Gene editing at CRISPR speed

Ease of use, economy and speed of targeting DNA has propelled the CRISPR-Cas system into the spotlight. Now, despite numerous gaps in our knowledge, commercial entities are looking for a piece of the action. Monya Baker reports.

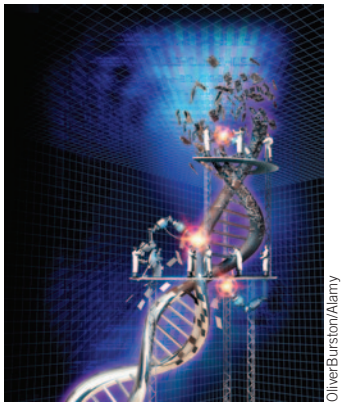
Research into the CRISPR-Cas gene editing system continues at a breakneck pace. The ease, low cost and speed of designing an RNA-guided endonuclease against a DNA target of interest has caught the imagination of researchers across the globe and spurred the publication of a slew of papers in the past 18 months. In recent weeks, for example, the crystal structure of Cas9 nuclease was published, offering for the first time the possibility of rational engineering of the complex based on structural information[1,2]. And the excitement isn't merely academic. Several startups have been created around the technology—the latest, venture-backed Cambridge, Massachusetts–based Editas Medicine, plans to use CRISPR-Cas to tackle human diseases. At the same time, supplier companies are rushing to create CRISPR reagents for the research community. As Kevin Bitterman, a principal at Polaris Partners and acting CEO of Editas, puts it, "It was hard to ignore the avalanche of papers on CRISPR-Cas…This is the type of technology that comes around every ten years."

But with the technology so young and posing so many questions, it remains to be seen whether CRISPR-Cas will supersede its predecessors, such as transcription activator–like effector nucleases (TALENs) or zinc finger nucleases (ZFNs), especially when the latter have decades of research behind them and are now showing promising clinical results (see p. 315).

A long silence and a big splash

The term CRISPR was introduced in 2002 to describe oddly repetitive sequences found across many bacteria and archaea species[3]. These sequences, called clustered, regularly interspaced, short palindromic repeats (CRISPRs), sit amid sequences that bear a striking resemblance to those of bacteriophages. This suggested a sort of immune system that could keep a DNA record of past viral attacks and use it to fend off similar invaders. All signs pointed to a DNA-cutting enzyme that could be programmed genetically, but it also seemed unworkably complicated. Several CRISPR-Cas systems exist, and most rely on protein complexes working with a CRISPR RNA element (crRNA) to silence the invader, but the function of essential components was unclear.

It wasn't until 2011 that CRISPR made its way out of obscurity when Emmanuelle Charpentier at Sweden's Laboratory for Molecular Infection Medicine in Umea discovered that this system requires a previously overlooked *trans*-encoded RNA (tracrRNA)[4]. Moreover, the bacteria Charpentier worked with, *Streptococcus pyogenes,* relies on a single protein, now called Cas9, to cut DNA. Charpentier teamed up with Jennifer Doudna at the University of California, Berkeley, who had been working on a multiprotein version of the system, and together they probed how the components worked: the protein Cas9 cut DNA, crRNAs determined the targeted DNA sequence and tracrRNA activated the system.



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Customize genes with CRISPR.

One day, as Doudna and postdoc Martin Jinek were sketching cartoons of Cas9 and the two RNAs, they realized the two RNAs could be combined into one, theoretically reducing the system to just two components. "Martin ran to the lab and did that experiment," Doudna recalls. In a *Science* paper published in 2012, Doudna and Charpentier showed that Cas9, programmed with various 'guide RNAs' (gRNA), could be directed to cut specific DNA sequences, at least inside a test tube[5].

At this point, multiple teams raced to create CRISPR-Cas systems that could be applied to eukaryotes. They tweaked bacterial genes to make them more suitable for eukaryotic cells—attached nuclear localization signals and swapped out certain codons. Five papers demonstrating this appeared within the first month of 2013, with teams led by four of the five Editas co-founders (Doudna[6], Keith Joung[7] and Feng Zhang[8], plus George Church[9] at Harvard University) as well as Jin-Soo Kim[10] at Seoul National University, who in 1999 founded ToolGen in Seoul to take advantage of burgeoning knowledge at the time about ZFNs.

