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CRISPR gets crunchy

New wrinkles on the Nobel prize-winning method are creating new efficiencies, and new research opportunities.

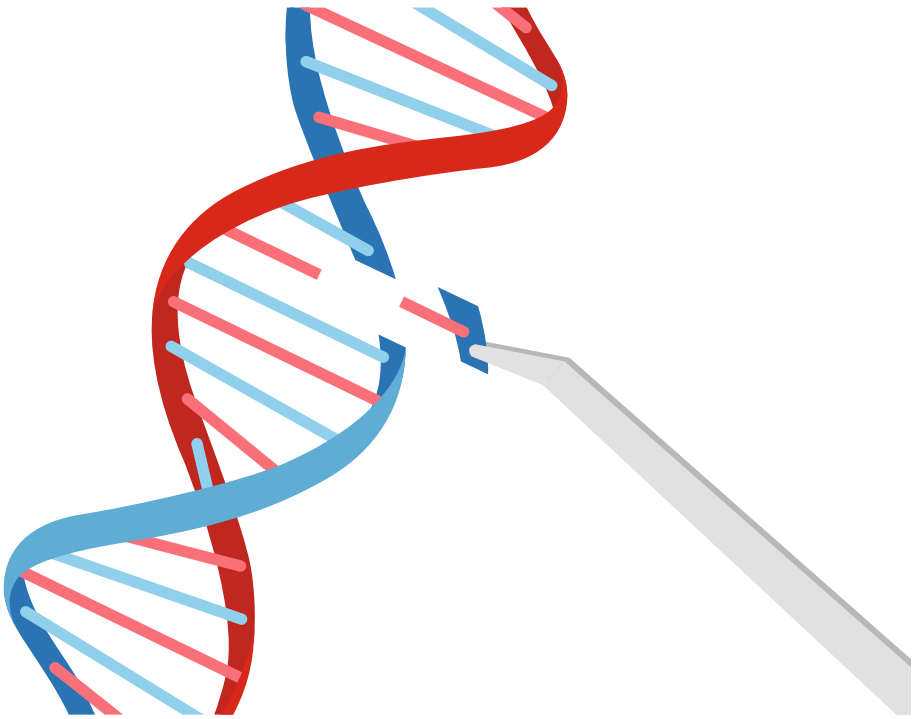
Jim Kling

In October, the Royal Swedish Academy of Sciences awarded the 2020 Nobel Prize in Chemistry to Emmanuelle Charpentier of the Max Planck Unit for the Science of Pathogens and Jennifer Doudna of the University California Berkeley for developing a cheap and easy genome editing technique called clustered regularly interspaced short palindrome repeat (CRISPR)-Cas9. The technique makes it much easier for researchers to disrupt or modify specific genes than previous methods, opening up the field of genetic modification to research groups that previously lacked the resources to pursue such research.

The prize is a fast recognition of the breakthrough, which Charpentier first published in 2011[1]. CRISPR-Cas9 is an adaptive immune mechanism found in bacteria that helps fend off viruses that previously infected them. The bacteria write bits of viral DNA into their own genomes which, once transcribed, form a CRISPR complex that scouts the cell for matching viral genetic material. The endonuclease Cas9 interacts with the complex and makes a cut in the invader's genome, incapacitating it.

Doudna and Charpentier realized CRISPR's potential to modify DNA of other organisms – including, potentially, that of humans. To make it practical, they simplified the native biology by fusing needed proteins, reducing the system to just two components: Cas9 endonuclease and a single guide RNA (sgRNA) that contains a sequence complementary to a DNA target. Cas9 and sgRNA first bind to one another, then bind to the target site.

Cas9 then unwinds the double helix and makes a double-stranded break, a potentially lethal development that cells rush to repair. But, the cell's repair machinery often makes an error, leading to an insertion or deletion that inactivates the gene. This capacity to 'knock out' a gene can have therapeutic benefit or aid in the creation of animal models. CRISPR can also be used to rewrite the genetic code, correcting a mutation or introducing a specific variant at the target site. It can accomplish this through homology-directed repair (HDR), which involves an additional, lengthier piece of DNA that uses sequence affinity close to the target site and a template to guide the cell's repair efforts.



