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CRISPR/Cas9 a Versatile Tool in Bio-Engineering

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Abstract

Clustered interspaced short palindromic repeats (CRISPR) with CRISPR associated 9 (Cas9) is a new bio-engineering tool originating from bacteria. It is a powerful and versatile tool to target and bind genes of interest using RNA guidance and is subsequently able to edit or regulate genes. Cas9 can be used as a machinery that induces double-stranded breaks or it can be used to regulate genes in various ways. This review covers various aspects such as basic mechanisms of CRISPR/Cas9, CRISPR/Cas9 experimental design, off-target effects caused by CRISPR/Cas9, and catalytically inactive Cas9 with its versatile functions.

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1. Introduction

Targeted changes in the genome have been a powerful way of studying the genome. By being able to change specific sequences within genes or regulatory sequences researchers will be able to unveil more of the complex mechanisms governing health and disease. So a tool which is able to accurately target specific sequences within the genome can prove useful in this field. Other nucleases that have been used for genome editing are transcription activator-like effector nucleases (TALENs) and zinc finger nucleases (ZFNs) which are able to introduce double stranded breaks (DSBs) by targeting the genome via protein-DNA interactions.[1] A more accurate and versatile tool of targeting has been found in bacteria and archaea; CRISPR/Cas9 which stands for clustered regularly interspaced palindromic repeats (CRISPR) combined with CRISPR associated 9 (Cas9).[2] This system is originally used by bacteria and archaea as defence mechanism against viruses.
The locus of CRISPR consists of multiple proteins and components such as non-adjacent direct repeats which are separated by spacers (variable sequences in-between the repeats). And a family of proteins that have several distinct functional domains as helicases, nucleases, polynucleotide-binding proteins, and polymerases.
A study was done to see whether CRISPR/Cas9 can be used in different organisms, besides its own specie. They found that the CRISPR/Cas system from *Streptococcus thermophilus* provided immunity when implanted in *Escherichia coli*.[3] Showing that CRISPR/Cas9 is able to function in a different bacterium. After the evidence that CRISPR/Cas9 from one prokaryote can function in the other prokaryote, the use of CRISPR/Cas9 was tested on eukaryotes as well. This works well and has been used for genome editing in various eukaryotes and eukaryotic cell lines such as yeast[4], mice[5], rats[6], frogs[7], fruit flies[8], and human pluripotent stem cells.[9]
This review will cover various aspects about CRISPR/Cas9 including off-target effects, different species that use CRISPR/Cas9, and the different methods based on CRISPR/Cas9.

2. CRISPR/Cas9

CRISPR/Cas9 is able to target and edit specific sequences in the genome using various strategies. Different bacteria have been screened for cas genes and it appears that Cas9 is a common protein found amongst bacteria.[10] Currently there are 10 identified 'core' Cas genes, Cas 1 to Cas10. But other genes are involved as well in this defence mechanism, such as the genes csn (1 and 2), RPT, cmr (1 to 6), and some others that are not noted. The genes Cas1 and Cas2 are thought to play the most prominent function in the degradation of injected viral DNA and spacer acquisition. Each bacteria has different Cas and Cas related genes, but most, if not all, contain both Cas1 and Cas2.[11,12]
Viruses infect bacteria by injecting its DNA in the bacterium. But, how the DNA is recognised and processed is not yet fully understood. The current hypothesis is that this DNA is recognised and cleaved by Cas. Cas is involved in cleaving this novel DNA, but how has not yet been determined.[12] It has been proposed that the gene Cas1 is involved in cleavage of double-stranded DNA (dsDNA) and that the gene Cas2 is involved in the cleavage of uracil-rich single-stranded RNA. Mutations in these 2 genes impair the bacterium's ability to acquire immunity against novel viral infections.[13,14] The degraded viral DNA (called spacer) is then incorporated in the CRISPR locus (see Figure 1A). The spacers are separated by repeat sequences (see Figure 1A). When the locus gets transcribed a pre-crRNA (pre-CRISPR RNA) is transcribed. It is a long strand consisting of multiple crRNA sequences that will be cleaved by RNAse III at the 5' end of each crRNA. [15] This cleaved strand will become crRNA after further processing (see Figure 1A). This is the guiding strand that guides CRISPR towards the homologous dsDNA (also called protospacer).[16–21]

