


# The epigenome editors:

## How tools such as CRISPR offer new details about epigenetics

By Cassandra Willyard



When neuroscientist Eric Nestler began his career 30 years ago, researchers were just coming to appreciate that addiction seems to indelibly alter the brain. Intense cravings persist even after an individual stops using drugs because, researchers realized, the cellular and molecular changes in the brain endure. Nestler, now at the Icahn School of Medicine at Mount Sinai in New York City, felt that he might have struck upon a possible explanation for this in the early 2000s, when he began reading papers about 'epigenetic' chemical tags found on DNA and the proteins around which it usually winds. These tags affect which genes get expressed and which ones remain silent. As Nestler pored over these seminal studies, he began to wonder whether repeated exposure to a drug such as cocaine might alter the epigenetic marks on brain cells, and in so doing, turn a normal neuron into an addicted one. Epigenetic alterations can permanently transform stem cells or cancer cells—so why not neurons?

Over the next several years, Nestler's lab began to amass evidence to support this hypothesis. He and his colleagues found that drugs such as cocaine can alter the epigenome. But with each new experiment, Nestler butted up against the same stubborn problem: he had tools for altering the epigenome, but they weren't precise. He could dramatically alter the expression of genes that add or remove marks. But this strategy changed the epigenome at hundreds or even thousands of sites. Nestler had no way to target epigenetic tags on specific genes.

Marianne Rots, an epigenetics researcher at the University of Groningen in the Netherlands, has come up against the same roadblock while working with epigenetic drugs such as broad-acting histone-deacetylase (HDAC) inhibitors, which disturb the entire genome. Although these drugs seem to work well for some diseases, such as cancer, "you do not really want to have this messy effect" if the goal is to understand epigenetic mechanisms, she says.

Now, however, researchers have tools for altering the epigenome with unprecedented precision. These techniques, developed over the past 15 years, have enabled them to add or remove chemical tags on histones or DNA at specific places in the genome. These precise alterations have made it possible to probe the epigenome in ways that would have been impossible a decade ago. For the longest time, epigenetics has been "guilty by association," Rots says. "This is really allowing us now to start to understand epigenetics."

As scientists race to deepen their understanding of the epigenetic code, clinical researchers are also taking baby steps toward testing these powerful new tools as therapies for various diseases, including HIV infection and cancer. "There are many different examples of diseases that are associated with these kind of changes," says Ahmad Khalil, a biomedical engineer at Boston University. And there's an added bonus. "We're not touching the underlying DNA sequence," he adds. "Those modifications are inherently reversible."

### Proof positive

Epigenetic marks modify gene expression in a variety of ways. Methyl groups on DNA tend to silence genes. And histones can carry several kinds of marks that alter the structure of chromatin, the complex of DNA and histones that loops and whorls through the cell's nucleus. Genes that inhabit tightly packed chromatin tend to stay silent. Genes that reside in more open stretches tend to be transcribed.

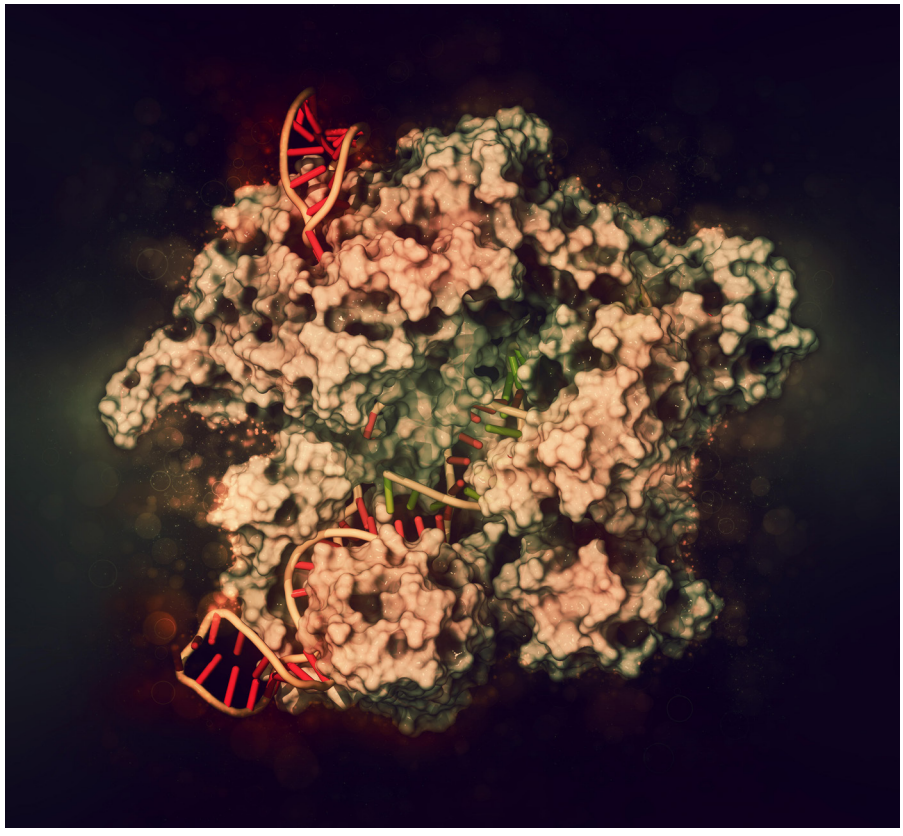
In the 1990s, epigenetics was still a young field, and some researchers questioned the importance of epigenetic modifications. Could they really be involved in gene regulation? "There was a hard-core transcription community who felt that chromatin architectures were largely irrelevant," says Philip Gregory, a former chromatin researcher who is now chief scientific officer at the Massachusetts-based gene-therapy company Bluebird Bio.

