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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
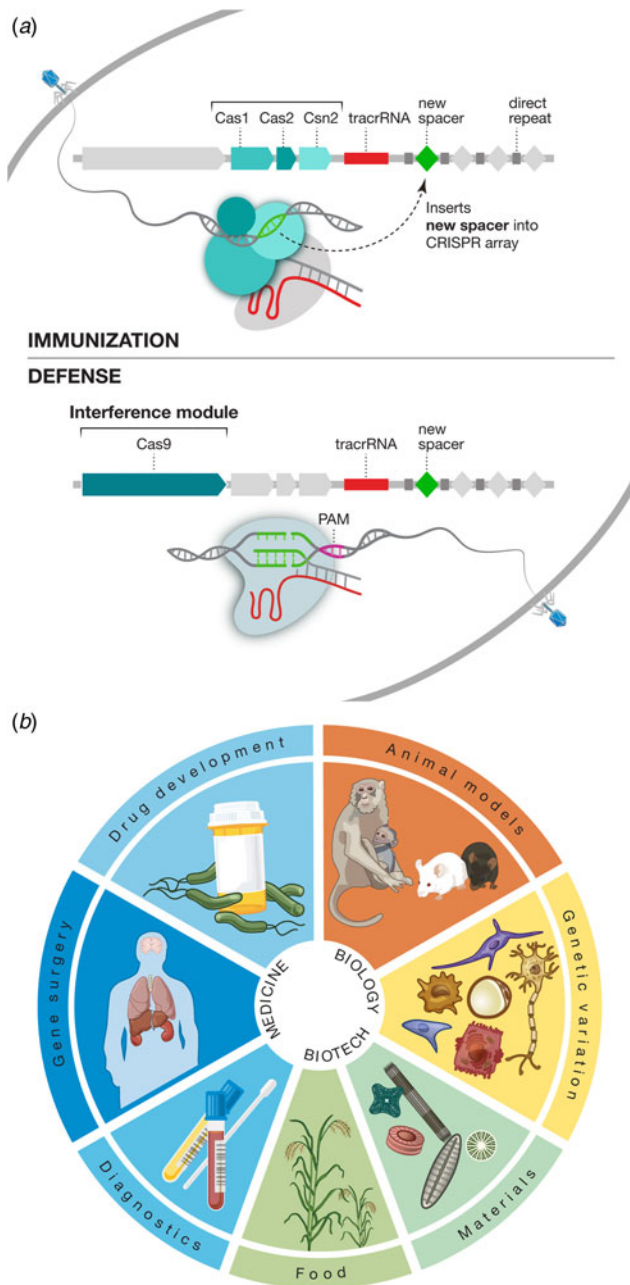
Fig. 1. Two aspects of CRISPR: biology and technology. (a) CRISPR-Cas adaptive immune systems help microbes defend against phages and other foreign genetic materials. During the immunization phase (top), an adaptation module inserts a new spacer, a stretch of DNA derived from the genome of the invader, 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).

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., 2013b) – 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.

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.

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

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.

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 first-hand 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
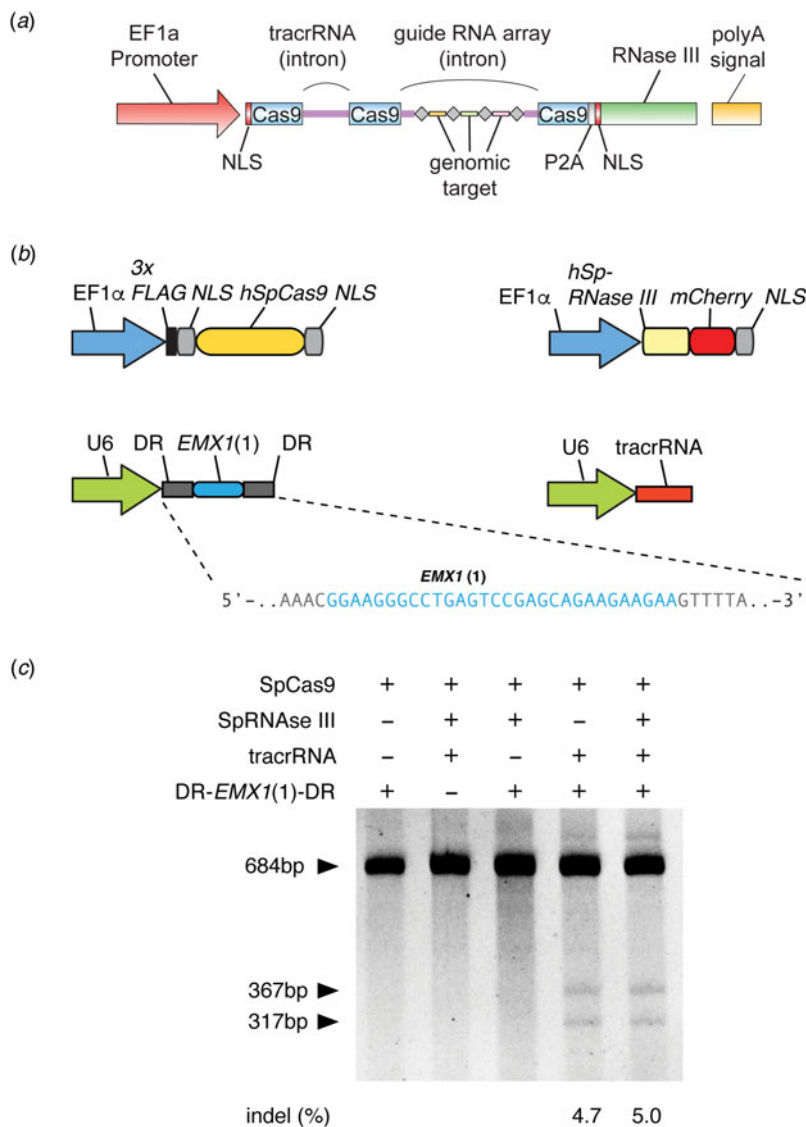
Fig. 3. Development of CRISPR-Cas9 for genome editing. (a) The design of a three-component system (Cas9, crRNA, and tracrRNA) for Cas9-mediated genome editing in eukaryotic cells, which was included in a grant submitted in January 2012 to the National Institutes of Health. In this design, the EF1a promoter 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

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., 2013b). 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

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.*, 2015a; 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.*, 2018b) 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, 2018a, 2018b).

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.*, 2015a). 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.*, 2015a). 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.*, 2015a). 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.*, 2016b; Kim *et al.*, 2017b), 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.*, 2018b).

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
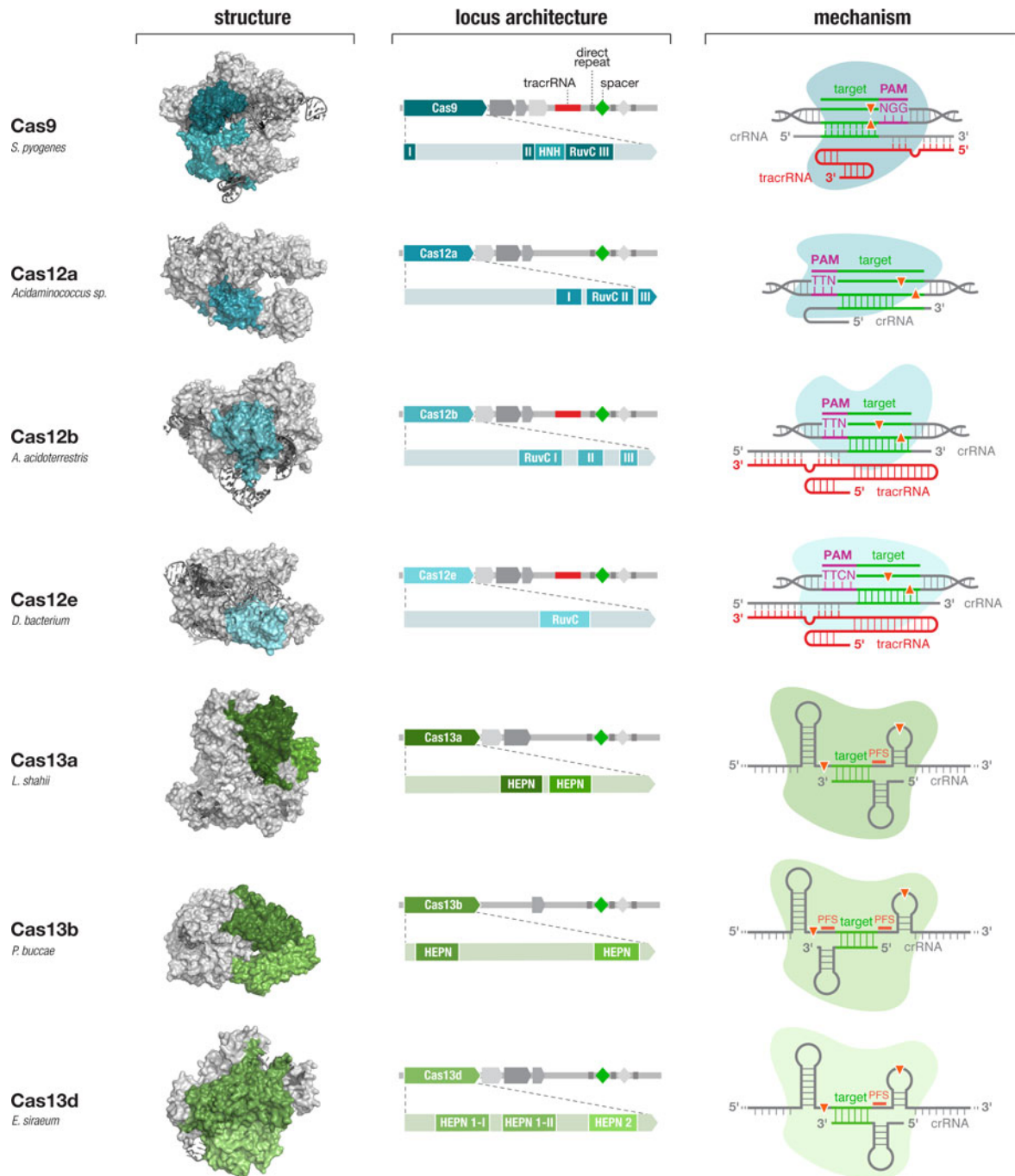
Fig. 4. Diverse class 2 CRISPR effectors have unique molecular features that contribute to an expansive toolbox for genome and transcriptome editing. To date, effectors from seven sub-types of CRISPR-Cas system have been developed for molecular technologies. These effectors differ in their locus architecture, 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' of Cas13 – recognition of the target RNA by

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.*, 2017a). 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.*, 2017a; 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.*, 2017a, 2017b; Zhang *et al.*, 2018a, 2018b; 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.*, 2016a).

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.*, 2015a; Abudayyeh *et al.*, 2017; Cox *et al.*, 2017; Kim *et al.*, 2017a; 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
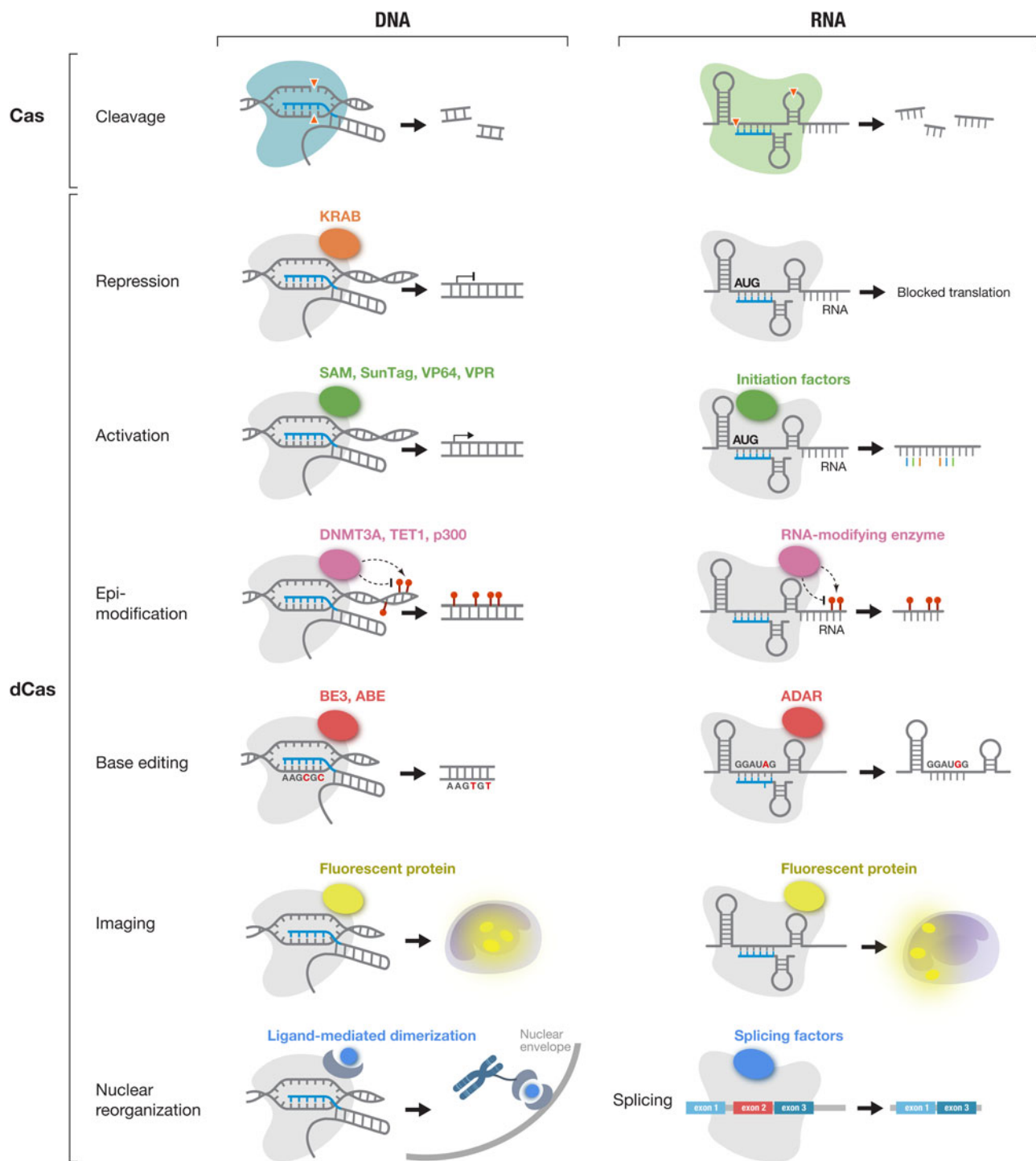
Fig. 5. The CRISPR-Cas toolbox enables a broad range of applications in eukaryotic cells. Applications of DNA-targeting Cas effectors (Cas9 and Cas12a-e) (left column) and applications of RNA-targeting Cas effectors (Cas13a-d) (right column) are shown. Active Cas effectors can be used for nucleic acid 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.

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

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.*, 2013a; 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.*, 2016a; 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, 2016b; Thakore *et al.*, 2015)). Similar to Cas9, the RuvC nuclease domain of Cas12 (Zetsche *et al.*, 2015a), 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.*, 2016a).