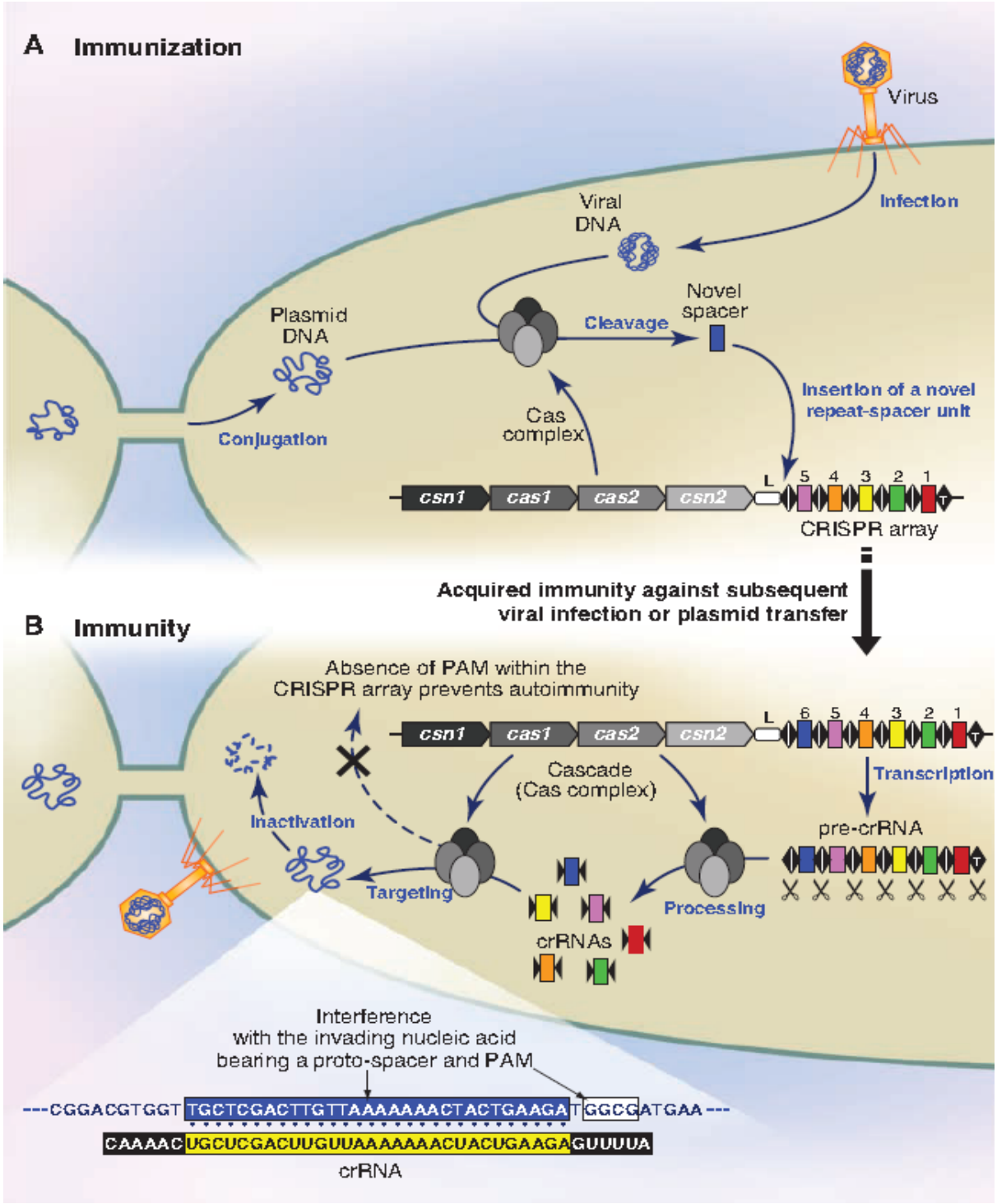

Figure 1 Overview of CRISPR/Cas immunization and acquired immunity in bacteria. (A) Immunization: Cas recognizes the invading exogenous DNA and degrades it. The degraded DNA can then be incorporated in the CRISPR locus. (B) The acquired spacers are transcribed to form pre-crRNA. This is processed after tracrRNA binds to the crRNA and Cas9 forms a complex with the formed pre-crRNA-tracrRNA complex. This is cleaved at the 5'ends via RNAse III, forming mature RNA. The mature crRNA-tracrRNA-Cas9 complex is able to recognise exogenous DNA and cleave that DNA. Adapted from Horvath, P. & Barrangou, R. CRISPR/Cas, the immune system of bacteria and archaea. *Science* 327, 167–170 (2010).

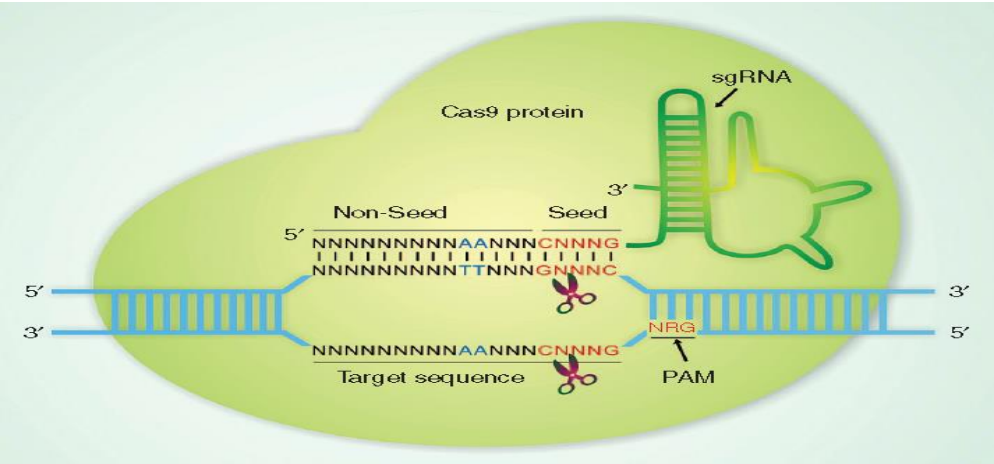

Figure 2 Cas9 complex with sgRNA sequence. Cas9 can bind DNA due to its RNA guiding strand called sgRNA. The first part of the sgRNA consists of the seed. The seed sequence has favourable positions on position 1(guanine) and 5(cytosine) for improved binding. Another factor for improved binding are the two adenosines in the middle of the strand. This recognition sequence is called the spacer and its complementary target strand is called the protospacer and it contains a PAM sequence at the 5'end. This sequence is used for distinction between self vs non-self and also helps improve binding specificity. Adapted from Zhang, X.-H., Tee, L. Y., Wang, X.-G., Huang, Q.-S. & Yang, S.-H. Off-target Effects in CRISPR/Cas9-mediated Genome Engineering. *Mol. Ther. Acids* 4, e264 (2015).

