Gene-editing nucleases

Precise ways to modify the genome arose from unexpected places. Monya Baker reports.

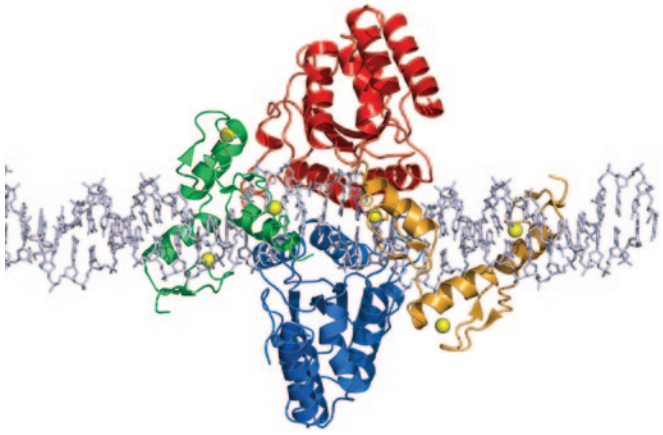
Around the time when the Human Genome Project and the Hubble Space Telescope were launched, Srinivasan Chandrasegaran, a newly hired assistant professor at Johns Hopkins University, asked his mentor, Nobel laureate Hamilton Smith, what project he thought would be complex enough to last a fledgling laboratory five years. Smith suggested a redesign of restriction enzymes: many attempts had been made to engineer the DNA-cutting enzymes to recognize and cleave new sequences, but few successes had been reported. Trained as a chemist, Chandrasegaran—or Chandra, as he is generally known—liked the idea of changing an enzyme's substrate.

Most scientists were focusing on the best-known class of enzymes, which recognize and cleave DNA at the same site, so Chandra decided to look into another type of enzyme, in which the recognition and cleavage sites are separated by a few base pairs. In his new lab, he flipped through the New England Biolabs catalog and found only two in a long list of restriction enzymes. Of the two, FokI, which is derived from a family of bacteria that infect freshwater fishes, was better characterized and seemed a good place to start.

Chandra's first research proposal was simple: he would physically separate the enzyme into two domains and test each for activity. Grant reviewers warned that the project had little chance of success—the separated units would certainly unfold. But they funded the project anyway. Failure, one wrote, would be very informative.

Chandra first used the digestive enzyme trypsin to cleave FokI. This produced an indiscriminate nuclease that chomped through DNA willy-nilly. But researchers have little use for nonspecific nucleases, so Chandra decided to reattach the FokI nuclease domain to new DNA-binding domains. After initial success with a fruit-fly homeodomain, Chandra decided to try a zinc-finger protein. Humans harbor hundreds of these modular proteins, each of which binds to a specific DNA sequence to turn genes on and off. Chandra visited Jeremy Berg, also at Johns Hopkins, who was working out ways to design new zinc fingers. Berg walked Chandra to his freezer and handed him two tiny tubes, each containing a plasmid encoding a zinc-finger protein that bound a specific sequence. Chandra used these to create two fusion proteins by combining each binding motif with the cleavage domain of FokI. The hybrid enzymes cut where expected. The first zinc-finger nuclease (ZFN) had been engineered, and the results were published in the *Proceedings of the National Academy of Sciences* in 1996.

This initial project—woven in with an understanding of DNA binding and DNA repair—would ultimately yield targeted ways to modify sequences in the genome, a powerful technique. Even as the genome sequences of many organisms are being revealed, understanding what those genes really do requires tweaking the sequences, deleting or modifying genes, and studying the consequences. With the exceptions of those from mice and yeast, however, eukaryotic genomes generally resist this type of precise tinkering.



E. Vanamee lab, Mount Sinai School of Medicine

A ZFN pair bound to DNA. Recognition units are green and gold; cutting units are blue and red.

