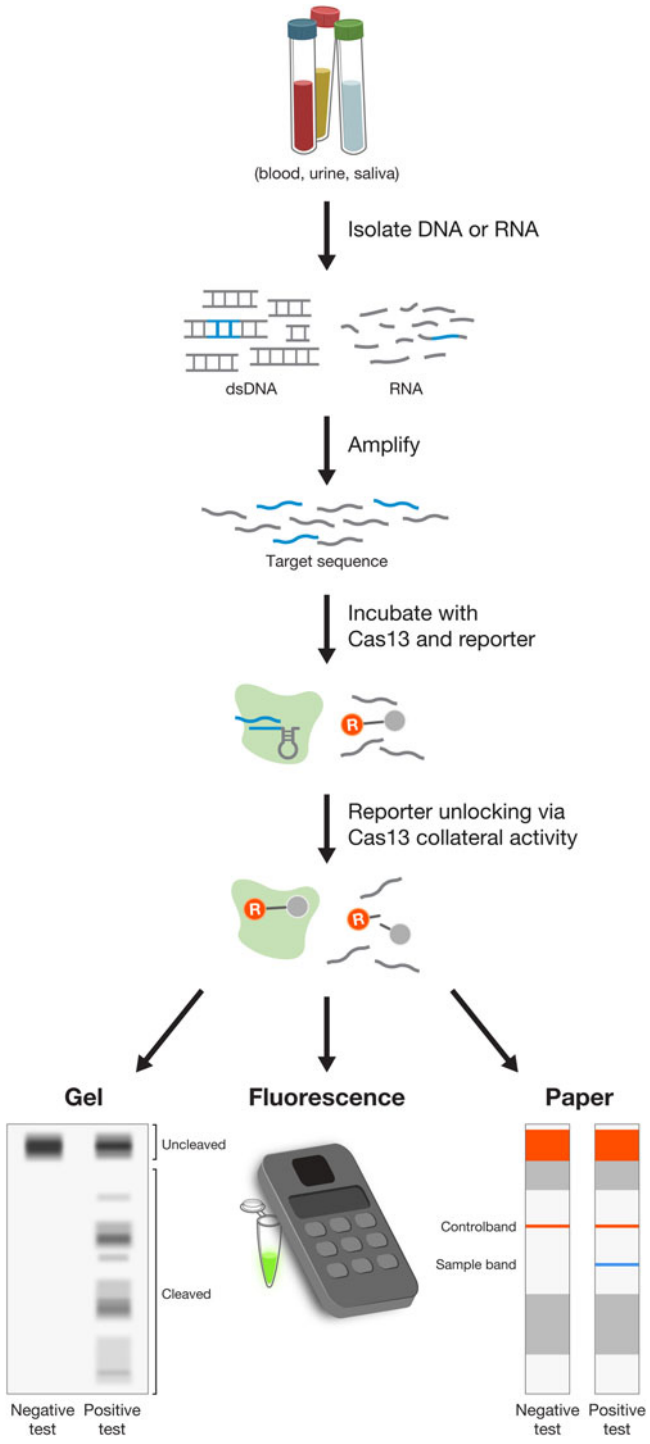


Fig. 7. Disease diagnostics using CRISPR-based detection of nucleic acids. The SHERLOCK (specific high sensitivity enzymatic reporter UnLOCKing) platform combines isothermal amplification with Cas13-mediated collateral cleavage of a reporter molecule to detect either DNA or RNA from blood, urine, or saliva. Depending on the design of the reporter molecule, SHERLOCK can be readout either through gel electrophoresis, fluorescence, or on a lateral flow strip, enabling instrument free-detection.

multiplex detection of multiple pathogens within the same test (Gootenberg *et al.*, 2018). To further increase the multiplexing scale, it has been found that distinct Cas13 family members have unique natural cleavage sequence preferences, enabling multiplex target detection in separate fluorescent color channels (Gootenberg *et al.*, 2018). Already, CRISPR-based tests are being optimized rapidly for a wide range of diagnostics applications outside the lab (Chen *et al.*, 2018; Myhrvold *et al.*, 2018) and have the potential to deliver affordable, sensitive, and rapid detection tools to the most needed areas of the world. Aside from Cas12 and Cas13, Cas9 has also been used to enrich or deplete specific sequences (Gu *et al.*, 2016) or to construct synthetic modules for integration with existing diagnostics methodologies (Pardee *et al.*, 2016). Recently, a CRISPR-CHIP electronic diagnostic platform was developed which uses dCas9 for rapid, sensitive detection of targeted DNA sequences (Hajian *et al.*, 2019).