Trans-activating crRNA (tracrRNA) hybridizes to the pre-crRNA on repeat regions after transcription of the CRISPR locus(see Figure 1B). RNAse III cleaves this hybridized crRNA-tracrRNA complex at the 5'end after Cas9 has attached to the crRNA-tracrRNA complex (forming the crRNA-tracrRNA-Cas9 complex). This is done in between each spacer resulting in multiple mature crRNA-tracrRNA-Cas9 complexes, which is able to locate its target sequence (see Figure 1B).[15]
In order to properly cleave the target sequence the spacer and its target the protospacer must be complementary and the protospacer must contain a sequence called PAM (protospacer adjacent motif) at the 3' end of the protospacer. Cas9 requires the PAM sequence for target recognition, Cas9 will not cleave if the PAM is not present next to the protospacer. The bacterium prevents cleaving its own DNA this way because the crRNA + PAM sequence is not present within the bacterium's genome, including within the repeats separating the spacers. The target DNA unwinds, starting next to the PAM, so that the sgRNA can hybridize to the DNA forming a RNA:DNA heteroduplex. The mechanism involved with sgRNA hybridization, that starts near the PAM and works its way to the 5'end, explains why the area proximal to the PAM is most important for stable Cas9 binding and cleavage. [22–24] It appears that the CRISPR/ Cas9 system has different PAM requirements, but the most commonly used system, which comes from *Streptococcus pyogenes* uses the PAM NGG, in which N can be either A, T, C, or G.[25]
Cas9 will cleave the targeted dsDNA once bound. This will then result in repairing the DNA via either non homologous end joining (NHEJ) or homology-directed repair (HDR).[1] NHEJ usually causes mutations, which can either be SNPs or insertions and deletions (INDELs).[26] HDR can be used to insert specific sequences within the gene of interest by supplying exogenous template DNA.[1,27,28]

3. Designing CRISPR/Cas9 experiments

It has already been shown that bacteria can readily use CRISPR/Cas9, even from different bacteria.[3] The usage of CRISPR/Cas9 system in human cells requires some modifications. The sequence must be designed in a way that it is translated properly. Human codons are needed for a functional Cas9, and a signal peptide must be attached to it so it remains in the nucleus.

Table 1 Web tools for CRISPR/Cas9 and sgRNA design. Adapted from Zhang, X.-H., Tee, L. Y., Wang, X.-G., Huang, Q.-S. & Yang, S.-H. Off-target Effects in CRISPR/Cas9-mediated Genome Engineering. *Mol. Ther. Acids* 4, e264 (2015).

Name	Web	Affiliation
CRISPR design	http://crispr.mit.edu/	Massachusetts Institute of Technology
E-Crisp	www.e-crisp.org/E-CRISP/designcrispr	DKFZ German Cancer Research Center
Cas-OFFinder	http://www.rgenome.net	Harvard Medical School

crRNA and tracrRNA can be expressed individually, but also combined, forming a chimera called short guide RNA (sgRNA). sgRNA is easier to implement because it has both the tracrRNA and crRNA combined. So there is no need for initial crRNA-tracrRNA hybridization.[29–31] The sgRNA strand is most commonly designed with about 100 nucleotides in length. The 20 nucleotides at the 5' end of the sgRNA guide Cas9 to its target via RNA-DNA complementary base pairing (see Figure 2).The rest of the sgRNA is involved in recruiting Cas9 as discussed earlier.[1]

Web tools are used in order to analyse off-target effects, but also to design the RNA strands that are to be used.[32] Table 1 has some web tools listed. http://crispr.mit.edu/ and www.e-crisp.org/E-CRISP/designcrispr are both sites that can be used for sgRNA design, whilst http://www.rgenome.net can be used to find potential off-target effects of the designed sgRNA.

The promoter that transcribes the RNAs can cause certain limitations. RNA polymerase III-dependant U6 or T7 promoter for example requires a G or GG added to the 5' end of the RNA sequence. This can be added to the designed sgRNA strand. This means that your strand will look like GN16-19NGG or GGN15-18NGG (a G or GG has been added to your designed sequence at the 5'end).[1] 2 methods to bypass these limitations have shown promise. One option would be to choose a site without regarding the 1st 1 or 2 nucleotides (G or GG) and thus making sgRNA with mismatches at these positions, or by adding the G or GG at the 5' end and thus have 1 or 2 extra nucleotides in your sgRNA. Both of these methods yield working sgRNA strands. [33–36]

Another factor to note is the seed region. This region is important for the specificity of target binding.[21,23,37] The nucleotides outside of this region also help with binding specificity, but less severe.[38,39] Mismatches within the seed sequence are poorly tolerated by Cas9. It was shown that these mismatches can lead to decreased levels of cleavage or no cleavage at all. [40]

Various methods have been used to deliver the CRISPR/Cas components. Electroporation[41], lipofectamine[29,31,42], and nucleofection[31,42] for transiently expressed plasmid transfection. Lentiviral vectors have been used for constitutive expression in human cells.[43,44] Although, with constitutive expression one might experience increased frequencies of off-target mutations, but also higher editing efficiency. [1]

4. Off-target effects

One problem with CRISPR/Cas9 is that it might bind and cleave an area in the genome that is not targeted, a so called off-target effect. Several researchers experienced these effects when using CRISPR/Cas9.[35,38,39,42,45,46] This is able to interfere with results so one has to account for these effects in order for it to be of maximum use when applying CRISPR/Cas9. Minimizing these effects is important for both research and clinical applications. Knowledge is needed about how this mechanism works, how to detect off-target effects, and lastly how to minimize said effects.

