Jennifer Doudna: CRISPR-Cas9

Rocío Mondaca Marcelo, M. Blanca Nieto Ponce, Paula Ochoa Mejía and Julio Ramírez Guerrero

Summary— Jennifer Doudna, a well-known scientist due to her excellent achievements, is a leading figure in what is referred to as the "CRISPR revolution" for her fundamental work and leadership in developing CRISPR-mediated genome editing. This was possible thanks to the previous description of the CRISPR system by Francisco Mojica. This article discusses some of the kinetics aspects related to the process in order to have a greater understanding of how this technique works. Furthermore, we have taken into account the ethical issues that arise with the creation of this innovative development.

Key words— Jennifer Doudna, Ribozyme, Hepatitis Delta, CRISPR-Cas9, Kinetic model, RGN, Kcat, Koff

1. Introduction

Jennifer Anne Doudna was born in Washington, D.C. on February 19, 1964. She is an American biochemist, Professor of Chemistry and Cellular and Molecular Biology at the University of California, Berkeley. She has been an investigator at the Howard Hughes Medical Institute (HHMI) since 1997 and since 2018 she has the position of principal investigator at the Gladstone Institutes, as well as a professor at the University of California, San Francisco [1].

Fig. 1. Jennifer Anne Doudna [2].

2. Achievements Prior to Crispr

Doudna first made her name uncovering the basic structure and function of the first ribozyme, a type of catalytic ribonucleic acid (RNA) that helps catalyse chemical reactions. This work helped lay the foundation for her future work pioneering CRISPR-Cas 9 [2].

One of Doudna's first breakthroughs occurred in 1989 in Jack Szostak's[1] lab while she was still a PhD student. She focused her doctoral research on ribozymes and helped show that RNA does not only carry instructions from DNA to synthesize proteins, but also helps catalyze the process [2].

In 1991 Doudna went to Thomas Cech's[2] laboratory at the University of Colorado Boulder to crystallize and determine the three-dimensional structure of a ribozyme for the first time. The project ended in 1996 at Yale University by announcing the three-dimensional structure of the catalytic core of the Tetrahymena Group I ribozyme, a particular type of catalytic RNA capable of removing introns through transesterifications and subsequently joining adjacent exons. It was a major achievement because before this, only a single RNA structure had been checked: transfer RNA (tRNA), which was much smaller and simpler than ribozyme [1], [2].

By 1998, Doudna and her team had determined the crystal structure of their first viral RNA - the hepatitis delta virus (HDV) [2].

Hepatitis D is a human disease that, in acute and chronic infections, can lead to increased chances of liver failure and liver cancer. It is caused by a small virus-like particle HDV, which only infects patients who have a hepatitis B infection. HDV has a circular RNA genome of 1.7 kb that is replicated inside the host cells into genomic and antigenomic (complementary to the original genome) RNA. The replication is carried out by a rolling circle mechanism that produces a linear RNA strand containing multiple copies of the genome. The catalytic activity of the HDV ribozyme is essential for viral replication and viral particle assembly inside the host cells. This is because it catalyses viral RNA self-cleavage through general acid-base chemistry in which an active-site cytidine and at least one metal ion are involved [3], [4].

This initial work to resolve large RNA structures led to further structural studies at an internal ribosome entry site (IRES) and protein-RNA complexes such as the Signal Recognition Particle [1].

3. Crispr-cas9 Technique

3.1. Evolution of the Study of this Technique

It is hard to establish the origin of CRISPR/Cas9 technology. Many minds had to come together in order to achieve such an innovative idea. CRISPRs were first reported almost 30 years ago and the term was pinned towards the beginning of the century. However, a good place to start is with the discovery of the genomic repeats and their subsequent characterization.

The first time these were seen was in *Haloferax medi-*

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terranei R-4. In 1989 the scientist Francisco Mojica started his PhD working with this strand. The thesis was focused on the salt dependence of a certain genome region. Since this region was not characterized, in 1992 one of the first sequencing experiments in the Universidad de Alicante was performed to do so. In this sequence, around 14 segments repeated in which short inverted repeats (palindromic) were found. They were spaced with regular intervals. Using Northern Blot, it was seen that this region was repeatedly transcribed, however, it did not appear to be translated into a known protein. These repeats would later be called TREPs after Tandem REPeats and they were found in other species of both archaea and bacteria.

The availability of sequencing techniques' growth was such that by the end of the century they were commonly used. However, they lacked the computer tools to be able to analyze these new genomes. César Díez-Villaseñor, from the same research team, created a high tolerance program to align the sequences. This led to the name we use today, CRISPR passing by SRSR, for Spacer-Repeat-Spacer-Repeat, and SPIDR, for SPacers Interspersed Direct Repeats.

