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THE ESTABLISHED, AND NOVEL DEVELOPMENTS IN CRISPR/CAS MEDIATED GENETIC ENGINEERING

by

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Abstract

Development of a precise and high-fidelity gene editing tool has become an important goal in microbiology, and other disciplines. CRISPR/Cas systems have garnered a lot of attention for their ability to make quick and efficient DNA edits. While they have matured to a viable genetic engineering tool in many regards, a great deal of research is still necessary to increase our understanding of their functioning and range of application. Originally studied as a bacterial immune system, recruitment and utilization of engineered CRISPR/Cas systems can be performed efficiently *in vitro* and *in vivo*, and is not restricted to any organism. This review serves to give an overview of the basic functioning of CRISPR/Cas systems, their current possible applications in genetic engineering and the most important developments in CRISPR/Cas mediated genetic engineering. Also passed under review are the possible problems associated with this type of gene editing compared to other methods and the efforts in circumventing these innate problems. An attempt is also made to give insight on the safety and viability of CRISPR/Cas based genome editing platforms.

Contents

Introduction

The understanding and applications of the CRISPR-genetic library and CRISPR-associated (Cas) proteins in genetic engineering have grown significantly over the last few years. From discovering newer, more precise nucleases[1], applying Cas-proteins in epigenetic modulation[2] to editing embryonal stem cells in mice[3], CRISPR/Cas has matured into a versatile genome editing tool, applicable in a range of different organisms. CRISPR/Cas finds its origin in a wide variety of both bacteria and archaea[4] and is generally defined as an adaptive immune system[5], playing an important role in both recognition, as well as degradation of hostile genetic vectors[6]. However, research has shown[7,8] that different Cas-proteins are also active in regulating innate virulence factors of the organism itself, suggesting a broader range of regulation than originally thought.

Phylogenetic analysis of different CRISPR-loci and associated nucleases, has shown a diverse collection of nucleases[4] divided in two distinct classes (class I & II), and six different types (I to VI), varying in their interaction and modulation between the Cas-proteins and the CRISPR-locus. Class I (type I, III, VI) regulates immunity and nuclease activity in a complex of multiple enzymes and accounts for 90% of all the described CRISPR/Cas systems[9]. Class II proteins (type II, IV, V) have evolved to a larger, single-enzyme constitution for DNA restriction[10] and are more often studied for genetic engineering purposes.

One of the best characterized Class II proteins is SpCas9. Cas9 from *Streptococcus pyogenes* was one of the first proteins observed[11,12], that could interact with previously discovered CRISPR loci. The function of CRISPR and SpCas9 as an immune system as well as their initial recruitment for DNA editing purposes will be documented here to present some background on the technical aspect of its applications and developments.

Cas9 as an immune system

The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) array is filled with protospacer motifs and repeats (*Figure 1a*). A protospacer is a piece of harmful DNA that is recognized and copied by integrase proteins Cas1 and Cas2, which are largely conserved in all types of CRISPR/Cas systems[13]. Their interaction with foreign DNA is mediated by effector protein Csn2[14] due to unknown binding mechanisms. Csn2 is hypothesized to recruit Cas9 to create a double stranded break, after which the integrase complex of Cas1-Cas2 facilitates insertion of a DNA fragment (i.e. spacer-acquisition) into the CRISPR array. Adjacent to the CRISPR array resides an independent, scaffold sequence, the trans-activating crRNA (tracrRNA)[15].

Inside the CRISPR array, protospacers are intertwined with repeats. Repeats are repetitive sequences, homologous to the tracrRNA. When part of the CRISPR array is transcribed into crRNA, homology of the repeats with tracrRNA causes duplex formation with the crRNA. Comaturation of the crRNA:tracrRNA is a requirement for successful association with Cas9[16]. RNAseIII and other, unknown enzymes[17] restrict the crRNA to a final length of 20 nt.

Specificity of Cas9 for the crRNA:tracrRNA hybrid is high, promoting a strong affinity[18]. The sequence homology of the crRNA causes efficient binding and double stranded breakage of the target gene, if a PAM sequence is present three nucleotides upstream 3' of the complementary crRNA sequence. A PAM (*Protospacer Adjacent Motif*)-sequence is a 3-8nt, Cas-protein specific recognition sequence which is hypothesized to be part of the protospacer that is removed in the maturation process of the crRNA[19]. Theorized to have evolved as a mechanism to prevent internal restriction of the CRISPR array, the presence of PAM sequences (for DNA targeted Cas-proteins), or PFS (*Protospacer Flanking Sequence)*-sequences (for Cas proteins that target RNA[20]) is essential for Cas-nuclease activity[21].

Nuclease activity of Cas9 is mediated by two different domains, HNH and RuvC, which are both highly conserved in different types of Cas-proteins[22]. They cut the complementary strand of the crRNA sequence and the target strand respectively.