4.1 Mechanism of off-target effects

Off-targets effects have been found whilst using CRISPR/Cas9 in eukaryotic cells. Researchers have been trying to find ways to minimize these effects, but to do so, the mechanism of off-target effects must be known.

The sgRNA sequence can contribute to off-target effects in various ways. The nucleotides furthest away from the PAM are better tolerated when mismatched. The amount of mismatches can range from 3 to 5 nucleotides at the 5'end (distal part to the PAM). Studies have reported that mismatch binding of these nucleotides are better tolerated than mismatches within the PAM or near the PAM, since mismatches in or near the PAM decreased binding and cleavage significantly and in other cases completely, whilst the nucleotides distal from the PAM showed less decrease in activity or even little to none when mismatched.[29,38,39,42,45]

Crystal structure studies suggest that PAM is essential for initiating Cas9 binding and that the seed region is very important for subsequent Cas9 binding, and activating the nuclease activity in Cas9.[47,48] A study involving sequenced chromatin immunoprecipitation (ChIP-Seq) of DNA bound to nuclease inactive Cas9 (dCas9) demonstrated that the seed region consists of only the first 5 nucleotides close to the PAM region.[49] Another ChIP-Seq study found similar results using CRISPR/Cas9.[50]

The seed sequence appears to be able to influence the binding specificity through various mechanisms.[32] If the seed + PAM sequence (seed + NGG) can bind to the genome often it will result in off-target effects. This can also control the effective concentration of the Cas9-sgRNA complex due to Cas9 binding and sgRNA abundance and specificity.[43,49] Increasing the amount of U's in the seed sequence can result in lower sgRNA levels due to termination of transcription, because a poly-U stretch is the termination signal for polymerase III.[43,49]

A high GC content in the most proximal region of the PAM was found to increase mutagenesis efficiency, this was found using a sequence that consisted of at least 4 GCs within the first 6 nucleotides proximal of the PAM region.[51]It is also important to list that guanine is favourable as 1st base next to the PAM region and cytosine is unfavourable, and on the 5th base it is the other way around. Also adenosine is preferred in the middle of the sgRNA sequence and cytosine does not go well at position 18 of the sequence. [43,49,52,53] The summary of these nucleotide positions are depicted in Figure 2.

The PAM sequence is able to influence sgRNA activity, it appears that NGG is a common PAM used by CRISPR/Cas9, but NRG (R stands for G or A) can be used as well, but with only a 5th of the efficiency of NGG.[19,39] But other orthologues of Cas9, for example that of *Streptococcus thermophilus* or *Staphylococcus aureus*, instead of *Streptococcus pyogenes*, can be used with different PAM sequences like NGA or NAC.[54]

Off-target effects can also vary between cell types, since each cell uses its own set of genes. The repair mechanism of the cell might be able to repair the DSB in a less error prone or a more error prone manner, depending on the cell type's repair pathway.[50] It has been shown in 2 different studies that human pluripotent stem cells with decent DSB repair mechanisms had fewer off-target mutations than a transformed human cell line with dysregulated DSB repair pathways.[55,56]

Methylation and small molecules can affect binding specificity as well. There have been reports showing that methylated DNA may impede Cas9 binding efficiency and small molecules can enhance genome editing of CRISPR/Cas9 by promoting genome editing via HDR (for example L755507, a 73-adrenergic receptor agonist) or promote knockout via NHEJ (for example azidothymineor Trifluridine), but these are still being studied.[49,50,57,58]

4.2 Off-target detection methods

Detecting whether there are off-target effects can be useful to test the designed sgRNA. There are various methods to detect these off-target effects.
The initial test was the T7 endonuclease assay, but this essay has a poor sensitivity (cannot detect mutations that occur at frequencies lower than 1%) and is not optimal to use when screening such large quantities.[35,59] Different advanced methods like deep sequencing, web-based prediction tools, ChIP-Seq, and other methods have been used to detect these off-target effects with higher frequencies(see Table 2).[32]
Genome-wide, unbiased identification of DSBs enabled by sequencing (GUIDE-Seq) is one of the tools used for off-target detection.This method is based on global capturing of DSBs made by RNA-guided nucleases (RGNs) and profiling them.[60] This is done in 2 steps. DSBs that are created by RGNs in human cells are tagged by integrating a blunt, 34bp double stranded oligodeoxynucleotide, via a mechanism that resembles non homologous end joining. These sites are then amplified and sequenced, giving this method a detection rate as low as 0.12%.[60]
High-throughput, genome-wide translocation sequencing identifies translocations by yeast I-scelmeganuclease-generated DSBs, named as 'bait' DSBs, at target sites. This was introduced in the mouse cell's genome, where the 'prey' DSBs are, and the formed translocations indicated where 2 DSBs of heterologous chromosomes could fuse. Showing DSBs and if done often enough this could lead to information about DSB hotspots.[61] This method is said to have a high enough resolution to spot nucleotide-level junctions.[61]
Web-based tools are used to screen for DSBs *in silico* and *in vitro*, but are not as good for predicting *in vivo* mutations, since sgRNA is able to hybridize with mismatches up to an extent.[35,38,39,42] So this method might be useful for preliminary analysis, but is not accurate enough to predict what will happen *in vivo*.
Another method, which has been used to detect cleave sites of ZFNs can also be used. Linear double-stranded integrase-defective lentiviral vectors incorporate themselves into DSBs and can identify cleavage sites with a frequency of at least 1% *in vivo*.[62,63]
The method called Digenome-seq (*in vitro* Cas9 digested whole-genome sequencing) can be used as a cost-effective, unbiased and sensitive method to detect genome-wide off target effects by RGNs.[64] Just as GUIDE-Seq this method is done in 2 steps. First DNA was isolated from transfected cells with either intact genomic DNA, without RGN, or with RGN . Both are then digested *in vitro* with RGNs separately followed by whole-genome sequencing. These digested strands have many DNA fragments with identical 5' ends. Which when read are vertically aligned at cleavage sites, whilst the others would be read in a staggered manner. The authors were then able to observe patterns in sequence alignments, at the on- and off-target sites, with Integrative Genomics Viewer after mapping the sequences.[64] Digenome-seq is said to be the best standard in detecting the DSBs to date.[32]
As a simple detection method for *in vitro* experiments one can also use FISH for quick and easy off-target detection. This works only when inserting exogenous DNA using CRISPR/Cas9, since you can use FISH to locate the locations of the exogenous DNA that is inserted. This method quick and cost efficient, but also less accurate than previously described methods.[32]
A peculiar finding is that *in vivo* experiments seem to have less DSBs than *in vitro* cellular experiments.[32]