The inner workings

CRISPR-Cas creates double-stranded cuts in DNA, triggering DNA repair mechanisms that can knock out a gene by breaking its sequence or, more rarely, tweak a gene by inserting an alternative DNA sequence from another template. It is not the only gene-editing technology that works this way. ZFNs, described first in 1996 (ref. 11) combine the DNA-cutting domain of a bacterial restriction enzyme with DNA-recognition elements; TALENs, reported in 2009, are also fusions (the same DNA-cutting domain combined with modular DNA recognition elements found in plant pathogens[12,13]).

Unlike ZFNs and TALENs, CRISPR-Cas has the distinction that DNA recognition depends not on protein, but rather on RNA. For ZFNs and TALENs, targeting a new DNA sequence of interest requires the rather onerous task of engineering a new protein, a process that relies on some heuristic rules in either case, but also a certain amount of trial and error (depending on the sequence). In contrast, the CRISPR-Cas system needs a simple oligonucleotide—targeting is mediated by Watson-Crick DNA base pairing.

Since its first demonstration in eukaryotic cells, the CRISPR-Cas system has been used to modify genes in a growing list of animal and plant species[14]. The addition of the first primate to be edited *in vivo* by Chinese researchers ratcheted the excitement another notch in January[15].

As with other gene-editing tools, CRISPR-Cas can be tweaked to perform additional functions, like modifying gene expression (**Box 1**). "It's a generalizable platform that lets you target anything you want to anywhere you want," says Feng Zhang at the Broad Institute in Cambridge, one of the first to show that CRISPR-Cas works in human cells[8].

Uptake of the CRISPR-Cas system by the community has been fast. Last year, the nonprofit distributor Addgene in Cambridge, Massachusetts, delivered CRISPR plasmids to over 3,000 different laboratories. The plasmids make up most of the top ten most-requested plasmids, says Melina Fan, a co-founder of Addgene. Keith Joung, a biologist at Massachusetts General Hospital in Boston, and his colleagues supply free online software

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Box 1 More than a cut

CRISPR-Cas can do more than cut a gene—the protein-RNA complex can label, repress and activate, too. "For me, what is really exciting is that they can deliver any regulator, any protein to a place on the genome," says Stanley Qi, a biologist at the University of California, San Francisco, and the first to show that CRISPR-Cas9 could be used to regulate gene transcription. Whereas techniques like RNA interference attack mRNAs, CRISPR-Cas9 acts before transcription occurs, and so can target regulatory and other elements on DNA[30].

For activating gene expression, the system works best when multiple gRNAs are made for different spots on the same gene, a fact that could make the system easier to control[31,32]. Fusing Cas9 with DNA-modifying enzymes could be used to examine the effects of methylating or demethylating specific DNA sequences (TALENs have also been used this way).

Qi has worked out ways to use CRISPR-Cas to deliver fluorescent markers to certain DNA sequences[33]. The technique is similar to fluorescence *in situ* hybridization (FISH) techniques, which visualize genes *in situ*, except FISH requires killing cells and CRISPR-imaging can be done with living ones. (Unlike FISH, however, it cannot yet label two sequences with different colors within the same cell.)

(http://zifit.partners.org/ZiFiT/) for CRISPR oligonucleotides, as well as for designing ZFN proteins and TALEN proteins. Similarly, his genome engineering newsgroup (http://www.egenome.org/) had about 700 members at the end of 2012, just before the first CRISPR demonstrations in cells were reported; as of the beginning of March, it had climbed to over 1,900, mostly due to CRISPR.

But along with the excitement comes unanswered questions. A spate of papers published last September suggests that CRISPR-Cas can have a wide range of off-target activity[16–19]. The best way to deliver CRISPR-Cas components to various cell types is still being worked out, and it is unclear how CRISPR-Cas might affect the health of organisms over extended periods, says Joung. But he also says that the current stream of research is making him optimistic. "At this point, for most people, CRISPRs do make sense."