In 1996, biologist David Allis, who was then at the University of Rochester, and his colleagues took an important step toward proving them wrong. They identified the first histone-modifying protein in a single-celled microbe[1]. This enzyme could add acetyl groups to histones, a modification that relaxes chromatin and makes DNA more accessible for transcription. That same year, another group identified an enzyme that could remove acetyl groups[2]. The enzymes seemed to be related to proteins that influence gene expression in yeast. By 2001, Allis and other researchers had uncovered many more enzymes able to add and remove histone marks. Allis proposed that this marking system might form a 'histone code,' a complex language that could be used to shape chromatin and regulate gene expression. This was one of the articles that piqued Nestler's interest.

Gregory and his colleagues, meanwhile, were interested in many of the same issues. They knew that certain epigenetic marks on histones were associated with gene activation or repression—but was this cause or effect? In the early 2000s, Gregory went to work for Sangamo BioSciences, a California-based company founded to explore the therapeutic potential of proteins called zinc fingers. These proteins can bind DNA and, when fused to a nuclease, snip it. Gregory and his colleagues wanted to use zinc fingers to add epigenetic tags to histones to determine whether adding a particular histone mark would be enough to repress the expression of an endogenous gene. In 2002, they showed that it could, further bolstering the importance of the epigenome[3].

At the time, "the zinc-finger platform provided really the only way to address that question," Gregory says. In the past decade, however, two other gene-editing technologies, both derived from bacteria, have become available: transcription activator-like effectors (TALEs) and CRISPR–Cas9. Each of these tools has advantages and disadvantages, but for ease of use and cost, nothing beats CRISPR. "It's straightforward, it's cheap, it's quick," Rots says. "That really makes life very easy."

In 2013, researchers in California hacked CRISPR to make it useful for epigenetic editing. Cas9 normally cleaves DNA, which is why it's often called 'molecular scissors.' The California team broke these scissors to create something they called 'dead' Cas9 (dCas9)[4]. dCas9 can still travel to any desired part of the genome with the help of a guide RNA, but it binds DNA without introducing a break. Since then, researchers have begun fusing dCas9 to epigenetic writers and erasers.



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**Editing the editor:** Standard CRISPR–Cas9 (shown here) has been retooled to modify epigenetics.

These new tools have allowed epigenetics researchers to delve into the mysteries of the field with even greater ease. For example, Rots and her colleagues used CRISPR to add a particular methyl mark to histones. The researchers targeted several genes and showed that when they added this mark to silenced genes, the genes became activated[5]. "We could re-express these genes just by writing one single mark," she says. And by combining several writers, they managed to get the marks to persist from one generation of cells to the next.

## Enhanced insight

Targeted epigenetic editing tools are also helping researchers to better understand the role of enhancers, short stretches of DNA that can activate genes from afar. An enhancer might be right next to a gene, or it might be on an entirely different chromosome. That makes it exceedingly difficult to tell which genes any given enhancer is controlling. "Up until now, it's been really difficult to study them and know what they do because there's no tool to go in and perturb them," says Charles Gersbach, a biomedical engineer at Duke University in Durham, North Carolina. "Now, with these epigenome editing tools, we can do that."