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.*, 2016a). 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

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.*, 2018c) 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.*, 2018d). 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

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.*, 2015b; 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,
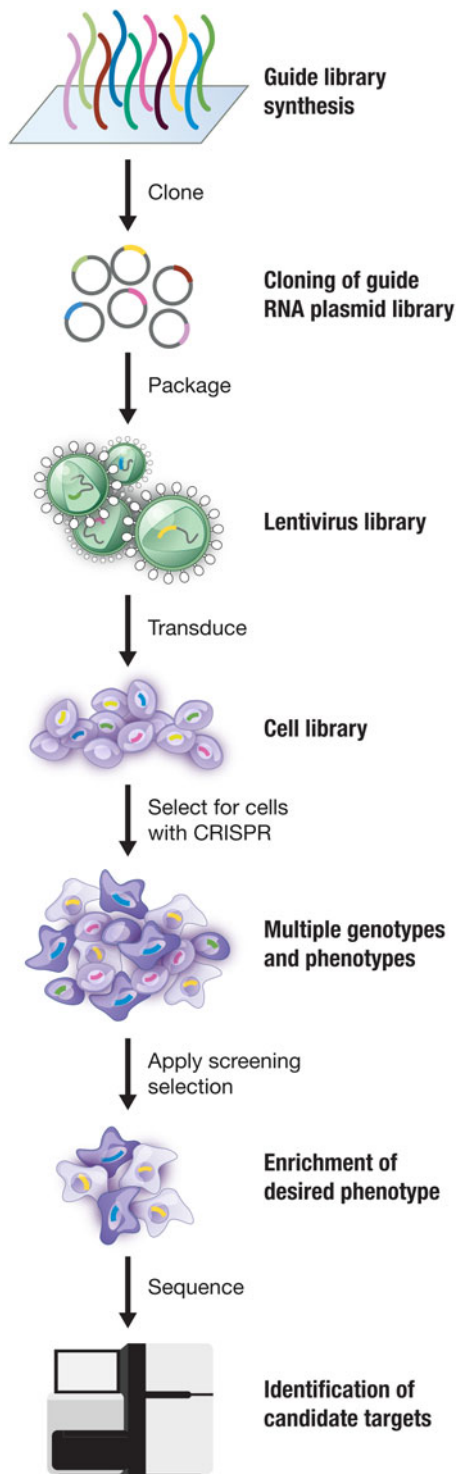
Fig. 6. CRISPR-mediated genome-wide screening allows large-scale interrogation of gene function. Cas9 can be combined with libraries of guide RNAs to facilitate gain- or loss-of-function genetic screening. First, guide RNAs designed to target coding or noncoding elements of the genome are synthesized 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

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.*, 2018a; 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 ($\sim$ 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.*, 2018b). 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.*, 2018b). Different types of Cas proteins with collateral activity can be combined to achieve
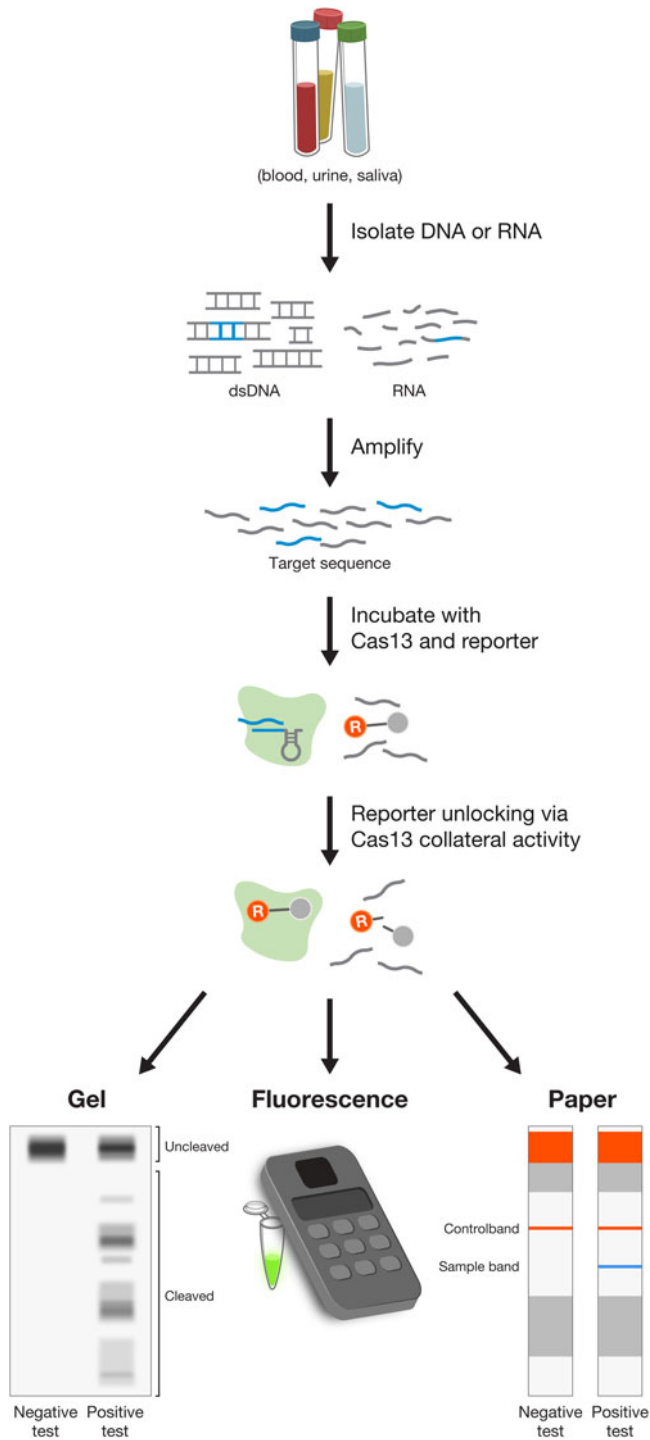
Fig. 7. Disease diagnostics using CRISPR-based detection of nucleic acids. The SHERLOCK (specific high sensitivity enzymatic reporter UnLOCKing) platform combines isothermal amplification with Cas13-mediated collateral cleavage of a reporter molecule to detect either DNA or RNA from blood, urine, or 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.

(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,

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

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.*, 2017a; 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, 2018a, 2018b). 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
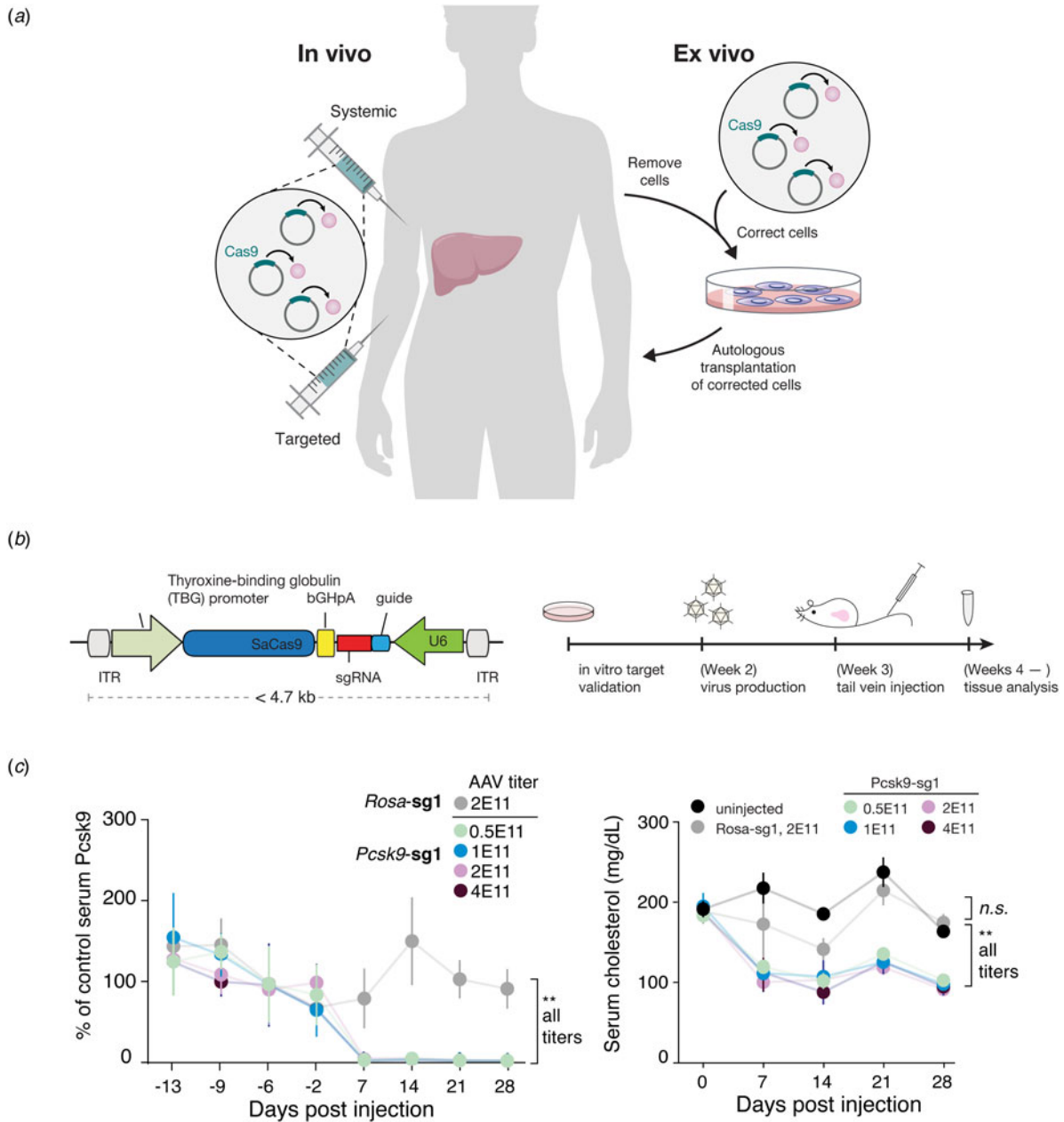
Fig. 8. CRISPR-based therapies can be used in multiple ways to treat genetic diseases. (a) CRISPR-based therapeutics can be administered either *in vivo* through targeted or systemic delivery or *ex vivo* in cells that have been removed from the body, manipulated in the lab, and then transplanted back 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

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
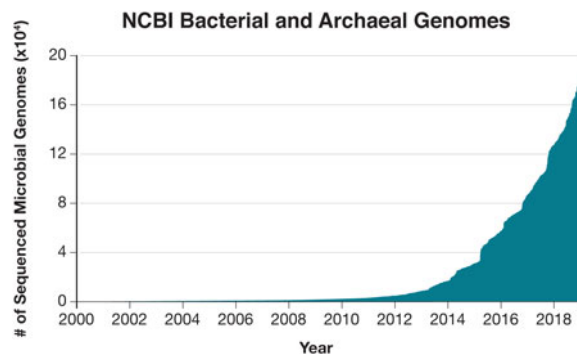

Fig. 9. The rapidly expanding database of microbial sequences holds many hidden treasures. Exploration of the natural bacterial and archaeal diversity will undoubtedly reveal new molecular systems that can provide the substrate for the development of new technologies. Data obtained from NCBI.

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.

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.

References

Abudayyeh OO, Gootenberg JS, Konermann S, Joung J, Slaymaker IM, Cox DBT, Shmakov S, Makarova KS, Semenova E, Minakhin L, Severinov K, Regev A, Lander ES, Koonin EV and Zhang F (2016) C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. *Science* **353**, aaf5573.

Abudayyeh OO, Gootenberg JS, Essletzbichler P, Han S, Joung J, Belanto JJ, Verdine V, Cox DBT, Kellner MJ, Regev A, Lander ES, Voytas DF, Ting AY and Zhang F (2017) RNA targeting with CRISPR–Cas13. *Nature* **550**, 280–284.

Adamson B, Norman TM, Jost M, Cho MY, Nuñez JK, Chen Y, Villalta JE, Gilbert LA, Horlbeck MA, Hein MY, Pak RA, Gray AN, Gross CA, Dixit A, Parnas O, Regev A and Weissman JS (2016) A multiplexed single-cell CRISPR screening platform enables systematic dissection of the unfolded protein response. *Cell* **167**, 1867–1882.

Adikusuma F, Piltz S, Corbett MA, Turvey M, Mccoll SR, Helbig KJ, Beard MR, Hughes J, Pomerantz RT and Thomas PQ (2018) Large deletions induced by Cas9 cleavage. *Nature* **560**, E8–E9.

Aguirre AJ, Meyers RM, Weir BA, Vazquez F, Zhang CZ, Ben-David U, Cook A, Ha G, Harrington WF, Doshi MB, Kost-Alimova M, Gill S, Xu H, Ali LD, Jiang G, Pantel S, Lee Y, Goodale A, Cherniack AD, Oh C, Kryukov G, Cowley GS, Garraway LA, Stegmaier K, Roberts CW, Golub TR, Meyerson M, Root DE, Tsherniak A and Hahn WC (2016) Genomic copy number dictates a gene-independent cell response to CRISPR/Cas9 targeting. *Cancer Discovery* **6**, 914–929.

Akbari OS, Bellen HJ, Bier E, Bullock SL, Burt A, Church GM, Cook KR, Duchek P, Edwards OR, Esvelt KM, Gantz VM, Golic KG, Gratz SJ, Harrison MM, Hayes KR, James AA, Kaufman TC, Knoblich J, Malik HS, Matthews KA, O'connor-Giles KM, Parks AL, Perrimon N, Port F, Russell S, Ueda R and Wildonger J (2015) BIOSAFETY. Safeguarding gene drive experiments in the laboratory. *Science* **349**, 927–929.

Akcakaya P, Bobbin ML, Guo JA, Malagon-Lopez J, Clement K, Garcia SP, Fellows MD, Porritt MJ, Firth MA, Carreras A, Baccega T, Seeliger F, Bjursell M, Tsai SQ, Nguyen NT, Nitsch R, Mayr LM, Pinello L, Bohlooly YM, Aryee MJ, Maresca M and Joung JK (2018) *In vivo* CRISPR editing with no detectable genome-wide off-target mutations. *Nature* **561**, 416–419.

ALLERGAN (2019) Single Ascending Dose Study in Participants With LCA10. ClinicalTrial.gov Identifier: NCT03872479 (clinicaltrials.gov/ct2/show/NCT03872479).

Aman R, Ali Z, Butt H, Mahas A, Aljedaani F, Khan MZ, Ding S and Mahfouz M (2018) RNA virus interference via CRISPR/Cas13a system in plants. *Genome Biology* **19**, 1.

Anders C, Niewoehner O, Duerst A and Jinek M (2014) Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. *Nature* **513**, 569–573.

Bae S, Park J and Kim JS (2014) Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. *Bioinformatics* **30**, 1473–1475.

Baltimore D, Berg P, Botchan M, Carroll D, Charo RA, Church G, Corn JE, Daley GQ, Doudna JA, Fenner M, Greely HT, Jinek M, Martin GS,

Penhoet E, Puck J, Sternberg SH, Weissman JS and Yamamoto KR (2015) Biotechnology. A prudent path forward for genomic engineering and germline gene modification. *Science* **348**, 36–38.

Barrangou R and Horvath P (2017) A decade of discovery: CRISPR functions and applications. *Nature Microbiology* **2**, 17092.

Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA and Horvath P (2007) CRISPR provides acquired resistance against viruses in prokaryotes. *Science* **315**, 1709–1712.

Bastakoty D and Young PP (2016) Wnt/beta-catenin pathway in tissue injury: roles in pathology and therapeutic opportunities for regeneration. *FASEB Journal* **30**, 3271–3284.

Benler S, Cobian-Guemes AG, Mcnair K, Hung SH, Levi K, Edwards R and Rohwer F (2018) A diversity-generating retroelement encoded by a globally ubiquitous Bacteroides phage. *Microbiome* **6**, 191.

Bewg WP, Ci D and Tsai CJ (2018) Genome editing in trees: from multiple repair pathways to long-term stability. *Frontiers in Plant Science* **9**, 1732.

Bikard D, Jiang W, Samai P, Hochschild A, Zhang F and Marraffini LA (2013) Programmable repression and activation of bacterial gene expression using an engineered CRISPR-Cas system. *Nucleic Acids Research* **41**, 7429–7437.

Bikard D, Euler CW, Jiang W, Nussenzweig PM, Goldberg GW, Duportet X, Fischetti VA and Marraffini LA (2014) Exploiting CRISPR-Cas nucleases to produce sequence-specific antimicrobials. *Nature Biotechnology* **32**, 1146–1150.

Birling MC, Herault Y and Pavlovic G (2017) Modeling human disease in rodents by CRISPR/Cas9 genome editing. *Mamm Genome* **28**, 291–301.

Blanc-Mathieu R, Krasovec M, Hebrard M, Yau S, Desgranges E, Martin J, Schackwitz W, Kuo A, Salin G, Donnadieu C, Desdevises Y, Sanchez-Ferandin S, Moreau H, Rivals E, Grigoriev IV, Grimsley N, Eyre-Walker A and Piganeau G (2017) Population genomics of picophytoplankton unveils novel chromosome hypervariability. *Science Advances* **3**, e1700239.

Boch J, Scholze H, Schornack S, Landgraf A, Hahn S, Kay S, Lahaye T, Nickstadt A and Bonas U (2009) Breaking the code of DNA binding specificity of TAL-type III effectors. *Science* **326**, 1509–1512.

Bolotin A, Quinquis B, Sorokin A and Ehrlich SD (2005) Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. *Microbiology (Reading England)* **151**(Pt 8), 2551–2561.

Boyden ES, Zhang F, Bamberg E, Nagel G and Deisseroth K (2005) Millisecond-timescale, genetically targeted optical control of neural activity. *Nature Neuroscience* **8**, 1263–1268.

Brooks C, Nekrasov V, Lippman ZB and Van Eck J (2014) Efficient gene editing in tomato in the first generation using the clustered regularly interspaced short palindromic repeats/CRISPR-associated9 system. *Plant Physiology* **166**, 1292–1297.

Brouns SJ, Jore MM, Lundgren M, Westra ER, Slijkhuis RJ, Snijders AP, Dickman MJ, Makarova KS, Koonin EV and Van Der Oost J (2008) Small CRISPR RNAs guide antiviral defense in prokaryotes. *Science* **321**, 960–964.

Burstein D, Harrington LB, Strutt SC, Probst AJ, Anantharaman K, Thomas BC, Doudna JA and Banfield JF (2017) New CRISPR-Cas systems from uncultivated microbes. *Nature* **542**, 237–241.