CRISPR-based therapeutic treatment strategies

CRISPR-based therapies cover a wide range of different treatment strategies, each with unique considerations. The discussion here is limited to applications in somatic cells in the body, whose DNA will not be passed on during reproduction. Applications in germline cells and embryos, whose DNA will be passed to future generations, have considerable ethical ramifications, and a community of scientists has recently called for a moratorium on germline editing for the purposes of implantation (see below) (Lander *et al.*, 2019). DNA targeting approaches, especially those that directly change the genome of targeted cells, provide the potential for one-time treatments with curative results. RNA targeting approaches, by contrast, do not permanently change the genome, and provide the potential for transient and reversible treatments. Together, DNA and RNA targeting approaches comprise a versatile toolbox for the development of a new generation of therapeutic options for improving human health.

Strategies for applying CRISPR-based technologies to treat diseases can be classified into three categories: First, treatment of monogenic diseases, such as hemophilia, sickle cell disease, and Duchenne muscular dystrophy, by rescuing a known pathogenic mutation. Second, treatment of common diseases by introducing beneficial natural genetic variants that have been identified in the human population and are thought to provide protective effects. Examples of this category include treatment of cardiovascular disease by mimicking the effect of a natural loss-of-function mutation in the gene *PCSK9*, which has been linked to low levels of cholesterol (Ran *et al.*, 2015), or mimicking the effect of a natural loss-of-function mutation in the gene *CCR5*, which has been found to confer protection against HIV in those individuals who are naturally CCR5-null (Mandal *et al.*, 2014). Third, treatment of diseases by introducing novel changes to cell types that can be harnessed to achieve a therapeutic benefit, a particularly rich strategy in the immune system. Examples of this category include engineering of immune cells to increase their tumor-killing efficiency by knocking out the immune check-point inhibitor PD1 or by knocking in a chimeric antigen receptor (Eyquem *et al.*, 2017).

For each of these three categories, CRISPR-based technologies can be deployed in several ways, at either the level of DNA or RNA. Gene inactivation, either through disruption of the open-reading frame through the generation of indels or through repression at the genetic, epigenetic or transcriptomic level, is the most straightforward application and may be useful particularly in the case of pathogenic gain-of-function mutations. The strategic placement of indels can also effect exon skipping to eliminate mutated segments of a protein. For example, Duchenne's muscular dystrophy, which can be caused by a number of mutations,

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including deletions and frame-shift mutations, can be treated with NHEJ-based exon skipping strategies, leading to restoration of the reading frame and a functional gene product (Long *et al.*, 2016; Nelson *et al.*, 2016; Tabebordbar *et al.*, 2016). In other situations, gene upregulation using dCas-activation platforms can achieve a therapeutic effect. This approach is appealing for X-linked dominant diseases, such as Fragile X, where upregulating the silent wild-type copy of the gene could ameliorate the phenotype; proof-of-concept experiments have shown that a dCas9-Tet fusion can erase methylation at the inactivated *FMR1* locus, leading to reactivation (Liu *et al.*, 2018b).

One particularly promising approach to treat a range of pathologies is base editing at either the DNA or RNA level. Because base editing does not introduce DSBs, it offers a safer, less restricted route to correcting pathogenic mutations, the majority of which are single-nucleotide changes that either disrupt regulatory regions or result in truncated or abnormal protein variants. Several proof-of-concept studies have been performed in human cells and in mice demonstrating that base editing can lead to measurable expression levels of the corrected transcript (Rees and Liu, 2018). The ability to edit RNA opens additional possibilities for treatment, including transient alterations to the transcriptome. Such transient RNA editing would allow reversible alteration of protein function, such as editing *β*-catenin to temporarily alter cellular signaling pathways to drive cellular regeneration (Bastakoty and Young, 2016).