Engineered gene-editing nucleases are now poised to change that. Over the past 15 years, researchers have been optimizing ZFNs. More recently, a DNA-binding motif that has been identified in proteins known as transcription activator–like effectors (TALEs) has also been attached to nucleases. Although they have not been as thoroughly tested, these nucleases, or TALENs, are so far proving to be easier to design and capable of performing the same feats.

And the feats are impressive: just this year, researchers working with human pluripotent stem cells used TALENs to replace disease alleles with healthy ones and to look for differences between otherwise genetically identical cells. In other work, genes from one species have been replaced with related genes from another to see how genomic context affects gene function. Specific genes in rats, roundworms and other organisms can now be targeted with engineered nucleases in ways once thought impossible.

The right break

Many attempts at targeted gene editing have met with limited success. That was certainly the initial experience of Dana Carroll at the University of Utah. From his and others'

Chandra Srinivasan at Johns Hopkins University invented ZFNs as part of a project to redesign DNA-cutting enzymes.

research, he knew that if he could just break both strands of DNA at a precise spot in the genome, then natural cell-repair machinery could produce the genetic changes he wanted.

The problem was breaking the DNA. Carroll had attached oligonucleotides to a chemical expected to cleave DNA, hoping to generate self-destructive triple helices, but the results weren't encouraging. "We were puttering along not getting anything dramatic, and we saw the *PNAS* paper," he says. Carroll had never met Chandra, but he picked up the phone and proposed a collaboration, explaining that he had a way to detect DNA repair in frog eggs.

It worked. Having shown that an engineered nuclease could cut DNA and trigger its repair within eggs, Carroll then wanted to create a nuclease that could knock out a gene in an organism. He consulted his University of Utah colleague Kent Golic, an expert in fruit fly genetics, who suggested an experiment involving a gene called *yellow*, which, when disrupted in fly larvae, causes the adult fly's brown cuticle to be flecked with amber specks. Carroll, along with his postdoctoral researcher Marina Bibikova and other lab members, began spending considerable time in Golic's lab. Relying on work from previous researchers about which zinc fingers would bind which sequences, they crafted genes encoding ZFNs designed to pluck *yellow* from cells. They injected fly larvae with the DNA and waited long days for the adults to emerge.

Peering through a microscope, Bibikova saw them first: tiny pale splotches on the dark abdomen of a fruit fly. Carroll saw the splotches too but was not sure that he could recognize the phenotype. He recalls calling Golic over to learn whether they should believe their eyes: "Kent bends over the microscope and then stands up and says, 'If I were you, I'd be pretty excited'." Subsequent studies showed that nucleases could do more than delete a gene. When homologous DNA was provided along with the nuclease, it could replace the endogenous DNA.

Meanwhile, Matthew Porteus had been enthusiastic about gene editing ever since learning about the creation of knockout mice. As a medical student treating patients with extreme sickle cell anemia, he had become frustrated that he could offer only palliative care. Gene editing promised a way to fix the disease-causing mutation. In the mid-1990s, as he began searching for laboratories in which to do a postdoc, he proposed genetically altering human cells as his project. The idea was met with little enthusiasm. "Engineering human cells was just not something that was considered possible," explains Porteus, who is now at Stanford University. But David Baltimore was willing to take the risk.

In Baltimore's lab at the California Institute of Technology, Porteus adapted an existing reporter system to human cells. Double-stranded breaks in DNA caused cells to produce green fluorescent protein, but the DNA-cutting enzyme he was using—a homing endonuclease—could cut only one particular sequence. He needed a way to more generalizably control where the DNA would break. A lab mate who had previously worked on zinc fingers introduced Porteus to the relevant literature, and soon he had a pair of ZFNs from Chandra, advice for designing a cutting site from Carroll, and a big experiment underway. "I remember it was a Saturday morning when I did the FACS analysis, and I started seeing green cells pop up," he says. "I'd done enough experiments not getting green cells to know this was real." In other words, the engineered ZFNs were cleaving specific sequences in human cells. A few years later, Porteus and collaborators at Sangamo BioSciences reported the use of ZFNs to modify an endogenous gene in human cells.