Table 2 Off-target detection methods with advantages and disadvantages listed. Adapted from Zhang, X.-H., Tee, L. Y., Wang, X.-G., Huang, Q.-S. & Yang, S.-H. Off-target Effects in CRISPR/Cas9-mediated Genome Engineering. *Mol. Ther. Acids* 4, e264 (2015).

Method	Advantage	Disadvantage
T7E1 assay	Simple	Poor sensitivity, not cost-effective
Deep sequencing	Precise	Biased, misses potential off-target sites elsewhere in the genome
In silico prediction	Predicts some off-target mutation sites	Fails to predict bona-fide off-target sites
GUIDE-seq	Unbiased, sensitive (0.1%), qualitative translocations, identifies breakpoint hotspots	False negatives present, limited by chromatin accessibility
HTGTS	Identifies translocations	False negatives present, limited by chromatin accessibility
IDLV	Programmable, sensitive (1%)	Many bona-fide off-target sites cannot be captured
Digenome-seq	Sensitive (0.1% or lower), unbiased and cost-effective	Not widely used
FISH	Quick	Less precise

FISH, fluorescence *in situ* hybridization; IDLV, integrase-defective lentiviral vectors.

4.3 Minimizing off-target effects

Multiple strategies are able to be used in order to minimize the off-target effects caused by RGNs. sgRNA can be edited for more specificity in various ways, like shortening the strand or adding guanine nucleotides at the 5' end of the sgRNA. Shortening the sgRNA strand (called tru-gRNA) at the 5' end of the sgRNA by 3 nucleotides can improve specificity significantly. These 2 strategies are capable of decreasing off-target effects by a 5000-fold at certain sites in the genome.[35,45,65] But, this also decreases on-target efficiency, as introduction of mismatches, or sgRNA shortening reduces the binding affinity. More research has to be conducted on these modifications in order to maintain the on-target effects or even increase on-target effects, but so far inconsistent data has been reported.[35,66]

It appears that chromatin accessibility seems to play a major role in *in vivo* experiments. Some genes have low mutate efficiencies due to the histones preventing dCas9 binding. [32] A way to circumvent this inhibition would greatly improve Cas9 function.

Another way of minimizing off-target effects could be by controlling the concentration of Cas9-sgRNA complexes, since higher concentrations could result in more off-target effects. This can be done by transfecting with less DNA. Less DNA containing the information of Cas9 results in less Cas9. This could result in less off-target binding due Cas9 binding to higher affinity targets, but this sacrifices on-target cleavage as well due to less Cas9 present. So more research has to be conducted in order to optimize this method.[39,42,45,57]

Cas9 cleavage can also be controlled by replacing wild-type Cas9 by the D10 mutant nickase version. If you pair 2 of these, both with a different sgRNA, but still in the same region of the target, one can induce 2 single stranded breaks, resulting in a DSB with overhang, or in the case of an off-target effect have a simple single stranded break which can be repaired more accurately, resulting in fewer off-target mutations (see Figure 3).[33] Due to the decreased off-target effects, this method can be used more easily when aiming to mutate multiple genes within a cell.[32] Web-tools are available to design paired sgRNA and predicting the off-target effects caused by the sgRNA pair.[67] The optimal proposed set up is by using two tail-to-tail orientated strands separated by about -10 to +30bp with the following sequence 5'-CCN(32-72)GG-3', this can be used for any model organism and results in fewer off-target effects.[32]

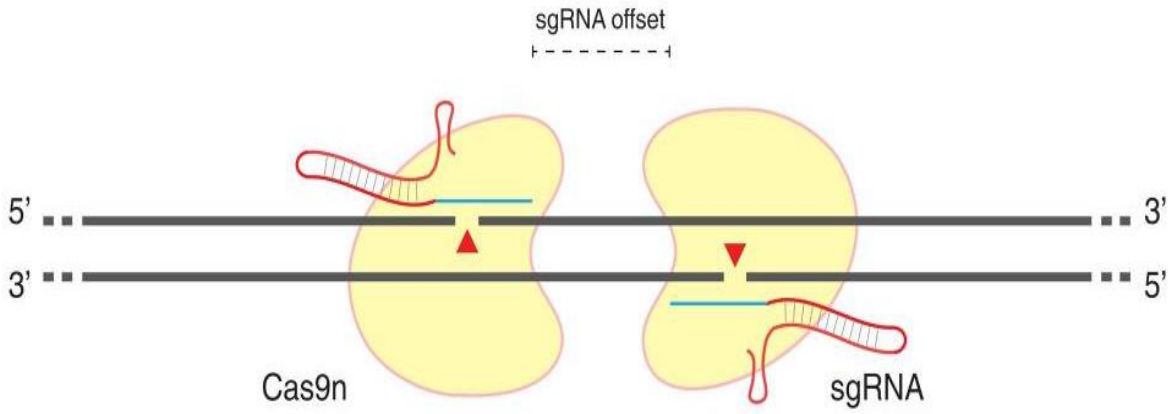

Figure 3 Nickase set-up. Cas9 has one of its catalytic domains mutated resulting in a single-stranded break instead of a double-stranded break. Double stranded breaks can also be introduced this way when using 2 nickases that target the same gene, but with some space in between. This results in fewer off-target effects. Adapted from Ran, F. A. *et al.* Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell* 154, 1380–9 (2013).

