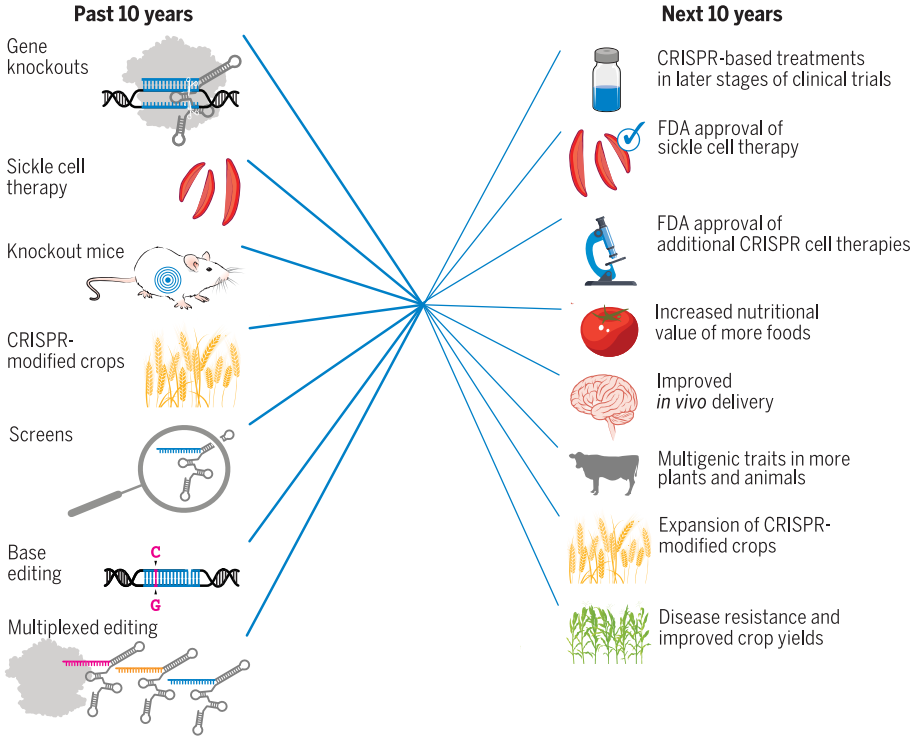
REVIEW SUMMARY

CRISPR

CRISPR technology: A decade of genome editing is only the beginning

Joy Y. Wang and Jennifer A. Doudna*

BACKGROUND: The fields of molecular biology, genetics, and genomics are at a critical juncture—a moment in history when a convergence of knowledge and methods has made it both technically possible and incredibly useful to edit specific base pairs or segments of DNA in cells and living organisms. The advent of clustered regularly interspaced short palindromic repeat (CRISPR) genome editing, coupled with advances in computing and imaging capabilities, has initiated a new era in which we can not only diagnose human diseases and even predict individual susceptibility based on personal genetics but also act on that information. Likewise, we can both identify and rapidly alter genes responsible for plant traits, transforming the pace of agricultural research and plant breeding. The applications of this technology convergence are profound and far reaching—and they are happening now. In the decade since the publication of CRISPR-Cas9 as a genome editing technology, the CRISPR toolbox and its applications have profoundly changed biological research, impacting not only patients with genetic diseases but also agricultural practices and products. As a specific example from the field of genomic medicine, it has become feasible to obtain a complete sequence of the human genome in less than 24 hours—a staggering advance considering the first such sequence took 5 years to generate. Notably, designing and putting to use a potent CRISPR genome editor to obtain clinically actionable information from that genome—previously a near-intractable challenge—now takes only a matter of days. For additional background and related topics, we refer readers to in-depth reviews of the microbiology and structural biology of CRISPR systems and to articles about the considerable ethical and societal challenges of this technology.



CRISPR: past, present, and future. The past decade of CRISPR technology has focused on building the platforms for generating gene knockouts, creating knockout mice and other animal models, genetic screening, and multiplexed editing. CRISPR's applications in medicine and agriculture are already beginning and will serve as the focus for the next decade as society's demands drive further innovation in CRISPR technology.

ADVANCES: The past decade has witnessed the discovery, engineering, and deployment of RNA-programmed genome editors across many applications. By leveraging CRISPR-Cas9's most fundamental activity to create a targeted genetic disruption in a gene or gene regulatory element, scientists have built successful platforms for the rapid creation of knockout mice and other animal models, genetic screening, and multiplexed editing. Beyond traditional CRISPR-Cas9–induced knockouts, base editing—a technology utilizing engineered Cas9's fused to enzymes that alter the chemical nature of DNA bases—has also provided a highly useful strategy to generate site-specific and precise point mutations. Over the past decade, scientists have utilized CRISPR technology as a readily adaptable tool to probe biological function, dissect genetic interactions, and inform strategies to combat human diseases and engineer crops. This Review covers the origins and successes of CRISPR-based genome editing and discusses the most pressing challenges, which include improving editing accuracy and precision, implementing strategies for precise programmable genetic sequence insertions, improving targeted delivery of CRISPR editors, and increasing access and affordability. We examine current efforts addressing these challenges, including emerging gene insertion technologies and new delivery modalities, and describe where further innovation and engineering are needed. CRISPR genome editors are already being deployed in medicine and agriculture, and this Review highlights key examples, including a CRISPR-based therapy treating sickle cell disease, a more nutritious CRISPR-edited tomato, and a high-yield, disease-resistant CRISPR-edited wheat, to illustrate CRISPR's current and potential future impacts in society.

OUTLOOK: In the decade ahead, genome editing research and applications will continue to expand and will intersect with advances in technologies, such as machine learning, live cell imaging, and sequencing. A combination of discovery and engineering will diversify and refine the CRISPR toolbox to combat current challenges and enable more wide-ranging applications in both fundamental and applied research. Just as during the advent of CRISPR genome editing, a combination of scientific curiosity and the desire to benefit society will drive the next decade of innovation in CRISPR technology. ■

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Cite this article as J. Y. Wang, J. A. Doudna, *Science* 379, eadd8643 (2023). DOI: 10.1126/science.add8643

REVIEW

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CRISPR technology: A decade of genome editing is only the beginning

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The advent of clustered regularly interspaced short palindromic repeat (CRISPR) genome editing, coupled with advances in computing and imaging capabilities, has initiated a new era in which genetic diseases and individual disease susceptibilities are both predictable and actionable. Likewise, genes responsible for plant traits can be identified and altered quickly, transforming the pace of agricultural research and plant breeding. In this Review, we discuss the current state of CRISPR-mediated genetic manipulation in human cells, animals, and plants along with relevant successes and challenges and present a roadmap for the future of this technology.

Starting with a 1987 report about repetitive DNA sequences in a bacterial genome (*1*), a small set of researchers working in the fields of microbiology and food science began studying mysterious DNA sequence arrays known as clustered regularly interspaced short palindromic repeats (CRISPRs), commonly found in microbial genomes together with genes encoding CRISPR-associated (Cas) proteins. The presence of short DNA sequences within CRISPRs matching those in viruses hinted at the function of these systems as adaptive immunity pathways used to prevent viral infection (Fig. 1A) (*2*). Curiosity-driven research ultimately showed how CRISPR systems use RNA molecules transcribed from the sequence arrays to guide Cas proteins to cut, and thereby destroy, viral DNA or RNA (*3*, *4*). Furthermore, this line of research showed how CRISPR's RNA-programmed cutting action (*5*, *6*) could be used to alter DNA sequences in any cell with unprecedented ease [reviewed in (*7*)]. Over the past decade, scientists around the world have rapidly adapted CRISPR to enable both fundamental research and wide-ranging applications in animals, plants, and humans.