Dana Carroll at the University of Utah showed that ZFNs could alter genes in whole organisms.

Well before these demonstrations, excitement around zinc fingers had already drawn commercial interest, although most plans involved engineering proteins to turn genes on and off rather than to change the underlying DNA. Edward Lanphier became intrigued by zinc fingers after reading Carl Pabo's 1991 publication describing the crystal structure of zinc fingers bound to DNA. The structure revealed how each finger domain contacted three nucleotides on a DNA strand. It filled researchers' minds with visions of made-to-order assemblies of fingers capable of recognizing any DNA sequence.

After interviewing top zinc-finger scientists, Lanphier, then head of commercial development at a gene-delivery company called Somatix, quit his job and launched Sangamo Biosciences in 1995, where he is still chief executive officer. Another company, Gendaq, was co-founded by Nobel laureate Aaron Klug, who discovered zinc fingers after realizing that many transcription factors contained similar domains. Describing the mood at the time, Keith Joung, who began his work on zinc fingers as a postdoc in Pabo's lab at the Massachusetts Institute of Technology, says, "I didn't know what they'd be useful for, but I figured if you could engineer binders to any sequence that that would be a very powerful technology."

Lanphier consolidated the intellectual property and know-how that were relevant to designing zinc fingers. Sangamo licensed technology from various universities, and it purchased Gendaq in 2001, acquiring a rich database detailing the construction of many zinc fingers, and retaining Klug on its scientific advisory board. That same year, Sangamo also recruited Pabo as chief scientific officer.

But zinc fingers did not immediately explode into extensive use. Sangamo kept a tight grip on the knowledge and intellectual property needed to make ZFNs work, and had a limited capacity for collaborating with academics. (The company estimates that the number of academic projects it has collaborated in is around 150.) Zinc-finger binding is subtle, and depends on more than simply matching triplets of DNA to the corresponding finger. Just as fingers on a hand have a certain order—pinky, ring, middle, index, thumb—zinc-finger proteins also fit together in a certain way. For example, a finger could bind tightly to one triplet of DNA when it is in the 'index' position, but have a less-secure grip when it is in other positions.

"The underlying constraint with zinc fingers that no one has been able to fully get around is that it takes some effort to make the best protein for the best site," explains Pabo, who returned to academia in 2003 and now runs a consulting company. "That

Plant pathologist Adam Bogdanove teamed up with bioinformatician Matt Moscou at the University of Utah to show how TALENs bind DNA.

kept ZFNs from immediately bursting out onto the world like [the polymerase chain reaction] or [RNA interference]," he says. The potential for more widespread use is still there, however. "TALENs have the possibility that they could break the field open," he says.

A simpler way to bind DNA

TALENs rely on a previously unknown way to bind DNA that is much simpler than that of ZFNs. Bacteria of the genus *Xanthomonas* ravage crops, infecting tomatoes, rice and some 200 other species. They inject virulence factors into plant cells as part of infection. Frank White and his postdoc Bing Yang at Kansas State University had shown that the injected proteins, the TALEs, bound DNA, but the exact sequences were unknown. Then, in 2007, researchers led by Ulla Bonas and Thomas Lahaye at Martin Luther University conducted a series of elegant and painstaking 'promoter-bashing' experiments, whittling away regulatory parts of plant genes activated by bell-pepper pathogens to reveal where exactly the protein bound. Investigation of other genes activated by this TALE identified a common sequence of 16 base pairs.