It's a cut up

Luciano Marraffini, now at Rockefeller University in New York, who worked on targeting DNA with the CRISPR-Cas system as a postdoc with Erik Sontheimer at Northwestern University[20], says he had no doubt the system could work in eukaryotic cells, given how well the bacterial system performs against swiftly replicating viruses. "To kill a phage is not a trivial thing," he says. "The protection that CRISPR provides to bacterial cells is amazing. If it is even one order of magnitude as active in eukaryotic cells, it would still be functional."

In fact, efficiency rates reported for CRISPR-Cas in eukaryotic cells are comparable to, or higher than, those reported for ZFNs and TALENs, though direct comparisons are problematic. And because adding a new cut requires adding just another small RNA, modifying several genes at once is feasible. By introducing Cas along with multiple gRNAs, researchers led by Rudolf Jaenisch at the Whitehead Institute in Cambridge, Massachusetts, disabled five genes in mouse embryonic stem cells with a single transfection of CRISPR-Cas components[21]. And by injecting CRISPR-Cas in newly fertilized eggs, the team produced mice in which both copies of two genes were modified, a task that could easily take a year by conventional techniques. "Labs that never would have thought about making a mouse model are going to consider it," says Doudna.

Last December, two groups reported using CRISPR-Cas to repair specific disease mutations. Researchers from the Shanghai Institutes for Biological Sciences corrected a cataract-causing dominant mutation in mouse zygotes[22]. In one set of experiments, 5 of 29 mice were born cataract-free and without the mutation, presumably because the healthy allele or an introduced oligo was used as a template when the DNA was repaired. Another 4 of the 29 mice were also born without cataracts because another DNA-repair process disrupted the gene. In a second paper, human intestinal stem cells carrying cystic fibrosis mutations were injected with CRISPR-Cas as well as DNA templates for the healthy sequence. Cells with the corrected allele exhibited behavior typical of nondisease cells[23]. No one on either paper had used CRISPR-Cas before. "Everything worked the first time," says Hans Clevers, the stem cell biologist at Hubrecht Institute in Utrecht, The Netherlands, who led the cystic fibrosis work.

Perhaps the broadest application of CRISPR-Cas will come from systematic analyses across genomes, demonstrated in three papers published in early 2014. Using nearly 65,000 gRNAs, Zhang targeted every annotated human gene in a series of cellular screens[24]. In one, the team treated cancer cell lines with the antimelanoma drug Zelboraf (vemurafenib) to see which gRNAs allowed cells to survive. This revealed the genes that, when mutated, likely help cancer cells resist treatment. To find genes essential for viability, the team applied CRISPR to over 18,000 genes, waited two weeks, and sequenced cells to see which gRNAs persisted; gRNAs that disabled essential genes were depleted. A team led by David Sabatani at the Whitehead Institute and Eric Lander at the Broad Institute, both in Cambridge, Massachusetts, targeted 7,000 genes with over 70,000 gRNAs in another screen for essential genes as well as for genes conferring resistance to several chemotherapy agents. They identified previously unknown pathways that might be involved with drug resistance[25]. A third group led by Kosuke Yusa at the Wellcome Trust Sanger Institute in Hinxton, UK, used nearly 88,000 gRNAs to target 19,150 mouse protein-coding genes in mouse embryonic stem cells expressing Cas9 and screened for genes that confer resistance to bacterial toxins[26].

Techniques like RNA interference (RNAi) that target mRNA can leave some residual protein in the cell, but DNA-targeting CRISPR can knock genes out completely, says Zhang. "CRISPR worked a lot better than the RNAi screen. We were able to find gene candidates that were not reported by RNAi, and the signal is much more robust, probably because it is more specific."

Specificity and other challenges

One way to cement CRISPR-Cas' place in the gene editing arsenal will be to establish rules for designing gRNAs. In their genome-wide screen, Lander and Sabatani looked at all possible guides against 84 ribosomal protein genes and found a range of efficacy, as well as some predictable patterns. "The sequence of the guide RNA can make a difference in terms of how well it cuts," says Tim Wang, first author on the paper.