Cameron P, Fuller CK, Donohoue PD, Jones BN, Thompson MS, Carter MM, Gradia S, Vidal B, Garner E, Slorach EM, Lau E, Banh LM, Lied AM, Edwards LS, Settle AH, Capurso D, Llaca V, Deschamps S, Cigan M, Young JK and May AP (2017) Mapping the genomic landscape of CRISPR-Cas9 cleavage. *Nature Methods* **14**, 600–606.

Canny MD, Moatti N, Wan LCK, Fradet-Turcotte A, Krasner D, Mateos-Gomez PA, Zimmermann M, Orthwein A, Juang YC, Zhang W, Noordermeer SM, Seclen E, Wilson MD, Vorobyov A, Munro M, Ernst A, Ng TF, Cho T, Cannon PM, Sidhu SS, Sicheri F and Durocher D (2018) Inhibition of 53BP1 favors homology-dependent DNA repair and increases CRISPR-Cas9 genome-editing efficiency. *Nature Biotechnology* **36**, 95–102.

Canver MC, Smith EC, Sher F, Pinello L, Sanjana NE, Shalem O, Chen DD, Schupp PG, Vinjamur DS, Garcia SP, Luc S, Kurita R, Nakamura Y, Fujiwara Y, Maeda T, Yuan G-C, Zhang F, Orkin SH and Bauer DE

(2015) BCL11A enhancer dissection by Cas9-mediated *in situ* saturating mutagenesis. *Nature* **527**, 192–197.

Carroll D (2012) A CRISPR approach to gene targeting. *Molecular Therapy* **20**, 1658–1660.

Casini A, Olivieri M, Petris G, Montagna C, Reginato G, Maule G, Lorenzin F, Prandi D, Romanel A, Demichelis F, Inga A and Cereseto A (2018) A highly specific SpCas9 variant is identified by *in vivo* screening in yeast. *Nature Biotechnology* **36**, 265–271.

Charlesworth CT, Deshpande PS, Dever DP, Camarena J, Lemgart VT, Cromer MK, Vakulskas CA, Collingwood MA, Zhang L, Bode NM, Behlke MA, Dejene B, Cieniewicz B, Romano R, Lesch BJ, Gomez-Ospina N, Mantri S, Pavel-Dinu M, Weinberg KI and Porteus MH (2019) Identification of preexisting adaptive immunity to Cas9 proteins in humans. *Nature Medicine* **25**, 249–254.

Chatterjee P, Jakimo N and Jacobson JM (2018) Minimal PAM specificity of a highly similar SpCas9 ortholog. *Science Advances* **4**, eaau0766.

Chen B, Gilbert LA, Cimini BA, Schnitzbauer J, Zhang W, Li GW, Park J, Blackburn EH, Weissman JS, Qi LS and Huang B (2013) Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. *Cell* **155**, 1479–1491.

Chen S, Sanjana NE, Zheng K, Shalem O, Lee K, Shi X, Scott DA, Song J, Pan JQ, Weissleder R, Lee H, Zhang F and Sharp PA (2015a) Genome-wide CRISPR screen in a mouse model of tumor growth and metastasis. *Cell* **160**, 1246–1260.

Chen Y, Zheng Y, Kang Y, Yang W, Niu Y, Guo X, Tu Z, Si C, Wang H, Xing R, Pu X, Yang SH, Li S, Ji W and Li XJ (2015b) Functional disruption of the dystrophin gene in rhesus monkey using CRISPR/Cas9. *Human Molecular Genetics* **24**, 3764–3774.

Chen B, Hu J, Almeida R, Liu H, Balakrishnan S, Covill-Cooke C, Lim WA and Huang B (2016) Expanding the CRISPR imaging toolset with Staphylococcus aureus Cas9 for simultaneous imaging of multiple genomic loci. *Nucleic Acids Research* **44**, e75–e75.

Chen JS, Dagdas YS, Kleinstiver BP, Welch MM, Sousa AA, Harrington LB, Sternberg SH, Joung JK, Yildiz A and Doudna JA (2017) Enhanced proof-reading governs CRISPR–Cas9 targeting accuracy. *Nature* **550**, 407–410.

Chen JS, Ma E, Harrington LB, Da Costa M, Tian X, Palefsky JM and Doudna JA (2018) CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity. *Science* **360**, 436–439.

Cho SW, Kim S, Kim JM and Kim JS (2013) Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. *Nature Biotechnology* **31**, 230–232.

Cho SW, Kim S, Kim Y, Kweon J, Kim HS, Bae S and Kim JS (2014) Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. *Genome Research* **24**, 132–141.

Chylinski K, Makarova KS, Charpentier E and Koonin EV (2014) Classification and evolution of type II CRISPR-Cas systems. *Nucleic Acids Research* **42**, 6091–6105.

Citorik RJ, Mimee M and Lu TK (2014) Sequence-specific antimicrobials using efficiently delivered RNA-guided nucleases. *Nature Biotechnology* **32**, 1141–1145.

Concordet JP and Haeussler M (2018) CRISPOR: intuitive guide selection for CRISPR/Cas9 genome editing experiments and screens. *Nucleic Acids Research* **46**, W242–W245.

Cong L, Ran FA, Cox D, Lin SL, Barretto R, Habib N, Hsu PD, Wu XB, Jiang WY, Marraffini LA and Zhang F (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819–823.

Courtier-Orgogozo V, Morizot B and Boete C (2017) Agricultural pest control with CRISPR-based gene drive: time for public debate: should we use gene drive for pest control? *EMBO Reports* **18**, 878–880.

Cox DB, Platt RJ and Zhang F (2015) Therapeutic genome editing: prospects and challenges. *Nature Medicine* **21**, 121–131.

Cox DBT, Gootenberg JS, Abudayyeh OO, Franklin B, Kellner MJ, Joung J and Zhang F (2017) RNA editing with CRISPR-Cas13. *Science* **550**, eaaq0180.

Cromwell CR, Sung K, Park J, Krysler AR, Jovel J, Kim SK and Hubbard BP (2018) Incorporation of bridged nucleic acids into CRISPR RNAs improves Cas9 endonuclease specificity. *Nature Communications* **9**, 1448.

Crosetto N, Mitra A, Silva MJ, Bienko M, Dojer N, Wang Q, Karaca E, Chiarle R, Skrzypczak M, Ginalski K, Pasero P, Rowicka M and Dikic I (2013) Nucleotide-resolution DNA double-strand break mapping by next-generation sequencing. *Nature Methods* **10**, 361–366.

Dahlman JE, Abudayyeh OO, Joung J, Gootenberg JS, Zhang F and Konermann S (2015) Orthogonal gene knockout and activation with a catalytically active Cas9 nuclease. *Nature Biotechnology* **33**, 1159–1161.

Davis KM, Pattanayak V, Thompson DB, Zuris JA and Liu DR (2015) Small molecule–triggered Cas9 protein with improved genome-editing specificity. *Nature Chemical Biology* **11**, 316–318.

Deltcheva E, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, Eckert MR, Vogel J and Charpentier E (2011) CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature* **471**, 602–607.

Deng W, Henriet S and Chourrout D (2018) Prevalence of mutation-prone microhomology-mediated end joining in a chordate lacking the c-NHEJ DNA repair pathway. *Current Biology* **28**, 3337–3341 e3334.

Deveau H, Barrangou R, Garneau JE, Labonte J, Fremaux C, Boyaval P, Romero DA, Horvath P and Moineau S (2008) Phage response to CRISPR-encoded resistance in Streptococcus thermophilus. *Journal of Bacteriology* **190**, 1390–1400.

Dever DP, Bak RO, Reinisch A, Camarena J, Washington G, Nicolas CE, Pavel-Dinu M, Saxena N, Wilkens AB, Mantri S, Uchida N, Hendel A, Narla A, Majeti R, Weinberg KI and Porteus MH (2016) CRISPR/cas9 beta-globin gene targeting in human haematopoietic stem cells. *Nature* **539**, 384–389.

Dicarlo JE, Norville JE, Mali P, Rios X, Aach J and Church GM (2013) Genome engineering in Saccharomyces cerevisiae using CRISPR-Cas systems. *Nucleic Acids Research* **41**, gkt135–gk4343.

Dixit A, Parnas O, Li B, Chen J, Fulco CP, Jerby-Arnon L, Marjanovic ND, Dionne D, Burks T, Raychowdhury R, Adamson B, Norman TM, Lander ES, Weissman JS, Friedman N and Regev A (2016) Perturb-Seq: dissecting molecular circuits with scalable single-cell RNA profiling of pooled genetic screens. *Cell* **167**, 1853–1866.

Doench JG (2017) Am I ready for CRISPR? A user's guide to genetic screens. *Nature Publishing Group* **19**, 67–80.

Doench JG, Hartenian E, Graham DB, Tothova Z, Hegde M, Smith I, Sullender M, Ebert BL, Xavier RJ and Root DE (2014) Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation. *Nature Biotechnology* **32**, 1262–1267.

Doench JG, Fusi N, Sullender M, Hegde M, Vaimberg EW, Donovan KF, Smith I, Tothova Z, Wilen C, Orchard R, Virgin HW, Listgarten J and Root DE (2016) Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. *Nature Biotechnology* **34**, 184–191.

Doetschman T, Gregg RG, Maeda N, Hooper ML, Melton DW, Thompson S and Smithies O (1987) Targetted correction of a mutant HPRT gene in mouse embryonic stem cells. *Nature* **330**, 576–578.

Dong D, Ren K, Qiu X, Zheng J, Guo M, Guan X, Liu H, Li N, Zhang B, Yang D, Ma C, Wang S, Wu D, Ma Y, Fan S, Wang J, Gao N and Huang Z (2016) The crystal structure of Cpf1 in complex with CRISPR RNA. *Nature* **532**, 522–526.

Donovan KF, Hegde M, Sullender M, Vaimberg EW, Johannessen CM, Root DE and Doench JG (2017) Creation of novel protein variants with CRISPR/Cas9-mediated mutagenesis: turning a screening by-product into a discovery tool. *PLoS One* **12**, e0170445.

Doron S, Melamed S, Ofir G, Leavitt A, Lopatina A, Keren M, Amitai G and Sorek R (2018) Systematic discovery of antiphage defense systems in the microbial pangenome. *Science* **359**, eaar4120.

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

Doulatov S, Hodes A, Dai L, Mandhana N, Liu M, Deora R, Simons RW, Zimmerly S and Miller JF (2004) Tropism switching in Bordetella bacteriophage defines a family of diversity-generating retroelements. *Nature* **431**, 476–481.

Dow LE, Fisher J, O'rourke KP, Muley A, Kastenhuber ER, Livshits G, Tschaharganeh DF, Socci ND and Lowe SW (2015) Inducible *in vivo* genome editing with CRISPR-Cas9. *Nature Biotechnology* **33**, 390–394.

Duan J, Lu G, Hong Y, Hu Q, Mai X, Guo J, Si X, Wang F and Zhang Y (2018) Live imaging and tracking of genome regions in CRISPR/dCas9 knock-in mice. *Genome Biology* **19**, 192.

East-Seletsky A, O'connell MR, Knight SC, Burstein D, Cate JHD, Tjian R and Doudna JA (2016) Two distinct RNase activities of CRISPR-C2c2 enable guide-RNA processing and RNA detection. *Nature* **538**, 270–273.

Egli D, Zuccaro MV, Kosicki M, Church GM, Bradley A and Jasin M (2018) Inter-homologue repair in fertilized human eggs? *Nature* **560**, E5–E7.

Esvelt KM, Mali P, Braff JL, Moosburner M, Yaung SJ and Church GM (2013) Orthogonal Cas9 proteins for RNA-guided gene regulation and editing. *Nature* **10**, 1116–1121.

Eyquem J, Mansilla-Soto J, Giavridis T, Van Der Stegen SJ, Hamieh M, Cunanan KM, Odak A, Gonen M and Sadelain M (2017) Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. *Nature* **543**, 113–117.

Fellmann C, Gowen BG, Lin PC, Doudna JA and Corn JE (2017) Cornerstones of CRISPR-Cas in drug discovery and therapy. *Nature Reviews Drug Discovery* **16**, 89–100.

Feng Z, Zhang B, Ding W, Liu X, Yang DL, Wei P, Cao F, Zhu S, Zhang F, Mao Y and Zhu JK (2013) Efficient genome editing in plants using a CRISPR/Cas system. *Cell Research* **23**, 1229–1232.

Finn JD, Smith AR, Patel MC, Shaw L, Youniss MR, Van Heteren J, Dirstine T, Ciullo C, Lescarbeau R, Seitzer J, Shah RR, Shah A, Ling D, Growe J, Pink M, Rohde E, Wood KM, Salomon WE, Harrington WF, Dombrowski C, Strapps WR, Chang Y and Morrissey DV (2018) A single administration of CRISPR/Cas9 lipid nanoparticles achieves robust and persistentiIn vivo genome editing. *Cell Reports* **22**, 2227–2235.

Fogarty NME, Mccarthy A, Snijders KE, Powell BE, Kubikova N, Blakeley P, Lea R, Elder K, Wamaitha SE, Kim D, Maciulyte V, Kleinjung J, Kim JS, Wells D, Vallier L, Bertero A, Turner JMA and Niakan KK (2017) Genome editing reveals a role for OCT4 in human embryogenesis. *Nature* **550**, 67–73.

Fonfara I, Le Rhun A, Chylinski K, Makarova KS, Lécrivain A-L, Bzdrenga J, Koonin EV and Charpentier E (2014) Phylogeny of Cas9 determines functional exchangeability of dual-RNA and Cas9 among orthologous type II CRISPR-Cas systems. *Nucleic Acids Research* **42**, 2577–2590.

Fonfara I, Richter H, Bratovič M, Le Rhun A and Charpentier E (2016) The CRISPR-associated DNA-cleaving enzyme Cpf1 also processes precursor CRISPR RNA. *Nature* **532**, 517–521.

Frieda KL, Linton JM, Hormoz S, Choi J, Chow KK, Singer ZS, Budde MW, Elowitz MB and Cai L (2017) Synthetic recording and *in situ* readout of lineage information in single cells. *Nature* **541**, 107–111.

Friedland AE, Tzur YB, Esvelt KM, Colaiácovo MP, Church GM and Calarco JA (2013) Heritable genome editing in C. elegans via a CRISPR-Cas9 system. *Nature Methods* **10**, 741–743.

Frock RL, Hu J, Meyers RM, Ho YJ, Kii E and Alt FW (2015) Genome-wide detection of DNA double-stranded breaks induced by engineered nucleases. *Nature Biotechnology* **33**, 179–186.

Fulco CP, Munschauer M, Anyoha R, Munson G, Grossman SR, Perez EM, Kane M, Cleary B, Lander ES and Engreitz JM (2016) Systematic mapping of functional enhancer-promoter connections with CRISPR interference. *Science* **354**, 769–773.

Fu Y, Foden JA, Khayter C, Maeder ML, Reyon D, Joung JK and Sander JD (2013) High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nature Biotechnology* **31**, 822–826.

Fu Y, Sander JD, Reyon D, Cascio VM and Joung JK (2014) Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nature Biotechnology* **32**, 279–284.

Gantz VM, Jasinskiene N, Tatarenkova O, Fazekas A, Macias VM, Bier E and James AA (2015) Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito Anopheles stephensi. *Proceedings of the National Academy of Sciences* **112**, E6736–E6743.

Gao C (2018) The future of CRISPR technologies in agriculture. *Nature Reviews Molecular Cell Biology* **19**, 275–276.

Gao L, Cox DBT, Yan WX, Manteiga JC, Schneider MW, Yamano T, Nishimasu H, Nureki O, Crosetto N and Zhang F (2017) Engineered Cpf1 variants with altered PAM specificities. *Nature Biotechnology* **163**, 759.

Garcia-Doval C and Jinek M (2017) Molecular architectures and mechanisms of class 2 CRISPR-associated nucleases. *Current Opinion in Structural Biology* **47**, 157–166.

Garneau JE, Dupuis ME, Villion M, Romero DA, Barrangou R, Boyaval P, Fremaux C, Horvath P, Magadan AH and Moineau S (2010) The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature* **468**, 67–71.

Gasiunas G, Barrangou R, Horvath P and Siksnys V (2012) Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proceedings of the National Academy of Sciences of the United States of America* **109**, E2579–E2586.

Gasperini M, Hill AJ, Mcfaline-Figueroa JL, Martin B, Kim S, Zhang MD, Jackson D, Leith A, Schreiber J, Noble WS, Trapnell C, Ahituv N and Shendure J (2019) A genome-wide framework for mapping gene regulation via cellular genetic screens. *Cell* **176**, 377–390.

Gaudelli NM, Komor AC, Rees HA, Packer MS, Badran AH, Bryson DI and Liu DR (2017) Programmable base editing of A•T to G•C in genomic DNA without DNA cleavage. *Nature* **551**, 464–471.

