Molecular Microbiology, GBB group, University of Groningen

Current and Future Applications of CRISPR/Cas in Eukaryotes

Meine Dominicus Boer, student number: s2540649
Supervisor: prof. dr. A.J.M Driessen
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Introduction

With the increasing knowledge of the structure of cells, there has been a growing demand for efficient techniques to restructure cells to possess <preferential> properties. For bacteria, several very efficient techniques have already been developed with the aid of the extensive library of restriction enzymes, the wide variety of genetic markers and premade plasmids. Unfortunately, the development of similar techniques as always been more difficult in eukaryotic cells. There are few genetic marker for eukaryotes and no extensive library of restriction enzymes. Still there are many applications both medical and biotechnological of nature, where the genetic modification of eukaryotes would be very beneficial. A second field where increasing understanding has led to a need for better techniques of modification is in epigenetics, the field of hereditary traits not encoded in the DNA-sequence. Until recently the target recognition necessary for epigenetic modification mostly relied on TALENs and Zinc-Fingers. These techniques are inefficient and time-consuming because for every target sequence a different protein is necessary and the selection methods often relies on trial and error. A third field where the search for effective methods continues is in the field of RNA interference, both of mRNA and viral RNA. Although these problems operate at different levels of the central biological dogma they might all have the same solution: CRISPR/Cas. CRISPR-Cas, an adaptive anti-viral immunity system from bacteria, has been developed in recent years to use its site-specific recognition and nuclease activity to improve current techniques. Recent studies have shown that CRISPR/Cas, and in particular Cas9 and variants thereon, could be the key to selective genomic editing because C9 possesses guided nuclease activity. If the nuclease domains of Cas9 are inactivated, the resulting dCas9 fused to other domains could also be an excellent epigenetic tool. Recent developments also showed that could be a suitable extension to or replacement of RNAi if cas9 is adapted to target RNA instead of DNA, and could also be used as a possible anti-viral treatment. This essay will not only discuss the origins of this CRISPR/Cas but also its development into a usable tool for applications in eukaryotes, and explores the myriad of possibilities with regards to current developments in genetics, epigenetics and RNA interference.

Background:

Origins of CRISPR/Cas and Cas9

Before continuing with the possibilities of CRISPR/Cas, it is necessary to understand the origin and nature of this system. This brings us to the antiviral immune-system of prokaryotes. Most systems found in the prokaryotic immune system are unspecific. CRISPR/Cas systems however, show that this is not always the case. The CRISPR/Cas system is not only able to recognize foreign DNA, but can also recollect earlier infections, and deal with them accordingly making it a very adaptive system.

This adaptability is regulated by an extensive cluster of genes and associated proteins which possess a wide variety of functions ranging from the fragmentation of invasive sequences to the incorporation of these fragments for later reference to the selective recognition and destruction of foreign sequences. All CRISPR/Cas systems contain a cluster of palindromic sequences separated by spacers which also explains its name. This cluster functions as a databank of sequences that have been previously encountered. Apart from this cluster there are several downstream located associated proteins, which proteins are present determines the classification of the two systems (I and II) and 5 classes.

The common theme among these associated protein is that they can be attributed to one of three phases: adaptation, expression, and interception. The proteins responsible for adaptation ensure that foreign sequences are cut up into small fragments and subsequently build into the clusters. Nucleases are required for this, as well as proteins that integrate small sequences. The second group, responsible for expression are tasked with recognition of invasions and expressing the part of the clusters with the corresponding sequences and producing crRNA-sequences. The final group, interception, use these crRNAs to intercept the hostile DNA/RNA, thereby neutralising the danger. This final group is the most interesting for the development of tools as they can recognize a specific target using the crRNA as a template, their task is intercept and digest the invading DNA to neutralise the danger.