The most widely used genome editor is the CRISPR-Cas9 protein complexed with its partner RNA (Fig. 1B). The power of CRISPR as a genome editing technology stems from its chemical mechanism of DNA cutting at a site dictated by RNA-determined sequence recognition. Because Cas proteins use RNA-DNA base pairings for DNA recognition, the same protein, such as Cas9, can target a wide range of DNA sequences by simply swapping guide RNAs (Fig. 1B). In eukaryotic cells, DNA breaks are efficiently repaired, enabling targeted changes to DNA sequences at will (Fig. 1B). Mutating the amino acids required for cleavage activity in the Cas9 active sites allows targeted DNA nicking (introducing a single-stranded DNA cut) or DNA binding by a catalytically inactive Cas9. As a result, the first examples of engineered CRISPR-Cas involved transcriptional repression or activation to silence or up-regulate specific genes (*8*, *9*). Other forms of engineered Cas9 are fused to enzymes that enable individual nucleobase editing, chromatin modification, or sequence insertion (*10*–*13*). Other Cas proteins, including RNA-targeting proteins, have been explored as genome-modifying tools, enabled by discovery efforts and extensive biochemical and structural characterization [reviewed in (*14*–*17*)]. Some of these enzymes have also been harnessed for the development of imaging methods (*18*–*20*) and diagnostic approaches (*21*, *22*).

Together, applications of CRISPR technology have provided the foundation for clinical trials of therapies to treat sickle cell disease, beta-thalassemia, the degenerative disease transthyretin (TTR) amyloidosis, and congenital eye disease, as well as planned clinical trials for both rare (progeria, severe combined immunodeficiency, familial hypercholesterolemia) and common (cancer, HIV infection) diseases. CRISPR technology has enabled agricultural advances including slick-coat cattle and a more nutritious tomato. It has spurred research across fields of molecular and cell biology, fueling the publication of thousands of research articles and providing a tool base for many companies focused on therapeutics, agriculture, and synthetic biology. However, CRISPR technology and its potential impact are still in their early stages. As we discuss in the next section, some genome editing applications have now become routine whereas others remain difficult due to limitations of today's tools. These genome editing challenges provide opportunities for new discoveries and engineering to advance the field by offering a more complete toolbox for genetic manipulation.

CRISPR-induced gene knockouts

The past decade has witnessed the astounding success of CRISPR-induced gene knockouts, which have transformed basic and translational research and demonstrate tremendous potential in agriculture and therapeutic development. Traditional CRISPR-induced knockout methods in eukaryotic cells involve the CRISPR-Cas9 ribonucleoprotein (RNP), composed of the Cas9 nuclease and an engineered single-guide RNA molecule (sgRNA) (*23*–*26*). The sgRNA directs Cas9 to the target site, where it creates a double-stranded DNA break (DSB) that is repaired by endogenous repair pathways including the nonhomologous end joining (NHEJ) and the microhomology-mediated end joining pathways and the more precise homology-directed repair (HDR) pathway that uses a repair template [reviewed in (*27*–*29*)] (Fig. 2A). Because of the high targeting specificity and efficacy of CRISPR-Cas9, such gene knockouts are now routine in research applications, providing a streamlined process to disrupt genes for functional study.

CRISPR-Cas9 has proven to be successful in enabling the rapid creation of knockout (KO) mice and other animal models (*30*, *31*) (Fig. 2B). Traditional gene targeting methods used inefficient homologous recombination in embryonic stem (ES) cells, followed by laborious screening of modified ES cells for the desired sequence change and injection into WT embryos (*32*, *33*). CRISPR-Cas9 provides a way to introduce DSBs in a one-cell-stage embryo, bypassing the screening stage of suitable targeted ES cells and greatly simplifying the production of gene-edited animals (*34*, *35*). This has reduced the time needed for generating genetically modified mice, from 1 year to as few as 4 weeks (*36*). As a result, the production of KO and transgenic mice has now become routine for research applications. Additionally, because most mammalian species lack established ES cell lines, CRISPR-Cas9 editing has facilitated the development of genetically engineered animal models in new species (*37*, *38*). The generation of KO and transgenic animal models has become even more efficient with advancements in strategies for introducing CRISPR-Cas9 components into zygotes, including CRISPR RNP electroporation of zygotes (CRISPR-EZ) (*39*), CRISPR RNP electroporation and AAV donor infection (CRISPR-READI) (*40*), and improved genome editing through oviductal delivery of

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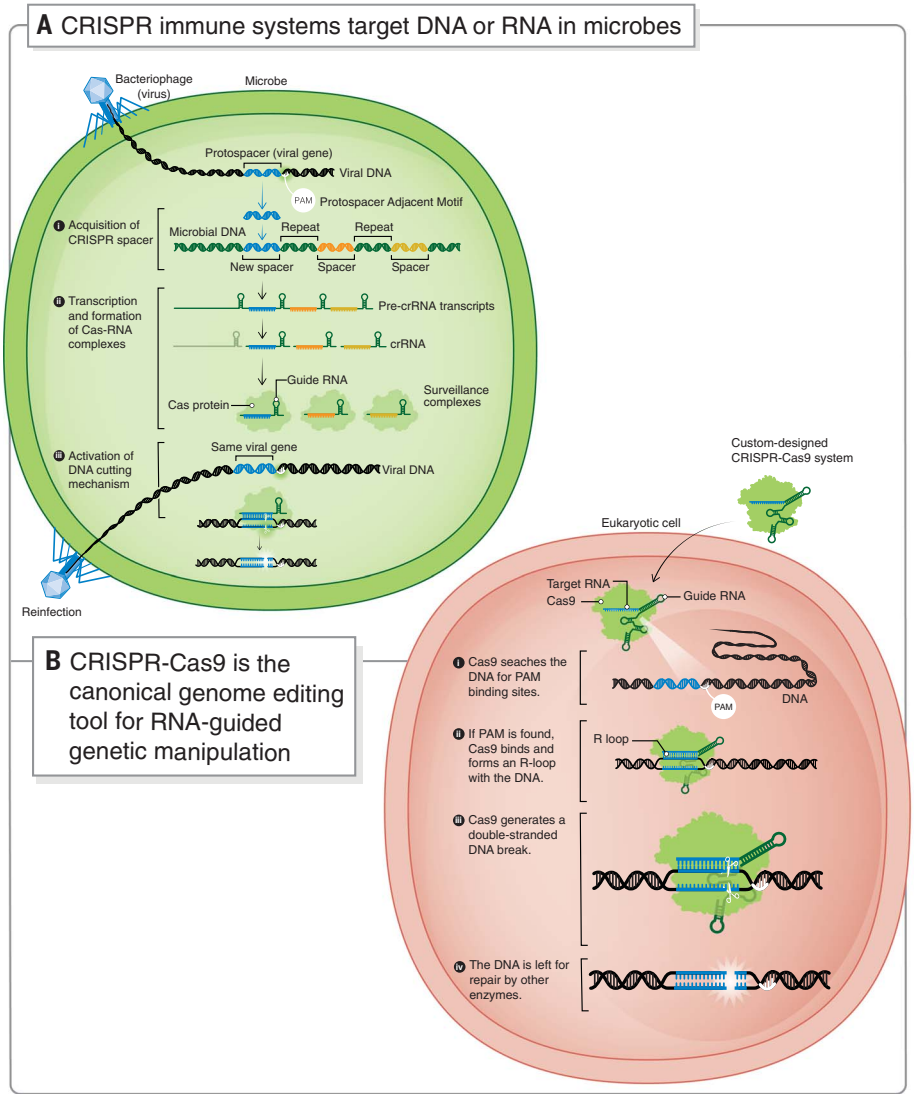

Fig. 1. CRISPR-based adaptive immunity provides programmable genome editing tools. (**A**) CRISPR immune systems target DNA or RNA in microbes (illustration depicts DNA targeting). Three steps to immunity include: (i) acquisition of CRISPR spacer sequence matching an infectious agent; (ii) transcription and formation of Cas-RNA complexes; (iii) seek-and-destroy surveillance mechanisms. (**B**) CRISPR-Cas9 is the canonical genome editing tool for RNA-guided genetic manipulation. Cas9 searches for target sites in a genome by engaging with PAM sequences, forming an R-loop with complementary DNA, generating a double-strand DNA (dsDNA) break, and finally releasing DNA for repair.

nucleic acids (i-GONAD) (*41*). Beyond germline editing, CRISPR-Cas9 is also used in somatic editing, which is useful in situations where whole-body knockouts are lethal for embryos and in many cases can more accurately model cancer progression and realistic modes of therapeutic treatment (*42*). Advancements in in vivo delivery strategies (discussed later in this review) have expanded the types of somatic animal models that can be created. Through CRISPR-genome engineering methods, animal models have been developed for many diseases, including tyrosinemia, Duchenne muscular dystrophy, cancer, osteoporosis, Huntington's disease, amyotrophic lateral sclerosis, Alzheimer's disease, and HIV-1/AIDS as just a few examples [reviewed in (*38*)]. The ability to rapidly create animal models has advanced and will continue to advance the study of genetic diseases, allowing researchers to study the causal relationships of specific genetic variations and disease and to develop and test new treatments for these diseases.