It is also possible to improve cleavage specificity by fusing dCas9 with FokI nuclease domain(fCas9). This targets DNA sites with higher specificity (>140 fold) than wild-type Cas9 and about a fourfold higher than using nickases with highly similar sites. This system is similar to nickases, but FokI is a nuclease that only works when forming a dimer. So FokI is able to form a dimer when dCas9 binds in the same region. The DNA is then cleaved once the dimer is formed. This system causes fewer off-target effects than nickases since nickases are able to cleave without a second nearby and thus causing mutations in unwanted regions. FokI requires that both dCas9 complexes bind in the same region before it can cleave, thus making this more specific.[68,69]

5. Different CRISPR/Cas9 applications

CRISPR/Cas9 has become a versatile method to perform various tasks like genome editing, but also genome regulation (see Figure 4). It can also promote interactions which other nucleases would not be able to do, such as fusing a protein to dCas9, or fusing transcription factors to promote or repress expression. Or simply inhibit transcription by just binding to the target gene.[70] The coming section will review the method dCas9 with its several different uses.

5.1 dCas9

Cas9 was made into dCas9 by introducing the mutations D10A and H840A (also called RuvC1- and HNH-like domains) to nullify its nuclease activity.[23,24] If one of either becomes inactivated it will turn Cas9 into a nickase.[11,71] Cas9's application diversity grows significantly once the nuclease activity has been nullified. dCas9 will still bind to its targets, but will not cleave it. dCas9 will simply bind, leaving lots of opportunities to regulate the genome or to determine loci of genes using fluorescent signals. All of these various possibilities will be reviewed below.

5.2 Transcription regulation

dCas9 can also be used to regulate transcription in various ways. It can activate, but also repress transcription. This can be done by fusing activating or repressor domains dCas9 (Figure 4A and B). The method of dCas9 mediated gene repression is also referred to as CRISPR-interference. This can be done in several ways as well. A simple method would be to use dCas9 binding to block RNA polymerase or to block transcription factor binding.[72–75] In bacteria this system works quite well and efficient. It can silence genes simultaneously (multiplexing) and it is reversible.[72] But, this can also lead to operon repression, which might be an off-target effect so to speak.[76] This method that uses dCas9 binding only gave moderate repression in mammal cells. [72] Improved repression was obtained by fusing Krüppel-associated box (KRAB) or four chain-linked mSin3 interaction domains (SID4X) to the carboxyl terminus of dCas9(Figure 4A).[77–79] Repression can be further enhanced by fusing KRAB to the amino terminus of dCas9.[80] Although, it has been suggested that chromatin structure can interfere when using CRISPR-interference because of the repression being dependent on whether the target gene is accessible or not.[76] The repressor MXl1 was used for effective repression in yeast(Figure 4A).[76]
Furthermore, transcription can also be regulated by placing or removal of epigenetic marks (Figure 4E). Histone demethylase LSD1 was fused to dCas9 of *Neisseria meningitidis* and was able to repress gene expression by removing the histone 3 lys4 dimethylation at an enhancer, leading to gene repression.[81] Another study showed that a dCas9-KRAB complex was able to trimethylate H3K9 at the enhancer of globin.[82]

Transcription can be activated by fusing the proteins VP64, and p65 activation domain (p65AD) to dCas9.[77,83–85] Another way is by fusing VP64 to both the C as the N terminus of dCas9 (Figure 4B).[86] But this required multiple sgRNAs for significant activation for some genes.[87] This inclines that recruitment of multiple transcription activators could enhance activation efficiency.[76] The method SunTag uses this knowledge as it, fused to the carboxyl terminal, consists of 10 small peptide epitopes. These peptides are then recognised by a cognate single-chain variable fragment (scFV) that is fused to super folded GFP (sfGFP) and VP64, making a scFV-sGFP-VP64 complex. This recruits VP64 proteins to the target site and also sfGFP to make visualization possible (see belowFigure 4C).[88]
A different activation method was found when screening for different activator domains. This screen has found that a tripartite activator domain, consisting of VP64, p65AD, and Epstein-Barr virus R transactivator RTA (VPR), showed improved activation of the target gene(Figure 4C).[89]
Another way to achieve enhanced specificity is by using protein interacting RNA aptamers that are incorporated in the sgRNA(Figure 4D).[38] The aptamer for MS2 is used to recruit its coat protein MCP that is fused to p65AD and heat shock factor 1 (HSF1). VP64 fusion with dCas9 further improves transcription activation using this method. [38] This method was able to activate 10 genes simultaneously.[90]
Fusing p300 to dCas9 can also regulate gene expression on an epigenetic level, when p300 is fused to the catalytic core of Cas9 (called dCas9-p300^{core}). This complex is able to acetylate histone3 lys27 at the promoters and enhancers of specific genes (Figure 4F).[82]

dCas9 is also able to activate and repress at the same time using scavenger RNA (scRNA) and by using multiple sgRNAs.[76]scRNA is made by extending the sgRNA at the 3'end with RNA domains that are able to recruit proteins. The sgRNA strand must first be extended by a hairpin domain followed by a 2 nucleotide long linker. Next is the RNA sequence required to recruit the regulating proteins of choice.[91] Transcription can be activated when adding an RNA aptamer for VP64 and can be repressed by adding an RNA aptamer recruiting KRAB. When more of these are used simultaneously, one will be able to research regulatory networks or pathways within a single cell by repressing or activating the genes of interest. This method can repress and activate different genes within a single

cell by targeting genes with either an RNA aptamer that recruits VP64 or KRAB to activate or silence the target gene(s) (see Figure 5).[91]