Ge Y, Gomez NC, Adam RC, Nikolova M, Yang H, Verma A, Lu CP, Polak L, Yuan S, Elemento O and Fuchs E (2017) Stem cell lineage infidelity drives wound repair and cancer. *Cell* **169**, 636–650.

Ghorbal M, Gorman M, Macpherson CR, Martins RM, Scherf A and Lopez-Rubio J-J (2014) Genome editing in the human malaria parasite plasmodium falciparum using the CRISPR-Cas9 system. *Nature Biotechnology* **32**, 819–821.

Giannoukos G, Ciulla DM, Marco E, Abdulkerim HS, Barrera LA, Bothmer A, Dhanapal V, Gloskowski SW, Jayaram H, Maeder ML, Skor MN, Wang T, Myer VE and Wilson CJ (2018) UDitas, a genome editing detection method for indels and genome rearrangements. *BMC Genomics* **19**, 212.

Gilbert LA, Larson MH, Morsut L, Liu Z, Brar GA, Torres SE, Stern-Ginossar N, Brandman O, Whitehead EH, Doudna JA, Lim WA, Weissman JS and Qi LS (2013) CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* **154**, 442–451.

Gilbert LA, Horlbeck MA, Adamson B, Villalta JE, Chen Y, Whitehead EH, Guimaraes C, Panning B, Ploegh HL, Bassik MC, Qi LS, Kampmann M and Weissman JS (2014) Genome-Scale CRISPR-mediated control of gene repression and activation. *Cell* **159**, 647–661.

Gootenberg JS, Abudayyeh OO, Lee JW, Essletzbichler P, Dy AJ, Joung J, Verdine V, Donghia N, Daringer NM, Freije CA, Myhrvold C, Bhattacharyya RP, Livny J, Regev A, Koonin EV, Hung DT, Sabeti PC, Collins JJ and Zhang F (2017) Nucleic acid detection with CRISPR-Cas13a/C2c2. *Science* **356**, 438–442.

Gootenberg JS, Abudayyeh OO, Kellner MJ, Joung J, Collins JJ and Zhang F (2018) Multiplexed and portable nucleic acid detection platform with Cas13, Cas12a, and Csm6. *Science* **360**, 439–444.

Gratz SJ, Cummings AM, Nguyen JN, Hamm DC, Donohue LK, Harrison MM, Wildonger J and O'connor-Giles KM (2013) Genome engineering of drosophila with the CRISPR RNA-guided Cas9 nuclease. *Genetics* **194**, 1029–1035.

Grunewald J, Zhou R, Garcia SP, Iyer S, Lareau CA, Aryee MJ and Joung JK (2019) Transcriptome-wide off-target RNA editing induced by CRISPR-guided DNA base editors. *Nature* **569**, 433–437.

Grunwald HA, Gantz VM, Poplawski G, Xu XS, Bier E and Cooper KL (2019) Super-Mendelian inheritance mediated by CRISPR-Cas9 in the female mouse germline. *Nature* **566**, 105–109.

Guilinger JP, Thompson DB and Liu DR (2014) Fusion of catalytically inactive Cas9 to FokI nuclease improves the specificity of genome modification. *Nature Biotechnology* **32**, 577–582.

Guo X, Chitale P and Sanjana NE (2017) Target discovery for precision medicine using high-throughput genome engineering. *Advances in Experimental Medicine and Biology* **1016**, 123–145.

Gu W, Crawford ED, O'donovan BD, Wilson MR, Chow ED, Retallack H and Derisi JL (2016) Depletion of abundant sequences by hybridization (DASH): using Cas9 to remove unwanted high-abundance species in sequencing libraries and molecular counting applications. *Genome Biology* **17**, 41.

Hajian R, Balderston S, Tran T, Deboer T, Etienne J, Sandhu M, Wauford NA, Chung J-Y, Nokes J, Athaiya M, Paredes J, Peytavi R, Goldsmith B, Murthy N, Conboy IM and Aran K (2019) Detection of

unamplified target genes via CRISPR–Cas9 immobilized on a graphene field-effect transistor. *Nature Biomedical Engineering.* http://doi.org/10.1038/s41551-019-0371-x.

Hale CR, Zhao P, Olson S, Duff MO, Graveley BR, Wells L, Terns RM and Terns MP (2009) RNA-guided RNA cleavage by a CRISPR RNA-Cas protein complex. *Cell* **139**, 945–956.

Halperin SO, Tou CJ, Wong EB, Modavi C, Schaffer DV and Dueber JE (2018) CRISPR-guided DNA polymerases enable diversification of all nucleotides in a tunable window. *Nature* **560**, 248–252.

Hammond A, Galizi R, Kyrou K, Simoni A, Siniscalchi C, Katsanos D, Gribble M, Baker D, Marois E, Russell S, Burt A, Windbichler N, Crisanti A and Nolan T (2015) A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector Anopheles gambiae. *Nature Biotechnology* **34**, 78–83.

Han BW, Herrin BR, Cooper MD and Wilson IA (2008) Antigen recognition by variable lymphocyte receptors. *Science* **321**, 1834–1837.

Han K, Jeng EE, Hess GT, Morgens DW, Li A and Bassik MC (2017) Synergistic drug combinations for cancer identified in a CRISPR screen for pairwise genetic interactions. *Nature Biotechnology* **35**, 463–474.

Han R, Li L, Ugalde AP, Tal A, Manber Z, Barbera EP, Chiara VD, Elkon R and Agami R (2018) Functional CRISPR screen identifies AP1-associated enhancer regulating FOXF1 to modulate oncogene-induced senescence. *Genome Biology* **19**, 118.

Harrington LB, Burstein D, Chen JS, Paez-Espino D, Ma E, Witte IP, Cofsky JC, Kyrpides NC, Banfield JF and Doudna JA (2018) Programmed DNA destruction by miniature CRISPR-Cas14 enzymes. *Science* **362**, 839–842.

Hart T and Moffat J (2016) BAGEL: a computational framework for identifying essential genes from pooled library screens. *BMC Bioinformatics* **17**, 164.

Hart T, Chandrashekhar M, Aregger M, Steinhart Z, Brown KR, Macleod G, Mis M, Zimmermann M, Fradet-Turcotte A, Sun S, Mero P, Dirks P, Sidhu S, Roth FP, Rissland OS, Durocher D, Angers S and Moffat J (2015) High-resolution CRISPR screens reveal fitness genes and genotype-specific cancer liabilities. *Cell* **163**, 1515–1526.

Hart T, Tong AHY, Chan K, Van Leeuwen J, Seetharaman A, Aregger M, Chandrashekhar M, Hustedt N, Seth S, Noonan A, Habsid A, Sizova O, Nedyalkova L, Climie R, Tworzyanski L, Lawson K, Sartori MA, Alibeh S, Tieu D, Masud S, Mero P, Weiss A, Brown KR, Usaj M, Billmann M, Rahman M, Constanzo M, Myers CL, Andrews BJ, Boone C, Durocher D and Moffat J (2017) Evaluation and design of genome-wide CRISPR/SpCas9 knockout screens. *G3 (Bethesda)* **7**, 2719–2727.

Heigwer F, Zhan T, Breinig M, Winter J, Brugemann D, Leible S and Boutros M (2016) CRISPR library designer (CLD): software for multispecies design of single guide RNA libraries. *Genome Biology* **17**, 55.

Heler R, Samai P, Modell JW, Weiner C, Goldberg GW, Bikard D and Marraffini LA (2015) Cas9 specifies functional viral targets during CRISPR-Cas adaptation. *Nature* **519**, 199–202.

Hess GT, Fresard L, Han K, Lee CH, Li A, Cimprich KA, Montgomery SB and Bassik MC (2016) Directed evolution using dCas9-targeted somatic hypermutation in mammalian cells. *Nature Methods* **13**, 1036–1042.

Hess GT, Tycko J, Yao D and Bassik MC (2017) Methods and applications of CRISPR-mediated base editing in eukaryotic genomes. *Molecular Cell* **68**, 26–43.

Hilton IB, D'ippolito AM, Vockley CM, Thakore PI, Crawford GE, Reddy TE and Gersbach CA (2015) Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. *Nature Biotechnology* **33**, 510–517.

Hirano H, Gootenberg JS, Horii T, Abudayyeh OO, Kimura M, Hsu PD, Nakane T, Ishitani R, Hatada I, Zhang F, Nishimasu H and Nureki O (2016) Structure and engineering of Francisella novicida Cas9. *Cell* **164**, 950–961.

Horlbeck MA, Gilbert LA, Villalta JE, Adamson B, Pak RA, Chen Y, Fields AP, Park CY, Corn JE, Kampmann M and Weissman JS (2016) Compact and highly active next-generation libraries for CRISPR-mediated gene repression and activation. *Elife* **5**, e19760.

Horlbeck MA, Xu A, Wang M, Bennett NK, Park CY, Bogdanoff D, Adamson B, Chow ED, Kampmann M, Peterson TR, Nakamura K, Fischbach MA, Weissman JS and Gilbert LA (2018) Mapping the genetic landscape of human cells. *Cell* **174**, 953–967.

Horvath P, Romero DA, Coute-Monvoisin AC, Richards M, Deveau H, Moineau S, Boyaval P, Fremaux C and Barrangou R (2008) Diversity, activity, and evolution of CRISPR loci in Streptococcus thermophilus. *Journal of Bacteriology* **190**, 1401–1412.

Horvath P, Coute-Monvoisin AC, Romero DA, Boyaval P, Fremaux C and Barrangou R (2009) Comparative analysis of CRISPR loci in lactic acid bacteria genomes. *International Journal of Food Microbiology* **131**, 62–70.

Hotta A and Yamanaka S (2015) From genomics to gene therapy: induced pluripotent stem cells meet genome editing. *Annual Review of Genetics* **49**, 47–70.

Hou Z, Zhang Y, Propson NE, Howden SE, Chu LF, Sontheimer EJ and Thomson JA (2013) Efficient genome engineering in human pluripotent stem cells using Cas9 from Neisseria meningitidis. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 15644–15649.

Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V, Li Y, Fine EJ, Wu X, Shalem O, Cradick TJ, Marraffini LA, Bao G and Zhang F (2013) DNA targeting specificity of RNA-guided Cas9 nucleases. *Nature Biotechnology* **31**, 827–832.

Hsu PD, Lander ES and Zhang F (2014) Development and applications of CRISPR-Cas9 for genome engineering. *Cell* **157**, 1262–1278.

Hua K, Tao X and Zhu JK (2019) Expanding the base editing scope in rice by using Cas9 variants. *Plant Biotechnology Journal* **17**, 499–504.

Hu W, Kaminski R, Yang F, Zhang Y, Cosentino L, Li F, Luo B, Alvarez-Carbonell D, Garcia-Mesa Y, Karn J, Mo X and Khalili K (2014) RNA-directed gene editing specifically eradicates latent and prevents new HIV-1 infection. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 11461–11466.

Hu JH, Miller SM, Geurts MH, Tang W, Chen L, Sun N, Zeina CM, Gao X, Rees HA, Lin Z and Liu DR (2018) Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. *Nature* **556**, 57–63.

Hwang WY, Fu Y, Reyon D, Maeder ML, Tsai SQ, Sander JD, Peterson RT, Yeh JR and Joung JK (2013) Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nature Biotechnology* **31**, 227–229.

Ibraheim R, Song CQ, Mir A, Amrani N, Xue W and Sontheimer EJ (2018) All-in-one adeno-associated virus delivery and genome editing by Neisseria meningitidis Cas9 in vivo. *Genome Biology* **19**, 137.

Ipsaro JJ, Shen C, Arai E, Xu Y, Kinney JB, Joshua-Tor L, Vakoc CR and Shi J (2017) Rapid generation of drug-resistance alleles at endogenous loci using CRISPR-Cas9 indel mutagenesis. *PLoS One* **12**, e0172177.

Ishino Y, Shinagawa H, Makino K, Amemura M and Nakata A (1987) Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. *Journal of Bacteriology* **169**, 5429–5433.

Ishino Y, Krupovic M and Forterre P (2018) History of CRISPR-Cas from encounter with a mysterious repeated sequence to genome editing technology. *Journal of Bacteriology* **200**, e00580.

Jackson RN and Wiedenheft B (2015) A conserved structural chassis for mounting Versatile CRISPR RNA-guided immune responses. *Molecular Cell* **58**, 722–728.

Jacob F (1977) Evolution and tinkering. *Science* **196**, 1161–1166.

Jain IH, Zazzeron L, Goli R, Alexa K, Schatzman-Bone S, Dhillon H, Goldberger O, Peng J, Shalem O, Sanjana NE, Zhang F, Goessling W, Zapol WM and Mootha VK (2016) Hypoxia as a therapy for mitochondrial disease. *Science* **352**, 54–61.

Jaitin DA, Weiner A, Yofe I, Lara-Astiaso D, Keren-Shaul H, David E, Salame TM, Tanay A, Van Oudenaarden A and Amit I (2016) Dissecting immune circuits by linking CRISPR-pooled screens with single-cell RNA-Seq. *Cell* **167**, 1883–1896.

Jansen R, Embden JD, Gaastra W and Schouls LM (2002) Identification of genes that are associated with DNA repeats in prokaryotes. *Molecular Microbiology* **43**, 1565–1575.

Jiang F and Doudna JA (2017) CRISPR-Cas9 Structures and mechanisms. *Annual Review of Biophysics* **46**, 505–529.

Jiang W, Zhou H, Bi H, Fromm M, Yang B and Weeks DP (2013) Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene

modification in Arabidopsis, tobacco, sorghum and rice. *Nucleic Acids Research* **41**, e188–e188.

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA and Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816–821.

Jinek M, East A, Cheng A, Lin S, Ma E and Doudna J (2013) RNA-programmed genome editing in human cells. *Elife* **2**, e00471.

Jinek M, Jiang F, Taylor DW, Sternberg SH, Kaya E, Ma E, Anders C, Hauer M, Zhou K, Lin S, Kaplan M, Iavarone AT, Charpentier E, Nogales E and Doudna JA (2014) Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. *Science* **343**, 1247997–1247997.

Jin S, Zong Y, Gao Q, Zhu Z, Wang Y, Qin P, Liang C, Wang D, Qiu J-L, Zhang F and Gao C (2019) Cytosine, but not adenine, base editors induce genome-wide off-target mutations in rice. *Science* **364**, 292–295.

Jost M and Weissman JS (2018) CRISPR approaches to small molecule target identification. *ACS Chemical Biology* **13**, 366–375.

Joung JK and Sander JD (2013) TALENs: a widely applicable technology for targeted genome editing. *Nature Reviews Molecular Cell Biology* **14**, 49–55.

Joung J, Engreitz JM, Konermann S, Abudayyeh OO, Verdine VK, Aguet F, Gootenberg JS, Sanjana NE, Wright JB, Fulco CP, Tseng Y-Y, Yoon CH, Boehm JS, Lander ES and Zhang F (2017) Genome-scale activation screen identifies a lncRNA locus regulating a gene neighbourhood. *Nature* **548**, 343–346.

Kalhor R, Mali P and Church GM (2017) Rapidly evolving homing CRISPR barcodes. *Nature Methods* **14**, 195–200.

Kang BC, Yun JY, Kim ST, Shin Y, Ryu J, Choi M, Woo JW and Kim JS (2018) Precision genome engineering through adenine base editing in plants. *Nat Plants* **4**, 427–431.

Kan Y, Ruis B, Takasugi T and Hendrickson EA (2017) Mechanisms of precise genome editing using oligonucleotide donors. *Genome Research* **27**, 1099–1111.

Karvelis T, Gasiunas G, Young J, Bigelyte G, Silanskas A, Cigan M and Siksnys V (2015) Rapid characterization of CRISPR-Cas9 protospacer adjacent motif sequence elements. *Genome Biology* **16**, 253.

Kearns NA, Pham H, Tabak B, Genga RM, Silverstein NJ, Garber M and Maehr R (2015) Functional annotation of native enhancers with a Cas9–histone demethylase fusion. *Nature Methods* **12**, 401–403.

Kim H and Kim JS (2014) A guide to genome engineering with programmable nucleases. *Nature Reviews Genetics* **15**, 321–334.

Kim D, Bae S, Park J, Kim E, Kim S, Yu HR, Hwang J, Kim J-I and Kim J-S (2015) Digenome-seq: genome-wide profiling of CRISPR-Cas9 off-target effects in human cells. *Nature Methods* **12**, 237–243.

Kim E, Koo T, Park SW, Kim D, Kim K, Cho HY, Song DW, Lee KJ, Jung MH, Kim S, Kim JH, Kim JH and Kim JS (2017a) In vivo genome editing with a small Cas9 orthologue derived from Campylobacter jejuni. *Nature Communications* **8**, 14500.

Kim H, Kim S-T, Ryu J, Kang B-C, Kim J-S and Kim S-G (2017b) CRISPR/Cpf1-mediated DNA-free plant genome editing. *Nature Communications* **8**, 14406.