Although the most technically challenging, HR-mediated approaches to achieve targeted insertion of the desired-DNA sequence have the potential to correct the broadest swath of pathogenic mutations. Currently, this approach is somewhat limited, although techniques for increasing the efficiency of gene insertion either through homologous recombination or independent pathways are continuing to be developed (Maruyama *et al.*, 2015; Paquet *et al.*, 2016; Richardson *et al.*, 2016, 2018; Schmid-Burgk *et al.*, 2016; Suzuki *et al.*, 2016; Kan *et al.*, 2017; Canny *et al.*, 2018). Gene insertion strategies may be most usefully deployed in disorders where a small increase in the corrected genotype is likely to have an outsized phenotypic impact, as may be the case when edited cells have a selection or fitness advantage over non-edited cells.

Any of the above CRISPR-based therapeutic strategies can be achieved either *ex vivo* or *in vivo* (Fig. 8). *Ex vivo* approaches offer substantial advantages in terms of safety and efficiency of editing but are limited to certain cell types that can be manipulated in the lab and subsequently engrafted, such as T cells (Roth *et al.*, 2018), hematopoietic stem cells (Dever *et al.*, 2016), and intestinal stem cell-derived organoids (Schwank *et al.*, 2013). By contrast, *in vivo* approaches may be applicable to a wider range of tissues, but the potential for off-targets, particularly if editing at the DNA level, is a safety concern. Currently, *in vivo* delivery modes for gene therapies typically rely on AAV vectors, which have been approved by the U.S. Food and Drug Administration (FDA). Although promising, AAV vectors have relatively limited cargo capacity, making it challenging to deliver SpCas9 and guide RNA effectively. Other Cas9 orthologs (SaCas9, CjCas9, and NmeCas9) are smaller than SpCas9, making them better suited for AAV delivery (Ran *et al.*, 2015; Kim *et al.*, 2017*a*; Ibraheim *et al.*, 2018). Recently, high levels of AAV integration into the genome have been reported, which may have long-term safety implications (Nelson *et al.*, 2019). Another challenge with AAV or any other type of *in vivo* delivery modality, such as lipid nanoparticles (Finn *et al.*, 2018), is achieving cell-type specificity to ensure that only the pathological tissues are targeted. *In vivo* applications must also take into account potential immunogenicity of the therapy, which may be particularly relevant for SpCas9 and SaCas9, as they are derived from pathogenic bacteria (Charlesworth *et al.*, 2019).

CRISPR in the clinic

Clinical trials to treat patients with CRISPR-based therapies are now underway to treat a handful of diseases. Currently, there are two sets of clinical trials entering phase 1 testing. The first set of trials are *ex vivo*, using SpCas9 to treat β-thalassemia and sickle cell disease (Vertex, 2018*a*, 2018*b*). The second trial is *in vivo*, using SaCas9 and delivery by AAV into the retina to treat Type 10 Leber congenital amaurosis (LCA10) (Allergan, 2019), which causes blindness. Pre-clinical studies have recently been published reporting the use of SaCas9 to correct a splice-site mutation causing LCA10, showing that in human retinal explants, humanized mice, and non-human primates, editing rates exceed the threshold of 10% thought to be clinically relevant for disease amelioration (Maeder *et al.*, 2019). A number of additional studies are developing Cas9, Cas12, and Cas13-based strategies to treat a wide array of diseases including genetic disorders and cancer, providing new hope for patients currently lacking treatment options.

Ethical considerations

In November 2018, it emerged that a scientist had reportedly used Cas9 to edit human embryos, creating at least two-genetically modified babies. This shocking news highlighted the far-reaching ethical challenges that CRISPR-based technologies present for society. Although a number of stakeholders, including ethicists, scientists, clinicians, and policy makers, have voiced concerns over the use of CRISPR-based technologies in germline genome editing (Baltimore *et al.*, 2015), the exact nature of whether clinical uses of germline editing should be permitted remains a contentious topic. To advance discussions around this topic, a group of specialists, myself included, from seven countries called for a 5-year moratorium for the use of clinical germline editing, arguing that given a combination of scientific, technical, medical, and moral considerations, society as a whole needs to wait and establish consensus before proceeding with any form of clinical germline editing (Lander *et al.*, 2019).