Of the three systems, type II was selected for further development as the interception stage in this system only requires one Cas: Cas9. This means that only one protein needs to be provided for the method to work. Cas9 itself requires only a guiding RNA, in native context this RNA is called crDNA but in when applied outside its native context it usually is called guideRNA (gRNA). This RNA dictates the target sequence where Cas9 binds and creates a double stranded break (DBS). Crucial in this target is the protospacer adjacent motive which is a short sequencing varying between the different organisms expressing Cas9. The Cas9 enzyme consists of two distinct lobes: a Nuclease lobe and a Recognition lobe. The Nuclease (NUC) lobe is responsible for the DSB and has two nuclease domains: s a HNH-nuclease domain which uses a two-metal mechanism and a RuvC domain which uses a single-metal mechanism. Apart from these nucleases the NUC lobe has a PAM-interacting domain and a Wedge domain that play a role in the binding of Cas9 to the target sequence. The recognition lobe contains the domains that the enzyme needs to bind the guide RNA and the enzyme to bind to the specific target[1].

Adaptation of CRISPR to be used as a method for modification in eukaryotes.

In order to create a usable method for the modification of eukaryotes from the CRISPR/Cas systems several steps had to be executed. The first step is selecting the enzyme or enzymes needed for our purposes. After that the organism needs to be selected to extract these enzymes from. The final step is adapting this enzyme to use it effectively in eukaryotes. Because the simple and well researched method at which Cas9 uses a guiding RNA to bind and digest a specific target on its own it was a logical choice. The organism from which to isolate was a more complicated task, as there are several bacteria that express Cas9. Most often a Cas9 is used that is derived from *Streptococcus pyogenes* as Cas9 was already researched in that organism and its PAM-sequence is simple a sequence (NGG).

The final step entailed the adaptations that need to be made for effective usage in eukaryotes. Several problems need to be resolved because of the bacterial origins of CRISPR/Cas. Therefore, differences between prokaryotes and eukaryotes need to be considered. The most obvious difference, as it enclosed in the definition of prokaryotes and eukaryotes, is that eukaryotes have a nucleus which harbours their DNA. This nucleus is absent in prokaryotes; therefore, prokaryotes do not need to tag a nuclear localisation signal (NLS) on Cas9, which is required to for successful transport the cas9-enzyme to the nucleus where the DNA can be found in eukaryotes. This transport and therefore the NLS tag is necessary if Cas9 is to be used for the modification of Eukaryotic DNA which was the intention. To resolve this a NLS was tagged on Cas9 so after translation it would be transported to the nucleus where it could perform its tasks appropriately [2].

A second concern that was raised is codon efficiency, in nature there are several codons that code for the same amino acids but their corresponding tRNAs are not equally abundant across species. This variation in the abundance of different tRNAs is reflected in the of codons as genes which are expressed using codons corresponding to the more abundant tRNAs of a certain species are more effectively translated. These differences can be found between most species but are distinct when comparing eukaryotes to prokaryotes. The solution to this problem is codon-optimisation, this technique adapts the different codons to their counterparts corresponding with the tRNAs that are more abundant in the organism of choice or eukaryotes in general. With this technique, it has proved possible to greatly improve the efficiency of translation.

Genetic modifications

Before continuing to the specific applications of genetic modification it is useful to explore the exact motivation for using Cas9 in the modification of eukaryotes. To understand this, the most common methods of genetic modification in eukaryotes needs to be explained. Genetic modification of eukaryotes usually uses the cells own repair mechanism for repairing double stranded breaks (DSB). The cell can repair these with Non-Homologous End Joining and Homologous recombination.

The mechanism of NHEJ starts with the recognition of a DBS and the assembly of the NHEJ-complex. This step is followed by the stabilisation and joining of the two ends of the DNA. After this the ends can be processed and finally ligated. After the DSB has been restored the NHEJ can be dissolved and its parts can find a new DBS. This mechanism is tricky and generally error-prone because of mistakes in the ligation[3].

Homologous recombination can be divided into three stages. During first phase called presynapsis, a DSB is recognized and both sides are trimmed to create single stranded ends. During the second phase named synapsis, the gap that was created is reconstructed either directly or by using the sequence on the other chromosome as a template. This second way leads to a holiday junction and is also the method to introduce mutations especially if the cell is provided with a synthetic template. During the final phase called the post-synaptic phase the repair is finished and the complex resolved [4].

