Examining CRISPR-Cas9: A Primer for "A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity."

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Abstract

Genome editing has the potential to transform the way we approach science and medicine. Clustered Regularly Interspaced Short Palindromic Repeat-Cas9 (CRISPR-Cas9), a new genome editing technique, has lowered the cost while increasing the specificity and simplicity of genome editing. This new method could drastically change how we treat and cure many different types of human diseases and disorders by correcting mistakes in human genes. The paper by Jinek and colleagues, "A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity", was the first to describe the functions of all the molecular components in the Cas9 complex and build a RNA that could be used by the complex for genome editing (Jinek et al., 2012). This primer will discuss the Jinek paper in order to provide baseline knowledge on CRISPR-Cas9 and help further understand why this method is causing a revolution in biology and disease treatment.

INTRODUCTION

Genome editing is a technique at the forefront of modern biology. Until recently, changing the genome of a desired organism has been relatively complicated and usually involved species-specific experimental systems. For example, knocking out genes using homologous recombination only works in a few species such as mice. In 2012, an international group of researchers showed that CRISPR-Cas9, composed of the Cas9 protein paired with RNA components, could bind to target gene sequences and cut the DNA of the targets with high specificity and fidelity. The cells' endogenous ability to repair DNA breaks then causes small deletions or insertions (indels) at the cut site while repairing the DNA. The CRISPR-Cas9 system is a powerful tool that has since been used to edit sequences in a wide variety of organisms. Consequently, CRISPR-Cas9 genome editing has opened up research in a wide array of fields. In humans CRISPR-Cas9 has been used in research for gene therapy for genetic disorders (Li et al., 2015; Schwank et al., 2013), HIV research (Liao et al., 2015), and even altering human embryonic stem cells (Kang et al., 2016).

Discovering and utilizing CRISPR-based techniques has been a decades-long process. CRISPR sequences are sequences of DNA that are repeated in bacterial genomes. They were originally discovered in the 1980s in certain bacteria (Ishino et al., 1987), but the purpose of the repeated sequences was not discernable at that time. The true role of CRISPRs was discovered in the 2000s when mounting evidence showed that CRISPRs, along with various proteins, act as a defense against incoming viruses and the inserted viral DNA (Barrangou et al., 2007; Garneau et al., 2010; Jansen et al., 2002). Bacteria take small pieces of the invading viral DNA and insert it into a specific region of the bacterial genome (Barrangou et al., 2007). These regions of viral DNA within the bacterial genome

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are flanked on either side by short repeating sequences—CRISPRs—that help the bacteria differentiate the viral DNA from their own DNA.

In conjunction with the CRISPR sequences, Cas endonuclease proteins in complex with RNA molecules then cut viral DNA, thereby protecting bacterial cells against viral infection. These Cas/RNA complexes are categorized into three types based on the Cas protein used. Type II CRISPR-Cas system, of which Cas9 is a part, is unique in that it always uses a Cas9 protein and two forms of noncoding RNA (Wiedenheft et al., 2012). Here lies the importance of the Jinek et al. 2012 paper. The paper's authors provide convincing evidence for the cleavage mechanism of CRISPR-Cas9, including the role of the non-coding RNAs. They then use that information to create the first human-designed CRISPR-Cas9 editing complex. Understanding exactly how bacteria cut up viral DNA with this Cas9 complex was an exciting advance and the missing link to the end goal of using CRISPR-Cas9 genome editing to modify almost all organisms, including humans.

OVERVIEW OF RESULTS

This primer will focus on the five most important experiments of the Jinek paper. Annotated versions of selected figures from the Jinek paper are used to aid understanding these experiments. The text will focus on both the experimental design and the relevance of each experiment's results.

The goal of the first experiment was to define the essential components needed to cause a double-stranded break in DNA. Molecular experiments were used to demonstrate that three components were required for the effective cleavage of DNA. In addition to the Cas9 protein, previously shown to be an endonuclease (Sapranauskas et al., 2011), the authors tested two noncoding RNAs, CRISPR RNA (crRNA) and trans-acting RNA (tracrRNA). These RNAs were chosen because previous work had implicated both in the cleavage of the target DNA (Deltcheva et al., 2011).