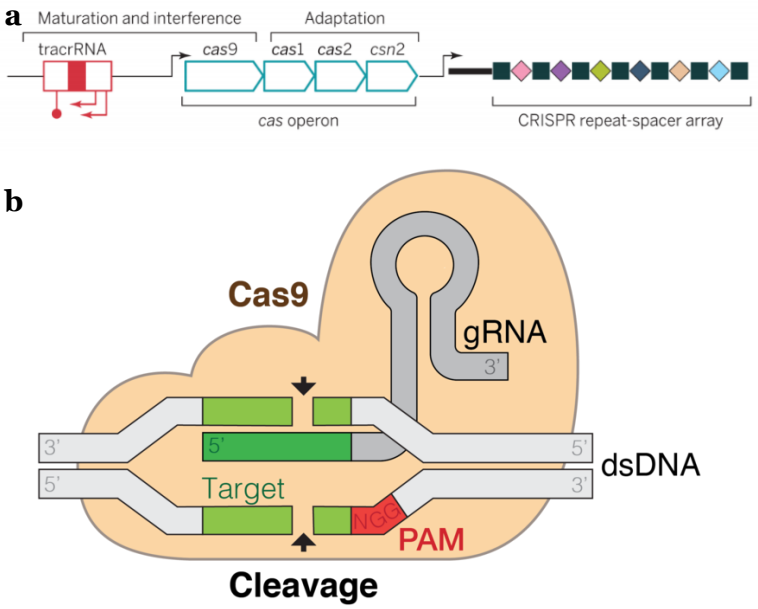

***Figure 1** | (**a**) CRISPR array, tracrRNA and Cas operon as found in S. pyogenes [23]. (**b**) Schematic of CRISPR/Cas9 when bound to target DNA by gRNA localization and PAM recognition [5]*

Adaptation for genome wide editing purposes
As more information about the functioning of CRISPR/Cas systems was published, its potential role in genetic engineering purposes was recognized. Since the only requirements (for Cas9) are expression of Cas9 in the target cell and a method of crRNA:tracrRNA hybrid transcription, attempts were successfully made to clone these components to a target organism without innate CRISPR resistance[24]. Later, it was shown that reprogramming the crRNA to change the site of the double stranded break was possible, the only requirement being a PAM sequence upstream[25]. To optimize the transcription of the crRNA:tracrRNA duplex in a vector, a linker between the two was implemented[16], the result of which is commonly denoted as guide RNA (gRNA), or single guide RNA(sgRNA, terms often interchangeable). Cas9 can now be recruited using a single expressed gRNA sequence (*Figure 1b)*, only changing 20nt that determines target recognition (*Figure 2)*.

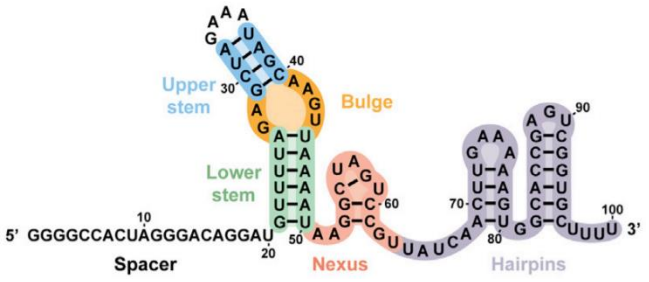

Figure 2 *| Structural representation of a hybrid gRNA. Visualized are the structural motifs in the crRNA (1-32nt), tracrRNA (37-100) and the GAAA linker in between. Target specificity is encoded by the Spacer sequence (1-20nt)[16]*

CRISPR/Cas as a genome wide editing tool has already been successfully employed for several purposes. A lot of information and publications about DNA editing techniques, transcriptional control, the development of improved nucleases, methods to increase fidelity *et cetera* is circulating. To give some clarity and an (incomplete) overview, the dominant applications in the field will be passed under review. These are genome editing techniques, largely based on the SpCas9 platform that have been shown to work reliably in microbiological organisms. The many developments in engineering of Cas9 and other nucleases are however more experimental and less well-characterized. Of these, the most relevant advancements and problems associated with them are discussed. The final chapter aims to give insight in the general disadvantages surrounding CRISPR/Cas based editing platforms. Comparisons with other techniques are drawn and the development of improved Cas-editors is discussed. Ultimately, an attempt is made to give a nuanced and broad perspective on the safety and applicability of modern CRISPR/Cas solutions in a microbiological context.