New techniques involving CRISPR/Cas still use these repair mechanisms as they are well studied and therefore familiar. Their most significant problem is efficiency, creating double stranded breaks at a desired location was always a difficult task. Luckily this is exactly where Cas9 is extremely proficient in, which also explains the strong motivation for researchers to develop a working technique using Cas9.

Delivery methods

Now that the motivation for using Cas9 is explained the next consideration is the delivery of three the components required for successful guided genetic modification. The Cas9 enzyme, the target sequence and the repair fragment. Delivery these different components in the cell can achieved through several different methods. The first common method entails the introduction of a plasmids encoding both the sgRNA and Cas9 simultaneously. This plasmid can be introduced by traditional methods such as electroporation or microinjection. This method is fast and can be applied to most cell lines, but the introduction of plasmids can be troublesome. For particularly large fragments or several targets, using multiple plasmids can be more stable. A second common technique is integrating parts into the host genome. Using this technique Cas9 and/or the sgRNA can be effectively expressed. A problem that needs to be avoided is that the control is lost. For this technique, the importance of codon-optimisation is crucial, as the host immune system can recognize the unmethylated CpG dinucleotides as originating from bacteria and react negatively. It is also possible to inject the sgRNA separately instead of expressing it [5].

Applications of Cas9 in the modification of the genome

Now that the background of genetic modification has been established the following section will continue with the specific applications. First the use of Cas9 for knockouts shall be discussed, followed by functional mutations and finishing with the insertion of entire genes. The different applications have in common that Cas9 binds to a target side and uses the RuvC and HNH domain to digest the Target DNA (figure 2).