The authors tested the two RNAs associated with the Cas9 complex using a technique called a metal dependent cleavage assay (Jinek et al., 2012, Figure 1). The metal dependent cleavage assay utilizes gel electrophoresis, a common molecular technique in which samples are run through a gel matrix, typically composed of agarose, and move due to an applied electric current. Gel electrophoresis works because DNA is a slightly negative molecule and moves toward the positive end of the gel apparatus when the current is applied. Higher molecular weight pieces of DNA travel more slowly than short pieces through the gel and as a result will be closer to the negative side of the gel.

In the metal dependent cleavage assay, test samples containing a known DNA test sequence with a specific

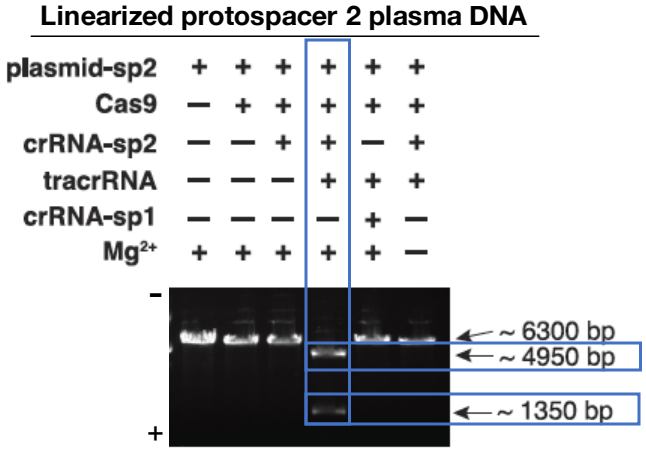

Figure 1. Adapted from Figure 1A of Jinek et al. 2012 paper showing a metal cation cleavage gel assay of different mixtures of possible molecular components tested for double-stranded DNA cutting ability. Lane 4 showed two bands of different lengths than the one band present in the control lane and in the lanes lacking an essential component. Figure modified from Jinek et al., 2012 with permission from original publishers.

protospacer adjacent motif sequence (PAM), a crRNA, tracrRNA, and the Cas9 protein were mixed with a metal required for catalysis. Samples were incubated for one hour and tested using gel electrophoresis for cutting activity. If the mixture was not competent to cut DNA, then the bands on the gel would have relatively high molecular weights and would be unchanged from control. If the mixture was competent to cut DNA, there would be two distinct DNA bands of lower molecular weight further down the gel. The authors only found cleavage of their DNA strand when an crRNA specific to the target DNA, a tracrRNA, and the Cas9 protein were in the same mixture (Figure 1). This indicated that for the Cas9 cleavage to work in vitro, all three components were needed.

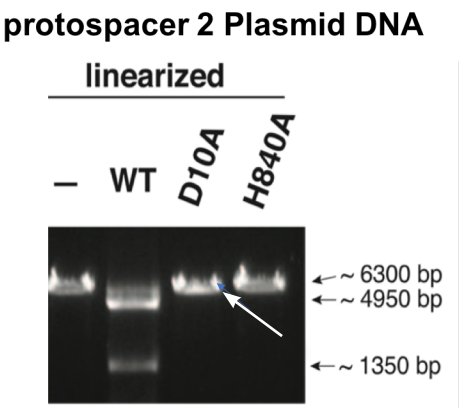

Figure 2. Domain cleavage of Cas9. Gel showing that the Cas9 mutated endonuclease domains D10A and H840A each cut one side of the linearized DNA strand targeted by the Cas9 complex. An example of fraying is indicated by the white arrow. Figure from Jinek et al., 2012 used with permission of the original publishers.