CRISPR screens

With the ease of introducing CRISPR-induced gene knockouts, researchers have successfully applied this technology to genetic screens, i.e., the systematic, targeted genetic alteration of a number of genes in parallel. Such CRISPR screens have become a powerful approach for understanding genetic interactions and dissecting biological pathways and have given rise to major advances in target discovery and drug development. The capabilities of CRISPR screens are continuing to expand, especially when combined with advancements in single-cell multiomics technologies. In general, genetic screens involve one or multiple gene perturbations, a model system such as engineered human cells, and a selection assay or readout to evaluate the effects of the perturbation(s) (*43*, *44*) (Fig. 2C). Because of its efficiency and flexibility, CRISPR editing is a powerful strategy for introducing perturbations that can be used for close study of how a single gene disruption affects a cell of interest, as well as high-throughput testing of thousands of perturbations in pooled screens (*45*, *46*). The ease of designing and cloning guide RNA (gRNAs) has enabled the development of gRNA libraries up to genome-wide scales, allowing researchers to perturb every gene in the human genome (*47*, *48*). CRISPR technology advancements have also expanded the types of CRISPR screens that researchers can use for different applications. Beyond CRISPR KO screens, CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa) screens have also become popular approaches that use reversible gene expression control [reviewed in (*49*, *50*)]. Saturation genome editing utilizing Cas9-mediated HDR enables the generation of all possible single-nucleotide polymorphisms (SNPs) for functional screening (*51*–*53*). More recently, as alternatives to Cas9-mediated HDR, researchers have also started applying CRISPR base and prime editing (discussed later in the Review) for genetic screens (*54*–*56*). Base editing, which can introduce point mutations more efficiently than Cas9-mediated HDR with minimal indel formation, may serve as an improved strategy for functional variant screening (*54*). Prime editing allows for the introduction of small insertions and deletions in addition to point mutations, which essentially enables saturation mutagenesis across residues. This technology is still relatively new, and it remains to be seen whether prime editing screens can achieve similar levels of flexibility as HDR with regard to target selection and targetable window size (*56*).

The successful editing of diverse cells and organisms using CRISPR technologies provides flexibility for choosing a model system for the genetic screen to best answer the relevant biological question. Beyond primary cells, CRISPR screens have been developed in more complex model systems including organoids, animals, and plants (*57*–*60*). CRISPR screens are now a common method for probing gene function in cancer and have allowed for identification of a variety of cancer drivers and regulators [reviewed in (*45*, *46*)].

Following the introduction of CRISPR components into the model, a variety of techniques

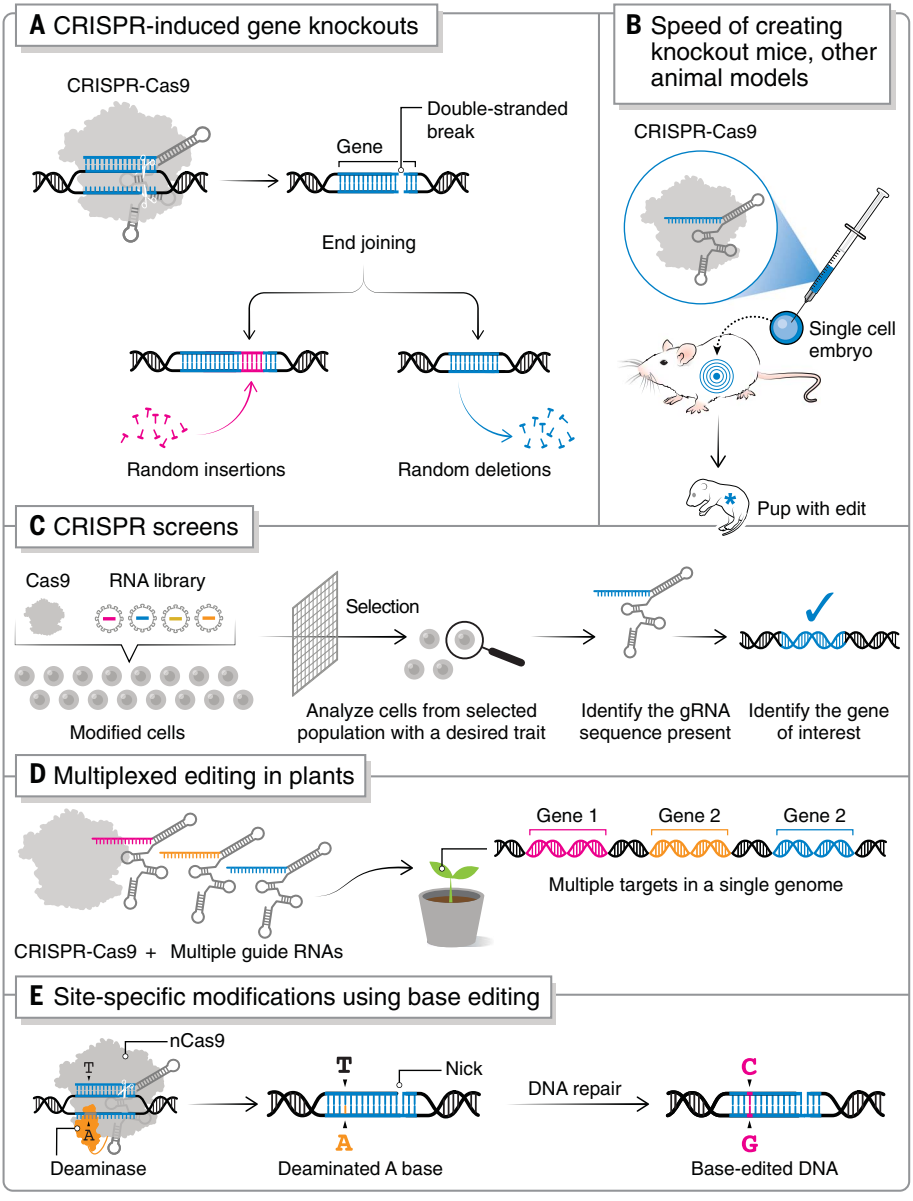

Fig. 2. The genome editing toolbox, part 1: what works well. (**A**) CRISPR-induced gene knockouts in eukaryotic cells result from the DNA DSB, created by the Cas9 RNP and then commonly repaired by endogenous end joining repair pathways. (**B**) The speed of creating KO mice and other animal models has greatly increased with the development of CRISPR-Cas9 editing technology, which can edit single-cell embryos to generate gene-modified mice. (**C**) CRISPR screens are utilized for functional genetic screening. CRISPR-Cas9 editors introduce genetic perturbations into a model, which is subjected to a selection assay, followed by a readout to evaluate the effects of the perturbations. (**D**) CRISPR-Cas9 is a platform for multiplexed editing in plants. Multiple gRNAs can be used with Cas9 to simultaneously edit multiple targets in a genome. (**E**) CRISPR base editors, typically composed of nCas9 or dCas9 fused to a deaminase, enable site-specific modifications without DSBs.