Kim D, Kim DE, Lee G, Cho SI and Kim JS (2019) Genome-wide target specificity of CRISPR RNA-guided adenine base editors. *Nature Biotechnology* **37**, 430–435.

Klann TS, Black JB, Chellappan M, Safi A, Song L, Hilton IB, Crawford GE, Reddy TE and Gersbach CA (2017) CRISPR-Cas9 epigenome editing enables high-throughput screening for functional regulatory elements in the human genome. *Nature Biotechnology* **35**, 561–568.

Kleinstiver BP, Prew MS, Tsai SQ, Nguyen NT, Topkar VV, Zheng Z and Joung JK (2015a) Broadening the targeting range of Staphylococcus aureus CRISPR-Cas9 by modifying PAM recognition. *Nature Biotechnology* **33**, 1293–1298.

Kleinstiver BP, Prew MS, Tsai SQ, Topkar VV, Nguyen NT, Zheng Z, Gonzales APW, Li Z, Peterson RT, Yeh J-RJ, Aryee MJ and Joung JK (2015b) Engineered CRISPR-Cas9 nucleases with altered PAM specificities. *Nature* **523**, 481–485.

Kleinstiver BP, Pattanayak V, Prew MS, Tsai SQ, Nguyen NT, Zheng Z and Joung JK (2016a) High-fidelity CRISPR–Cas9 nucleases with no detectable genome-wide off-target effects. *Nature* **529**, 490–495.

Kleinstiver BP, Tsai SQ, Prew MS, Nguyen NT, Welch MM, Lopez JM, Mccaw ZR, Aryee MJ and Joung JK (2016b) Genome-wide specificities of CRISPR-Cas Cpf1 nucleases in human cells. *Nature Biotechnology* **34**, 869–874.

Kleinstiver BP, Sousa AA, Walton RT, Tak YE, Hsu JY, Clement K, Welch MM, Horng JE, Malagon-Lopez J, Scarfo I, Maus MV, Pinello L, Aryee MJ and Joung JK (2019) Engineered CRISPR-Cas12a variants with increased activities and improved targeting ranges for gene, epigenetic and base editing. *Nature Biotechnology* **37**, 276–282.

Knight SC, Xie L, Deng W, Guglielmi B, Witkowsky LB, Bosanac L, Zhang ET, El Beheiry M, Masson JB, Dahan M, Liu Z, Doudna JA and Tjian R (2015) Dynamics of CRISPR-Cas9 genome interrogation in living cells. *Science* **350**, 823–826.

Knott GJ, East-Seletsky A, Cofsky JC, Holton JM, Charles E, O'connell MR and Doudna JA (2017) Guide-bound structures of an RNA-targeting A-cleaving CRISPR-Cas13a enzyme. *Nature Structural &Molecular Biology* **24**, 825–833.

Kocak DD, Josephs EA, Bhandarkar V, Adkar SS, Kwon JB and Gersbach CA (2019) Increasing the specificity of CRISPR systems with engineered RNA secondary structures. *Nature Biotechnology*. http://doi.org/10.1038/s41587-019-0095-1.

Koike-Yusa H, Li Y, Tan EP, Velasco-Herrera Mdel C and Yusa K (2014) Genome-wide recessive genetic screening in mammalian cells with a lentiviral CRISPR-guide RNA library. *Nature Biotechnology* **32**, 267–273.

Komor AC, Kim YB, Packer MS, Zuris JA and Liu DR (2016) Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature* **533**, 420–424.

Konermann S, Brigham MD, Trevino AE, Hsu PD, Heidenreich M, Cong L, Platt RJ, Scott DA, Church GM and Zhang F (2013) Optical control of mammalian endogenous transcription and epigenetic states. *Nature* **500**, 472–476.

Konermann S, Brigham MD, Trevino AE, Joung J, Abudayyeh OO, Barcena C, Hsu PD, Habib N, Gootenberg JS, Nishimasu H, Nureki O and Zhang F (2014) Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature* **517**, 583–588.

Konermann S, Lotfy P, Brideau NJ, Oki J, Shokhirev MN and Hsu PD (2018) Transcriptome engineering with RNA-targeting type VI-D CRISPR effectors. *Cell* **173**, 665–676.

Koonin EV and Makarova KS (2017) Mobile genetic elements and evolution of CRISPR-Cas systems: all the way there and back. *Genome Biology and Evolution* **9**, 2812–2825.

Kosicki M, Tomberg K and Bradley A (2018) Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements. *Nature Biotechnology* **36**, 765–771.

Labun K, Montague TG, Gagnon JA, Thyme SB and Valen E (2016) CHOPCHOP v2: a web tool for the next generation of CRISPR genome engineering. *Nucleic Acids Research* **44**, W272–W276.

Lander ES, Baylis F, Zhang F, Charpentier E, Berg P, Bourgain C, Friedrich B, Joung JK, Li J, Liu D, Naldini L, Nie JB, Qiu R, Schoene-Seifert B, Shao F, Terry S, Wei W and Winnacker EL (2019) Adopt a moratorium on heritable genome editing. *Nature* **567**, 165–168.

Lee CM, Cradick TJ and Bao G (2016) The Neisseria meningitidis CRISPR-Cas9 system enables specific genome editing in mammalian cells. *Molecular Therapy* **24**, 645–654.

Lee JK, Jeong E, Lee J, Jung M, Shin E, Kim Y-H, Lee K, Jung I, Kim D, Kim S and Kim J-S (2018) Directed evolution of CRISPR-Cas9 to increase its specificity. *Nature Communications* **9**, 3048.

Lemay ML, Horvath P and Moineau S (2017) The CRISPR-Cas app goes viral. *Current Opinion in Microbiology* **37**, 103–109.

Liang Z, Zhang K, Chen K and Gao C (2014) Targeted mutagenesis in Zea mays using TALENs and the CRISPR/Cas system. *Journal of Genetics and Genomics=Yi chuan xue bao* **41**, 63–68.

Liang P, Xu Y, Zhang X, Ding C, Huang R, Zhang Z, Lv J, Xie X, Chen Y, Li Y, Sun Y, Bai Y, Songyang Z, Ma W, Zhou C and Huang J (2015) CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes. *Protein Cell* **6**, 363–372.

Liang Z, Chen K, Li T, Zhang Y, Wang Y, Zhao Q, Liu J, Zhang H, Liu C, Ran Y and Gao C (2017) Efficient DNA-free genome editing of bread wheat

using CRISPR/Cas9 ribonucleoprotein complexes. *Nature Communications* **8**, 14261.

Liao HK, Hatanaka F, Araoka T, Reddy P, Wu MZ, Sui Y, Yamauchi T, Sakurai M, O'keefe DD, Nunez-Delicado E, Guillen P, Campistol JM, Wu CJ, Lu LF, Esteban CR and Izpisua Belmonte JC (2017) *In vivo* target gene activation via CRISPR/Cas9-mediated trans-epigenetic modulation. *Cell* **171**, 1495–1507.

Link KH and Breaker RR (2009) Engineering ligand-responsive gene-control elements: lessons learned from natural riboswitches. *Gene Therapy* **16**, 1189–1201.

Lin S, Staahl BT, Alla RK and Doudna JA (2014) Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery. *Elife* **3**, e04766.

Listgarten J, Weinstein M, Kleinstiver BP, Sousa AA, Joung JK, Crawford J, Gao K, Hoang L, Elibol M, Doench JG and Fusi N (2018) Prediction of off-target activities for the end-to-end design of CRISPR guide RNAs. *Nature Biomedical Engineering* **2**, 38–47.

Liu KI, Ramli MNB, Woo CWA, Wang Y, Zhao T, Zhang X, Yim GRD, Chong BY, Gowher A, Chua MZH, Jung J, Lee JHJ and Tan MH (2016a) A chemical-inducible CRISPR-Cas9 system for rapid control of genome editing. *Nature Chemical Biology* **12**, 980–987.

Liu XS, Wu H, Ji X, Stelzer Y, Wu X, Czauderna S, Shu J, Dadon D, Young RA and Jaenisch R (2016b) Editing DNA methylation in the mammalian genome. *Cell* **167**, 233–247.

Liu L, Li X, Ma J, Li Z, You L, Wang J, Wang M, Zhang X and Wang Y (2017a) The molecular architecture for RNA-guided RNA cleavage by Cas13a. *Cell* **170**, 714–726.

Liu L, Li X, Wang J, Wang M, Chen P, Yin M, Li J, Sheng G and Wang Y (2017b) Two distant catalytic sites are responsible for C2c2 RNase activities. *Cell* **168**, 121–134.

Liu SJ, Horlbeck MA, Cho SW, Birk HS, Malatesta M, He D, Attenello FJ, Villalta JE, Cho MY, Chen Y, Mandegar MA, Olvera MP, Gilbert LA, Conklin BR, Chang HY, Weissman JS and Lim DA (2017c) CRISPRi-based genome-scale identification of functional long noncoding RNA loci in human cells. *Science* **355**, eaah7111.

Liu N, Lee CH, Swigut T, Grow E, Gu B, Bassik MC and Wysocka J (2018a) Selective silencing of euchromatic L1s revealed by genome-wide screens for L1 regulators. *Nature* **553**, 228–232.

Liu XS, Wu H, Krzisch M, Wu X, Graef J, Muffat J, Hnisz D, Li CH, Yuan B, Xu C, Li Y, Vershkov D, Cacace A, Young RA and Jaenisch R (2018b) Rescue of Fragile X syndrome neurons by DNA methylation editing of the FMR1 gene. *Cell* **172**, 979–992.

Liu Y, Yu C, Daley TP, Wang F, Cao WS, Bhate S, Lin X, Still 2nd C, Liu H, Zhao D, Wang H, Xie XS, Ding S, Wong WH, Wernig M and Qi LS (2018c) CRISPR activation screens systematically identify factors that drive neuronal fate and reprogramming. *Cell Stem Cell* **23**, 758–771.

Liu Z, Lu Z, Yang G, Huang S, Li G, Feng S, Liu Y, Li J, Yu W, Zhang Y, Chen J, Sun Q and Huang X (2018d) Efficient generation of mouse models of human diseases via ABE- and BE-mediated base editing. *Nature Communications* **9**, 2338.

Liu J-J, Orlova N, Oakes BL, Ma E, Spinner HB, Baney KLM, Chuck J, Tan D, Knott GJ, Harrington LB, Al-Shayeb B, Wagner A, Brötzmann J, Staahl BT, Taylor KL, Desmarais J, Nogales E and Doudna JA (2019) Casx enzymes comprise a distinct family of RNA-guided genome editors. *Nature* **566**, 218–223.

Li J-F, Norville JE, Aach J, Mccormack M, Zhang D, Bush J, Church GM and Sheen J (2013) Multiplex and homologous recombination-mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. *Nature Biotechnology* **31**, 688–691.

Li W, Xu H, Xiao T, Cong L, Love MI, Zhang F, Irizarry RA, Liu JS, Brown M and Liu XS (2014) MAGeCK enables robust identification of essential genes from genome-scale CRISPR/Cas9 knockout screens. *Genome Biology* **15**, 554.

Li X, Fan D, Zhang W, Liu G, Zhang L, Zhao L, Fang X, Chen L, Dong Y, Chen Y, Ding Y, Zhao R, Feng M, Zhu Y, Feng Y, Jiang X, Zhu D, Xiang H, Feng X, Li S, Wang J, Zhang G, Kronforst MR and Wang W (2015) Outbred genome sequencing and CRISPR/Cas9 gene editing in butterflies. *Nature Communications* **6**, 8212.

Li M, Au LYC, Douglah D, Chong A, White BJ, Ferree PM and Akbari OS (2017) Generation of heritable germline mutations in the jewel wasp Nasonia vitripennis using CRISPR/Cas9. *Scientific Reports* **7**, 901.

Li C, Zong Y, Wang Y, Jin S, Zhang D, Song Q, Zhang R and Gao C (2018a) Expanded base editing in rice and wheat using a Cas9-adenosine deaminase fusion. *Genome Biology* **19**, 59.

Li S-Y, Cheng Q-X, Wang J-M, Li X-Y, Zhang Z-L, Gao S, Cao R-B, Zhao G-P and Wang J (2018b) CRISPR-Cas12a-assisted nucleic acid detection. *Cell Discovery* **4**, 20.

Li X, Wang Y, Liu Y, Yang B, Wang X, Wei J, Lu Z, Zhang Y, Wu J, Huang X, Yang L and Chen J (2018c) Base editing with a Cpf1-cytidine deaminase fusion. *Nature Biotechnology* **36**, 324–327.

Loenen WA, Dryden DT, Raleigh EA, Wilson GG and Murray NE (2014) Highlights of the DNA cutters: a short history of the restriction enzymes. *Nucleic Acids Research* **42**, 3–19.

Long C, Amoasii L, Mireault AA, Mcanally JR, Li H, Sanchez-Ortiz E, Bhattacharyya S, Shelton JM, Bassel-Duby R and Olson EN (2016) Postnatal genome editing partially restores dystrophin expression in a mouse model of muscular dystrophy. *Science* **351**, 400–403.

Lunshof J (2015) Regulate gene editing in wild animals. *Nature* **521**, 127.

Maddalo D, Manchado E, Concepcion CP, Bonetti C, Vidigal JA, Han YC, Ogrodowski P, Crippa A, Rekhtman N, De Stanchina E, Lowe SW and Ventura A (2014) *In vivo* engineering of oncogenic chromosomal rearrangements with the CRISPR/Cas9 system. *Nature* **516**, 423–427.

Maeder ML, Linder SJ, Cascio VM, Fu Y, Ho QH and Joung JK (2013) CRISPR RNA-guided activation of endogenous human genes. *Nature Methods* **10**, 977–979.

Maeder ML, Stefanidakis M, Wilson CJ, Baral R, Barrera LA, Bounoutas GS, Bumcrot D, Chao H, Ciulla DM, Dasilva JA, Dass A, Dhanapal V, Fennell TJ, Friedland AE, Giannoukos G, Gloskowski SW, Glucksmann A, Gotta GM, Jayaram H, Haskett SJ, Hopkins B, Horng JE, Joshi S, Marco E, Mepani R, Reyon D, Ta T, Tabbaa DG, Samuelsson SJ, Shen S, Skor MN, Stetkiewicz P, Wang T, Yudkoff C, Myer VE, Albright CF and Jiang H (2019) Development of a gene-editing approach to restore vision loss in Leber congenital amaurosis type 10. *Nature Medicine* **25**, 229–233.

Makarova KS, Aravind L, Grishin NV, Rogozin IB and Koonin EV (2002) A DNA repair system specific for thermophilic archaea and bacteria predicted by genomic context analysis. *Nucleic Acids Research* **30**, 482–496.

Makarova KS, Grishin NV, Shabalina SA, Wolf YI and Koonin EV (2006) A putative RNA-interference-based immune system in prokaryotes: computational analysis of the predicted enzymatic machinery, functional analogies with eukaryotic RNAi, and hypothetical mechanisms of action. Biology Direct **1**, 7.

Makarova KS, Haft DH, Barrangou R, Brouns SJJ, Charpentier E, Horvath P, Moineau S, Mojica FJM, Wolf YI, Yakunin AF, Van Der Oost J and Koonin EV (2011) Evolution and classification of the CRISPR–Cas systems. *Nature Reviews Microbiology* **9**, 467–477.

Makarova KS, Wolf YI, Alkhnbashi OS, Costa F, Shah SA, Saunders SJ, Barrangou R, Brouns SJJ, Charpentier E, Haft DH, Horvath P, Moineau S, Mojica FJM, Terns RM, Terns MP, White MF, Yakunin AF, Garrett RA, Van Der Oost J, Backofen R and Koonin EV (2015) An updated evolutionary classification of CRISPR-Cas systems. *Nature Reviews Microbiology* **13**, 722–736.

Mali P, Aach J, Stranges PB, Esvelt KM, Moosburner M, Kosuri S, Yang L and Church GM (2013a) CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. *Nature Biotechnology* **31**, 833–838.

Mali P, Yang LH, Esvelt KM, Aach J, Guell M, Dicarlo JE, Norville JE and Church GM (2013b) RNA-guided Human genome engineering via Cas9. *Science* **339**, 823–826.

Mandal PK, Ferreira LM, Collins R, Meissner TB, Boutwell CL, Friesen M, Vrbanac V, Garrison BS, Stortchevoi A, Bryder D, Musunuru K, Brand H, Tager AM, Allen TM, Talkowski ME, Rossi DJ and Cowan CA (2014) Efficient ablation of genes in human hematopoietic stem and effector cells using CRISPR/Cas9. *Cell Stem Cell* **15**, 643–652.

Mandegar MA, Huebsch N, Frolov EB, Shin E, Truong A, Olvera MP, Chan AH, Miyaoka Y, Holmes K, Spencer CI, Judge LM, Gordon DE,

Eskildsen TV, Villalta JE, Horlbeck MA, Gilbert LA, Krogan NJ, Sheikh SP, Weissman JS, Qi LS, So PL and Conklin BR (2016) CRISPR interference efficiently induces specific and reversible gene silencing in human iPSCs. *Cell Stem Cell* **18**, 541–553.