Enhancers are silenced or activated by the same epigenetic marks that control the expression of genes. In 2015, Gersbach and his colleagues showed that they could turn individual enhancers on by hitching dCas9 to enzymes that add acetyl groups on histones, and then guiding these fusion proteins to particular enhancers[6]. However, the human body could contain as many as 2 million different enhancers. "It's going to take us forever if we go in and try to manipulate these things one at a time," Gersbach says.

So the researchers developed a high-throughput screen[7]. Rather than creating a single guide RNA and targeting a single enhancer, they manufactured tens of thousands to take dCas9 to many different regions of the genome likely to include enhancers. That allowed them to identify the regions that altered the expression of particular genes. In this case, the team focused on two genes: beta-globin and human epidermal growth factor

receptor 2 (*HER2*). Because the enhancers for beta-globin are well characterized, the team used this gene to validate the system. The researchers also looked at *HER2,* which is often overexpressed in cancer. "We wanted to get a better idea of what are the regulatory elements that are driving the most well-known oncogenes," Gersbach says. The screen picked up several potential enhancers that had never before been linked to *HER2*. Gersbach hopes to eventually combine this technique with DNA sequencing to search for cancer-driving mutations in enhancers.

Enhancers control genes by taking advantage of chromatin's loopy structure. Intersecting loops bring enhancers into contact with various genes. An enhancer can boost expression of the genes within its own loop, but it can't regulate genes in other loops because proteins called insulators separate one loop from the next. In glioma cells, abnormal methylation prevents insulators from binding properly and allows two neighboring loops that are normally separate to combine[8]. "The loop sort of unfolds and becomes one big loop," says Bradley Bernstein, a pathologist at Massachusetts General Hospital in Boston. The missing insulator allows an enhancer on one loop to activate an oncogene nearly a million base pairs away. Bernstein thinks that researchers might be able to use epigenetic editing to leverage this mechanism by adding methyl groups that unite previously separate loops, a strategy that might turn genes on and keep them on.

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A recent paper from the laboratory of Rudolf Jaenisch, a biologist at the Massachusetts Institute of Technology in Cambridge, provides evidence that this epigenetic editing strategy might work. Last year, Jaenisch and his colleagues fused dCas9 to an enzyme that adds methyl marks, and steered this molecule to two insulator binding sites in mouse embryonic stem cells[9]. Methylation of these sites disrupted insulator binding, enabling enhancers to turn on genes in neighboring loops. "You can change the whole chromatin configuration, but very surgically, very specifically," Jaenisch says.

Having the ability to perturb enhancers using targeted epigenetic modifiers is a huge advance, Gersbach says. Hundreds of genome-wide association studies have revealed that more than half of disease-associated variation occurs in enhancers, not in genes. That makes some sense, he adds, because mutations in genes can have devastating consequences. Mutations in enhancers might have subtler effects. "You're dialing a gene up or down a little bit more than it should be," he says.

## Rewriting the epigenome

In the race to bring gene-editing technologies to the clinic, targeted epigenetic editing has been largely overshadowed. In some ways, altering the epigenome by targeting epigenetic markers on specific genes could be a safer option than altering the genome. Epigenetic editing can be, in principle, reversible, Jaenisch says. "You change gene expression by changing the epigenome, not by changing the sequence." Marks can be added or removed by simply swapping the enzyme. And using CRISPR for targeted epigenetic editing allows researchers to potentially target multiple genes at once.

But some research suggests that using CRISPR in combination with the special dCas9 might have more off-target effects than genome-snipping with the regular Cas9 protein. In 2014, researchers created a binding map of dCas9. It showed that dCas9 bound between 2,000 and 20,000 sites in the genome, depending on the guide RNA used. Mismatches also occur with active Cas9, but there seems to be a mechanism for preventing erroneous cuts. The researchers found that active Cas9 made a snip at only one of 295 possible off-target sites[10]. There's also another challenge related to dCas9: scientists tend to package DNA-editing CRISPR into viruses, but CRISPR when engineered for targeted epigenetic editing is substantially larger because it also contains an epigenetic writer or eraser. It won't fit inside the small adeno-associated virus that researchers often use to deliver gene therapies. Nestler's group has sidestepped this issue by using larger herpes simplex viruses, but those vectors might not be as safe to use in humans.