One worry, particularly for therapeutic applications, is that CRISPR can cut DNA at unintended places. Joung found cutting at genomic sites with as many as five mismatches from the RNA guide sequence[16]. And whereas certain positions in the gRNA seem to be more tolerant of mismatches, the patterns are unclear. "My read of all that data collectively is that there are no simple rules," he says.

"People will start evaluating their projects with CRISPR, screen target sequences, make their proof of concept and then, probably, will come back to 'cleaner' nucleases like TALEN

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Table 1 Select companies with CRISPR-Cas tools

Company (location)	Product description
Applied StemCells (Menlo Park, California)	Genome engineering, gene editing and knock-in cell lines
GenScript (Piscataway, New Jersey)	gRNA design, transfection and cell pool evaluation, single-cell clone generation and validation
GeneCopoeia (Rockville, Maryland)	Research tools, Cas9 nuclease expression, genome-CRISPR sgRNA design and cloning services
Horizon Discovery	Gene-editing tools, validated gRNAs, Cas9 vectors, cell line generation kit, delivery vectors
Life Technologies (Carlsbad, California)	CRISPR Nuclease Vector Reporter Kit, genome-CRISPR sgRNA design and cloning services
OriGene (Rockville, Maryland)	CRISPR cloning kits, CRISPR-Cas9 custom services
Sage Labs	Transgenic animal models, knockout, gene replacement, targeted transgenics
Sigma-Aldrich (St. Louis)	Plasmid expressing Cas9, GFP and customizable gRNA under U6 pol III promoter
ToolGen	Custom-designed crRNA for target with tracrRNA and Cas expression systems

for the 'real' work," predicts Jean-Charles Epinat, deputy CEO of Cellectis bioresearch, which began offering custom-designed TALENs shortly after they were reported.

Several groups are working on ways to make the system more accurate. Erik Sontheimer recently reported that the CRISPR-Cas9 system from the bacterium *Neisseria meningitides* uses a longer recognition sequence, which could help design more accurate systems[27]. Editas co-founder David Liu, of Harvard University, and Doudna suggested that lower concentrations of the CRISPR-Cas9 reagents in a cell reduces cleaving in off-target sites more than they reduce cleaving in on-target sites[19].

Another technique, reported separately by Church and Zhang, is to modify Cas9 so that it nicks only one strand of DNA[18,28]. The nickase is introduced into the cell along with two gRNAs that direct a pair of nickases to separate, nearby sites—effectively introducing a double-strand break and a longer targeted sequence. The technique will work only for CRISPR applications that require cutting DNA. Nonetheless, the studies found off-target rates 50 to 1,500 times lower than a nuclease targeting a similar sequence.

And Joung found that gRNAs can be made more specific if they are two or three nucleotides shorter, particularly at certain positions[29]. "It's counterintuitive, but the standard system has more complementarity than it needs, and so mismatches are tolerated." The solution is to truncate the gRNAs. This doesn't reduce interaction with target sites but cuts down interactions with off-target sites, he says, "in some cases to undetectable levels with deep sequencing."

Nonetheless, assessing off-target effects thoroughly is a huge burden because off-target effects could vary cell to cell. The task is less like looking for a needle in a haystack than making sure none of the haystacks contains a needle. "There is no unbiased genome-wide way to assess off-target effects for gene-editing nucleases period. That's part of the problem," Joung adds.

Tools to better measure both efficiency and specificity will be necessary to improve the system, says Zhang. So will a better mechanistic understanding. "We need to understand more about this enzyme, how does it bind to DNA, how does it search for the right target site, what are the off-target activities, how well is it tolerated in cells over long periods of time."

The recent crystal structures of Cas9 protein bound to DNA and RNA could provide some of the answers. "They're beautiful," says Dana Carroll, at the University of Utah in Salt Lake City, and a pioneer of genome editing. He believes knowing how the functional parts of the protein are positioned could guide efforts to boost specificity in genome editing as well as to make the protein smaller and so easier to apply to nonediting applications.