In the next phase of the study, the authors showed that both of the endonuclease domains of the Cas9 protein were involved with cutting DNA, with each domain cutting a single strand of a double-stranded DNA molecule (Figure 2). To do this, they modified the Cas9 protein sequence to ascertain the effects on the protein's activity. The two domains of the Cas9 protein were modified and activity of the altered proteins compared to the wild type (WT), normal Cas9 by running another metal dependent cleavage assay. While the wild type sample effectively cut both strands of the DNA sequence, the samples with only one normal domain were shown to only cut one strand. The double-stranded breaks appeared as two bands on the gel and the single-stranded breaks appeared one DNA band with fraying on the edges of the DNA band (Figure 2). This was strong evidence that each domain cut only one side of the double-stranded target DNA. To augment this analysis, the authors ran the experiment again with single-stranded DNA to determine which domain cuts which side of the DNA, finding that each domain could effectively cut one of the single-stranded DNA samples.

Having determined which RNAs were needed for Cas9 to work and the relevance of the Cas9 domains, the researchers determined the exact roles of the crRNA and tracrRNA, the two noncoding RNA sequences already associated with the complex. crRNA was shown

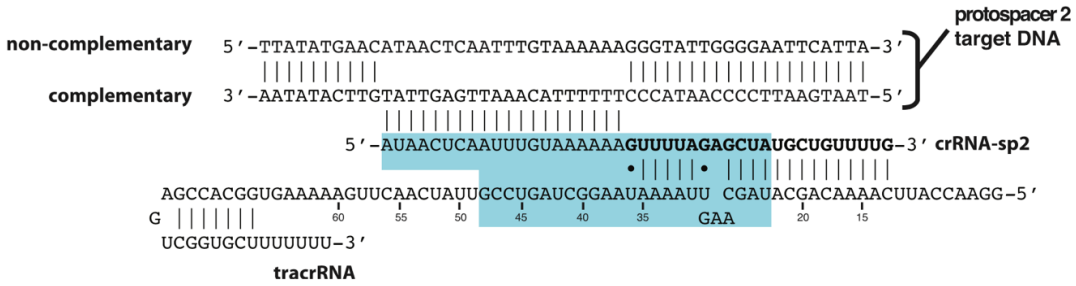

Figure 3. Schematic showing how crRNA and tracrRNA work to bind to target DNA. Blue marks the regions of both RNAs that the authors found necessary for effective DNA cleavage of target. The non-blue sequences could be mutated or removed without effecting cleavage ability. Figure from Jinek et al., 2012 used with permission of the original publishers.

to guide Cas9 to DNA sequences via base sequence pairing the DNA target while tracrRNA was shown to be necessary for the Cas9 complex to cleave the target DNA (Figure 3).

The authors next mutated various parts of both the tracrRNA and crRNA to see which were required for function (Figure 3). The authors were able to show that up to 6 bases could be removed from either side of the tracrRNA (5' or 3') but only from the 3' side of the crRNA. The similarity of the sequences of the tracrRNA and crRNA led the researchers to hypothesize that the two RNAs would then be linked together to form a hairpin structure. This hairpin structure could be bound by the Cas9 protein.

The researchers next focused on how the Cas9-RNA complex bound to the DNA. They found that a specific sequence called PAM in the target DNA was needed for accurate Cas9 attachment. PAM had been shown to be necessary to Cas9 binding (Sapranauskas et al., 2011), but the mechanism for the binding was not known. As the PAM sequence was only three nucleotides long, Jinek and colleagues were able to mutate the sequence and run gel assays with mutated PAM sequences against controls to test the function of the PAM on the target DNA (Figure 4).