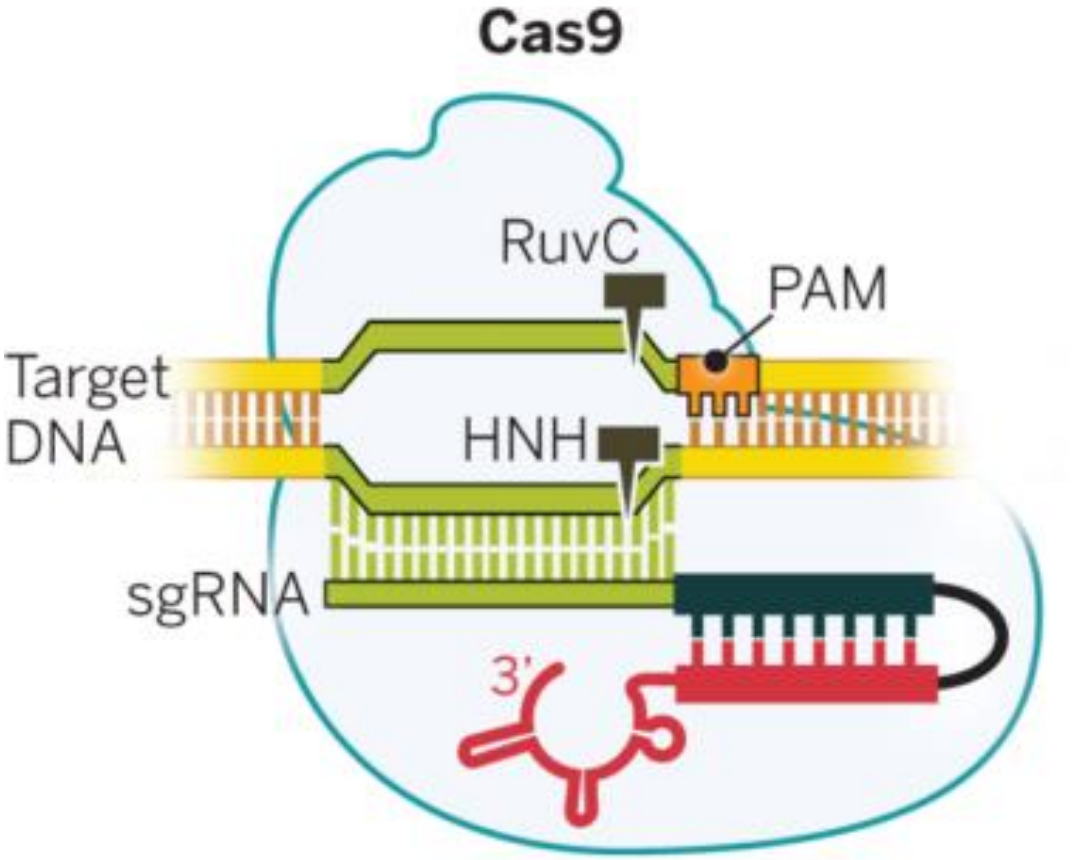

Figure 1 Schematic representation of the functioning of Cas9 [21].

Cas-9 mediated gene inactivation:

As was explained previously a sgRNA sequence is used to target a sequence of 20 bp in the gene of interest. Cas9 then induces a DSB at the targeted locus which the cell repairs. For Cas9 mediated knock-outs no repair fragment is included, this way the cell uses the error-prone method of non-homologous end joining. Because this system has a high occurrence of deletions and insertions it is an effective method for knocking out genes (figure 2). Yang et al. showed in 2015 that with an Cas9 expressing plasmid it is possible to knock outs several genes simultaneously and removed all antigens from the heart of a pig making the first step in the long way towards possible cross-species transplantations [6]. Using a similar technique Dicarlo et al. deleted all genes associated with the biosynthesis of Cortisol: ATF2 , GCY1 , and YPR1 over a period of 6 days with an efficiency of 100% [7].

Mutations

If a repair fragment is provided the cell is more likely to depend on Homologous Recombination (HR) to repair the DSB. With HR, the cell uses the repair fragment as template and replaces the original sequence effectively introducing a mutation or other alteration in a gene of choice (figure 2). The simplest application of this is the introduction of interesting mutations. In this method Cas9 is predominately used to improve the efficiency of the classical methods of genetic modification discussed earlier and but is also negates the necessity of genetic markers which allows to the modification of several genes simultaneously, as well as modifying larger genes for which inclusion of a marker constitutes a problem.

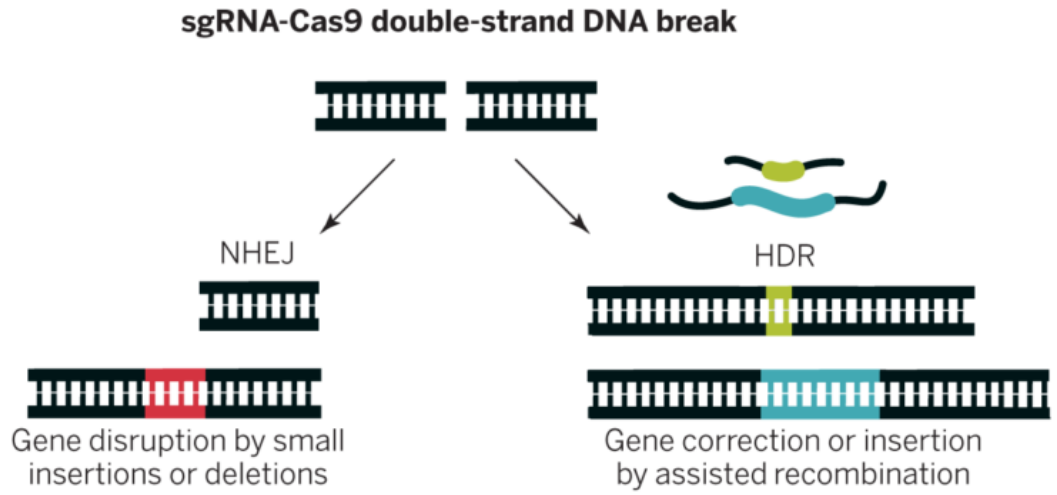

Figure 2: Method of genetic modification through Cas9 mediated DSB. (Source: Doudna & Charpentier 2014)

Insertion of genes:

However, besides the introduction of single point mutations, it is also possible to transform larger fragments of DNA. This allows the transformation of entire genes. This is very useful as it allows the introduction of parts or even entire metabolic pathways, which is extremely time-consuming with classical methods. This allows a plurality of possibilities, such as accessing new food sources, synthesising new metabolites, or eliminating side products.