can be used for the selection assay and readout. Common selection strategies include viability- or proliferation-based screens, fluorescence-activated cell sorting or microfluidics-assisted cell screening-based screens using cell surface proteins as markers (i.e., PD1, PDL1, MHC), and in vivo screens assaying phenotypes, such as tumor growth or sensitivity/resistance to immunotherapy (*45*, *46*, *61*). One exciting area of development in the readout of CRISPR screens is new methods that provide simultaneous proteomic, epigenetic, and/or transcriptomic analyses, such as Procode (*62*), Perturb-ATAC (*63*), Perturb-seq (*64*), and ECCITE-seq (*65*), which can provide a wealth of information. The successes of CRISPR screens will continue to accelerate with new technologies that improve the sensitivity of these assays and readouts. We are only beginning to see the impact of combining CRISPR screens and single-cell multiomics modalities with the rapidly advancing infrastructure for big data collection and analysis (*64*, *66*, *67*). Further discovery and engineering, including the enhancement of orthologous Cas9 enzymes or other RNA-guided nucleases such as Cas12a [reviewed in (*7*)], can also greatly increase the potential of combinatorial or multiplex CRISPR screens, which can reveal novel and complex genetic interactions.

Multiplexed editing in plants and beyond

Multiplex genome editing, or the simultaneous targeting of multiple specific DNA loci in a genome, represents another area where CRISPR-induced gene knockout technology has been scaled up and adapted into a successful platform—particularly in the plant science fields [reviewed in (*68*, *69*)] (Fig. 2D). Over the past decade, CRISPR-Cas9 has become a popular tool for plant editing. Traditional crop trait engineering methods involved random mutagenesis (e.g., with radiation) or transgenesis with Agrobacterium followed by laborious crossing and screening to identify a plant with a new trait of interest. These processes are lengthy and hard to control in addition to facing substantial regulatory hurdles. By contrast, CRISPR-induced modifications are targeted, can be made rapidly, and generally represent small indels, i.e., insertions and/or deletions, or point mutations at locations specified by the trait engineer [reviewed in (*70*)]. CRISPR-Cas9 has been adapted for simultaneous multilocus editing, which is especially useful for editing crop species that can carry multiple copies of the target gene (e.g., hexaploid wheat) (*71*) and for crop domestication, which involves targeting multiple different genes [reviewed in (*72*)]. One advantage of CRISPR-Cas9 in regards to multiplexed editing is separation of the nuclease and gRNA, such that multiple gRNAs can be used with one Cas protein to edit different targets (*23*). The gRNAs can be provided as multiple individual expression cassettes, each transcribed from their own promoters (*73*–*75*), or as a single polycistronic cassette that is processed posttranscriptionally (*76*–*78*). In the past few years, CRISPR multiplex genome editing has become a successful strategy for creating new crop genotypes and agriculturally useful traits in a single generation. One area where multiplexed CRISPR-Cas9 editing has achieved successful results is in crop domestication and improvement. Examples include the use of multiplexed editing to disrupt domestication genes, introduce characteristics such as herbicide resistance, and increase crop yield and quality (*68*, *79*–*81*).

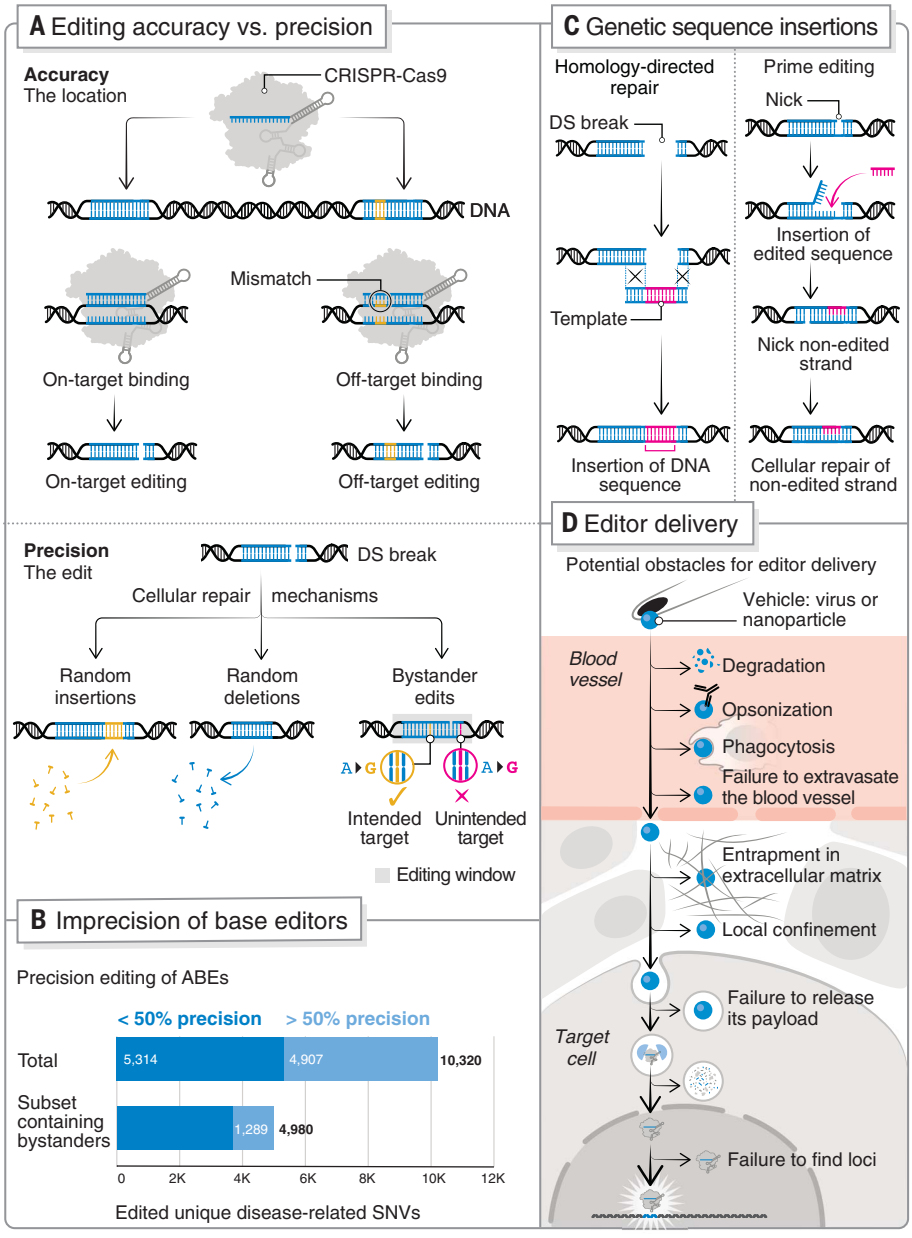

Fig. 3. The genome editing toolbox, part 2: challenges and emerging tools. (**A**) Editing accuracy versus editing precision. Editing accuracy involves specificity for the target location, which is challenged by off-target binding by the Cas9 RNP. Editing precision involves producing the correct intended edit with no unintended edits and is challenged by undesired indels and bystander edits. (**B**) Imprecision of correcting most SNPs using current base editors [data from (*130*), chart contributed by P. H. Yoon]. Correction precision is defined as the fraction of edited reads in their library of disease-associated SNVs correctable by adenine base editors that consists of an exact single-nucleotide correction (*130*). (**C**) Genetic sequence insertions by HDR-mediated CRISPR-Cas9 editing (left) and by prime editing (right) (*195*). (**D**) Challenges of gene editor delivery, including potential biological obstacles that can prevent the gene editor from reaching its target site.