They found that while the PAM was not needed for single-stranded DNA cutting, the PAM region acted as a start region for the Cas9 complex to bind. Cas9 had limited targeting ability to DNA sequences with even one changed PAM nucleotide. The researchers accomplished this using an electrophoretic shift mobility assay (ESMA) (Hellman and Fried, 2007). This specialized method shows whether or not a protein is bound to DNA by putting a mixture of known DNA and the protein of interest on a gel. As with the previous experiments, a current was applied to the gel. If the protein is attached the DNA, the DNA

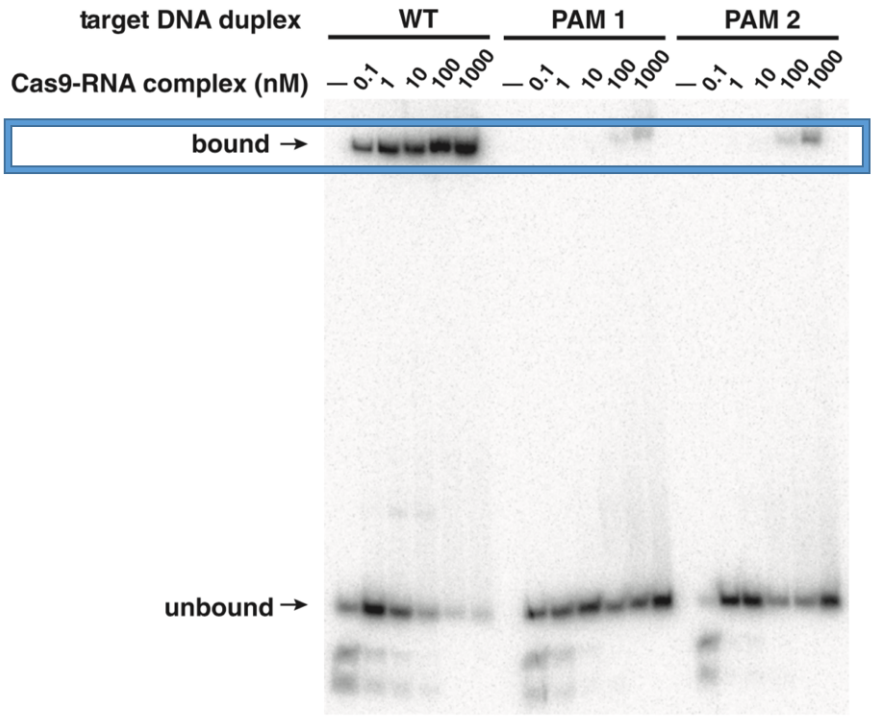

Figure 4. EMSA gel showing PAM sequence is needed for effective DNA cleavage by Cas9 complex. Gel indicates that the two PAM mutants, PAM1 and PAM2, did not allow for effective binding of the Cas9 complex to the target DNA as comparted to the wild type, as indicated by lack of banding within blue box. Figure from Jinek et al., 2012 used with permission from original publishers.

will be hindered and will not travel as far down the gel as similar control strands of DNA. When WT PAM was used, the Cas9 bound to the DNA and the DNA migration was hindered. When the mutant PAMs were used, Cas9 did not bind to the DNA and the DNA traveled farther down the gel (Figure 4).

To see if they could program the Cas9 complex to target desired DNA sequences, the researchers built what they called a chimeric RNA (now called a single-guide RNA: sgRNA) by attaching a truncated tracrRNA to a strand of crRNA complementary to the target DNA(Figure 5). They were successful. Their chimeric RNA, along with Cas9, was able to cut the targeted DNA with high accuracy. This technique is now known as the famous and highly effective CRISPR-Cas9 genome editing technique that has been used in labs across the world.