From scissors to scalpels | CRISPR-based approaches to editing genes in different organisms are advancing. Credit: Traffic_analyzer / DigitalVision Vectors / Getty

Previous techniques such as zinc finger nucleases and transcription-activator-like effector nucleases (TALENs) were also powerful, but these required a brand new protein for every base change or modification a researcher wanted to make. In contrast, sgRNA is simple to design using the known target genomic sequence and simple to make, and Cas9 can be readily expressed by the target cell.

Those advances were revolutionary, but the method nevertheless has shortcomings. For clinical applications, in which researchers envision using CRISPR to correct genetic errors among patients, knock out rogue genes, or potentially even target viruses or bacteria, the method has some troubling issues with accuracy. The system can sometimes attack regions of the genome that share some similarity to the target sequence, inactivating or modifying unintended genes; within the correct target, large insertions or deletions can also have negative consequences.

When it comes to using CRISPR to produce new animal models, the issue isn't quite so dramatic, as edited cells can be screened for off target effects before injection into a developing embryo. The issue is throughput: even though it can be easier to use than earlier genome engineering technologies such as zinc finger nucleases and TALENs, CRISPR still requires a lot of work – but improvements are on the way.

Building on the original

A key advance arrived in 2016, when David Liu and his colleagues at Harvard Medical School developed a method called base editing[2]. Base editing uses a Cas9 enzyme fused to another enzyme, called a deaminase, which can directly convert one cytidine to uridine. This effectively changes a cytosine (C), one of the four bases that make up DNA, to thymine (T) (or uridine

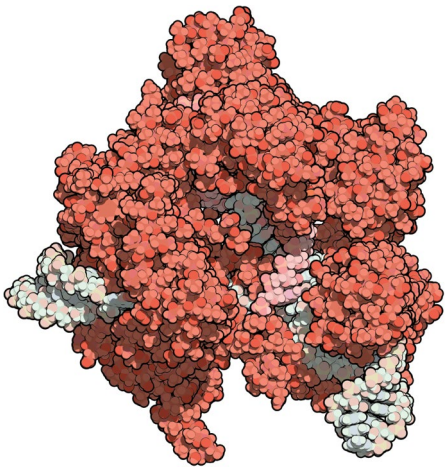
(U), in RNA versions). Another deaminase can change an adenine (A) to a guanine (G)[3]. Unlike the original CRISPR system, base editing does not produce a double-stranded break, making it less prone to insertions or deletions that often occur with HDR. That should make it less prone to insertions or deletions that often occur with HDR.

Base editing opened up many new doors, according to Lukas Dow, an assistant professor of biochemistry and medicine at Cornell University. "Until base editing came along, the only real option we had to mutate genes precisely was to use HDR, which just has a low and quite variable effect depending on the cell you're targeting. The activation energy for people to want to do that was quite high, and you wouldn't think of doing more than a couple (modifications) at a time," said Dow, whose group fine-tuned the genes encoding base editing enzymes so that they could be more readily expressed in mice[4].

While base editing was one of the first big modifications to CRISPR, it is hardly the last. A study from a team headed by Norbert Perrimon, a geneticist at Harvard Medical School, recently applied a CRISPR-based method called prime editing to *Drosophila*[5]. Prime editing[6] was introduced in 2019 by Liu's group at Harvard, and it uses a version of the Cas9 enzyme with an inactivating mutation preventing it from making double-stranded cuts in the target DNA. The enzyme has two separate domains, one for cleaving each strand of double-stranded DNA, and researchers created a mutant inactivating one of the domains. Prime editing uses one of these versions of Cas9 – a 'nickase' that cuts only one strand. The altered Cas9 is also fused to a reverse transcriptase – an enzyme that copies an RNA template into DNA. The guide RNA includes both the sequence that determines the genomic target as well as a template that instructs the reverse transcriptase to insert a specific sequence.