Beyond its success in plants, multiplexed CRISPR-Cas9 editing has been extended to other cell types and organisms. One notable example is the use of multiplexed editing to generate porcine endogenous retrovirus-inactivated pigs, addressing a major safety concern of transplanting pig organs into humans (*82*). Multiplexed editing has the potential to be particularly useful in engineering cell therapy products for cancer and studying the effects of complex polygenic diseases. However, a challenge of CRISPR-Cas9 editing in mammalian cells that is exacerbated by simultaneous DNA cleavages in multiplexed editing is the possibility of triggering DNA damage-response mechanisms governed by transcription factor p53 that can lead to cellular senescence or apoptosis (*82*–*84*). Addressing this challenge and extending the applications of multiplexed editing may become easier with the advancement of newer CRISPR precision editing technologies that do not involve DSBs, including base editing and prime editing (discussed further below), which have already been adapted for multiplexed editing (*85*, *86*).

Site-specific modifications using base editing

Beyond traditional CRISPR-induced knockouts involving double-stranded DNA cutting, base editing (*11*)—which uses Cas effectors fused to enzymes that alter the chemical nature of DNA bases—has provided a successful strategy to generate site-specific and precise point mutations without DSBs, eliminating the need for repair templates and limiting undesired byproducts during editing (Fig. 2E). CRISPR base editors generally consist of fusions between a Cas9 nickase (nCas9), a Cas9 variant that produces a single-stranded rather than a double-stranded break (or a catalytically inactive or "dead" Cas protein, such as dCas9, dCas12a, or dCas13b), and an enzyme that catalyzes a nucleobase deamination reaction [reviewed in (*87*, *88*)]. The sgRNA directs the nCas9-deaminase fusion to the genomic target, where ternary complex formation exposes a region of ssDNA to the deaminase for chemical modification. The resulting base mismatch is then resolved through cellular repair mechanisms. Over the past few years, the toolbox of DNA and RNA base editors has expanded to enable C>T, A>G, C>G, A>I, and C>U conversions (*10*, *11*, *89*–*93*), though there is still need for further improvement, especially for C>G editing. Site-specific modifications broaden the abilities of researchers to study the effects of mutations within genes and can treat genetic disorders by correcting point mutations, which represent the largest class of human pathogenic genetic variants (*94*–*97*). Furthermore, base editing can introduce modifications in dividing and nondividing cells, providing an advantage over HDR, which is restricted to dividing cells (*98*). Base editing has already shown promising results in correcting loss-of-function mutations in a number of mouse models [reviewed in (*87*, *88*)], with a notable example being the recent use of in vivo base editing to correct Hutchinson-Gilford progeria syndrome in mice (*99*). By eliminating the introduction of DSBs, the development of base editing technology represents an important step in precision editing. Refining these tools to the levels of accuracy and precision needed for treatments in humans will be one of the main challenges in the next decade. An early-stage clinical trial using base editing for familial hypercholesterolemia has already begun, and others using base editing for sickle cell disease are set to begin this year (*100*).

Editing accuracy and precision

As we transition into the next decade of CRISPR genome editing, several key challenges demand innovative solutions. Two of these are editing accuracy (i.e., specificity for the target site) and precision (i.e., producing the exact desired editing outcome) (Fig. 3A). To reduce the off-target effects of CRISPR-Cas nucleases resulting from unintended binding and cleavage, researchers have utilized a combination of rational design and selection to develop high-fidelity Cas variants [such as SpCas9-HF1 (*101*), evoCas9 (*102*), HiFiCas9 (*103*)] and the Cas9_R63A/Q768A variant (*104*) and guide optimization methods [such as E-Crisp (*105*, *106*), CasOFFinder (*107*), and sgDesigner (*108*)]. These efforts have been productive: Neither the CRISPR Therapeutics/Vertex nor the Intellia sgRNAs used in clinics today have measurable off-target sites using US Food and Drug Administration (FDA)–grade assays (*109*, *110*). However, off-target editing inaccuracies can also occur as a result of the Cas9-independent behavior of effector domains including deaminases, reverse transcriptases, and transcriptional regulators, as exemplified by analysis of base editing outcomes (*111*, *112*). Progress is currently being made through use of high-fidelity Cas variants and rational engineering of the deaminase domain to reduce nucleic acid binding without Cas assistance (*111*–*115*), and early-stage clinical trials of base editing (*31*, *116*) offer encouragement in this regard. At the same time, innovating new methods to deliver Cas editors to the target site and refining existing ones (described further below) can also minimize off-target effects. Editing precision poses a larger challenge. In traditional CRISPR-Cas9 editing in eukaryotic cells, the scientist still does not fully control the editing outcome following the introduction of the DSB. Recent machine learning tools have been developed to help predict repair outcomes, although these have yet to be demonstrated for in vivo applications (*117*–*119*). Following the DSB, NHEJ—the default repair pathway for human cells—competes with the less-efficient HDR pathway and results in a spectrum of indels at the target site (*120*, *121*). Although this may be acceptable for a number of CRISPR-induced knockout applications (including clinical), many therapeutic applications require much higher levels of precision and cannot afford undesired indels. Increasing editing precision requires a better understanding of DNA repair processes and a combination of innovation and engineering. One approach is improving HDR efficiency and/or suppressing NHEJ. Strategies that have been developed include chemically inhibiting key enzymes in the NHEJ pathway, using single-stranded oligodeoxynucleotide templates (which have been shown to increase HDR efficiency to 60% in human cells for a single-nucleotide substitution) (*122*), utilizing cell cycle stage control to favor HDR repair (*123*–*125*) and using site-specific Cas9-oligonucleotide conjugates to recruit the donor DNA template to the target site (*126*). Even with these strategies, there are still risks of large deletions and chromosomal rearrangements associated with DSB formation that can lead to genome instability (*127*, *128*). Base editing and prime editing (addressed further below) represent another approach for precision editing intended to avoid DSB formation. Base editing and prime editing have reduced indel formation compared with classic Cas9-mediated editing. However, in some cases, unintentional DSBs can still form at the editing site and lead to indels. It has been demonstrated that fusing base editors to Gam—a bacteriophage Mu protein that binds DSBs—can minimize indel formation during base editing (*129*). For base editing, editing precision is also challenged by bystander editing (or undesired conversions of neighboring editable bases within or near the editing window in addition to the target base) (*87*, *88*). The correction precision of base editors decreases by a large margin when there is more than one target base in the editing window, limiting their therapeutic potential (*130*) (Fig. 3B). In a recent study, about half of the pathogenic single-nucleotide variants (SNVs) correctable by adenine base editors revealed ≥50% correction precision (*130*) (Fig. 3B). However, of the subset of SNVs containing more than one target base in the editing window, only 26% revealed ≥50% correction precision (Fig. 3B). Unfortunately, with current base editors, bystanders are a fairly common occurrence. In a recent study of 21 different base editing systems, about half of the targetable pathogenic point mutations had bystanders in the editing window (*131*). Reducing the size of the editing window can increase precision; however, this also limits the genomic sites that can be targeted because of PAM constraints. A variety of strategies involving structure-guided mutagenesis, directed evolution, and computational-aided design are currently being employed to increase the targeting scope of CRISPR-Cas9 and reduce the bystander effects of base editors (*132*–*134*). For base editing to be an effective strategy for a wider breadth of applications, further engineering is required to build on current strategies to develop base editors with narrower editing windows and different PAM compatibilities without compromising on efficiency and targeting specificity.