Manguso RT, Pope HW, Zimmer MD, Brown FD, Yates KB, Miller BC, Collins NB, Bi K, Lafleur MW, Juneja VR, Weiss SA, Lo J, Fisher DE, Miao D, Van Allen E, Root DE, Sharpe AH, Doench JG and Haining WN (2017) *In vivo* CRISPR screening identifies Ptpn2 as a cancer immunotherapy target. *Nature* **547**, 413–418.

Mao Y, Zhang H, Xu N, Zhang B, Gou F and Zhu JK (2013) Application of the CRISPR-Cas system for efficient genome engineering in plants. *Molecular Plant* **6**, 2008–2011.

Marceau CD, Puschnik AS, Majzoub K, Ooi YS, Brewer SM, Fuchs G, Swaminathan K, Mata MA, Elias JE, Sarnow P and Carette JE (2016) Genetic dissection of Flaviviridae host factors through genome-scale CRISPR screens. *Nature* **535**, 159–163.

Marino ND, Zhang JY, Borges AL, Sousa AA, Leon LM, Rauch BJ, Walton RT, Berry JD, Joung JK, Kleinstiver BP and Bondy-Denomy J (2018) Discovery of widespread type I and type V CRISPR-Cas inhibitors. *Science* **362**, 240–242.

Marraffini LA (2015) CRISPR-Cas immunity in prokaryotes. *Nature* **526**, 55–61.

Marraffini LA and Sontheimer EJ (2008) CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. *Science* **322**, 1843–1845.

Martin A, Serano JM, Jarvis E, Bruce HS, Wang J, Ray S, Barker CA, O'connell LC and Patel NH (2016) CRISPR/cas9 mutagenesis reveals versatile roles of Hox genes in crustacean limb specification and evolution. *Current Biology: CB* **26**, 14–26.

Maruyama T, Dougan SK, Truttmann MC, Bilate AM, Ingram JR and Ploegh HL (2015) Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. *Nature Biotechnology* **33**, 538–542.

Mastroianni M, Watanabe K, White TB, Zhuang F, Vernon J, Matsuura M, Wallingford J and Lambowitz AM (2008) Group II intron-based gene targeting reactions in eukaryotes. *PLoS One* **3**, e3121.

Ma H, Naseri A, Reyes-Gutierrez P, Wolfe SA, Zhang S and Pederson T (2015) Multicolor CRISPR labeling of chromosomal loci in human cells. *Proceedings of the National Academy of Sciences of the United States of America* **112**, 3002–3007.

Ma H, Tu LC, Naseri A, Huisman M, Zhang S, Grunwald D and Pederson T (2016a) CRISPR-Cas9 nuclear dynamics and target recognition in living cells. *Journal of Cell Biology* **214**, 529–537.

Ma H, Tu LC, Naseri A, Huisman M, Zhang S, Grunwald D and Pederson T (2016b) Multiplexed labeling of genomic loci with dCas9 and engineered sgRNAs using CRISPRainbow. *Nature Biotechnology* **34**, 528–530.

Ma H, Marti-Gutierrez N, Park SW, Wu J, Lee Y, Suzuki K, Koski A, Ji D, Hayama T, Ahmed R, Darby H, Van Dyken C, Li Y, Kang E, Park AR, Kim D, Kim ST, Gong J, Gu Y, Xu X, Battaglia D, Krieg SA, Lee DM, Wu DH, Wolf DP, Heitner SB, Belmonte JCI, Amato P, Kim JS, Kaul S and Mitalipov S (2017) Correction of a pathogenic gene mutation in human embryos. *Nature* **548**, 413–419.

Ma H, Marti-Gutierrez N, Park SW, Wu J, Hayama T, Darby H, Van Dyken C, Li Y, Koski A, Liang D, Suzuki K, Gu Y, Gong J, Xu X, Ahmed R, Lee Y, Kang E, Ji D, Park AR, Kim D, Kim ST, Heitner SB, Battaglia D, Krieg SA, Lee DM, Wu DH, Wolf DP, Amato P, Kaul S, Belmonte JCI, Kim JS and Mitalipov S (2018) Ma *et al.* Reply. *Nature* **560**, E10–E23.

Mckenna A, Findlay GM, Gagnon JA, Horwitz MS, Schier AF and Shendure J (2016) Whole-organism lineage tracing by combinatorial and cumulative genome editing. *Science* **353**, aaf7907.

Meeske AJ and Marraffini LA (2018) RNA guide complementarity prevents self-targeting in type VI CRISPR systems. *Molecular Cell* **71**, 791–801.

Meier JA, Zhang F and Sanjana NE (2017) GUIDES: sgRNA design for loss-of-function screens. *Nature Methods* **14**, 831–832.

Miyaoka Y, Berman JR, Cooper SB, Mayerl SJ, Chan AH, Zhang B, Karlin-Neumann GA and Conklin BR (2016) Systematic quantification of HDR and NHEJ reveals effects of locus, nuclease, and cell type on genome-editing. *Scientific Reports* **6**, 23549.

Miyaoka Y, Mayerl SJ, Chan AH and Conklin BR (2018) Detection and quantification of HDR and NHEJ induced by genome editing at endogenous gene loci using droplet digital PCR. *Methods in Molecular Biology* **1768**, 349–362.

Mojica FJ and Rodriguez-Valera F (2016) The discovery of CRISPR in archaea and bacteria. *FEBS Journal* **283**, 3162–3169.

Mojica FJ, Diez-Villasenor C, Garcia-Martinez J and Soria E (2005) Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *Journal of Molecular Evolution* **60**, 174–182.

Mojica FJ, Diez-Villasenor C, Garcia-Martinez J and Almendros C (2009) Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiology (Reading England)* **155**(Pt 3), 733–740.

Moreno-Mateos MA, Fernandez JP, Rouet R, Vejnar CE, Lane MA, Mis E, Khokha MK, Doudna JA and Giraldez AJ (2017) CRISPR-Cpf1 mediates efficient homology-directed repair and temperature-controlled genome editing. *Nature Communications* **8**, 2024.

Morgan SL, Mariano NC, Bermudez A, Arruda NL, Wu F, Luo Y, Shankar G, Jia L, Chen H, Hu JF, Hoffman AR, Huang CC, Pitteri SJ and Wang KC (2017) Manipulation of nuclear architecture through CRISPR-mediated chromosomal looping. *Nature Communications* **8**, 15993.

Morgens DW, Wainberg M, Boyle EA, Ursu O, Araya CL, Tsui CK, Haney MS, Hess GT, Han K, Jeng EE, Li A, Snyder MP, Greenleaf WJ, Kundaje A and Bassik MC (2017) Genome-scale measurement of off-target activity using Cas9 toxicity in high-throughput screens. *Nature Communications* **8**, 15178.

Moscou MJ and Bogdanove AJ (2009) A simple cipher governs DNA recognition by TAL effectors. *Science* **326**, 1501.

Munoz DM, Cassiani PJ, Li L, Billy E, Korn JM, Jones MD, Golji J, Ruddy DA, Yu K, Mcallister G, Deweck A, Abramowski D, Wan J, Shirley MD, Neshat SY, Rakiec D, De Beaumont R, Weber O, Kauffmann A, Mcdonald 3rd ER, Keen N, Hofmann F, Sellers WR, Schmelzle T, Stegmeier F and Schlabach MR (2016) CRISPR screens provide a comprehensive assessment of cancer vulnerabilities but generate false-positive hits for highly amplified genomic regions. *Cancer Discovery* **6**, 900–913.

Myers SA, Wright J, Peckner R, Kalish BT, Zhang F and Carr SA (2018) Discovery of proteins associated with a predefined genomic locus via dCas9-APEX-mediated proximity labeling. *Nature Methods* **15**, 437–439.

Myhrvold C, Freije CA, Gootenberg JS, Abudayyeh OO, Metsky HC, Durbin AF, Kellner MJ, Tan AL, Paul LM, Parham LA, Garcia KF, Barnes KG, Chak B, Mondini A, Nogueira ML, Isern S, Michael SF, Lorenzana I, Yozwiak NL, Macinnis BL, Bosch I, Gehrke L, Zhang F and Sabeti PC (2018) Field-deployable viral diagnostics using CRISPR-Cas13. *Science* **360**, 444–448.

Nakamura M, Srinivasan P, Chavez M, Carter MA, Dominguez AA, La Russa M, Lau MB, Abbott TR, Xu X, Zhao D, Gao Y, Kipniss NH, Smolke CD, Bondy-Denomy J and Qi LS (2019) Anti-CRISPR-mediated control of gene editing and synthetic circuits in eukaryotic cells. *Nature Communications* **10**, 194.

Nakayama T, Fish MB, Fisher M, Oomen-Hajagos J, Thomsen GH and Grainger RM (2013) Simple and efficient CRISPR/Cas9-mediated targeted mutagenesis in Xenopus tropicalis. *Genesis (New York N.Y. : 2000)* **51**, 835–843.

Nelles DA, Fang MY, O'connell MR, Xu JL, Markmiller SJ, Doudna JA and Yeo GW (2016) Programmable RNA tracking in live cells with CRISPR/Cas9. *Cell* **165**, 488–496.

Nelson CE, Hakim CH, Ousterout DG, Thakore PI, Moreb EA, Rivera RMC, Madhavan S, Pan X, Ran FA, Yan WX, Asokan A, Zhang F, Duan D and Gersbach CA (2016) *In vivo* genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy. *Science* **351**, 403–407.

Nelson CE, Wu Y, Gemberling MP, Oliver ML, Waller MA, Bohning JD, Robinson-Hamm JN, Bulaklak K, Castellanos Rivera RM, Collier JH, Asokan A and Gersbach CA (2019) Long-term evaluation of AAV-CRISPR genome editing for Duchenne muscular dystrophy. *Nature Medicine* **25**, 427–432.

Nguyen DP, Miyaoka Y, Gilbert LA, Mayerl SJ, Lee BH, Weissman JS, Conklin BR and Wells JA (2016) Ligand-binding domains of nuclear

receptors facilitate tight control of split CRISPR activity. *Nature Communications* **7**, 12009.

Nihongaki Y, Kawano F, Nakajima T and Sato M (2015) Photoactivatable CRISPR-Cas9 for optogenetic genome editing. *Nature Biotechnology* **33**, 755–760.

Nishida K, Arazoe T, Yachie N, Banno S, Kakimoto M, Tabata M, Mochizuki M, Miyabe A, Araki M, Hara KY, Shimatani Z and Kondo A (2016) Targeted nucleotide editing using hybrid prokaryotic and vertebrate adaptive immune systems. *Science* **353**, aaf8729–aaf8729.

Nishimasu H, Ran FA, Hsu PD, Konermann S, Shehata SI, Dohmae N, Ishitani R, Zhang F and Nureki O (2014) Crystal structure of Cas9 in complex with guide RNA and target DNA. *Cell* **156**, 935–949.

Nishimasu H, Cong L, Yan WX, Ran FA, Zetsche B, Li Y, Kurabayashi A, Ishitani R, Zhang F and Nureki O (2015) Crystal structure of Staphylococcus aureus Cas9. *Cell* **162**, 1113–1126.

Nishimasu H, Shi X, Ishiguro S, Gao L, Hirano S, Okazaki S, Noda T, Abudayyeh OO, Gootenberg JS, Mori H, Oura S, Holmes B, Tanaka M, Seki M, Hirano H, Aburatani H, Ishitani R, Ikawa M, Yachie N, Zhang F and Nureki O (2018) Engineered CRISPR-Cas9 nuclease with expanded targeting space. *Science* **361**, 1259–1262.

Nissim L, Perli SD, Fridkin A, Perez-Pinera P and Lu TK (2014) Multiplexed and programmable regulation of gene networks with an integrated RNA and CRISPR/Cas toolkit in human cells. *Molecular Cell* **54**, 698–710.

Niu Y, Shen B, Cui Y, Chen Y, Wang J, Wang L, Kang Y, Zhao X, Si W, Li W, Xiang AP, Zhou J, Guo X, Bi Y, Si C, Hu B, Dong G, Wang H, Zhou Z, Li T, Tan T, Pu X, Wang F, Ji S, Zhou Q, Huang X, Ji W and Sha J (2014) Generation of gene-modified Cynomolgus monkey via Cas9/RNA-mediated gene targeting in one-cell embryos. *Cell* **156**, 836–843.

Niu D, Wei HJ, Lin L, George H, Wang T, Lee IH, Zhao HY, Wang Y, Kan Y, Shrock E, Lesha E, Wang G, Luo Y, Qing Y, Jiao D, Zhao H, Zhou X, Wang S, Wei H, Guell M, Church GM and Yang L (2017) Inactivation of porcine endogenous retrovirus in pigs using CRISPR-Cas9. *Science* **357**, 1303–1307.

Noordermeer SM, Adam S, Setiaputra D, Barazas M, Pettitt SJ, Ling AK, Olivieri M, Álvarez-Quilón A, Moatti N, Zimmermann M, Annunziato S, Krastev DB, Song F, Brandsma I, Frankum J, Brough R, Sherker A, Landry S, Szilard RK, Munro MM, Mcewan A, De Rugy TG, Lin Z-Y, Hart T, Moffat J, Gingras A-C, Martin A, Van Attikum H, Jonkers J, Lord CJ, Rottenberg S and Durocher D (2018) The shieldin complex mediates 53BP1-dependent DNA repair. *Nature* **560**, 117–121.

Nunez JK, Harrington LB, Kranzusch PJ, Engelman AN and Doudna JA (2015) Foreign DNA capture during CRISPR-Cas adaptive immunity. *Nature* **527**, 535–538.

Nymark M, Sharma AK, Sparstad T, Bones AM and Winge P (2016) A CRISPR/Cas9 system adapted for gene editing in marine algae. *Scientific Reports* **6**, 24951.

Paquet D, Kwart D, Chen A, Sproul A, Jacob S, Teo S, Olsen KM, Gregg A, Noggle S and Tessier-Lavigne M (2016) Efficient introduction of specific homozygous and heterozygous mutations using CRISPR/Cas9. *Nature* **533**, 125–129.

Pardee K, Green AA, Takahashi MK, Braff D, Lambert G, Lee JW, Ferrante T, Ma D, Donghia N, Fan M, Daringer NM, Bosch I, Dudley DM, O'connor DH, Gehrke L and Collins JJ (2016) Rapid, low-cost detection of Zika virus using programmable biomolecular components. *Cell* **165**, 1255–1266.

Park RJ, Wang T, Koundakjian D, Hultquist JF, Lamothe-Molina P, Monel B, Schumann K, Yu H, Krupzcak KM, Garcia-Beltran W, Piechocka-Trocha A, Krogan NJ, Marson A, Sabatini DM, Lander ES, Hacohen N and Walker BD (2017) A genome-wide CRISPR screen identifies a restricted set of HIV host dependency factors. *Nature Genetics* **49**, 193–203.

Parnas O, Jovanovic M, Eisenhaure TM, Herbst RH, Dixit A, Ye CJ, Przybylski D, Platt RJ, Tirosh I, Sanjana NE, Shalem O, Satija R, Raychowdhury R, Mertins P, Carr SA, Zhang F, Hacohen N and Regev A (2015) A genome-wide CRISPR screen in primary immune cells to dissect regulatory networks. *Cell* **162**, 675–686.

Patel SJ, Sanjana NE, Kishton RJ, Eidizadeh A, Vodnala SK, Cam M, Gartner JJ, Jia L, Steinberg SM, Yamamoto TN, Merchant AS, Mehta GU, Chichura A, Shalem O, Tran E, Eil R, Sukumar M, Guijarro EP, Day CP, Robbins P, Feldman S, Merlino G, Zhang F and Restifo NP (2017) Identification of essential genes for cancer immunotherapy. *Nature* **548**, 537–542.

Pattanayak V, Lin S, Guilinger JP, Ma E, Doudna JA and Liu DR (2013) High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. *Nature Biotechnology* **31**, 839–843.

Pawluk A, Amrani N, Zhang Y, Garcia B, Hidalgo-Reyes Y, Lee J, Edraki A, Shah M, Sontheimer EJ, Maxwell KL and Davidson AR (2016) Naturally occurring off-switches for CRISPR-Cas9. *Cell* **167**, 1829–1838.

Perez-Pinera P, Kocak DD, Vockley CM, Adler AF, Kabadi AM, Polstein LR, Thakore PI, Glass KA, Ousterout DG, Leong KW, Guilak F, Crawford GE, Reddy TE and Gersbach CA (2013) RNA-guided gene activation by CRISPR-Cas9-based transcription factors. *Nature Methods* **10**, 973–976.

Perli SD, Cui CH and Lu TK (2016) Continuous genetic recording with self-targeting CRISPR-Cas in human cells. *Science* **353**, aag0511.