Indeed, this is an enormously complicated issue. Clinical germline editing applications can be divided into two types: genetic correction and genetic enhancement, as described above. With increasing knowledge about human genetic variation at the population level, it is reasonable to expect that the outcome of converting a rare disease-causing variant to a common variant that does not lead to disease will be predictable and beneficial. By contrast, genetic enhancement relies on information about rare variants in the human population, such as APOE-4 or CCR5 null, which are much less well understood. For example, although loss of CCR5 appears to prevent HIV infection, it increases susceptibility to West Nile virus, and the selective pressures on this allele are not well understood (Telenti, 2009). Introducing such changes to the genome will likely have unpredictable consequences. Even in the future, when significant advances in our understanding of biology and human genetic variation become sufficient to predict the outcome of genome editing for enhancement, whether society as a whole should adopt germline editing still needs to be vigorously debated. The moral quandaries are

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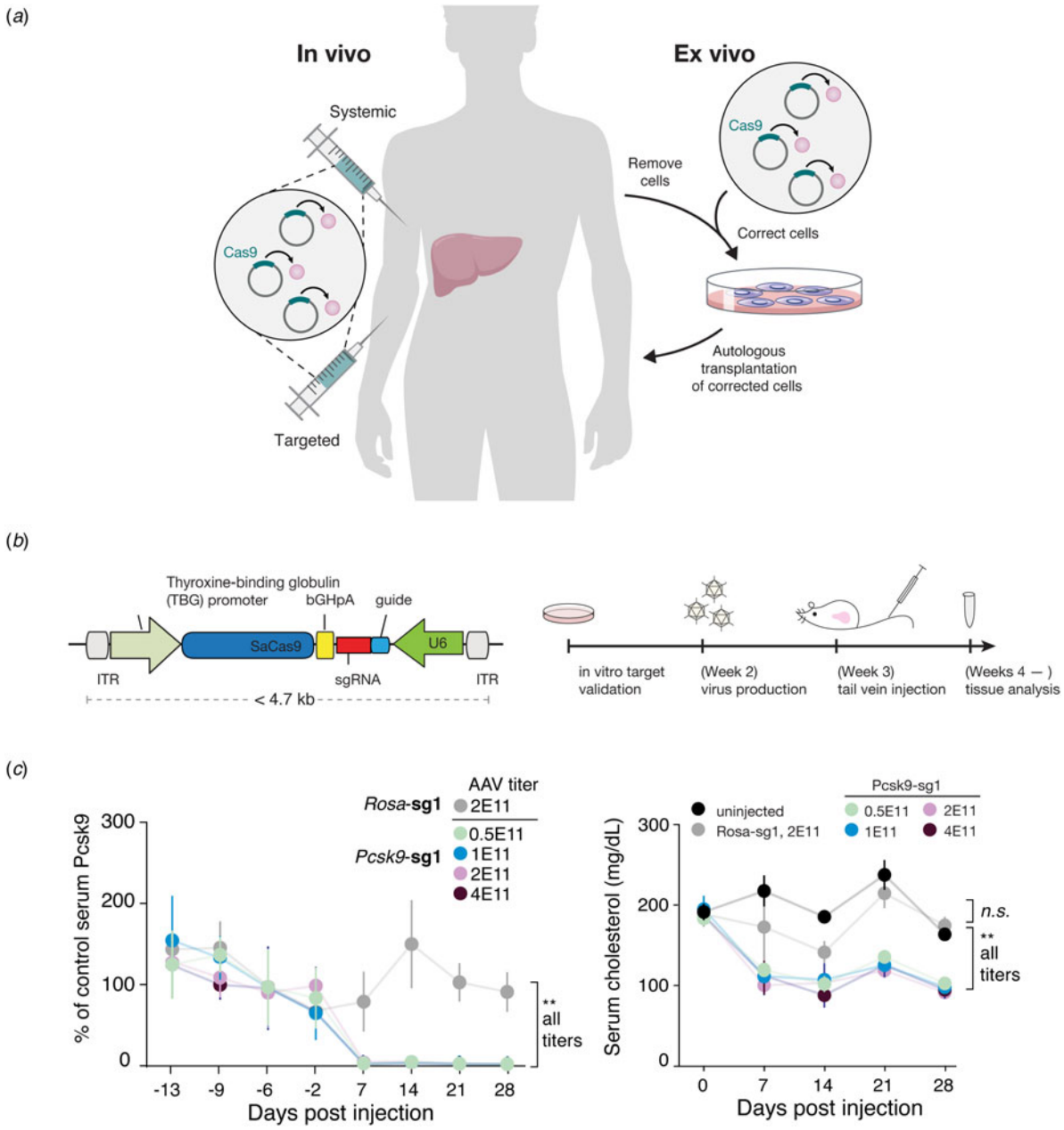