With these methods, several synergistic alleles and pathways have been transformed to yeast among which the pathway for the synthesis of Muconic Acid. This pathway had already been introduced to *S. cerevisiae* [8] but now with the use of multiplex-integration all three required genes coding for the enzymes required for a high production of Muconic acid could be introduced in a single transformation [9].

Another specific application of the transformation of entire genes is TAR-cloning. With this technique genes with unknown functionality are transformed from larger and more complex organisms such as humans to simpler eukaryotes which simplifies research and avoids ethical concerns. Because the model-organisms are also eukaryotic, Important aspects such as post-transcriptional adaptations are conserved which is not possible with similar experiments in prokaryotes. As a result, TAR-cloning is already widely used but again the was trouble with low transformation rates (0.5 – 2%). A recent study showed that CRISPR-Cas can increase this efficiency to 32% [10].

Modification of gene expression

Similarly, to genetic modification, before going into the specificities of gene expression modification the required modifications need to be explained to use Cas9 without its nucelatic activity, which is essential if it is to be used for epigenetic modifications instead of genetic. Once these modifications have been established, it will be explained how Cas9 can be used both for direct gene regulation as well as the modification of epigenetic factors such as DNA-methylation and histone modifications.

The development of dCas9 from Cas9

To use Cas9 for the modification of gene expression it is necessary to eliminate the nuclease activity of Cas9, this way Cas9 can be used to recognize a target without the creation of double stranded breaks and resulting unwanted genetic modifications through NHEJ. Already established in the description of Cas9 in the background is that Cas9 contains two nuclease domains: RuvC and HNH.

A mutation in one of the domains, such as D10A for RuvC or H847A for HNH creates a variant of Cas9 that only nicks the DNA instead of the DSB and is therefore named Cas9 nickase (Cas9n). the two different Cas9n's can be used simultaneously with two different sgRNAs. This way a staggered DSB can be induced. A staggered DSB allows for even more efficient and specific application of the CRISPR/Cas system.

It is also possible to inactivate both domains with a mutation of the same Cas9 completely inactivating all nuclease activity. The resulting nuclease-null or dead Cas9 (dCas9) is the version necessary for the intended applications.

Direct modification of gene expression

Apart from the epigenetic applications that shall be discussed shortly this dCas9 can also be used to directly target genes. To do this the Cas9 has to be fused with a domain that has a direct effect on the system, also referred to as CRISPR interference (CRISPRi), provides two functions: fusion-mediated activation (CRISPRa) and fusion-mediated inhibition (CRISPRi)[11] (figure 4).

Li et al. reported on a tuneable CRISPR/Cas system, which they named tCRISPRi. This system allows for a very precise and reversible control of gene expression. The researchers fused dCas9 to a tuneable arabinose operon promotor (PBAD) which allowed for an efficient control of the expression of any target, and with minimal leaky expression. Again, one of the greater advantages is that for the changing of target only some recombination of gRNA is required [12].