The method avoids the double-stranded breaks that occur with HDR, which can lead to random insertions and deletions with potentially wide-ranging consequences. "Depending on what that does to the framework of the protein or the structure of a gene, you can have a lot of phenotypic outcomes in each cell. With base editing, 80 to 90% of the edits you get are the same thing. The better you can make it work, the easier it is to study," said Dow.

It also simplifies experimental design. HDR requires creation of a sgRNA, which isn't difficult, but it also requires creation of a donor plasmid that provides a template for the new sequence. This step is a bit more challenging, and it is slow. Base editing and prime editing do away with the need to design and clone that DNA sequence, according to Justin Bosch, a postdoc with Perrimon at Harvard Medical School who is interested in identifying novel cell-to-cell signaling molecules. He wants to study proteins in the blood with unknown function, using precise edits in the genes that make these blood proteins in fruit flies – for example, adding sequence to track their location in the animal or removing sequence to prevent their secretion from cells. This could be accomplished with HDR, but that requires laborious computer design and bench work to create a donor plasmid for each desired change. "It's more time planning your experiment, more time waiting to receive some primer DNAs from a company, more time to set up the reactions on your bench, more days waiting for these donor DNAs to be sequenced and carefully vetted and verified that they are the right sequence," said Bosch.



Cas9 & beyond | Cas9 is just one endonuclease that can modify DNA; variants of it, as well as different enzymes entirely, are changing how researchers can edit genes. Credit: MOLEKUUL / Science Photo Library / Getty

With base and prime editors, "those steps are simply faster and less prone to errors. The RNA sequence that encodes your edit is very short, whereas sometimes the DNA sequences you create for HDR can be very long and prone to mistakes, and you have to screen for the right one. For making small precise edits, prime editing has the potential to help target more genes, spend less money, or have fewer people work on the project," he said. In the *Drosophila* paper, the group was the first to apply prime editing to fruit flies, using it to truncate three visible marker genes in both cultured cells and somatic cells.

The work illustrates the potential of prime editing to do more than just alter gene function. It can also be used to insert epitope tags that allow researchers to follow the fate of proteins in an *in vivo* system. For example, researchers could track putative messaging proteins through the bloodstream and even pinpoint their destination organs, using antibodies or antibody fragments against the inserted epitopes, according to Bosch.

The ability to precisely target specific genes can be useful for understanding function. If a protein is normally secreted into the bloodstream, for example, researchers can truncate the protein in such a way as to preserve the protein's function, but prevent its secretion. "The mutant phenotype of that case might be illustrative of what the extracellular function of that protein is," Bosch said. Such experiments are possible with traditional CRISPR-Cas9, but experimental design takes longer, making it difficult to survey large numbers of candidate genes.

CRISPR isn't restricted to targeting DNA. It can also be used to knock down the messenger RNA that guides the synthesis of proteins. This can be useful for research questions involving developing embryos, where there is no transcription of an organism's DNA in the earliest stages. In that early time window, everything depends on RNA provided by the mother. Determining the function of maternal RNAs during development means selectively knocking them out and observing the consequences.

That could already be done with RNA interference in various organisms, but the technique doesn't work well in vertebrate embryos such as zebrafish or amphibians. Synthetic analogs of RNA called morpholinos can do the job, but these can lead to off-target effects, immune reactions, and toxicity. That forces researchers to do a great deal of work to ensure that a morpholino is only affecting the target gene.

To study embryonic development in zebrafish, Ariel Bazzini, an investigator at Stowers Institute for Medical Research, has turned to another Cas enzyme called Cas13d, which had been previously used to edit RNA in yeast, plants, and mammalian cell lines. In embryos, Cas13d binds to a target sequence and destroys it, leading to a depletion of the target RNA within the embryo. In a recent paper[7], Bazzini and his colleagues demonstrated that the technique works in zebrafish, killifish, and Japanese rice fish (medaka), as well as mouse embryos, with no sign of toxicity. By working with RNA, it's possible to directly observe reduced expression of the gene through a simple real-time quantitative reverse transcription PCR. The potential off-target effects can be revealed by RNA

sequencing (RNA-seq) "It's a very clean technique," said Bazzini.