Pinello L, Canver MC, Hoban MD, Orkin SH, Kohn DB, Bauer DE and Yuan GC (2016) Analyzing CRISPR genome-editing experiments with CRISPResso. *Nature Biotechnology* **34**, 695–697.

Platt RJ, Chen S, Zhou Y, Yim MJ, Swiech L, Kempton HR, Dahlman JE, Parnas O, Eisenhaure TM, Jovanovic M, Graham DB, Jhunjhunwala S, Heidenreich M, Xavier RJ, Langer R, Anderson DG, Hacohen N, Regev A, Feng G, Sharp PA and Zhang F (2014) CRISPR-Cas9 knockin mice for genome editing and cancer modeling. *Cell* **159**, 440–455.

Plessis A, Perrin A, Haber JE and Dujon B (1992) Site-specific recombination determined by I-SceI, a mitochondrial group I intron-encoded endonuclease expressed in the yeast nucleus. *Genetics* **130**, 451–460.

Porteus MH (2015) Towards a new era in medicine: therapeutic genome editing. *Genome Biology* **16**, 286.

Pourcel C, Salvignol G and Vergnaud G (2005) CRISPR elements in Yersinia pestis acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies. *Microbiology (Reading England)* **151**(Pt 3), 653–663.

Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP and Lim WA (2013) Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* **152**, 1173–1183.

Quiberoni A, Moineau S, Rousseau GM, Reinheimer J and Ackermann HW (2010) Streptococcus thermophilus bacteriophages. *International Dairy Journal* **20**, 657–664.

Ramanan V, Shlomai A, Cox DBT, Schwartz RE, Michailidis E, Bhatta A, Scott DA, Zhang F, Rice CM and Bhatia SN (2015) CRISPR/cas9 cleavage of viral DNA efficiently suppresses hepatitis B virus. *Scientific Reports* **5**, 10833.

Ran FA, Hsu PD, Lin C-Y, Gootenberg JS, Konermann S, Trevino AE, Scott DA, Inoue A, Matoba S, Zhang Y and Zhang F (2013) Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell* **154**, 1380–1389.

Ran FA, Cong L, Yan WX, Scott DA, Gootenberg JS, Kriz AJ, Zetsche B, Shalem O, Wu X, Makarova KS, Koonin EV, Sharp PA and Zhang F (2015) *In vivo* genome editing using Staphylococcus aureus Cas9. *Nature* **520**, 186–191.

Rauch BJ, Silvis MR, Hultquist JF, Waters CS, Mcgregor MJ, Krogan NJ and Bondy-Denomy J (2017) Inhibition of CRISPR-Cas9 with bacteriophage proteins. *Cell* **168**, 150–158.

Rees HA and Liu DR (2018) Base editing: precision chemistry on the genome and transcriptome of living cells. *Nature Publishing Group* **19**, 770–788.

Richardson CD, Ray GJ, Dewitt MA, Curie GL and Corn JE (2016) Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-Cas9 using asymmetric donor DNA. *Nature Biotechnology* **34**, 339–344.

Richardson CD, Kazane KR, Feng SJ, Zelin E, Bray NL, Schafer AJ, Floor SN and Corn JE (2018) CRISPR-Cas9 genome editing in human cells occurs via the Fanconi anemia pathway. *Nature Genetics* **50**, 1132–1139.

Rodriguez EA, Campbell RE, Lin JY, Lin MZ, Miyawaki A, Palmer AE, Shu X, Zhang J and Tsien RY (2017) The growing and glowing toolbox

of fluorescent and photoactive proteins. *Trends in Biochemical Sciences* **42**, 111–129.

Rose JC, Stephany JJ, Valente WJ, Trevillian BM, Dang HV, Bielas JH, Maly DJ and Fowler DM (2017) Rapidly inducible Cas9 and DSB-ddPCR to probe editing kinetics. *Nature Methods* **14**, 891–896.

Roth TL, Puig-Saus C, Yu R, Shifrut E, Carnevale J, Li PJ, Hiatt J, Saco J, Krystofinski P, Li H, Tobin V, Nguyen DN, Lee MR, Putnam AL, Ferris AL, Chen JW, Schickel JN, Pellerin L, Carmody D, Alkorta-Aranburu G, Del Gaudio D, Matsumoto H, Morell M, Mao Y, Cho M, Quadros RM, Gurumurthy CB, Smith B, Haugwitz M, Hughes SH, Weissman JS, Schumann K, Esensten JH, May AP, Ashworth A, Kupfer GM, Greeley SAW, Bacchetta R, Meffre E, Roncarolo MG, Romberg N, Herold KC, Ribas A, Leonetti MD and Marson A (2018) Reprogramming human T cell function and specificity with non-viral genome targeting. *Nature* **559**, 405–409.

Rouet P, Smih F and Jasin M (1994) Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. *Molecular and Cellular Biology* **14**, 8096–8106.

Rudin N, Sugarman E and Haber JE (1989) Genetic and physical analysis of double-strand break repair and recombination in Saccharomyces cerevisiae. *Genetics* **122**, 519–534.

Sanchez-Rivera FJ, Papagiannakopoulos T, Romero R, Tammela T, Bauer MR, Bhutkar A, Joshi NS, Subbaraj L, Bronson RT, Xue W and Jacks T (2014) Rapid modelling of cooperating genetic events in cancer through somatic genome editing. *Nature* **516**, 428–431.

Sanjana NE, Wright J, Zheng K, Shalem O, Fontanillas P, Joung J, Cheng C, Regev A and Zhang F (2016) High-resolution interrogation of functional elements in the noncoding genome. *Science* **353**, 1545–1549.

Sanson KR, Hanna RE, Hegde M, Donovan KF, Strand C, Sullender ME, Vaimberg EW, Goodale A, Root DE, Piccioni F and Doench JG (2018) Optimized libraries for CRISPR-Cas9 genetic screens with multiple modalities. *Nature Communications* **9**, 5416.

Sapranauskas R, Gasiunas G, Fremaux C, Barrangou R, Horvath P and Siksnys V (2011) The Streptococcus thermophilus CRISPR/Cas system provides immunity in Escherichia coli. *Nucleic Acids Research* **39**, gkr606–gk9282.

Schindele P, Wolter F and Puchta H (2018) Transforming plant biology and breeding with CRISPR/Cas9, Cas12 and Cas13. *FEBS letters* **592**, 1954–1967.

Schmid-Burgk JL, Schmidt T, Gaidt MM, Pelka K, Latz E, Ebert TS and Hornung V (2014) Outknocker: a web tool for rapid and simple genotyping of designer nuclease edited cell lines. *Genome Research* **24**, 1719–1723.

Schmid-Burgk JL, Honing K, Ebert TS and Hornung V (2016) CRISPaint allows modular base-specific gene tagging using a ligase-4-dependent mechanism. *Nature Communications* **7**, 12338.

Schmidt F, Cherepkova MY and Platt RJ (2018) Transcriptional recording by CRISPR spacer acquisition from RNA. *Nature* **562**, 380–385.

Schunder E, Rydzewski K, Grunow R and Heuner K (2013) First indication for a functional CRISPR/Cas system in Francisella tularensis. *International Journal of Medical Microbiology* **303**, 51–60.

Schwank G, Koo BK, Sasselli V, Dekkers JF, Heo I, Demircan T, Sasaki N, Boymans S, Cuppen E, Van Der Ent CK, Nieuwenhuis EE, Beekman JM and Clevers H (2013) Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. *Cell Stem Cell* **13**, 653–658.

Scott DA and Zhang F (2017) Implications of human genetic variation in CRISPR-based therapeutic genome editing. *Nature Medicine* **23**, 1095–1101.

Shalem O, Sanjana NE, Hartenian E, Shi X, Scott DA, Mikkelsen TS, Heckl D, Ebert BL, Root DE, Doench JG and Zhang F (2014) Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science* **343**, 84–87.

Shalem O, Sanjana NE and Zhang F (2015) High-throughput functional genomics using CRISPR-Cas9. *Nature Reviews Genetics* **16**, 299–311.

Shan Q, Wang Y, Li J, Zhang Y, Chen K, Liang Z, Zhang K, Liu J, Xi JJ, Qiu J-L and Gao C (2013) Targeted genome modification of crop plants using a CRISPR-Cas system. *Nature Biotechnology* **31**, 686–688.

Shapiro RS, Chavez A and Collins JJ (2018) CRISPR-based genomic tools for the manipulation of genetically intractable microorganisms. *Nature Reviews Microbiology* **16**, 333–339.

Shimatani Z, Kashojiya S, Takayama M, Terada R, Arazoe T, Ishii H, Teramura H, Yamamoto T, Komatsu H, Miura K, Ezura H, Nishida K, Ariizumi T and Kondo A (2017) Targeted base editing in rice and tomato using a CRISPR-Cas9 cytidine deaminase fusion. *Nature Biotechnology* **35**, 441–443.

Shipman SL, Nivala J, Macklis JD and Church GM (2016) Molecular recordings by directed CRISPR spacer acquisition. *Science* **353**, aaf1175.

Shipman SL, Nivala J, Macklis JD and Church GM (2017) CRISPR-Cas encoding of a digital movie into the genomes of a population of living bacteria. *Nature* **547**, 345–349.

Shi J, Wang E, Milazzo JP, Wang Z, Kinney JB and Vakoc CR (2015) Discovery of cancer drug targets by CRISPR-Cas9 screening of protein domains. *Nature Biotechnology* **33**, 661–667.

Shmakov S, Abudayyeh OO, Makarova KS, Wolf YI, Gootenberg JS, Semenova E, Minakhin L, Joung J, Konermann S, Severinov K, Zhang F and Koonin EV (2015) Discovery and functional characterization of diverse class 2 CRISPR-Cas systems. *Molecular Cell* **60**, 385–397.

Shmakov S, Smargon A, Scott D, Cox D, Pyzocha N, Yan W, Abudayyeh OO, Gootenberg JS, Makarova KS, Wolf YI, Severinov K, Zhang F and Koonin EV (2017) Diversity and evolution of class 2 CRISPR-Cas systems. *Nature Reviews Microbiology* **15**, 169–182.

Shmakov SA, Makarova KS, Wolf YI, Severinov KV and Koonin EV (2018) Systematic prediction of genes functionally linked to CRISPR-Cas systems by gene neighborhood analysis. *Proceedings of the National Academy of Sciences of the United States of America* **115**, E5307–E5316.

Sidik SM, Huet D, Ganesan SM, Huynh M-H, Wang T, Nasamu AS, Thiru P, Saeij JPJ, Carruthers VB, Niles JC and Lourido S (2016) A genome-wide CRISPR screen in toxoplasma identifies essential Apicomplexan genes. *Cell* **166**, 1423–1435.

Silas S, Mohr G, Sidote DJ, Markham LM, Sanchez-Amat A, Bhaya D, Lambowitz AM and Fire AZ (2016) Direct CRISPR spacer acquisition from RNA by a natural reverse transcriptase-Cas1 fusion protein. *Science* **351**, aad4234.

Slaymaker IM, Gao L, Zetsche B, Scott DA, Yan WX and Zhang F (2015) Rationally engineered Cas9 nucleases with improved specificity. *Science* **351**, 84–88.

Slaymaker IM, Mesa P, Kellner MJ, Kannan S, Brignole E, Koob J, Feliciano PR, Stella S, Abudayyeh OO, Gootenberg JS, Strecker J, Montoya G and Zhang F (2019) High-resolution structure of Cas13b and biochemical characterization of RNA targeting and cleavage. *Cell Reports* **26**, 1–11.

Smargon AA, Cox DBT, Pyzocha NK, Zheng K, Slaymaker IM, Gootenberg JS, Abudayyeh OA, Essletzbichler P, Shmakov S, Makarova KS, Koonin EV and Zhang F (2017) Cas13b Is a type VI-B CRISPR-associated RNA-guided RNase differentially regulated by accessory proteins Csx27 and Csx28. *Molecular Cell* **65**, 618–630.

Smith JJ, Timoshevskaya N, Ye C, Holt C, Keinath MC, Parker HJ, Cook ME, Hess JE, Narum SR, Lamanna F, Kaessmann H, Timoshevskiy VA, Waterbury CKM, Saraceno C, Wiedemann LM, Robb SMC, Baker C, Eichler EE, Hockman D, Sauka-Spengler T, Yandell M, Krumlauf R, Elgar G and Amemiya CT (2018) The sea lamprey germline genome provides insights into programmed genome rearrangement and vertebrate evolution. *Nature Genetics* **50**, 270–277.

Sollelis L, Ghorbal M, Macpherson CR, Martins RM, Kuk N, Crobu L, Bastien P, Scherf A, Lopez-Rubio J-J and Sterkers Y (2015) First efficient CRISPR-Cas9-mediated genome editing in Leishmania parasites. *Cellular Microbiology* **17**, 1405–1412.

Sontheimer EJ and Barrangou R (2015) The bacterial origins of the CRISPR genome-editing revolution. *Human Gene Therapy* **26**, 413–424.

Sorek R, Kunin V and Hugenholtz P (2008) CRISPR--a widespread system that provides acquired resistance against phages in bacteria and archaea. *Nature Reviews Microbiology* **6**, 181–186.

Soyk S, Lemmon ZH, Oved M, Fisher J, Liberatore KL, Park SJ, Goren A, Jiang K, Ramos A, Van Der Knaap E, Van Eck J, Zamir D, Eshed Y and Lippman ZB (2017) Bypassing negative epistasis on yield in tomato imposed by a domestication gene. *Cell* **169**, 1142–1155 e1112.

Stella S, Alcón P and Montoya G (2017) Structure of the Cpf1 endonuclease R-loop complex after target DNA cleavage. *Nature* **546**, 559–563.

Sternberg SH, Redding S, Jinek M, Greene EC and Doudna JA (2014) DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. *Nature* **507**, 62–67.

Strecker J, Jones S, Koopal B, Schmid-Burgk J, Zetsche B, Gao L, Makarova KS, Koonin EV and Zhang F (2019) Engineering of CRISPR-Cas12b for human genome editing. *Nature Communications* **10**, 866.

Strohkendl I, Saifuddin FA, Rybarski JR, Finkelstein IJ and Russell R (2018) Kinetic basis for DNA target specificity of CRISPR-Cas12a. *Molecular Cell* **71**, 816–824.

Suzuki K, Tsunekawa Y, Hernandez-Benitez R, Wu J, Zhu J, Kim EJ, Hatanaka F, Yamamoto M, Araoka T, Li Z, Kurita M, Hishida T, Li M, Aizawa E, Guo S, Chen S, Goebl A, Soligalla RD, Qu J, Jiang T, Fu X, Jafari M, Esteban CR, Berggren WT, Lajara J, Nunez-Delicado E, Guillen P, Campistol JM, Matsuzaki F, Liu GH, Magistretti P, Zhang K, Callaway EM, Zhang K and Belmonte JC (2016) In vivo genome editing via CRISPR/Cas9 mediated homology-independent targeted integration. *Nature* **540**, 144–149.

Swarts DC, Van Der Oost J and Jinek M (2017) Structural basis for guide RNA processing and seed-dependent DNA targeting by CRISPR-Cas12a. *Molecular Cell* **66**, 221–233.

Tabebordbar M, Zhu K, Cheng JKW, Chew WL, Widrick JJ, Yan WX, Maesner C, Wu EY, Xiao R, Ran FA, Cong L, Zhang F, Vandenberghe LH, Church GM and Wagers AJ (2016) In vivo gene editing in dystrophic mouse muscle and muscle stem cells. *Science* **351**, 407–411.

Tak YE, Kleinstiver BP, Nuñez JK, Hsu JY, Horng JE, Gong J, Weissman JS and Joung JK (2017) Inducible and multiplex gene regulation using CRISPR-Cpf1-based transcription factors. *Nature Methods* **14**, 1163–1166.

Tambe A, East-Seletsky A, Knott GJ, Doudna JA and O'connell MR (2018) RNA binding and HEPN-nuclease activation Are decoupled in CRISPR-Cas13a. *Cell Reports* **24**, 1025–1036.

Tanenbaum ME, Gilbert LA, Qi LS, Weissman JS and Vale RD (2014) A protein-tagging system for signal amplification in gene expression and fluorescence imaging. *Cell* **159**, 635–646.

Tang W and Liu DR (2018) Rewritable multi-event analog recording in bacterial and mammalian cells. *Science* **360**, eaap8992.

Telenti A (2009) Safety concerns about CCR5 as an antiviral target. *Curr Opin HIV AIDS* **4**, 131–135.

Teng F, Cui T, Feng G, Guo L, Xu K, Gao Q, Li T, Li J, Zhou Q and Li W (2018) Repurposing CRISPR-Cas12b for mammalian genome engineering. *Cell Discovery* **4**, 63.

Thakore PI, D'ippolito AM, Song L, Safi A, Shivakumar NK, Kabadi AM, Reddy TE, Crawford GE and Gersbach CA (2015) Highly specific epigenome editing by CRISPR-Cas9 repressors for silencing of distal regulatory elements. *Nature Methods* **12**, 1143–1149.