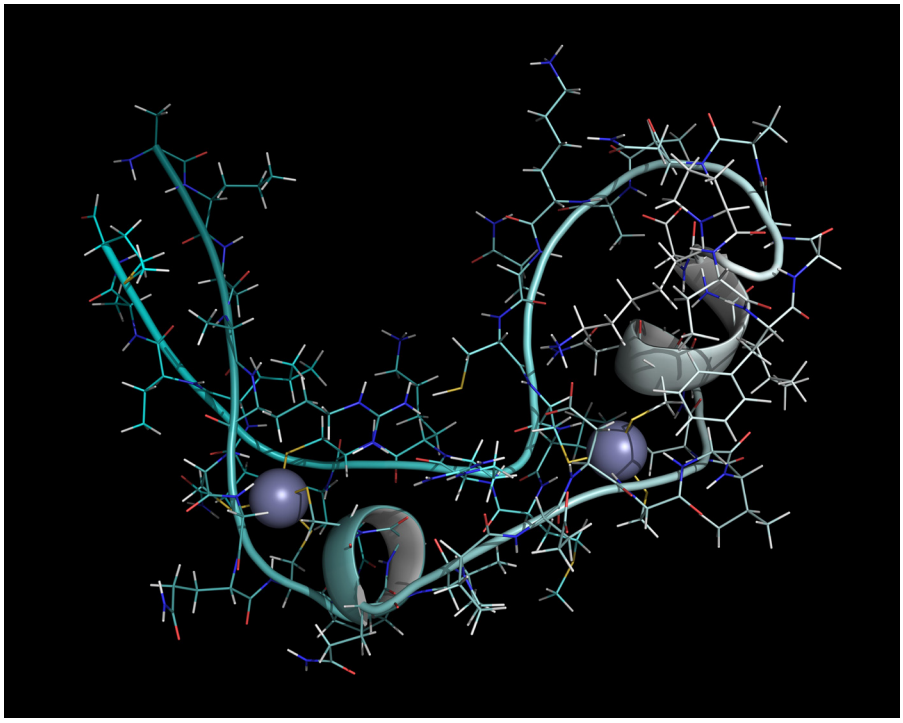
Despite these challenges, researchers still think that dCas9 and similar epigenetic editing technologies have promise. Rots and her colleagues investigated the possibility of using targeted epigenetic editing to curtail mucus production in cells. That could be a way to help people with diseases such as chronic obstructive pulmonary disease and asthma, conditions that often result in an overproduction of mucus. A vast network of genes is responsible for mucus production and secretion. Rots and her team went after a crucial one called sterile alpha-motif pointed domain-containing Ets transcription factor, or *SPDEF*[11]. The researchers hoped that if they could turn off *SPDEF* in mucin-secreting lung cells, they might be able to staunch the flow of mucus.

There are a variety of ways one might use epigenetic editing to shut down *SPDEF*. Rots and her team have tried nearly all of them. The most successful strategy involved hooking either zinc fingers or dCas9 to G9a, an enzyme that adds methyl marks to histones. The team used three guide RNAs to steer the fusion protein to sequences in the promoter region of *SPDEF*, and then used a virus or plasmid to get these proteins into lung cells. The process not only shut down *SPDEF*, it indirectly reduced the expression of other mucus-related genes. Scientists have other ways, such as RNA interference, of turning off genes in cells, but one potential advantage of epigenetic editing is that it could be long-lasting or even permanent. Cells have mechanisms for maintaining their epigenetic marks as they divide, and Rots says that the marks added by G9a did seem to persist from one generation of cells to the next. But that's not always the case. No one yet understands the rules that govern which engineered epigenetic changes will persist in daughter cells, she adds. The rules seem to vary depending on gene and cell type.

Other groups, meanwhile, are using targeted epigenetic editing to turn specific genes on. David Schaffer, a bioengineer at the University of California, Berkeley, hopes that this strategy will help to eradicate HIV from the body. Antiretrovirals "have been miracle drugs," Schaffer says. But they aren't a cure for the disease, because HIV can integrate into regions of the genome that aren't being transcribed. Antiretrovirals can't touch this latent pool of virus. "Because that latent pool is always hiding around waiting to wake up, that means you need to keep those antiretrovirals on permanently," he says.