The second part of the technique, Cas proteins, were described in 2002. They were linked to CRISPR because of the similarity in their sequences. Their biological function was not discovered until Mojica's research team checked for homologous regions of *Escherichia coli* K12-derivative strains' CRISPRs in a public nucleotide database. The results are the key to discovering their role in nature. Parts of the spacers (the DNA in between the palindromic repeats) were seen to be present in the genome of coliphages and plasmids. "The CRISPR meaning suddenly clicked into place; these arrays are crisper-like compartments for storing DNA chunks of invaders, to keep a fresh memory of past infections" [5]. Experiments checking for the immune response were successfully performed on many different bacteria, showing that this was true across the domain. With the rise of bioinformatics and the publication of similar results in other species, CRISPR began to be accepted and cherished.

It was with this serendipitous discovery of the biological mechanism described that Doudna and her team found the genome editing application of the technique. In 2012 Charpentier and Doudna published their research showing the possibilities of the system. It was published in science, as a research article titled "A Programmable Dual-RNA–Guided DNA Endonuclease in Adaptive Bacterial Immunity". In this study they explained how Cas9 functions exactly, proving that cas9 proteins cut at specific sites using their RuvC and HNH domains. More importantly, they showed that crRNA (the RNA from CRISPR sequences that guides Cas9) can be minimized to 20 base pairs and even changed to a different sequence. The key to be able to take advantage of this biological system is the ability to synthesize guide RNA that Cas will latch on to. This means that it is a completely customizable genome editing technique.

3.2. What Does it Consist Of?

Today, this mechanism is used worldwide as one of the cheapest, easiest, and quickest ways to edit DNA. In the lab, this technique starts by synthesizing a guide RNA specific to the gene that is being altered. This acts like a synthetic crRNA and attaches to cas9, like it does in nature. When the Cas9 protein enters the cell with the desired gene, it acts as molecular scissors and cuts at the specific site. Besides from cas9, a DNA sequence (that could be a separate gene or a non-mutated gene in case of a genetic disease), is also introduced in the cell. This is done in order to guide the repairing process using homology directed repair. CRISPR-Cas9 system has an immense variety of applications, from treating genetic diseases to developing plague resistant crops. The main difficulty is in the process of getting these molecules into the cell and into the organism. This, along with the potential side effects is the main reason why it is not normally performed at a clinical level yet.

3.3. Kinetics Associated to the Process

Here, we describe a kinetic model that is broadly applicable to any RNA-guided nuclease (RGN) and should enhance our understanding of on- and off-target binding when applied to CRISPR-Cas systems. CRISPR-Cas9 is one of the RGN systems that provide sequence-specific gene regulation through base-pairing interactions between a small RNA guide and target RNA or DNA [6], [7].

The kinetic models described below provide a foundation for understanding RGN targeting specificities.

Specificity is determined by the ratio of k_{cat}/K_M (K_M is the Michaelis constant and k_{cat} the catalytic constant) values for a reaction with two different substrates, in this case, a matched (S_{Match}) versus a mismatched target (S_{MM}) (Fig. 2).

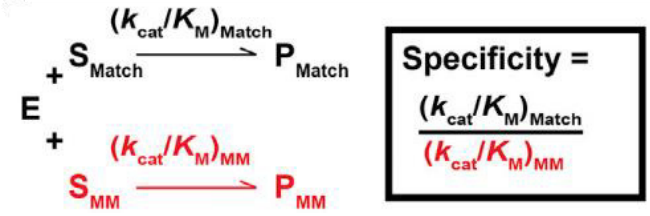

Fig. 2. Adapted image from [7].

k_{cat}/K_M represents the overall rate of the reaction and takes into account the rate constants with which the enzyme binds (k_{on}), dissociates from (k_{off}), and, for RGNs, cleaves a target (k_{cat}) (Fig. 3).

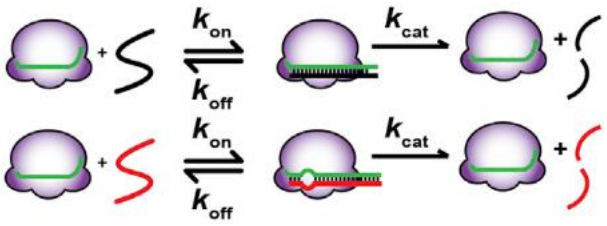

Fig. 3. Adapted image from [7].

The relative magnitudes of the rate constants that comprise k_{cat}/K_M (i.e., k_{on}, k_{off}, and k_{cat}) determine the extent of discrimination. Two kinetic regimes can be defined based on the relative magnitudes of k_{off}, k_{cat} and the Michaelis-Menten model [7].