Thomas KR and Capecchi MR (1987) Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* **51**, 503–512.

Toledo CM, Ding Y, Hoellerbauer P, Davis RJ, Basom R, Girard EJ, Lee E, Corrin P, Hart T, Bolouri H, Davison J, Zhang Q, Hardcastle J, Aronow BJ, Plaisier CL, Baliga NS, Moffat J, Lin Q, Li XN, Nam DH, Lee J, Pollard SM, Zhu J, Delrow JJ, Clurman BE, Olson JM and Paddison PJ (2015) Genome-wide CRISPR-Cas9 screens reveal loss of redundancy between PKMYT1 and WEE1 in Glioblastoma stem-like cells. *Cell Reports* **13**, 2425–2439.

Truong D-JJ, Kühner K, Kühn R, Werfel S, Engelhardt S, Wurst W and Ortiz O (2015) Development of an intein-mediated split-Cas9 system for gene therapy. *Nucleic Acids Research* **43**, 6450–6458.

Tsai SQ and Joung JK (2016) Defining and improving the genome-wide specificities of CRISPR-Cas9 nucleases. *Nature Reviews Genetics* **17**, 300–312.

Tsai SQ, Wyvekens N, Khayter C, Foden JA, Thapar V, Reyon D, Goodwin MJ, Aryee MJ and Joung JK (2014) Dimeric CRISPR RNA-guided FokI nucleases for highly specific genome editing. *Nature Biotechnology* **32**, 569–576.

Tsai SQ, Zheng Z, Nguyen NT, Liebers M, Topkar VV, Thapar V, Wyvekens N, Khayter C, Iafrate AJ, Le LP, Aryee MJ and Joung JK (2015) GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nature Biotechnology* **33**, 187–197.

Tsai SQ, Nguyen NT, Malagon-Lopez J, Topkar VV, Aryee MJ and Joung JK (2017) CIRCLE-seq: a highly sensitive in vitro screen for genome-wide CRISPR-Cas9 nuclease off-targets. *Nature Methods* **14**, 607–614.

Tso CF, Simon T, Greenlaw AC, Puri T, Mieda M and Herzog ED (2017) Astrocytes regulate daily rhythms in the Suprachiasmatic nucleus and behavior. *Current Biology* **27**, 1055–1061.

Tycko J, Barrera LA, Huston NC, Friedland AE, Wu X, Gootenberg JS, Abudayyeh OO, Myer VE, Wilson CJ and Hsu PD (2018) Pairwise library screen systematically interrogates Staphylococcus aureus Cas9 specificity in human cells. *Nature Communications* **9**, 2962.

Urnov FD, Rebar EJ, Holmes MC, Zhang HS and Gregory PD (2010) Genome editing with engineered zinc finger nucleases. *Nature Reviews Genetics* **11**, 636–646.

Vakulskas CA, Dever DP, Rettig GR, Turk R, Jacobi AM, Collingwood MA, Bode NM, Mcneill MS, Yan S, Camarena J, Lee CM, Park SH, Wiebking V, Bak RO, Gomez-Ospina N, Pavel-Dinu M, Sun W, Bao G, Porteus MH and Behlke MA (2018) A high-fidelity Cas9 mutant delivered as a ribonucleoprotein complex enables efficient gene editing in human hematopoietic stem and progenitor cells. *Nature Medicine* **24**, 1216–1224.

Van Der Oost J, Westra ER, Jackson RN and Wiedenheft B (2014) Unravelling the structural and mechanistic basis of CRISPR-Cas systems. *Nature Reviews Microbiology* **12**, 479–492.

VERTEX (2018a) A Safety and Efficacy Study Evaluating CTX001 in Subjects With Severe Sickle Cell Disease. ClinicalTrial.gov Identifier: NCT03745287, (clinicaltrials.gov/ct2/show/NCT03745287).

VERTEX (2018b) A Safety and Efficacy Study Evaluating CTX001 in Subjects With Transfusion-Dependent β-Thalassemia. ClinicalTrial.gov Identifier: NCT03655678 (clinicaltrials.gov/ct2/show/NCT03655678).

Vestergaard G, Garrett RA and Shah SA (2014) CRISPR adaptive immune systems of Archaea. *RNA Biology* **11**, 156–167.

Villiger L, Grisch-Chan HM, Lindsay H, Ringnalda F, Pogliano CB, Allegri G, Fingerhut R, Haberle J, Matos J, Robinson MD, Thony B and Schwank G (2018) Treatment of a metabolic liver disease by in vivo genome base editing in adult mice. *Nature Medicine* **24**, 1519–1525.

Vinayak S, Pawlowic MC, Sateriale A and Brooks CF (2015) Genetic modification of the diarrhoeal pathogen cryptosporidium parvum. *Nature* **523**, 477–480.

Vojta A, Dobrinić P, Tadić V, Bočkor L, Korać P, Julg B, Klasić M and Zoldoš V (2016) Repurposing the CRISPR-Cas9 system for targeted DNA methylation. *Nucleic Acids Research* **44**, 5615–5628.

Voytas DF and Gao C (2014) Precision genome engineering and agriculture: opportunities and regulatory challenges. *PLoS Biology* **12**, e1001877.

Wang H, Yang H, Shivalila CS, Dawlaty MM, Cheng AW, Zhang F and Jaenisch R (2013) One-Step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* **153**, 910–918.

Wang T, Wei JJ, Sabatini DM and Lander ES (2014) Genetic screens in human cells using the CRISPR-Cas9 system. *Science* **343**, 80–84.

Wang T, Birsoy K, Hughes NW, Krupczak KM, Post Y, Wei JJ, Lander ES and Sabatini DM (2015) Identification and characterization of essential genes in the human genome. *Science* **350**, 1096–1101.

Wang H, Xu X, Nguyen CM, Liu Y, Gao Y, Lin X, Daley T, Kipniss NH, La Russa M and Qi LS (2018) CRISPR-mediated programmable 3D genome positioning and nuclear organization. *Cell* **175**, 1405–1417.

Wang B, Wang M, Zhang W, Xiao T, Chen CH, Wu A, Wu F, Traugh N, Wang X, Li Z, Mei S, Cui Y, Shi S, Lipp JJ, Hinterdorfer M, Zuber J, Brown M, Li W and Liu XS (2019) Integrative analysis of pooled CRISPR genetic screens using MAGeCKFlute. *Nat Protoc* **14**, 756–780.

Wan H, Feng C, Teng F, Yang S, Hu B, Niu Y, Xiang AP, Fang W, Ji W, Li W, Zhao X and Zhou Q (2015) One-step generation of p53 gene biallelic mutant Cynomolgus monkey via the CRISPR/Cas system. *Cell Research* **25**, 258–261.

Watters KE, Fellmann C, Bai HB, Ren SM and Doudna JA (2018) Systematic discovery of natural CRISPR-Cas12a inhibitors. *Science* **362**, 236–239.

Winter J, Breinig M, Heigwer F, Brugemann D, Leible S, Pelz O, Zhan T and Boutros M (2016) Carpools: an R package for exploratory data analysis and documentation of pooled CRISPR/Cas9 screens. *Bioinformatics* **32**, 632–634.

Wong AS, Choi GC, Cui CH, Pregernig G, Milani P, Adam M, Perli SD, Kazer SW, Gaillard A, Hermann M, Shalek AK, Fraenkel E and Lu TK (2016) Multiplexed barcoded CRISPR-Cas9 screening enabled by CombiGEM. *Proceedings of the National Academy of Sciences of the United States of America* **113**, 2544–2549.

Woo JW, Kim J, Kwon SI, Corvalan C, Cho SW, Kim H, Kim SG, Kim ST, Choe S and Kim JS (2015) DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. *Nature Biotechnology* **33**, 1162–1164.

Wright AV, Sternberg SH, Taylor DW, Staahl BT, Bardales JA, Kornfeld JE and Doudna JA (2015) Rational design of a split-Cas9 enzyme complex. *Proceedings of the National Academy of Sciences of the United States of America* **112**, 2984–2989.

Wu Y, Liang D, Wang Y, Bai M, Tang W, Bao S, Yan Z, Li D and Li J (2013) Correction of a genetic disease in mouse via use of CRISPR-Cas9. *Cell Stem Cell* **13**, 659–662.

Wu D, Guan X, Zhu Y, Ren K and Huang Z (2017) Structural basis of stringent PAM recognition by CRISPR-C2c1 in complex with sgRNA. *Cell Research* **27**, 705–708.

Xiong X, Chen M, Lim WA, Zhao D and Qi LS (2016) CRISPR/cas9 for human genome engineering and disease research. *Annual Review of Genomics and Human Genetics* **17**, 131–154.

Xu R, Li H, Qin R, Wang L, Li L, Wei P and Yang J (2014) Gene targeting using the Agrobacterium tumefaciens-mediated CRISPR-Cas system in rice. *Rice (N Y)* **7**, 5.

Xu X, Tao Y, Gao X, Zhang L, Li X, Zou W, Ruan K, Wang F, Xu G-L and Hu R (2016) A CRISPR-based approach for targeted DNA demethylation. *Cell Discovery* **2**, 16009.

Yamada M, Watanabe Y, Gootenberg JS, Hirano H, Ran FA, Nakane T, Ishitani R, Zhang F, Nishimasu H and Nureki O (2017) Crystal structure of the minimal Cas9 from Campylobacter jejuni reveals the molecular diversity in the CRISPR-Cas9 systems. *Molecular Cell* **65**, 1109–1121.

Yamano T, Nishimasu H, Zetsche B, Hirano H, Slaymaker IM, Li Y, Fedorova I, Nakane T, Makarova KS, Koonin EV, Ishitani R, Zhang F and Nureki O (2016) Crystal structure of Cpf1 in complex with guide RNA and target DNA. *Cell* **165**, 949–962.

Yamasaki T, Hoyos-Ramirez E, Martenson JS, Morimoto-Tomita M and Tomita S (2017) GARLH family proteins stabilize GABAA receptors at synapses. *Neuron* **93**, 1138–1152.

Yang H, Wang H, Shivalila CS, Cheng AW, Shi L and Jaenisch R (2013) One-Step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. *Cell* **154**, 1370–1379.

Yang L, Guell M, Niu D, George H, Lesha E, Grishin D, Aach J, Shrock E, Xu W, Poci J, Cortazio R, Wilkinson RA, Fishman JA and Church G (2015) Genome-wide inactivation of porcine endogenous retroviruses (PERVs). *Science* **350**, 1101–1104.

Yang H, Gao P, Rajashankar KR and Patel DJ (2016) PAM-dependent target DNA recognition and cleavage by C2c1 CRISPR-Cas endonuclease. *Cell* **167**, 1814–1828.

Yan WX, Mirzazadeh R, Garnerone S, Scott D, Schneider MW, Kallas T, Custodio J, Wernersson E, Li Y, Gao L, Federova Y, Zetsche B, Zhang F, Bienko M and Crosetto N (2017) BLISS is a versatile and quantitative method for genome-wide profiling of DNA double-strand breaks. *Nature Communications* **8**, 15058.

Yan S, Tu Z, Liu Z, Fan N, Yang H, Yang S, Yang W, Zhao Y, Ouyang Z, Lai C, Yang H, Li L, Liu Q, Shi H, Xu G, Zhao H, Wei H, Pei Z, Li S, Lai L and Li XJ (2018a) A Huntingtin Knockin Pig model recapitulates features of selective neurodegeneration in Huntington's disease. *Cell* **173**, 989–1002.

Yan WX, Chong S, Zhang H, Makarova KS, Koonin EV, Cheng DR and Scott DA (2018b) Cas13d Is a compact RNA-targeting type VI CRISPR effector positively modulated by a WYL-domain-containing accessory protein. *Molecular Cell* **70**, 327–339.

Yan WX, Hunnewell P, Alfonse LE, Carte JM, Keston-Smith E, Sothiselvam S, Garrity AJ, Chong S, Makarova KS, Koonin EV, Cheng DR and Scott DA (2019) Functionally diverse type V CRISPR-Cas systems. *Science* **363**, 88–91.

Zaidi SS-E-A, Mahfouz MM and Mansoor S (2017) CRISPR-Cpf1: a new tool for plant genome editing. *Trends in Plant Science* **22**, 550–553.

Zalatan JG, Lee ME, Almeida R, Gilbert LA, Whitehead EH, La Russa M, Tsai JC, Weissman JS, Dueber JE, Qi LS and Lim WA (2015) Engineering complex synthetic transcriptional programs with CRISPR RNA scaffolds. *Cell* **160**, 339–350.

Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, Volz SE, Joung J, Van Der Oost J, Regev A, Koonin EV and Zhang F (2015a) Cpf1 Is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* **163**, 759–771.

Zetsche B, Volz SE and Zhang F (2015b) A split-Cas9 architecture for inducible genome editing and transcription modulation. *Nature Biotechnology* **33**, 139–142.

Zetsche B, Heidenreich M, Mohanraju P, Fedorova I, Kneppers J, Degennaro EM, Winblad N, Choudhury SR, Abudayyeh OO, Gootenberg JS, Wu WY, Scott DA, Severinov K, Van Der Oost J and Zhang F (2016) Multiplex gene editing by CRISPR–Cpf1 using a single crRNA array. *Nature Biotechnology* **35**, 31–34.

Zetsche B, Strecker J, Abudayyeh OO, Gootenberg JS, Scott DA and Zhang F (2017) A survey of genome editing activity for 16 Cpf1 orthologs. *bioRxiv.* https://doi.org/10.1101/134015.

Zhang F, Wang LP, Brauner M, Liewald JF, Kay K, Watzke N, Wood PG, Bamberg E, Nagel G, Gottschalk A and Deisseroth K (2007) Multimodal fast optical interrogation of neural circuitry. *Nature* **446**, 633–639.

Zhang F, Cong L, Lodato S, Kosuri S, Church GM and Arlotta P (2011) Efficient construction of sequence-specific TAL effectors for modulating mammalian transcription. *Nature Biotechnology* **29**, 149–153.

Zhang Y, Heidrich N, Ampattu BJ, Gunderson CW, Seifert HS, Schoen C, Vogel J and Sontheimer EJ (2013) Processing-independent CRISPR RNAs limit natural transformation in Neisseria meningitidis. *Molecular Cell* **50**, 488–503.

Zhang H, Zhang J, Wei P, Zhang B, Gou F, Feng Z, Mao Y, Yang L, Zhang H, Xu N and Zhu JK (2014) The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. *Plant Biotechnology Journal* **12**, 797–807.

Zhang R, Miner JJ, Gorman MJ, Rausch K, Ramage H, White JP, Zuiani A, Zhang P, Fernandez E, Zhang Q, Dowd KA, Pierson TC, Cherry S and Diamond MS (2016) A CRISPR screen defines a signal peptide processing pathway required by flaviviruses. *Nature* **535**, 164–172.

Zhang S, Takaku M, Zou L, Gu AD, Chou WC, Zhang G, Wu B, Kong Q, Thomas SY, Serody JS, Chen X, Xu X, Wade PA, Cook DN, Ting JPY and Wan YY (2017) Reversing SKI-SMAD4-mediated suppression is essential for TH17 cell differentiation. *Nature* **551**, 105–109.

Zhang B, Ye W, Ye Y, Zhou H, Saeed AFUH, Chen J, Lin J, Perčulija V, Chen Q, Chen C-J, Chang M-X, Choudhary MI and Ouyang S (2018a) Structural insights into Cas13b-guided CRISPR RNA maturation and recognition. *Cell Research* **28**, 1198–1201.

Zhang C, Konermann S, Brideau NJ, Lotfy P, Wu X, Novick SJ, Strutzenberg T, Griffin PR, Hsu PD and Lyumkis D (2018b) Structural basis for the RNA-guided ribonuclease activity of CRISPR-Cas13d. *Cell* **175**, 212–223.

Zhou Y, Zhu S, Cai C, Yuan P, Li C, Huang Y and Wei W (2014) High-throughput screening of a CRISPR/Cas9 library for functional genomics in human cells. *Nature* **509**, 487–491.

Zhu S, Li W, Liu J, Chen C-H, Liao Q, Xu P, Xu H, Xiao T, Cao Z, Peng J, Yuan P, Brown M, Liu XS and Wei W (2016) Genome-scale deletion screening of human long non-coding RNAs using a paired-guide RNA CRISPR-Cas9 library. *Nature Biotechnology* **34**, 1279–1286.

Zong Y, Wang Y, Li C, Zhang R, Chen K, Ran Y, Qiu JL, Wang D and Gao C (2017) Precise base editing in rice, wheat and maize with a Cas9-cytidine deaminase fusion. *Nature Biotechnology* **35**, 438–440.

Zuo E, Sun Y, Wei W, Yuan T, Ying W, Sun H, Yuan L, Steinmetz LM, Li Y and Yang H (2019) Cytosine base editor generates substantial off-target single-nucleotide variants in mouse embryos. *Science* **364**, 289–292.