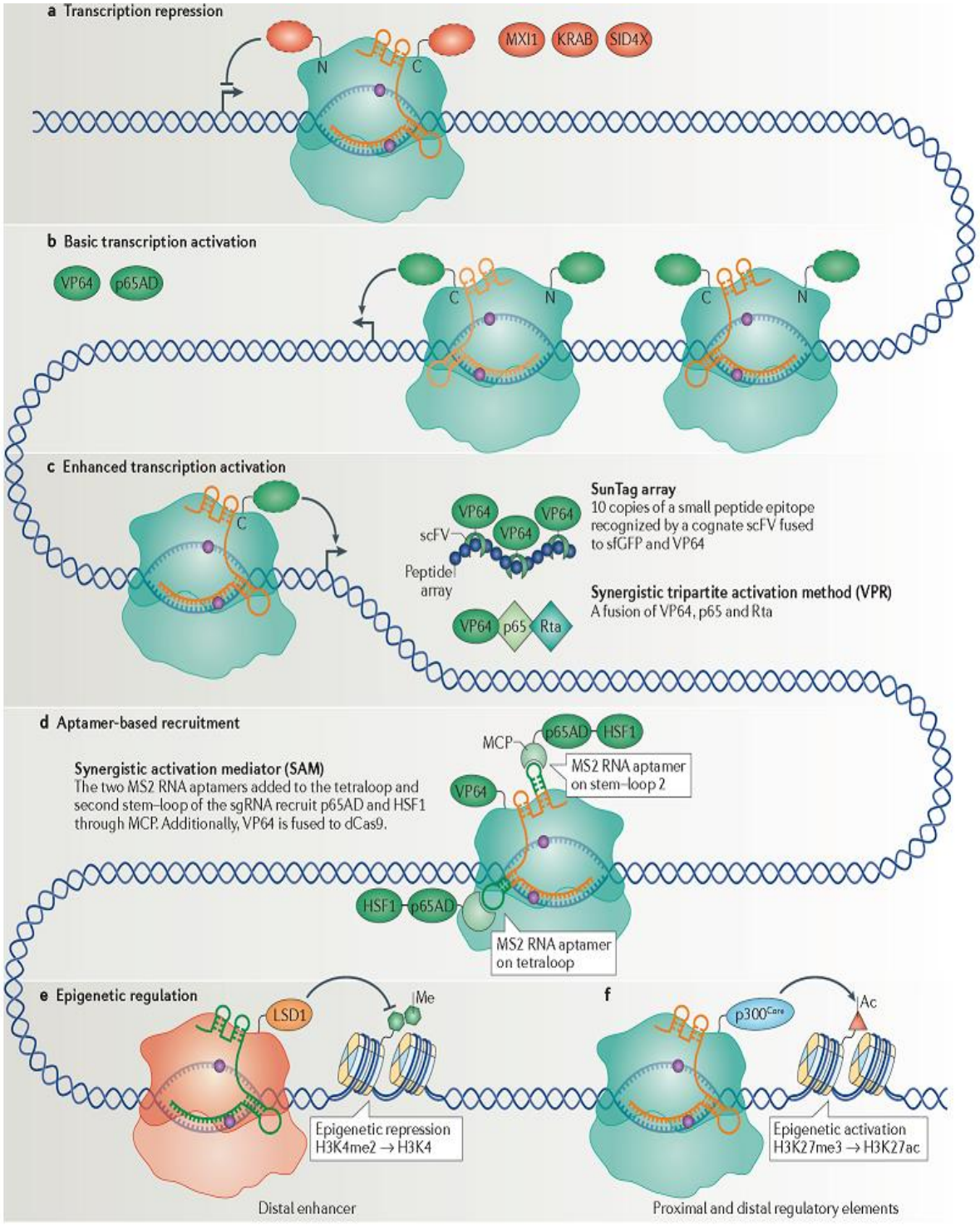

Figure 4 Gene transcription regulation methods using dCas9. (A) Transcription can be repressed by fusing dCas9 with either MXI1, KRAB or SID4X, better activation is achieved by fusing it at both carboxyl as amine terminal.(B) Transcription can be activated by fusing dCas9 to VP64 or p65AD, better activation is achieved by fusing it at both carboxyl as amine terminal.(C) SunTag is another method for transcription activation. It employs 10 small epitopes of peptides that are targeted by scFVs fused with VP64 and sfGFP. (D) MS2 RNA aptamers are incorporated in the sgRNA to recruit the MS2 protein, which recruits VP64 fused to HSF1 for transcription activation.(E) Epigenetic marks can be used as well by fusing an epigenetic regulator, such as LSD1, or p300 (see F). Adapted from Dominguez, A. A., Lim, W. A. & Qi, L. S. Beyond editing: repurposing CRISPR–Cas9 for precision genome regulation and interrogation. *Nat. Rev. Mol. Cell Biol.* (2015). doi:10.1038/nrm.2015.2).