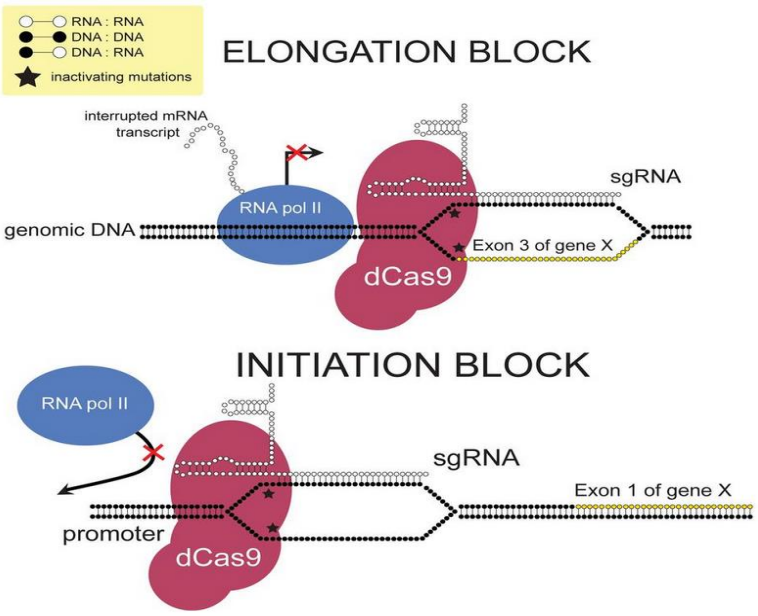

Figure 3: Schematic overview of CRISPRa (top) and CRISPRi(bottom)

Source: https://en.wikipedia.org/wiki/File:CRISPR_Sterics.pdf

Explanation of epigenetics

Most of the regulation of gene-expression can be found in epigenetics. Therefore, epigenetics needs to be explained before understanding the most effective methods for gene expression modification. Epigenetics entails all inheritable characteristics that are not encoded by the DNA-sequence. Two common examples are (de)methylation of DNA and histone-modifications. Before explaining how these factors can be used to modify expression with Cas9 it will be useful to explain trough which mechanisms these factors function.

DNA (de)methylation

DNA-methylation is a broadly characterized epigenetic modification consisting of the linking of a methyl group to DNA. This methyl group protrudes into the major groove, the wider gap where the sequence can be read. As the major groove is the place where transcription factors bind, the methylation of DNA inhibits their effects by blocking their binding. The most frequently described example of methylation is the covalent addition of methyl at the 5-carbon of the cytosine ring which transforms it into 5-methylcytosine [13].

Histone modification

Histones are involved with the packaging of DNA which is coiled around the histones. If the DNA is very tightly packed around the histones DNA-transcriptase and other DNA-binding proteins bind with more difficulty. Histone-modification modify the tails of the histones, with for instance an acetyl group. These modifications tend to change the charge of the histones, the negatively charged backbone of DNA will be attracted to the positively charged histones making a tighter package and repelled by negatively charged histones making the DNA packing looser and the DNA better accessible. Histone modifying proteins such as acetyltransferases catalyse these processes [14].

Applications of dCas9 in epigenetics

Epigenetics plays are important role in current research. By combining the significant current knowledge of epigenetics with the efficient and precise recognition of sequences of Cas9, a world of possibilities opens for the modification of epigenetics in organisms. Currently the most significant limitation is that target recognition until recently was limited to Zinc-fingers and TALENs, even though these can indeed recognize specific sequences they require specific proteins which development requires extensive screening and often rely on trial and error. The major advantage of dCas9 is that, provided there is a pam-site in the proximity of the target, every locus can be targeted using a template to recognize the sequence, without extensive modification of the Cas9. This means that once coupled to the expression modifying domain the system can be adapted for different genes by simply altering the gRNA and even be used for several targets simultaneously by providing multiple gRNAs

DNA (de)methylation

The first epigenetic that is going to be discussed is the (de)methylation of DNA. The viability of using dCas9 for this purpose has been shown [15]. By fusing dCas9 to the catalytic domain of de novo DN methyltransferase (DNMT3A), which catalyses the cytosine-5 methylation, a DNA-methylation can be induced of the 50 bp directly surrounding the sgRNA target site. This induced methylation proved to be sufficient to lower expression of all the targeted genes and had only minor off target effects [15]. The demethylation of DNA can also be achieved in similar fashion by fusing dCas9 to Ten-Eleven translocation dioxygenase 1 (TET1), a maintenance DNA demethylase that prevents abnormal hypermethylation of CpG islands. Using this fusion it proved possible to successfully upregulate the BRCA1 promotor [15].