It was the first time a secreted protein from a bacterium had been shown to directly activate plant genes. Adam Bogdanove at Iowa State University was intrigued. He studies two rice pathogens, each of which makes around 20 TALEs. The proteins from each pathogen regulate different sets of rice genes, but their structures are very similar, except for one section in the middle. This section is highly repetitive, made up of sequences that are each about three dozen amino acids long. Within each protein, repeating sequences differ from each other by just two residues in the middle. White had developed a system of classifying TALEs according to these differing residues (called repetitive variable di-residues or RVDs). Bogdanove, who was accustomed to thinking of TALEs in terms of RVDs, noticed that the number of repeats in the bell-pepper TALE was approximately equal to the number of nucleotides to which it bound. "I thought, 'Could it really be so simple?'" Bogdanove says.

At this point, the researchers knew the exact binding site for only one TALE, but Bogdanove knew the promoter sequences of many genes affected by TALEs, and he knew the RVD sequences of those proteins. He also had a very special resource: a sort of bioinformatics brain trust, an entrepreneurial group of graduate students at Iowa State who take on small computational projects for fun and experience.

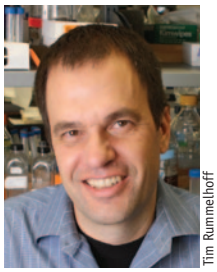
As it happened, Matthew Mouscou, a founding member of this group, worked in a lab just down the hall. Bogdanove told Mouscou about his hypothesis—that each variable di-residue in a TALE repeat bound to a single nucleotide—and said that he thought it could be tested computationally. The idea was to run RVDs along the promoter regions, looking for areas where the association between protein unit and nucleotide aligned. Within a week, they had found positions on the plant promoters where the RVDs locked in. Further, the association between nucleotide and RVD was constant over some 20 TALE-promoter pairings. The code was clear.

Meanwhile, Jens Boch, Sebastian Schomack and others on Bonas's team at Martin Luther University were also figuring out how TALEs recognize DNA. They noticed that TALEs with similar RVDs induce the same plant promoter. Wherever there was a continuous string of nucleotides in the binding site, there was also a continuous string of RVDs. Using those associations, Boch and his colleagues were able to find binding sites for seven other TALEs. When the researchers made artificial promoters designed to match particular TALEs, the proteins bound and activated reporter genes. Boch and his team were also able to construct artificial TALEs that bound predicted sequences of DNA. The implications were striking: proteins could easily be made to bind to any DNA sequence. "The potential was very clear right from the beginning," says Boch. "I couldn't sleep for two nights."

Bogdanove and Boch published papers showing how TALEs recognize specific nucleotides in the same issue of *Science* in 2009. By then, both were already at work on applications. Bogdanove teamed up with Dan Voytas at the University of Minnesota, who had done pioneering work with ZFNs in plants. They designed a new TALE and attached it to the DNA-cleaving domain of FokI, the same enzyme that Chandra had originally picked from a reagents catalog.

Maike Glöckner, Martin Luther University Halle-Wittenberg

Jens Boch and Sebastian Schomack redesigned proteins from plant pathogens to bind desired DNA sequences.


Tim Rummelhoff

Dan Voytas at the University of Minnesota found designing TALENs surprisingly easy after his work with ZFNs.

Thanks to Voytas's previous work with zinc fingers, the infrastructure and reagents needed to check for the requisite double-strand breaks were already in place.

Voytas was expecting a long, grueling haul. "I was very skeptical at first, having struggled so long to engineer the ZFNs, and then I finally got around to doing the experiment." He and his lab members set up a simple system in yeast such that if the nuclease cut as expected, clear fluid in a reaction tube would turn yellow. Voytas was surprised by the results, which were consistent and obvious. Still, he suspected an artifact. They repeated the experiment with another binding site and then others, continuing to accumulate positive results. He continues to use zinc fingers in his laboratory, but says that his lab members already prefer TALENs. All of his first-year graduate students are assigned the project of making and validating a gene-editing nuclease. This year's students usually have one within six weeks, says Voytas. Two years ago, when he asked new students to make ZFNs, they either never got past the validation stage or, if they did, the ZFN didn't work, he says.