Fig. 8. CRISPR-based therapies can be used in multiple ways to treat genetic diseases. (a) CRISPR-based therapeutics can be administered either *in vivo* through targeted or systemic delivery or *ex vivo* in cells that have been removed from the body, manipulated in the lab, and then transplanted back into the patient. Image modified based on Cox *et al.*(2015). (b) For *in vivo* use of CRISPR therapeutics, a major challenge is efficient delivery. The most promising delivery vehicle for gene therapy is the AAV vector, but SpCas9 is too large to be packaged into AAV along with a guide RNA and regulatory sequences. We identified a smaller ortholog of Cas9, SaCas9, which we developed for *in vivo* use via AAV delivery. Shown on the left is a schematic of the AAV containing thyroxine-binding globulin promoter driving SaCas9 and the guide RNA driven by the U6 promoter, separated by the bGHpA terminator and flanked by inverted terminal repeat, which are required for packaging. Guides were used to target either *Pcsk9* or the Rosa26 locus as a control. Shown on the right is the experimental timeline. Images adapted from Ran *et al*. (2015). (c) One week after injecting mice with AAV carrying SaCas9 and a guide targeting *Pcsk9*, we observed complete loss of *Psck9* (left) and a concomitant reduction in serum cholesterol levels (right). SaCas9 is now being developed as the first *in vivo* genome editing medicine for humans. Images adapted from Ran *et al*. (2015).

numerous. For example, allowing genetic enhancement may further exacerbate social inequality and reduce the rich and treasured diversity of the human population.

Ethical considerations surrounding CRISPR-based technologies extend to other arenas as well. For example, Cas9-based tools have also accelerated the development of gene drives, elements in the genome that bias inheritance in their favor, resulting in non-Mendelian transmission and their rapid spread throughout a population. Gene drives could potentially be used to control the spread of certain diseases, such as malaria and Lyme disease, which are carried by insect vectors, or combat invasive species (Gantz *et al.*, 2015; Hammond *et al.*, 2015). Although gene drives have not yet been applied in the real world, and scientists are working on improved version of gene drives with better control and containment strategies (Akbari *et al.*, 2015), the potentially significant and irreversible environmental and

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ecological consequences of gene drives also demand careful consideration (Lunshof, 2015; Courtier-Orgogozo *et al.*, 2017).

Looking ahead

The accumulation of genomic sequences, which initially powered the discovery of CRISPR systems, has continued apace (Fig. 9). The availability of microbial sequences, driven in large part through developments in metagenomics, is particularly compelling. Until quite recently, our sampling of microbial genomics was severely taxonomically limited compared to the predicted diversity of organisms. Even today, roughly 50% of the ~200 000 available bacterial genomes encompass just 20 species, leaving out a vast swath of diversity. Concerted efforts, such as the Earth Microbiome Project (earthmicrobiome.org), are underway to systematically sample genomes across microbial taxa to gain a more comprehensive understanding of prokaryotic natural diversity.

It is clear that we have only begun to scratch the surface of the full microbial diversity. For example, the discovery of single-effector RNA targeting systems highlights the diversity within CRISPR-Cas systems, which themselves are only a sliver of the microbial defense systems that exist in nature. The continued search for CRISPR effectors as well as the large diversity of auxiliary proteins associated with CRISPR loci remain a rich source for exploration and development. Further revealing the sophistication of the arsenal used in microbial warfare, numerous anti-Cas9 and anti-Cas12 proteins (Pawluk *et al.*, 2016; Rauch *et al.*, 2017; Marino *et al.*, 2018; Watters *et al.*, 2018) as well as novel bacterial anti-phage defense systems (Doron *et al.*, 2018) have recently been discovered. These findings prefigure yet-to-be-discovered adaptive immune systems hidden within the immense diversity of the microbial world. Hints that this may be the case lie in the diversity of immune systems in animals – while most vertebrates have an antibody-based adaptive immune system, immunity in even the jawless vertebrates is based on different distinct antibody-like proteins called variable lymphocyte receptors (VLRs) (Han *et al.*, 2008). The existence of VLRs within a narrow branch of the animal kingdom suggests that perhaps within similarly narrow phyla of the microbial diversity there may be unique adaptive immune systems that operate through distinct mechanisms. Perhaps some bacteria have evolved protein-based adaptive immune systems that employ powerful diversification mechanisms to generate diverse proteins that provide critical survival functions for those microbes in their native environments. The technological potential of novel adaptive immune systems is also tantalizing – by virtue of being adaptive, these systems are naturally reprogrammable for the recognition of diverse substrates. Harnessing such reprogrammable systems could likely provide many new biotechnological platforms for the recognition of proteins, metabolites, or patterns of glycosylation.