Histone Modifications

The second major epigenetic factor that will be discussed are histone modifications. Epigenome editing technologies have demonstrated the feasibility of targeting histone epigenetic modifications. Two studies from 2015 explored the possibility of repurposing the CRISPR-Cas9 to trigger histone modifications and subsequently changes in chromatin structure and accessibility. The first study used dCas9 fused with an acetyltransferase (HAT) enzyme that catalyses the acetylation of lysine residues on histone tails (figure 3)[16]. The second study demonstrated gene silencing using dCas9 fused to the Krüpel-associated box (KRAB), which is a naturally-occurring transcriptional repression domain that mediates histone methylation and deacetylation [17].These two studies exemplify that dCas9-fusion can be used both with domains that have a direct catalytic effect (HAT) and indirectly with a domain that recruits other enzymes (KRAB)

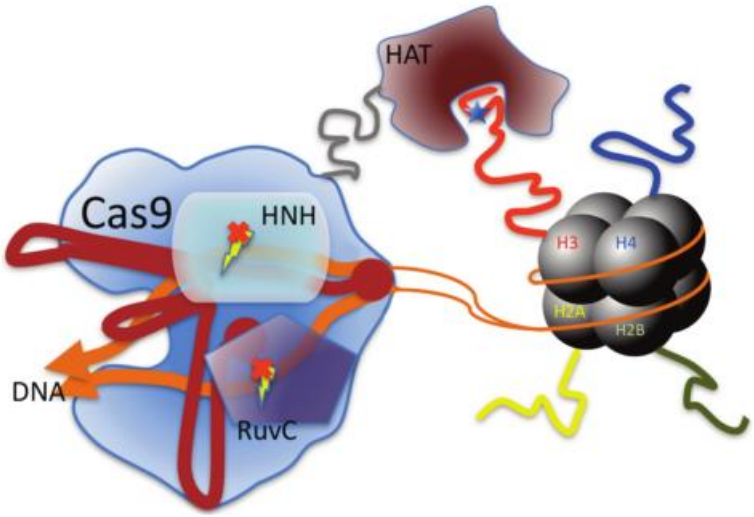

Figure 4: Schematic representation of epigenome editing using dCas9 fused to an epigenetic effector. Source: [15]

RNA Modification

However, natively the cas9 enzyme only binds to DNA as it requires a PAM-sequence. In a recent study [18] it was shown that by presenting the PAM-sequence in trans as a separate oligonucleotide, it is not only possible to induce high-affinity binding to ssRNA targets but also to stimulate site-specific cleavage. By using special PAMmers, Cas9 can cleave specific RNA target while leaving corresponding DNA-sequences intact. This means that it could be used as an efficient method for RNA interference that could replace or complement siRNA [11].

Another application for the targeting of RNA is the targeting of viral RNA. A possible method for achieving this depends on the cas9 from the pathogenic *Francisella novicida* which targets and degrades mRNA in order to suppress its host's immune system [19]. Using an adapted version of this system, it was possible to target the RNA of the hepatitis C virus. The FnCas9 was introduced together with a synthetically designed RNA-targeting guide RNA (rgRNA). The complex that these components formed bound directly to the HCV RNA and inhibited both the translation and replication. An Interesting observation was that FN Cas9 mediated inhibition does not depend on its nuclease-activity and does not require a PAM-sequence which negates the problem previously discussed [19].

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Limitations of CRISPR/Cas

Practical concerns

Despite the many advantages of this system, there are, as usual, still challenges remaining to the current Cas9-based tools. The most prevalent of these are off-target effects and the balance of HDR/NHEJ pathways.