Rapid-Equilibrium Regime: $k_{off} > k_{cat}$: Figure 4 illustrates the reaction using a free energy reaction diagram, where the valleys represent states and the peaks represent the barriers for transitions between those states. The heights of individual barriers are inversely proportional to the logarithm of the rate constant for each step, and k_{cat}/K_M corresponds to the free energy difference between the unbound state (E + S) and the highest reaction barrier, or transition state (z). The RGN ("E") can bind the matched (black curve) or the mismatched (red curve) substrate. Once bound, there are peaks on either side the E·S complex: one for dissociation to E and S (k_{off}) and one for cleavage to E + P (k_{cat}). The central feature of "rapid-equilibrium" kinetics is that the peak for cleavage is higher relative to dissociation—both for the matched and the mismatched target (i.e., cleavage is the rate-limiting step). This means that the E·S_{Match} and E·S_{MM} complexes dissociate faster than they are cleaved, such that E can equilibrate its binding before cleavage occurs. As a result, there is a preference for cleaving S_{Match} over S_{MM} that matches the thermodynamic preference for binding S_{Match} over S_{MM} [7].

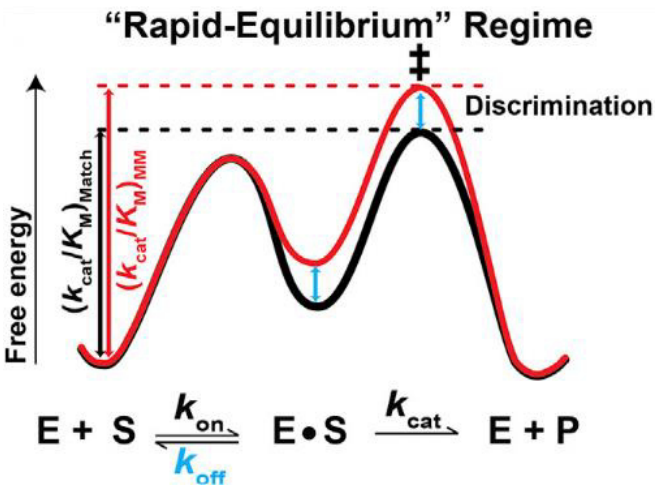

Fig. 4. Adapted image from [7].

Sticky Regime: $k_{off} < k_{cat}$: In the sticky kinetic regime (Fig. 5), the barrier for dissociation of S from the E·S complex is higher than the barrier for cleavage. Thus, once either S_{Match} or S_{MM} bind, they are "stuck" to the enzyme and are cleaved essentially every time they bind. Because neither substrate has an opportunity to dissociate and achieve equilibration before cleavage, there is no discrimination between S_{Match} or S_{MM}. These models can be expanded to take into account more complex scenarios, including binding-site accessibility (e.g., determined by chromatin state), conformational changes, and non-cleavage activities of engineered RGNs [7].

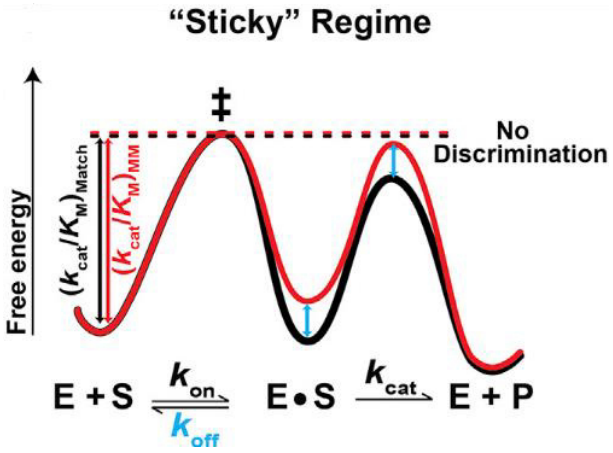

Fig. 5. Adapted image from [7].

Figure 6 illustrates the predictions of a simple kinetic model for specificity between a target and a potential off-target. In this model, there are two steps, binding and cleavage, for both a target and off-target sequence. In the simplest scenario, we consider that the target and off-targets have the same on-rate (k_{on}) and forward reaction rate (k_{cat}) constant and that there is a 100-fold difference in dissociation rate constant (k_{off}) between the target and off-target. As the absolute affinity for the target sequence is varied (e.g., by changing the GC content or length of the guide RNA), this model predicts two regimes of specificity. When targets have high binding affinities (i.e., slow dissociation rate constants), specificity between a target and potential off-target is absent or low (Fig. 6, left). However, as the dissociation rates for both the target and the off-target increase (i.e., affinities decrease), specificity increases (Fig. 6, right), eventually reaching the maximal level of 100-fold. Thus, the "excess energy" model can be rationalized by a shift from the sticky enzyme regime to the rapid equilibrium regime as the dissociation rate constant increases with an improvement in specificity as a consequence [7].

