Genome Engineering with Targetable Nucleases

Dana Carroll
University of Utah School of Medicine
Salt Lake City, Utah

dana@biochem.utah.edu

It is amazing what we can do with current tools of molecular biology. I often tell students we can answer questions today that we couldn't even phrase 30 years ago. For example, we can query whole genomes in a variety of ways, from sequencing to gene expression to chromatin status. In addition, for the last dozen years or so we have had the capability to modify genomes with great range, subtlety and specificity, and with striking efficiency. Part of this meeting is dedicated to describing the tools for making such modifications, and I will do my part. I will also offer a few thoughts about the applications of this technology in ways that have great potential for improving the human condition.

Zinc Fingers

First, a little history. As implied by their "nuclease" designations, the genome-engineering tools act by making breaks in DNA. Many years of research on many different organisms and in many laboratories led to an understanding by the mid-1990s that double-strand breaks are repaired by cellular mechanisms that rely on homologous recombination or error-prone end joining. This suggested that the very low frequency of "classical" gene targeting could be improved by making targeted double-strand breaks. Meanwhile, Chandrasegaran and colleagues discovered that the recognition and cleavage activities of the Type IIS restriction endonuclease, FokI, could be physically separated (Kim *et al.*, 1996). They replaced the natural recognition domain with alternatives from other sources and showed that cleavage was redirected to new sites. When they linked the cleavage domain to a set of zinc fingers, they opened the door to designing reagents that would be directed to completely novel targets. Zinc fingers were identified in natural eukaryotic sequence-specific transcription factors and were known to bind DNA in very modular fashion—one finger per three base pairs—and fingers had already been identified for a number of different triplets. Thus, in addition to basic advances in DNA manipulation, three specific research threads led to the production of the first zinc-finger nucleases (ZFNs).

In a nutshell, early work with ZFNs showed that it takes two such constructs to cut DNA efficiently, since the cleavage domain must dimerize, and the dimer interface is quite weak. An optimal linker between the binding and cleavage domains was identified. Despite the fact that the nuclease domain came from a bacterium, ZFNs effectively cleaved targets that were assembled into chromatin.

The next major step was the demonstration of ZFN activity in whole cells and organisms. The first such experiments were done with *Drosophila melanogaster*, demonstrating both targeted mutagenesis by simple cleavage and reliance on inaccurate repair and homologous gene replacement when an appropriate donor DNA was supplied. Additional work showed efficacy in cultured human cells, in plants and in nematodes. By now, ZFNs and other nucleases have been used successfully to modify the genomes of more than 50 distinct organisms.

A drawback to the use of ZFNs was the difficulty of deriving reliable designs for new targets. Assembling new combinations of fingers from existing libraries was successful in some cases, but not in others. Methods for selecting novel combinations for individual new targets from mutagenized pools helped, but were not widely adopted for various reasons. Very reliable constructs were and are available commercially, but at prices inaccessible to some. Nonetheless, ZFNs have been used widely.

TALENs

The discovery of a simpler DNA-recognition code in transcription activator-like effectors (TALEs) in *Xanthomonas* bacteria led to a new platform for targeted cleavage. In these proteins, each module binds a single base pair, and individual modules reliably recognize each of the four types of base pair. Thus, new recognition domains could be assembled simply by reading the target DNA sequence and inserting the appropriate TALE subunit. Linkage to the FokI cleavage domain created TALE nucleases (TALENs) that, like ZFNs, needed to be provided in pairs to ensure cleavage. It is perhaps fortunate that, again like zinc fingers, the TALE modules naturally bind DNA in a chromatin context, in this case targets in plant-host chromosomes. TALENs were rapidly adopted in preference to ZFNs, particularly in research labs, for the simplicity of their design, for the higher rate of success of new designs, and the apparent sequence specificity of the ultimate reagents.

CRISPRs

The latest additions to the tool kit are the CRISPR/Cas RNA-guided nucleases that I like to simply call CRISPRs. In this case, recognition is mediated by a guide RNA, using Watson-Crick base pairing, making designs for new targets even simpler than for TALENs. In addition, a single protein, Cas9, is required, and it doesn't need to be modified when the target is changed. These features have led to very rapid adoption of CRISPR nucleases.

Common Characteristic

An important characteristic common to these three platforms is that DNA recognition and cleavage are mediated by functionally separable domains. This means that each can be manipulated independent of the other, which is, of course, critical for attacking new

targets by changing binding specificity. The nuclease domains have also been manipulated in various fashions, notably to produce single- rather than double-strand breaks at the target.

Ultimately, these targetable nucleases make breaks only at the desired DNA targets. Everything that happens afterward depends on cellular DNA-repair activities. This means that the outcomes of a nuclease-mediated targeting event may be different in different organisms or cell types, depending on the status of those repair activities. In addition, the ways in which the nucleases—and donor DNA, when homologous repair is sought—are delivered will depend on what is appropriate in any application.

Model Fruit Fly

We have investigated a number of these repair parameters using *Drosophila* as the model organism. We asked, *What gene products participate in nuclease-mediated targeting, and what are the consequences of disabling each of them?* Not surprisingly, we found that the Rad51 and Rad54 homologues were required for most (but not quite all) of homologous repairs. More importantly, in the absence of DNA ligase IV (Lig4), a dedicated component of nonhomologous end joining (NHEJ), the majority of repairs proceeded by homologous recombination (HR). In many experimental situations, HR is the desired pathway. It is not possible to propagate Lig4 mutants of quite a number of organisms, so alternative means of disabling the enzyme, or other NHEJ activities, are being sought.

Another important question regarding homologous repair is, *How much homology is needed to support efficient sequence replacement?*

Reference

Kim YG *et al.* (1996) Hybrid restriction enzymes: zinc finger fusions to FokI cleavage domain. Proceedings of the National Academy of Sciences of the USA 93(3) 1156–1160.

DANA CARROLL received a bachelor's degree in chemistry from Swarthmore College, and a PhD in biophysical chemistry from the University of California at Berkeley in the laboratory of Ignacio Tinoco, Jr. He was a postdoctoral fellow in Glasgow with John Paul, and at the Carnegie Institution Department of Embryology with Donald Brown. He has been on the faculty at the University of Utah School of Medicine since 1975, where he served as chair of the Department of Biochemistry from 1985 to 2009.

DR. CARROLL has a long-term interest in molecular mechanisms of DNA repair and recombination. He was a pioneer in the use of zinc-finger nucleases (ZFNs) for targeted genome modifications. For this work he received the 2012 Novitski Prize from the Genetics Society of America and the 2014 Sober Lectureship Award from the American Society for Biochemistry and Molecular Biology. His current research focuses on genome engineering using ZFNs, TALENs, and CRISPR/Cas nucleases.