Off target effects are consistently mentioned when discussing the limitations of Cas9. Even though Cas9 has a relatively great precision when it comes to target recognition there are always several other sites similar in sequence for Cas9 to show activity, due to the repetitive nature of DNA. A possible explanation for the presence of these off-target effects is that a certain variability in the recognition of viral nucleic acids or plasmid DNA is beneficial to bacteria and archaea[20]. For application in biological studies and genetic therapies, these off-target effects result in undesired mutations at random sites, which has a significant effect on the precision, and therefore need to be addressed to avoid side effects.

The balance between Homologous Recombination and NHEJ is a second concern. Because these different repair mechanisms have different outcomes generally one of the two repair mechanism would be optimal for a certain technique. Unfortunately, the system governing which method is used to repair DSBs, although the subject of cell-repair is well-studied, remains difficult to predict. This is detrimental to the efficiency and precision of methods using CRISPR/Cas.

Ethical Concerns

There are off course not only technical concerns but also ethical concerns that are raised with regards to CRISPR/Cas[19]. The most important ethical concern, especially if Cas9 is cooperated into the genomic DNA, is the possible leakage into nature. Although unlikely, this possibility cannot be entirely ruled out. This uncertainty is problematic as it would be disastrous if, due to poor understanding of the mechanism, entire species would change and the ecological balance disrupted. A second major concerns the ethical complexity of gene editing in human embryos. Both the editorial team of Nature and Science argue that progressive policy needs to be developed. In comparison, the editor-in-chief of Cell Emilie Marcus stated that the journal would not reject articles containing human germline modification, if they meet high technical and ethical standards. The editor-in-chief stresses that this should not be considered as an endorsement of human germline editing, but rather to encourage an ethical discussion. The editing or engineering of human fetuses remains a very controversial and has already led to restriction or even bans on the use of CRISPR/Cas for use in humans in several countries. However, it can be argued that this discussion mostly focuses on the ethical concerns of modification in humans itself rather than the technique and is only mentioned in regards to Cas9 because it the technique seems to have accelerated the field making these ethical dilemmas more pressing.

Conclusion and discussion

Though the need will probably remain to improve the techniques in the biological toolbox it is evident from the shown examples that with CRISPR/Cas researchers have found a system that could provide solutions on several different levels of the central biological dogma. The system found in the antiviral defense system has shown many remarkable capabilities one of which is RNA-guided target recognition. Through the selection of Cas9 from system II in organisms like *S. pyrogenes* an enzyme is found that guided by an RNA-sequence can make a target specific DSB. After this enzyme was isolated it could be adapted for eukaryotic applications by tagging on a Nuclear Localisation Signal and codon-optimisation. This optimized version could be used to greatly aid the editing of genes in eukaryotes trough HR and NHEJ. With Cas9 researchers have shown that it is possible to create one or several knock-outs simultaneously, to introduce specific mutations allowing for the extensive modification of metabolic routes and even that entire genes can be inserting allowing the creation of new pathways or the research of genes in different ethically more manageable organisms through TAR-cloning. However, the applications of Cas9 are not limited to genetic modifications alone, after inactivation of the nuclease domains a special version of Cas9 could be created named dCas9. This version of Cas9 still possessed the target-recognition of Cas9 but could no longer create a DSB. Not only is it possible to fuse dCas9 with gene regulating domains but also with epigenetic modifying domains which can make adaptations to major epigenetic factors such as histone modifications and DNA-methylation. The final field discussed in this essay where Cas9 has proven to be useful is in RNA-interference, by making adaptations to Cas9 causing it to target RNA rather than DNA or using a different Cas9 from *F. novicida* it is possible to interfere with RNA-sequences. This way the interception of mRNA and the subsequent translation as well as the elimination of viral RNA is possible. Of course, there are still limitations both practical and ethical. The off-target effects continue to be a problem, as well as the balance between NHEJ and HR. There are also still ethical concerns with regards to possible leakage into nature and human embryo editing. In the future, the limitations of CRISPR/Cas might be resolved and new policy might aid resolving the ethical concerns. Either way future research will continue to improve on the already widely applicable CRISPR/Cas system.

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