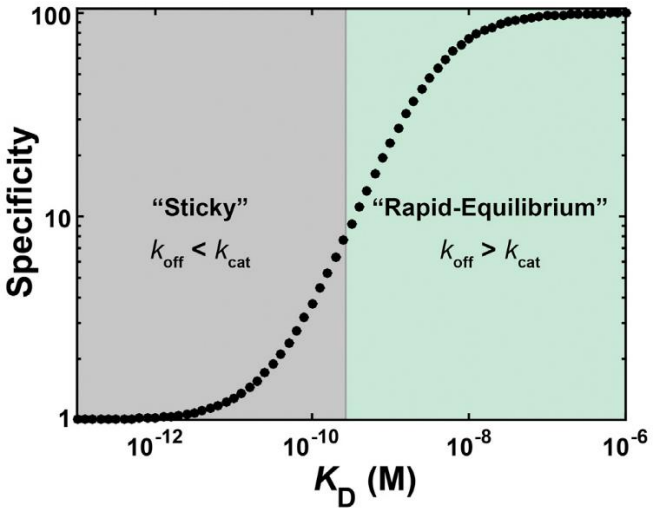

Fig. 6. Adapted image from [7].

4. Awards

Since her beginnings, Doudna has received many awards to reward her research work. Some of her honors include the Johnson Foundation Prize for Innovative Research (1996), the Beckman Young Investigator Award (1996), the David and Lucille Packard Foundation Fellow Award (1996), the Lucille P. Markey Scholar Award in Biomedical Science (1991), and the National Research Service Award in Biomedical Science (1986). Doudna also got the NAS prize for research initiatives in 1999. In 2000, The National Science Foundation (NSF) chose her to receive its most prestigious prize for young researchers, the Alan T. Waterman Award [8].

In 2014, she received the Lurie award in biomedical sciences. With her colleague Emmanuelle Charpentier, they received many awards like in 2014 they got the Dr. Paul Janssen award for biomedical research and the innovation in life sciences award. In 2015 they got the Princesa de Asturias award and the Gruber award in genetics. In 2016 the BBVA foundation: frontiers of knowledge award and she also got the L'Oréal-UNESCO award for women in science. In 2020 they got the Wolf award in medicine and the Nobel Prize in Chemistry for their discovery of the CRISPR-Cas9 technique.

5. Ethical Issues

This last discovery, the CRISPR-Cas9 can bring up some ethical problems associated with genome editing that need to be considered. This technology can be used not only in adult cells but also in embryos of organisms, including humans. Doudna expressed the necessity to have a global conversation to discuss all the ethical and social implications of this technique. With this technology we are able to engineer humans that have improved characteristics such as stronger bones or less susceptibility to certain diseases, or even qualities that we find desirable like an eye color, being taller, etc. We could get "designer humans" if we would like. This supposes a great ethical problem and that is why Doudna and Emmanuel call for a global pause in any clinical application in human embryos until the ethical limits are established [9], [10].

We can consider the event below as a clear example of the ethical conflicts of this technique.
In 2018, the Chinese scientist He Jiankui claimed that he had created the first genetically edited human babies, the twins known as Lulu and Nana. For this purpose, He took sheep sperm and eggs, and he performed an in vitro fertilization. Then he edited the embryos genome using CRISPR/Cas9. He edited the CCR5 gene, which codes for proteins used by HIV to enter human cells. Therewith he was trying to create the mutation in CCR5 gene that some people had naturally developed, acquiring HIV immunity. For these actions, He was sentenced to three years in jail.

6. Conclusions

Jennifer Doudna is a well-known scientist thanks to her exceptional achievements. She is the leading figure for the so called "CRISPR revolution" that allow us to edit genomes. This accomplishment has a large number of applications such as genomic diseases treatment. With all this, we must consider the legal and bioethical aspects related to make good use of this discovery.

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[1] Jack Szostak is an English molecular biologist, famous for his work on telomerase, an enzyme that forms telomeres during DNA duplication. Together with Elizabeth Blackburn and Carol Greider, he received the 2009 Nobel Prize in Medicine.

[2] Thomas Robert Cech is an American chemist, biochemist and university professor who was awarded the Nobel Prize in Chemistry in 1989 "for discoveries of the chemical processes of catalytic properties of ribonucleic acid".