Genetic sequence insertions

In recent years, emerging technologies are expanding the functional capabilities of the CRISPR toolbox to make precise programmable genetic sequence insertions, and an important challenge in the next decade will be refining and effectively implementing these technologies for genome engineering applications. Traditional Cas9 editing can introduce transgenes by relying on HDR to incorporate genetic material from a co-delivered donor template into the target site (Fig. 3C) (*27*). Currently, this approach is being widely used in many areas of genome engineering. A notable recent example of its application is the use of targeted integration to fluorescently tag more than 1000 human proteins to study their localization and interactions (*135*). HDR-mediated CRISPR-Cas9 editing has also shown promising results in preclinical and clinical testing for therapeutic development, with key examples in correcting alpha1 antitrypsin deficiency (*136*) and in cancer immunotherapy (*137*, *138*). Despite these successes, HDR-mediated CRISPR-Cas9 editing has its limitations, including being restricted to dividing cells (*123*), the difficulty of donor template delivery, and the precision-related challenges introduced by the DSB. Although certain single-nucleotide mutations can be addressed by base editing, many human pathogenic genetic variants require a small sequence insertion to repair an indel, calling for high-precision alternatives to HDR-mediated CRISPR-Cas9.

Prime editing represents one such alternative that can insert and delete DNA sequences without introducing DSBs (*12*), though this technology still needs further refinement (Fig. 3C). Prime editors consist of nCas9 fused to a reverse transcriptase (RT) and a prime editing gRNA (pegRNA) that serves both to direct nCas9 to the target site and act as a template containing the desired edit for the RT (*12*). Unlike HDR, prime editing can introduce modifications in both dividing and nondividing cells, which is useful for correcting mutations in quiescent cells, such as neurons or hematopoietic stem cells (*139*). Prime editing also provides advantages over base editing in situations where there are multiple target bases in the editing window (*132*) and where a PAM sequence is not immediately adjacent to the desired editing site (*140*). Currently, prime editing has shown promise as an accurate and fairly precise editing tool that has been demonstrated to work in multiple cell types, organoids, mouse embryos, and plants but is still limited in its applications as a result of low editing efficiency [reviewed in (*139*, *141*)]. In two separate demonstrations of prime editing in organoids and mice, there were no detectable off-target edits (*142*, *143*). Although low levels of undesired indel formation have been reported, the ratio of correct editing to indel formation was ~30 times higher for prime editing than for HDR (*12*, *142*). Unfortunately, prime editing efficiencies are low for many applications. In one study, prime editing was more than 30 times less efficient than HDR in repairing a mutation in intestinal CF organoids (*144*). Although more efficient prime editors have been developed, these

also result in higher rates of indel formation (*12*). Currently, base editors still have an advantage over prime editors in editing efficiency and precision (*139*). A main goal for prime editing in the next decade is improving efficiency without compromising editing product purity—an outcome that has the potential to turn prime editing into one of the most versatile tools for precision editing. Future studies should also address remaining uncertainties about the mechanism of prime editing. Recent results show that physical untethering of Cas9 and the RT has no effect on prime editing levels in cells, suggesting that the RT could engage the editing site without being fused to Cas9 and raising questions about whether it could induce unintended integration at other RNA-DNA hybrid sites (*13*). Refining prime editing tools will require engineering and optimizing the different constituent components, including the pegRNAs (*145*).

For large gene insertions, an emerging area in CRISPR genome engineering is RNA-guided DNA transposition. CRISPR-associated transposons (CASTs) enable the precise RNA-guided integration of large DNA cargo up to 10 kb (*146*–*149*). So far, this has only been demonstrated in a few prokaryotes and has not yet been reported to work in mammalian cells (*146*, *147*, *150*, *151*). There is potential for new developments as this area is still in the early stages of research with limited mechanistic understanding of how these systems work (*15*), and very few computationally predicted CAST systems have been characterized (*149*). Further discovery, testing, and engineering will be required to harness the potential of CASTs for genome engineering applications.

Recombinases, which perform a wide array of activities, including insertions, deletions, inversions, and replacements (*152*, *153*), are another area of tool development with the potential to combine with Cas proteins and may be able to further diversify the CRISPR toolbox (*154*). This has recently been demonstrated with the development of two new approaches that have enabled programmable integration of large DNA sequences in human cells (*155*, *156*). One approach, programmable addition through site-specific targeting elements, uses engineered fusion proteins of Cas9, a reverse transcriptase, and a serine integrase that have enabled multiplexed insertions of large DNA cargo, including the fluorescent tagging of different endogenous genes (*155*). Another approach uses twin prime editing, which involves a prime editor and two prime editing guide RNAs that have enabled large gene insertions and inversions when combined with a site-specific serine recombinase (*156*). This approach was used to correct a large sequence inversion associated with Hunter syndrome in human cells with up to ~9% efficiency (*156*). Notably, these studies report no detectable off-target insertions. These approaches for programmable gene insertions will require further characterization and refinement to increase editing efficiencies and to serve as potential therapeutic strategies. The potential impact of programmable gene insertion for genome engineering will continue to motivate discovery and innovation in search of new strategies in addition to improving existing technologies.

Delivery of editors ex vivo and in vivo

Despite all of the recent advances in CRISPR editors, delivery of editors remains a major bottleneck for genome editing in organisms; both innovation and engineering are needed to ensure high delivery efficiency, target specificity, and safety. Advances in delivery technologies have played a large role in developing CRISPR-based therapeutics. The liver represents a clear example where efficient delivery of CRISPR editors has been a clinically tractable challenge (*110*). However, for less accessible organs, the feasibility of CRISPR therapeutics is limited by low delivery efficiencies and will largely depend on improved delivery strategies. Current delivery strategies for potential CRISPR-based treatments in humans are divided into two types of approaches: ex vivo, where cells are isolated from and modified outside of the patient before being reintroduced, and in vivo, where cells are edited directly in the patient following delivery of CRISPR components [reviewed in (*157*–*160*)]. Ex vivo approaches, often used for editing hematopoietic stem and progenitor cells and leukocytes, offer higher cell-type specificity and tighter quality control of editing; however, they are limited to cell types that can survive and be expanded in culture (to achieve a minimum number for reengraftment) and retain in vivo function. In vivo approaches expand CRISPR editing to cell types where ex vivo approaches are not possible, allowing CRISPR to treat a wider range of genetic diseases. Two notable examples where in vivo delivery has had some success in humans are the treatment of transthyretin amyloidosis, which represents the first systemic in vivo delivery of CRISPR to the liver using targeted lipid nanoparticle (LNP) delivery (*110*), and treatment of Leber congenital amaurosis type 10, which involves direct injection of an adeno-associated viral vector harboring the RNA-guided enzyme into the eye (*161*, *162*).

These successes show the tremendous potential of in vivo therapeutic genome editing; however, in general, in vivo delivery of CRISPR editors remains a formidable challenge. Many biological obstacles stand in the way of effective in vivo delivery of editors to targets. In the case of systemic delivery, delivery vehicles need to prevent degradation of the cargo, opsonization, and phagocytosis extravasate from the blood vessel; pass efficiently through the interstitial space; and effectively release cargo upon endocytosis (Fig. 3D). Once the CRISPR cargo is released, it also needs to localize to the nucleus and access the target locus in the

Box 1. Cost, regulation, and access.

With the growing therapeutic potential of CRISPR technologies, other important considerations include affordability, regulation, and access. For a more in-depth examination of the ethical and societal challenges of these technologies, we refer readers to refs. (*196*, *197*). One main challenge to the development and accessibility of CRISPR therapeutics is cost. In many cases, manufacturing costs encompass the expenses of producing both the CRISPR editors and the delivery vehicles, which can be difficult to scale up. For instance, viral-based delivery is a popular strategy for developing CRISPR therapeutics, but the manufacturing of viral vectors depends on expensive culture systems and facilities to produce required quantities of virus (*175*). Improving process development and providing the infrastructure to scale up the production of viral vectors will be important to reduce costs. Treatment administration can also be expensive, especially for ex vivo approaches, which consume time and resources to expand cells in culture and, in cases of bone marrow transplantation with autologous hematopoietic stem cells, require preconditioning patients with chemotherapy (*198*).