The method opens up the possibility of an in-depth study of maternal RNA in development, because the reagents are cheap and easy to make. "For us, it's a very exciting moment. In zebrafish you have roughly five thousand maternal genes, so you can start knocking down one, two, three at a time. You can't order five thousand morpholinos - that would cost a fortune," said Bazzini.

He is currently generating zebrafish lines that express Cas13d in every cell, or in specific tissues, along with a range of other zebrafish lines that are engineered with unique guide RNAs. Then it would be possible, for example, to cross a fish expressing Cas13d in a specific organ like the heart with other fish that produce guide sequences suspected of being involved in heart development. Offspring bearing both Cas13d and the RNA sequence of interest, could reveal hints of its function. The ease and low cost of the Cas13d system makes such a grand scale project feasible.

CRISPR'ing continues

While base editing and prime editing improve the ability to induce specific changes to the genome, other emerging approaches have expanded CRISPR's scope. One new technique alters the specific locations that Cas enzymes can target on a genome. The enzymes already depend on the guide sequence to reach the target, but there is an additional requirement: Close proximity to a protospacer-adjacent motif (PAM). It's like an extra tether that Cas9 needs before it can cut DNA. For Cas9, the PAM is NGG, where N can be any nucleotide and G is guanine. Cas9 won't target a sequence that isn't within 12-20 base pairs of a naturally occurring NGG sequence. The limitation exists in the natural bacterial system to prevent Cas9 from attacking the bacteria's own genome, where the record of past viral infection is stored with no NGG sequences in its vicinity.

A practical consequence of this is that many regions within a specific gene are off-limits because they are aren't close enough to an NGG sequence. That's not really an issue when the goal is to knock out a gene, since statistically almost any gene will have an NGG sequence somewhere within it, and to inactivate a gene, most targets within it will do. But attempts to use HDR, base editing, or prime editing to create a mutation or truncation at a specific location require this physical proximity. If an NGG sequence isn't close enough to the specific positions researchers want to alter, they have to look for another location to intervene, or to another Cas9 enzyme dependent on a different PAM that might fall into the required range of the target.

Ben Kleinstiver and his team decided to fix that by engineering Cas9 so that it could bind to DNA without a PAM requirement[8]. They replaced the amino acids in Cas9 responsible for binding specifically to NGG with amino acids capable of binding non-specifically to DNA. The resulting modified Cas9 proved capable of targeting DNA with no PAM requirement. The group used it on human cell lines, both alone as a nuclease and combined with various deaminase domains to transform it into base editors.

In some cases, the modified version can be less efficient than its wild-type relative, possibly because the amino acids altered to bind any nucleotide just don't bind as tightly, or because the new Cas9 variants have a much larger genomic space to search versus Cas9s that require a specific PAM. "That being said, with these new proteins, you can edit sites that you couldn't before. These proteins are definitely enabling in their capabilities compared to what was available before," said Kleinstiver, an assistant professor of pathology at Harvard Medical School and an assistant investigator in the Center for Genomic Medicine at Massachusetts General Hospital.

Base editors, prime editors, and more have also opened new avenues to research, and the plethora of emerging CRISPR technologies seem poised to transform animal studies; already, there is a growing record of success in applying them to animal models. "New genome engineering systems developed in mammalian cell culture can get ported to model organisms very quickly," said Bosch.

That's all thanks to the work of Charpentier and Doudna, but the Nobel Prize recognizing their work has had its detractors. "Online, I've seen people say, 'why would you give the Nobel Prize for CRISPR-Cas9? It's old news,' said Bosch. "They're implying that people won't use CRISPR-Cas9 in the future. It'll be prime editing and these other techniques. But that's not true. The work to show that CRISPR-Cas9 was a method for genome editing, that is the achievement, and the blossoming of all of these other CRISPR-based techniques is just little details on top of that."

Taken together, these new tools are poised to greatly expand researchers' abilities to understand biological and genetic mechanisms. Bosch is banking his career on it, with various projects using different techniques. He hopes the expertise he gains in various methods will pay off. "You could say that I'm a cook with many pots and pans going, and I'm just getting started on prime editing while simultaneously using older engineering techniques to accomplish my goals. It's like an investment in my future." ❐

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