Spoiled for choice

In just two years, TALENs have been used for many of the tasks that have been accomplished with ZFNs, including modification of human pluripotent stem cells and genome editing in rats, worms and zebrafish. This ease of use has led to a surge in numbers of scientists ready to try their hands at gene editing, says Joung, who is now at Massachusetts General Hospital. "People wanted to do this and thought it was too difficult, and so they were ready to jump in with TALENs," he says. "The pent-up demand is coming out."

Companies are eager to commercialize the potential of TALENs. Life Technologies licensed TALE technology from Martin Luther University. Paris-based Cellectis licensed it from Iowa State University and is already selling custom-made TALENs. And even as Philip Gregory, who is Sangamo's chief scientific officer, declares ZFNs the better-characterized technology, the company's researchers have done much to push forward TALEN technology, creating reagents and collaborating with scientists to show that TALENs can edit mammalian genes as well as engineering rats and human pluripotent stem cells.

But it is still far too early to declare TALENs the technology of choice. Zinc-finger nucleases have been around for more than a decade, but only this year have researchers examined nonspecific cutting across the entire genome. Some preliminary work indicates that TALENs might be more specific and less toxic than ZFNs, but they have not been studied as extensively. "Off-targeting is a key question," says Bogdanove. "You don't want to fix one gene and break another."


A. Bekker, MGH

Keith Joung at Massachusetts General Hospital develops nonproprietary techniques for designing gene-editing nucleases.

Another key question is how efficiently a newly designed nuclease will cut the intended sequence, a factor that becomes particularly important for researchers hoping to change several genes within the same cell, to completely knock out a pathway, for example. At this stage, researchers are still publishing successful firsts with TALENs, not reporting how frequently a TALEN fails to work. "The idea that ZFNs should be shunted to the side because TALENs are easier, I'm not sure I agree with that," Joung says. "At the end of the day, you want the platform with the least off-target effects and confounding variables, and the highest success rates." And both TALENs and ZFNs will benefit from a better understanding of how to deliver enzymes and donor DNA sequence. At this point, cutting at a desired sequence is easier than prompting the kind of DNA repair that will result in the desired edit. There is a lot of underlying biology that we still need to learn. "It's delivery, delivery, delivery: how do you get everything to happen at the right time within the cells?" says Voytas. "We have to create the break and slip in the donor template. A straightforward solution hasn't presented itself yet. The standardized, optimized protocols for specific organisms haven't been forthcoming, but it is coming."

Meanwhile, ZFNs are more accessible than they have ever been. Although the labor required is still significant, Joung and other researchers are working out public, nonproprietary methods for designing them and are creating protocols, reagents and design tools. The first protocol was published in 2008, just weeks before Sigma-Aldrich, which has a license agreement with Sangamo for selling research products, launched a program making ZFNs available for purchase. (Sangamo began a clinical trial in people infected with HIV in 2008, and the company is now focusing on clinical programs.) Although validated, custom-made ZFNs once cost tens of thousands of dollars apiece, Sigma now offers them at around $12,000 each. Ready-made ZFNs cost $6,000. The company also sells rats, mice and cell lines that carry specific mutations made using ZFNs. Researchers may worry about restrictions on the products, such as limitations on breeding or sharing animals with collaborators, but they also buy them.

The availability of ZFNs is starting to shape the way scientists do research. "Most scientists don't think of editing the human genome as an immediate possibility when creating experimental schemes to verify a hypothesis," says Keith Hansen, product manager for functional genomics at Sigma Life Sciences, but that's changing quickly. "While most of the earlier ZFN requests were for constitutive gene knockout applications in cell lines, researchers have become more sophisticated and demanding in the types of genetic changes that they want to create." More subtle questions can be addressed by changing a gene rather than deleting it. Ultimately, the question of whether TALENs are superior to ZFNs may not be as important as the fact that they get more people thinking about new kinds of experiments.

Monya Baker is technology editor for *Nature* and *Nature Methods* (m.baker@us.nature.com).