Manufacturers also face the burden of regulatory costs to provide extensive characterization and strict safety and quality controls, which can be challenging for investigational or academic manufacturing facilities. As increasing numbers of CRISPR-based treatments move to later stages of clinical trials, manufacturers will need to build the infrastructure and bear the costs to support a current good manufacturing practice–compliant operation. The challenges of bearing these costs and getting FDA approval can lead to abandonment of the therapeutic development in a for-profit setting, as was recently the case for a gene therapy for adenosine deaminase severe combined immunodeficiency (ADA-SCID) that had promising long-term results (*199*, *200*). Even if a treatment passes through all clinical trial phases and gets FDA approval, the potential retail price charged to cover manufacturing costs may be unaffordable to most patients without changes to the current health care infrastructure. Although many of the costs associated with developing new therapies are unavoidable, the motivation to make future CRISPR therapeutics widely accessible to those who need them will drive innovation of more efficient and cost-effective strategies for large-scale production that can meet regulatory standards.

chromosome. Each of these intermediary steps or requirements between the initial intravenous injection and the actual editing of the target locus has its own set of challenges that will necessitate both engineering and innovation to overcome. One of these is controlling delivery vehicle size (often restricted by cargo size), which may present a challenge for bypassing the vascular endothelium and interstitial space between blood vessels and target cells (*157*). This challenge has motivated efforts to engineer and discover smaller CRISPR editors that can be delivered more efficiently (*163*–*167*). Another challenge is preventing uptake and editing in off-target cells; this could be addressed by utilizing a targeting molecule, such as a single-chain variable fragment or glycoprotein, through conjugation to the CRISPR RNP (*168*). Other approaches include engineering or evolving the delivery vehicle to target specific cells (*160*). An alternative strategy to systemic delivery is direct injection to a specific tissue, which largely avoids editing other unintended tissues and organs. However, direct injection results in genome editing of relatively few cells within a localized space and is practical only for organs directly accessible to such injection (*161*, *169*).

In these various approaches, the cargo is delivered as one of three forms: a plasmid DNA (>6400 kDa for a 10-kb plasmid DNA) encoding the CRISPR-Cas9 and gRNA (either together or separately), the Cas9 mRNA (1400 kDa) and gRNA (34 kDa), or the Cas9-gRNA RNP (194 kDa) [reviewed in (*158*, *170*)]. DNA cargo is relatively stable compared with RNA or protein, but its delivery results in the slowest initiation of editing and offers lower control over the functional RNP concentration in the system at any given time. In some cases, DNA cargo can permit prolonged expression of Cas9, but this increases the probability of off-target effects and immunogenic reactions (*170*, *171*). Of the three, RNP delivery results in the fastest initiation of editing and generally results in lower off-target effects, but methods for delivering RNPs are still limited.

Currently, a variety of delivery methods exist for CRISPR gene editing in mammalian systems, though each has its own set of challenges and limitations. These are broadly divided into physical delivery, viral-based delivery, and synthetic material–based delivery [reviewed in (*158*, *159*)]. Common physical delivery approaches include microinjection and electroporation, which can deliver CRISPR editors in all three forms of cargo (*170*). These methods allow for controlled dosage and high-efficiency delivery; however, both methods are effectively limited to ex vivo delivery. Viral-based delivery methods include adeno-associated viruses (AAVs), adenoviruses (AdVs), and lentiviruses, which deliver CRISPR cargo in the form of plasmid DNA and offer high delivery efficiencies by harnessing delivery vehicles that viruses have had thousands of years to evolve. Of these, AAVs are the most promising for in vivo clinical use for CRISPR therapeutics, with notable examples being an ongoing clinical trial for Leber congenital amaurosis type 10 (*161*), a soon-to-begin trial for HIV (*172*), and advanced preclinical work for Hutchinson–Gilford progeria syndrome (*99*). However, one main limitation of AAVs is their low packaging capacity (*173*). Compared with AAVs, AdVs and lentiviruses offer higher packaging efficiencies and have also been used for CRISPR-Cas9 delivery but have faced other challenges, including immunogenicity concerns [reviewed in (*157*, *159*, *174*)]. Another important consideration for viral-based delivery is the high cost and labor-intensive production, especially for AAVs and AdVs (*175*), which represents a major challenge for large-scale manufacturing and patient treatment (Box 1). Compared with viral-based delivery, synthetic material–based delivery methods, which include LNPs (*176*), cationic polymers and peptides (*177*–*181*), and gold nanoparticles (*182*), are often safer and offer high levels of control and flexibility because they can be more easily tailored to all three forms of cargo (DNA, mRNA, and RNP) and be optimized for immunocompatibility (*157*). Notably, as mentioned above, LNPs are the delivery strategy used in the first systemic in vivo delivery of CRISPR in humans for the successful treatment of transthyretin amyloidosis (*110*). In general, however, these synthetic material–based delivery methods offer lower delivery efficiencies compared with viral-based methods (*159*), limiting their effectiveness for in vivo delivery to less accessible target organs. Although improvements can be made with further optimization, their maximum efficiency may be limited by the materials' bulky size and cationic nature, which result in poor interstitial dispersion. Recently, extracellular vesicles and virus-like particles

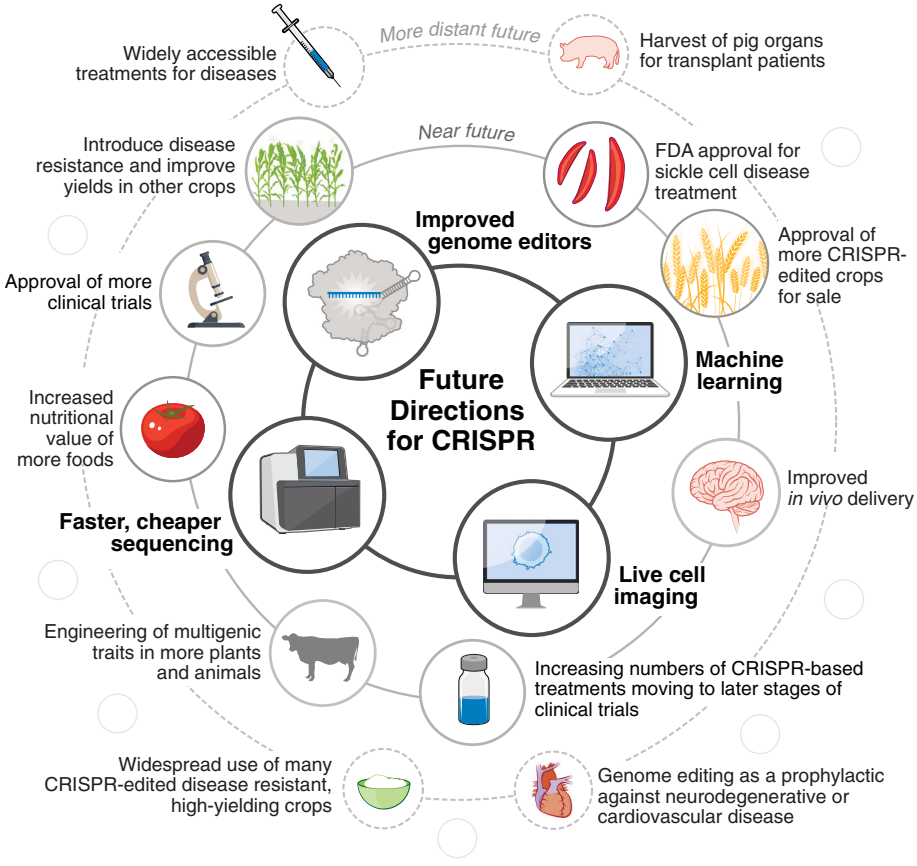

Fig. 4. Future directions: where we could be 10 years from now. The future of CRISPR genome editing will intersect with advances in technologies such as machine learning, live-cell imaging, and sequencing. In the near future, we may witness FDA approval for the first CRISPR-based medicine, as well as increasing numbers of CRISPR treatments moving to later stages of clinical trials and approval of new clinical trials using improved in vivo delivery methods. We expect approval of more CRISPR-edited crops for sale and more demonstrations of CRISPR used to engineer multigenic traits in plants and animals. In the more distant future, we may one day see many widely accessible CRISPR-based treatments and even use genome editing to safely harvest pig organs for transplant patients or as a prophylactic against disease. In agriculture, CRISPR may be routinely used to generate disease-resistant, high-yield crops to increase global food supply and security.