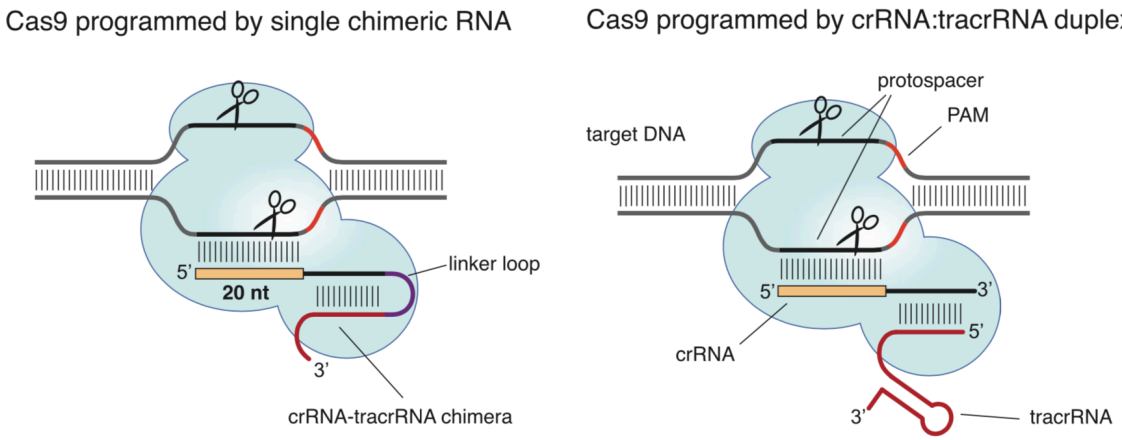

Figure 5. Authors' schematic of the the WT Cas9 complex and lab-created sgRNA. Figure depicts the spatial arrangement of the different molecular components of the native Cas9 complex and the Cas9 complex that was shown to utilize the chimeric RNA created by the researchers. Figure from Jinek et al., 2012 used with permission of original publishers.

DISCUSSION

The 2012 paper by Jinek et al. demonstrated which components the Cas9 complex were required to accurately target and cleave target DNA. The authors found that the Cas9 complex cuts both strands of DNA with targeted endonuclease activity guided by RNAs. They used this information to build a single RNA construct that had full activity when combined with Cas9 (Figure 5). However, the paper did not discuss the limitations of CRISPR-Cas9. For instance, CRISPR-Cas9 cannot add DNA sequences to a genome, nor can it degrade sequences it has cleaved. CRISPR-Cas9 depends on cellular machinery for linking the two ends of DNA on either side of the cleavage site as well as the degradation of the excised DNA fragment. CRISPR-Cas9 alone cannot be used to insert new DNA sequences. The main advantages of CRISPR-Cas9 are its accuracy and low cost due to the relatively low cost of the sgRNA sequence construction as compared to other methods.

CRISPR-Cas9 has the potential to change medicine, but it cannot do so yet. It is noteworthy that Jinek et al. (2012) did not quantify the specificity CRISPR-Cas9, and knowing an exact rate for CRISPR-Cas9 mistargeting is vital in transitioning to medical research. Another issue that has not been thoroughly addressed since the publication of the Jinek et al. 2012 paper is the problem of delivery of the CRISPR-Cas9 protein and RNAs to specific tissues. Viral delivery systems have been used and there has been at least one successful attempt to design nanoparticles to deliver the needed components of the CRISPR-Cas9 complex in mice (Miller et al., 2017). We are still years away from CRISPR-Cas9 being used in hospitals as a routine treatment.

The ethics and politics behind CRISPR-Cas9 have been hotly debated by the scientific community and the general public. The ability of science to edit the genome is the Pandora's box of biology. Combined with other genome editing techniques, CRISPR-Cas9 has vast implications on agriculture, genetics, medicine, and many other fields (Hsu et al., 2014). But caution must be used; because CRISPR-Cas9 is still a new technology, few countries have adequate laws and regulations for its use (Lanphier et al., 2015). Countries such as China and the United Kingdom have already started experimentation on vertebrate embryos using CRISPR-Cas9 and have had limited success transitioning to human embryos despite the lack of guidelines (Kang et al., 2016). More data on the reliability of CRISPR-Cas9 is needed before enough is known to safely proceed with medical applications of CRISPR-Cas9.

The Jinek et al. 2012 paper has been cited over 3,000 times since its original publication. CRISPR-Cas9 technology also economically important, as evidenced by a lengthy and bitter patent lawsuit between University of California, Berkeley and Harvard University (Ledford, 2016). Dispute its minor drawbacks and limitations, CRISPR-Cas9 genome editing already has changed biology and will continue to hugely impact the future of science and medicine.

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