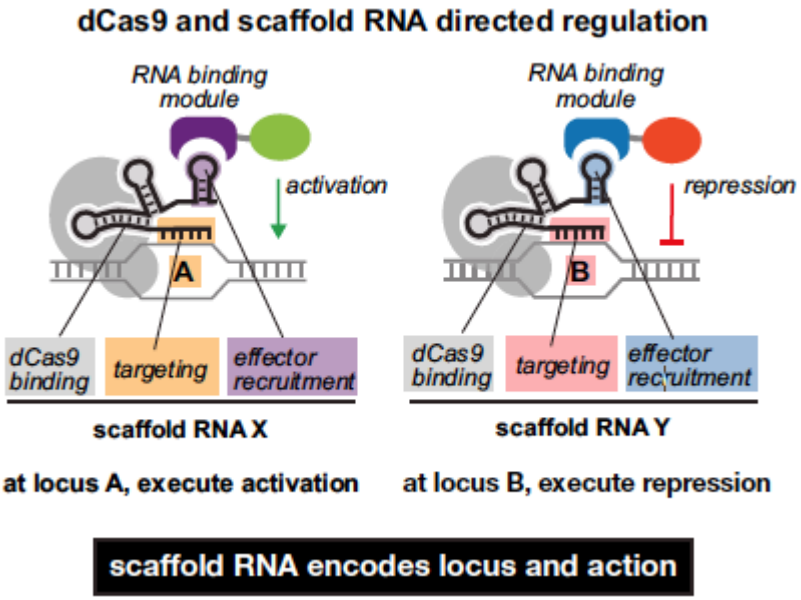

Figure 5 scaffold RNA set-up. dCas9 can be used to recruit activator or repressor proteins by extending the sgRNA at the 3'end with a hairpin domain and a linker followed by the RNA sequence that recruits the effector adapted from Zalatan, J. G. *et al.* Engineering Complex Synthetic Transcriptional Programs with CRISPR RNA Scaffolds. *Cell* 160, 339–50 (2014).

5.3 Non-transcription regulating functions

Non coding RNA (ncRNA) has also been delivered to targets via dCas9. This was done by inserting the ncRNA sequence within the sgRNA or it was implemented at one of the termini.[92] The study showed that this method was able to display long ncRNA (lncRNA) up to at least 4.8kb long. Its set up is like using scRNA, but without the protein recruiting RNA sequences. Using this one could see the effect of ncRNA and lncRNA on target genes within the cell.
Another function of dCas9 is fusing it with a fluorescent protein, like GFP, to locate certain genes and its loci. GFP was used to study telomere dynamics in live retinal pigments epithelium or HeLa cells by tagging dCas9 with enhanced GFP (eGFP).[93] SunTag was used to further enhance this signal by recruiting scFVs via 10 small peptide epitopes fused to dCas9.[88] Multicolour imaging is also an option by using dCas9 from different species of bacteria such as *Streptococcus pyogenes*, *Neisseria meningitidis*, and *Streptococcus thermophilus*.[94]
dCas9 is also able to 'staple' pieces of the genome together, so to speak. This can be done by designing 2 sgRNA strands with complementary 3' regions, causing the 2 strands to bind to one another and also to dCas9, that binds the target genes.[95] And if dCas9 is fused to GFP, this can even be visualized.[70]
dCas9 could even function as a RNA-guided-recombinase by fusing the catalytic domain of a recombinase to dCas9.[96] But this method would probably need optimization due to the low activity of the fused recombinase.[70]

6. Discussion

CRISPR-Cas9 is a great tool to use in genome editing, regulating and can even be used for imaging loci. Many forms of Cas9 can be used for specific purposes such as Cas9 from different bacteria, such as *Streptococcus pyogenes*, *Neisseria meningitidis*, and *Streptococcus thermophilus*, that contain small differences in efficiencies or PAMs making them original. It seems that *Streptococcus pyogenes*

is the most commonly used when using CRISPR-Cas9 type II. Although more bacteria could be screened in order to enhance specificity. It would be ideal if a more efficient Cas9 can be designed, but this could pose quite the challenge.
Another beautiful aspect of CRISPR/Cas9 is the fact that it is RNA guided for specific targeting, but also that it is able to be fused with proteins and that the sgRNA strand can be edited to hold different RNAs that are able to regulate genes. This makes CRISPR/Cas9 a versatile method in molecular biology.
But, there are still a lot of challenges when it comes to optimizing CRISPR/Cas9. CRISPR/Cas9's sgRNA binding area is only about 20bases long making it a sequence of which complementary sequences can be found multiple times within the genome. When including the finding that the first 5 base pairs, that cause mismatches at the 5'end of the sgRNA, are more readily tolerated, it becomes more challenging to avoid off-target effects.[42] Because the sgRNA will be able to hybridize to similar sequences that only have a few mismatches. A few strategies have been proposed and suggested in order to minimize these off-target effects. The most important ones include using nickases instead of regular Cas9, or dCas9 fused with FokI. Even when using these strategies it is still recommendable to screen for off-target effects when using CRISPR/Cas9.
The advantage of CRISPR/Cas9 in comparison to other nucleases like ZFNs and TALENs is that it is able to fuse with ncRNAs, proteins, transcription factors. Another reason is its ability to multiplex, it can induce different edits in a cell, as is done in rats[97], mice,[5] and zebrafish[98] But CRISPR/Cas9 has the same flaw as ZFNs and TALENs as well due to all nucleases having off-target effects.[1]
CRISPR/Cas9 is already a powerful application in research and certainly has the potential to also become a very useful tool for the development of novel therapeutic approaches.
CRISPR/Cas9's applications will grow even more diverse over time when more research is conducted on different applications.

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