Many researchers, including Schaffer, are trying a novel solution: wake up the virus and purge it all at once. Researchers have tried to do this by using HDAC inhibitors, which strip the genome of epigenetic silencing markers. But these drugs can be toxic, and the strategy doesn't seem to work well in people with HIV. In 2014, two human trials of such drugs showed that they didn't do much to shrink the reservoir of infected cells.

Schaffer and his colleagues realized that if they could use a more targeted tool to remove some of the epigenetic marks that keep HIV quietly hiding in the genome, they might



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**Within reach:** Epigenetic editing made inroads in the lab with zinc fingers.

have a shot at wiping it out. Once the virus is actively replicating, infected cells quickly die. And antiretrovirals can prevent the virus from infecting new cells in the meantime. So the researchers fused dCas9 either to viral gene activators, which alter the epigenome indirectly by recruiting epigenetic editing proteins, or to p300, an enzyme that adds acetyl groups to histones[12]. They used guide RNAs to direct these proteins to portions of HIV's long-terminal-repeat promoter. Both strategies worked in cell-line models of HIV latency, but Schaffer's best results came when he combined targeted epigenetic editing with the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA). Combining the two strategies might allow for the use of less-potent drugs or lower doses, and help avoid unwanted side effects. Antiretrovirals work best in combination, Schaffer says, and tools to activate latent HIV might, too. "We may need combinations of perturbations to activate the HIV genome," he says.

And these applications are just the beginning. Targeted epigenetic editing could address a whole range of other disorders, and lab experiments in cells and animals are already under way. Grant Challen, a researcher at the Washington University School of Medicine in St. Louis, is working to develop these tools as possible cancer therapies. Jaenisch is attempting to tackle autism by activating silenced genes in cells and rodents. And Rots is exploring the possibility of soon using epigenetic editing to silence genes associated with fibrosis in transplanted organs. Transplanted kidneys, for example, often fail even if they aren't rejected. Over time, the kidneys develop too much tough collagen tissue. Rots sees a possible fix. "There are so few good organs," she says. And delivery of such a therapy would be relatively straightforward. "You have the organ outside of the donor before you transplant it. So, you can easily perfuse it and have the cells take up the editors," she says. "If the cells do remember their epigenetic programming, then this might prolong the functionality of the transplanted organs."

None of these strategies is ready for human testing. But it's only a matter of time. Researchers have already launched clinical trials to test the use of zinc fingers to edit the genomes of people with HIV infection, hemophilia, or lysosomal-storage disorders. And in 2016, scientists in China began injecting a person with CRISPR-edited cells. If gene-editing therapies pan out, targeted editing of epigenomes in people may come next.

In the meantime, scientists such as Nestler are using this targeted approach to better understand the molecular underpinnings of diseases such as addiction. One of the key genes in cocaine addiction is the FosB proto-oncogene, AP-1 transcription factor subunit (*FosB*). Cells in certain regions of the brain express *FosB* normally, but in mice that are chronically exposed to cocaine, a truncated variant of FosB accumulates to high levels. Nestler and others think that *FosB* constitutes a molecular addiction switch.

In 2014, Nestler's team used zinc fingers and TALEs to add methyl groups to histones at the promoter region of *FosB* in one particular reward region of the brain[13]. The researchers found that this methylation blocked expression of the gene in mice and prevented cocaine from activating *FosB*. Moreover, the strategy helped them to decipher the underlying mechanism: the added methyl groups prevented the activation of another transcription factor that has a key role in the process. "That was really exciting," says Elizabeth Heller, a former postdoc in Nestler's lab and now an addiction researcher at the University of Pennsylvania's Perelman School of Medicine in Philadelphia. "I don't think we could have discovered that mechanism any other way."

Nestler compares the methods of the recent work with the less precise tools of the past: "For the past 20 years or so, anyone who wanted to study the role of a gene in the brain would overexpress that gene or knock it out," Nestler says. Using these older tools, researchers could crank gene expression way up or eliminate a gene completely. "That's very different from what we see in biological regulation," which involves subtle fine-tuning—something more akin to what's possible using targeted epigenetic editing.

"These types of epigenetic editing approaches will become the norm of gene-regulation studies in brain," he adds. They may even eventually grow into therapies for addiction.

For targeted epigenetic editing to happen in humans, researchers must still solve some of the technical challenges of delivery and safety. But these seem to be surmountable barriers. "The field really is very young, but it's highly exciting," Rots says. "I'm quite sure it's here to stay."

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