Fully understanding and harnessing this natural diversity will require solving a number of open challenges, both computational and experimental. For example, further understanding of the molecular diversity of microbial species will require comprehensive genome sequencing and repeated sampling of diverse environments to capture the population dynamics in microbial communities. How do we deconvolute and assemble metagenomic data to obtain more refined genetic sequence and information about these organisms? How do we more accurately predict the function of novel protein sequences? Could protein structure help predict protein function? Perhaps advances in artificial intelligence may be applied to better infer the function of microbial proteins. Indeed, one of the key assumptions often used for the study of bacterial protein sequences is the idea of 'guilt by association', where genes located within the same neighborhood or operon are likely related to each other. This functional organization by neighborhood may perhaps suggest that there is a certain syntax or grammar to the organization of bacterial genomes, and approaches developed for natural language processing and deep learning may be borrowed to make advances here.

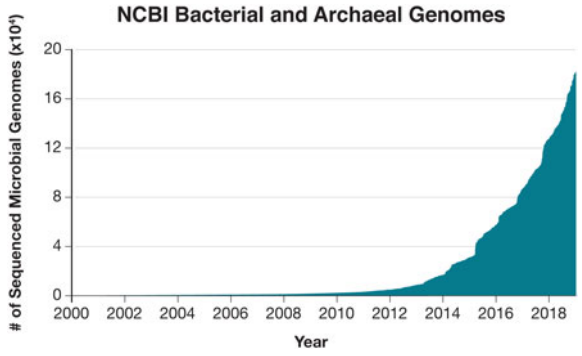


Fig. 9. The rapidly expanding database of microbial sequences holds many hidden treasures. Exploration of the natural bacterial and archaeal diversity will undoubtedly reveal new molecular systems that can provide the substrate for the development of new technologies. Data obtained from NCBI.

There are also experimental challenges that need to be further resolved. How can we study microbes at scale without cultivating them? How do we cultivate those microbes that we cannot currently culture so that we can study them comprehensively? How do we further accelerate some of the basic molecular biology techniques, like gene assembly, protein purification and structure determination? Solutions to some of these problems may arise through the work itself, much as CRISPR-based technologies are now being used to manipulate microbial genomes in the lab, while some may come through the intersection of molecular biology with other research fields, notably nanoscience and miniaturization and parallelization.

Indeed, it often seems that some of the biggest leaps forward in science are arrived at tangentially, and this should encourage all of us to probe the literature of unrelated fields for information that may be applicable in new situations and dare to move our own research programs not just forward, but also sideways. In February 2011, through a serendipitous encounter, I learned about CRISPR, and my imagination was captured by this fascinating and elegant mechanism of microbial adaptive immunity. This path has taken me from the development of CRISPR-Cas9 for genome editing to the exploration of the expansive world of CRISPR-Cas systems and microbial diversity. It has been particularly exciting to witness how this technology has flourished through the contributions of so many talented scientists who share the same spirit of openness and generosity.

This is a time of plenty for curious biologists – we have only glimpsed a tiny sliver of the diversity of life at the molecular level, and every few months, new genetic 'wonders' are reported: plankton with shattered chromosomes (Blanc-Mathieu *et al.*, 2017), lamprey genome rearrangements (Smith *et al.*, 2018), phage-encoded diversity generating retroelements (Doulatov *et al.*, 2004; Benler *et al.*, 2018), and marine microorganisms that use novel DNA repair systems (Deng *et al.*, 2018). Each natural system tempts us to explore new paths, determining the mechanism and function behind these systems, and opening new opportunities to tinker, ultimately leading to a healthier and more sustainable future.

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