Commercial interests

A cluster of research tool companies have already launched products for CRISPR-Cas (**Table 1**). These generally involve web-based bioinformatic tools to design gRNAs, plus RNA or plasmid vectors that encode Cas9, along with fluorescent proteins or other expression markers.

Why would companies invest in offering tools for a system that has proven so easy to use? "The way I think about this has a lot to do with the way I think about the PCR market," Rachel Haurwitz, who co-founded genome engineering company Caribou with Doudna in Berkeley. "In principle, researchers can make their own polymerases, they can design their own primers, but they don't."

ToolGen offers ZFNs as well as TALENs, but company director Seokjoong Kim expects CRISPR products to reach 80% of revenues next year. His company offers validated RNAs for carefully chosen target DNA sequence. "Not all guide RNA provide the same high level of activity," he says, "about 10% show no activity."

Scientists steeped in gene knockdown who were reluctant to try other genome-editing tools are turning to CRISPR-Cas, according to Gregory Davis, principal scientist at Sigma Aldrich in St. Louis, which commercialized ZFNs. They will notice several differences, he says. Instead of considering the mRNA—commonly targeted to knock down a gene—researchers designing gRNAs will instead be working with genomic DNA, such as introns and exons and single nucleotide polymorphisms that might not be apparent in a transcript's sequence.

Still other companies like Horizon Discovery in Cambridge, UK, are setting up the system to modify animal models and cell lines. "Since it's so easy to implement, there's no reason not to give it a try," says Eric Rhodes, the company's chief technology officer.

The company has already genetically customized cell lines using a recombinant adeno-associated virus, which can insert a genetic payload into cells. When CRISPR and virus are

Box 2 The CRISPR IP land grab

It remains to be seen how the CRISPR patent landscape will unfold, and what entities will have access to the technology. Both SAGE Labs and Horizon Discovery chose not to work with TALENs because of issues accessing intellectual property, and both companies have licensed CRISPR technology, albeit from different patents that have been filed but not yet granted. And both anticipate that future licensing may be necessary. Many patent applications are still unpublished, so it is hard to know what uses inventors have claimed. Horizon Discovery's Rhodes' prediction is that the situation will be analogous to that of RNA interference, several years ago. Many groups claimed diverse rights, and for a while it seemed no one would have freedom to operate. Eventually, he says, "people did a lot of cross-licensing and people got access to it. I hope it will play out that way." [see p. 331]

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used together, both technologies seem to work better. Because CRISPR makes double-stranded breaks at sites of insertion, the rate of payload incorporation can be as much as tenfold higher, cutting down the screening required. The company and others are making cells carrying an inducible form of the Cas9 gene. Researchers could launch experiments simply by adding gRNA and a small-molecule drug to activate the gene. That would allow precise time control and avoid ill effects of continual Cas9 expression, a problem that some researchers have noted anecdotally.

Another application is made-to-order genetically modified animals. ToolGen injects the Cas9 protein and gRNAs directly into zygotes, which increases the likelihood that a genome modification will take place before the zygote starts dividing, reducing the chances of genetic mosaicism. And as this technique doesn't involve DNA, the potential for misincorporating DNA is avoided.

SAGE Labs in St. Louis has already used ZFNs to make a suite of knockout rats. Having a CRISPR reagent ready to target a particular gene takes about a week, compared with a month or two for ZFNs, says CEO David Smoller. But preparing a reagent and knowing that it will work as intended are two different things, he says. "There are some speed advantages to CRISPR, but there are some unknowns" (**Box 2**).

Broadening options

CRISPR-Cas9 has made an impressive entry on to the gene-editing stage in the past 18 months. But many aspects of the system remain to be worked out. That means that other gene-targeting technologies like ZFNs and TALENs will remain relevant. "For people who want something fast and cheap, CRISPR is a good option," Davis says. "If people want to go down a path that has more of a proven track record, then ZFNs are the way to go."

But although improvements in CRISPR tools are sure to come, the technology is already being put to use. 2013, Doudna says, was the year that people did a lot of experiments, not to ask questions, but to show that the technology was working. "My guess is that we are cresting that wave at this point; next we will see people using the system to do real biology."

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