(VLPs) (assemblies of the viral envelope and/or structural proteins that can transduce cells but lack viral genetic material) are emerging as promising delivery platforms that utilize the strengths from both viral-based delivery and synthetic material–based delivery (*183*–*186*). These have the potential to achieve the high-delivery efficiencies of viral-based methods without the safety concerns of random transgene integration or prolonged expression of the editors. One especially exciting area of development is programming the cellular tropism of VLPs by using different envelope glycoproteins to target specific cell types, as recently demonstrated for ex vivo and in vivo delivery and editing (*185*, *186*). The future of CRISPR treatments in humans will largely depend on improving current delivery strategies, innovating new delivery modalities, discovering and engineering more compact CRISPR editors, or a combination of the above.

Beyond mammalian systems, advances are also being made for CRISPR reagent delivery in plants. The plant's cell wall, which has a size exclusion limit of 5 to 20 nm, presents a major challenge for cargo to pass through (*187*). The two predominant methods are delivery of plasmid DNA using agrobacterium, which integrates the transfer DNA into the plant genome, and particle bombardment, which physically breaches the cell wall barrier to introduce the cargo [reviewed in (*187*)]. The main drawback of these methods is random integration of the CRISPR cassette into the plant genome. The goal of enabling transgene-free breeding has motivated alternative approaches, including direct RNP delivery by polyethylene glycol–mediated cell transfection, particle bombardment, electroporation, or lipofection [reviewed in (*188*)]. Though these methods hold promise, the broad application of RNPs in many plant species requires further work in improving delivery efficiency and plant regeneration from edited protoplasts.

Current and future applications

The advent of programmable genome editing technologies has paved the way for applications of cell and gene therapy to treat and even cure disease. Although applications of CRISPR are too numerous to list comprehensively, the treatment of sickle cell disease (SCD) provides an excellent example of the value and risks ahead. There are now at least eight FDA-approved clinical trials of CRISPR-based therapies for SCD and related blood disorders that are ongoing or soon to begin, and FDA approval of the first is expected in 2023 (*189*). However, the challenges to widespread deployment of CRISPR cures for SCD remain formidable. For genome editing to become a standard of care, the field will need to address the difficulty in manufacturing edited cells for each individual patient, the logistical limitations conferred by the need for bone marrow transplantation, and the cost, which can run up to $2,000,000 per patient (*190*). But suppose a new delivery modality for genome editors obviates the need for both ex vivo cell editing and bone marrow transplantation? Such an advance would be game changing, propelling the field into a new era where much broader deployment of genomic therapies would be possible.

Beyond clinical applications, CRISPR is beginning to have impacts in agriculture and animal husbandry. CRISPR-edited foods are already beginning to enter the market. This includes a CRISPR-created tomato with enhanced nutritional qualities and two CRISPR-edited fish (a faster-growing tiger puffer and a red sea bream with greater edible yield), which have been approved for sale in Japan (*191*, *192*). Among these agronomic applications are many examples where CRISPR has enabled the precise "transfer" of small genetic changes that confer desirable traits from one variety of a species to another—a transfer that is either impossible or impractical using any other approach. Beyond small perturbations, CRISPR has also shown potential to generate new genetic variation and complex editing previously not seen in nature. A key example is the recent use of multiplexed editing to simultaneously knock out and activate different genes to introduce disease resistance in wheat and restore growth and yield (*193*). These constitute just the beginning of a wide range of genome editing advances that will increasingly affect our lives in the coming years.

CRISPR-Cas9, part of a bacterial immune system, uses an RNA-guided mechanism to recognize and cut DNA sequences. This fundamental biochemical activity forms the basis for genome editing technology that spans all realms of basic and applied biological research, ranging from developmental biology and plant genetics to sickle cell disease and animal husbandry. The discovery of new CRISPR-based and CRISPR-related enzymes has accelerated rapidly, leading to continued expansion of understanding about the natural biology of these systems in microbes and their utility for genome editing in other cells and organisms. CRISPR-Cas9 and CRISPR-Cas12a, the most widely adopted genome editing enzymes, have become workhorses in research laboratories worldwide. Fundamental research enabled by genome editing speaks to the cross-cutting nature of CRISPR technology and to the timeliness of its arrival as a readily adaptable tool. These wide-ranging applications have in turn enabled expansion of the CRISPR toolbox to enable more precise editing of specific nucleotides or the targeted integration of new genetic information. In the decade ahead, genome editing research and applications will continue to accelerate and will increasingly intersect with technologies including machine learning, live cell imaging, and faster, cheaper DNA sequencing (Fig. 4). Just as the past decade has focused on CRISPR platforms, the decade ahead will increasingly apply those platforms for real-world impacts. In the clinic, we will undoubtedly see increased numbers and types of clinical trials, providing data that will guide next-generation gene and cell therapies. As clinical applications expand, there may be an opening for CRISPR to be used to protect health. For example, as safety and efficacy are established for disease treatment, genome editing might become a prophylactic against neurodegenerative or cardiovascular disease (*31*). Such opportunities would require detailed knowledge of the genetics of multigenic disease and the means to deliver to organs including the brain and heart—neither of which are small tasks. But the potential benefits may drive innovation in these areas well beyond what is possible today. In agriculture, CRISPR screening will provide increasing insights into paths to engineering multigenic traits in both plants and animals. Products generated using CRISPR—whether pig organs for transplant patients (*194*), rice that resists drought with increased yield, or microbiomes fine-tuned for health using CRISPR editing—may all become routine. CRISPR also serves as a notable example of the connection between curiosity-driven research, innovation, and technological breakthroughs. By continuing to explore the natural world, we will discover what cannot be imagined and put it to real-world use for the benefit of the planet.

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ACKNOWLEDGMENTS

Many people have made important contributions to the CRISPR field, and we apologize to those whose work was not cited due to space limitations. We thank T. Tolpa for helpful discussions and artistic renderings of the figures. We thank P. H. Yoon for contributing the analysis of the imprecision of correcting most SNPs using current base editors and M. J. Kan and M. Trinidad for helpful discussions. We thank P. H. Yoon, M. J. Kan, C. A. Tsuchida, F. D. Urnov, and J. H. D. Cate for critical reading of the draft and thoughtful feedback. **Funding:** J.Y.W. was supported by a US National Science Foundation Graduate Fellowship. J.A.D. is a Howard Hughes Medical Institute investigator. **Author contributions:** Both authors contributed to writing and editing the manuscript. **Competing interests:** J.A.D. is a cofounder of Caribou Biosciences, Editas Medicine, Scribe Therapeutics, Intellia Therapeutics and Mammoth Biosciences. J.A.D. is a scientific advisory board member of Vertex, Caribou Biosciences, Intellia Therapeutics, Scribe Therapeutics, Mammoth Biosciences, Algen Biotechnologies, Felix Biosciences, The Column Group and Inari. J.A.D. is the chief science advisor of Sixth Street, is on the board of directors at Altos, Johnson & Johnson, and Tempus, and has research projects sponsored by AppleTree Partners and Roche. The Regents of the University of California have patents issued and pending for CRISPR technologies on which J.A.D. is named as an inventor. J.Y.W. declares no competing interests. **Data and materials availability:** All data are available from the authors.

Submitted 9 July 2022; accepted 12 December 2022
10.1126/science.add8643