1. Current applications of CRISPR/Cas

Creating gene knockouts
Since wild type Cas9 (as well as other Cas-nucleases) creates double stranded breaks (DSB) upon recruitment by gRNA, cellular repair mechanisms are recruited in vivo to minimize damage. This can be utilized to create gene knockouts efficiently, compared to other methods (see *chapter 3*). As *Zhang et al* point out[26], there is a competition between homologous directed recombination (HDR) and the more error-prone, non-homologous end joining (NHEJ) as a response to DSB damage. For HDR to occur, a template with high sequence similarity is required to incorporate DNA without frameshifting[27], in diploid eukaryotes this is usually the sister chromatid, often putting a requirement on mitosis for most applications in mammals[32].

When a small template (5-25nt) with homology close to the DSB is present, repair mechanisms can cause microhomology-mediated end joining (MMEJ), which is even more error-prone than NHEJ[28]. The small homology causes sticky overhangs, which have to be degraded by a MMEJ-specific nuclease. This extra nuclease activity causes a high chance for extra insertions and deletions. NHEJ occurs through an independent, different pathway and directly ligates the blunt ends of the chromosome without the requirement for a template sequence to be present[29]. This often causes frameshifts, by insertions or deletions (indels) and can be problematic in preventing premature stop codons, or other truncation mechanisms[30]. Thus, when aiming for high efficiency edits using Cas9 (e.g. creating a knockout), most applications use the addition of a small (50-200nt[29]) double stranded repair fragment that is homologous to part of the organism's own genome to optimize HDR contribution, although the maximum efficiency achieved is still roughly 10% [31]. In some organisms, mitosis is critical for the overall success rate of CRISPR/Cas induced dsDNA repair, as mitosis suppresses a lot of NHEJ pathway components to minimize damage in telomeric regions[32]. However, in microbiological applications like in *E. coli* or haploid yeast, this is not really an issue.

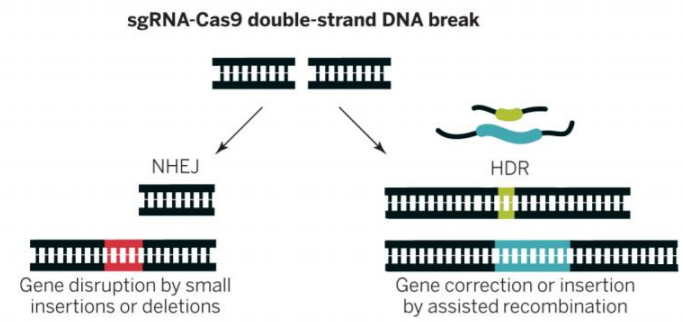

Figure 3 *| Repair of dsDNA breaks when induced by Cas9 will cause either NHEJ recruitment leading to frameshifts in the target gene, or HDR recruitment which will cause blunt insertion by template recognition, leaving the flanking sequences intact.*

Reports have emerged which show an antiproliferative cell response in mammalian cells[33] to dsDNA damage induced by CRISPR/Cas. Multicopy areas would especially be sensitive[33] and might show deleterious effects, especially under sustained dsDNA damage. Other research also emphasizes the same risk factors in mammalian cells[34], but no concluding work on these same effects in prokaryotes and lower eukaryotes has been published.

Some argue that using single base editing to create stop codons is a safer and more effective method than relying on intracellular recombination pathways[102], which could be an alternative to knocking out genes should any problems rise to the surface during screening.

Single base editing

Precision base editing is a valuable tool for generating premature stop codons or targeted amino-acid mutations amongst others. CRISPR/Cas9 has been successfully employed in several methods to optimize editing efficiency and margin of error[35]. One of the first developments was that of Cas9 nickases, mutated to be catalytically inactive in one domain, these enzymes generate only a single stranded cut on the activation location of Cas9. By mutating catalytic residues in the RuvC, or HNH domain (D10A[16] and H840A[22], *Figure 4*), all other basal Cas9 functions are maintained, but catalytic activity is partly restricted.

However, when employing a nickase, the cut is quickly filled in by recruitment of the base-excision repair pathway[36]. When recruiting the two different nickases to the same location though, a sticky overhang which is only dependent on the offset in the different gRNAs can be created[37]. The flexibility created by this type of DSB is far larger than that of the wild type. Either NHEJ-processes will clear up the sticky ends with 100x less indels events, or homology directed repair can be more efficiently utilized if a repair fragment is available[38]. By varying in repair fragment size and sequence, point mutations can be implemented. The efficiency of HDR in multiplexing (using multiple gRNAs at the same time to target DNA) however at the time was very low, only around 0.1-0.5% of all repair events showed the correct sequence insertion[39]. Development in this area was thus quickly superseded by the release of a fusion between a base-exchange protein and a dead Cas9 (dCas9)[40].

Development of this BE1 protein removed the risks associated with dsDNA breaks and allowed for very efficient, single-stranded DNA (ssDNA) breaks[41]. Previously, a catalytically inactive, dead Cas9 (dCas9) had already been created, combining both RuvC and HNH inactivity without the loss of affinity for DNA[42]. By fusing this dCas9 to a cytidine deaminase, C:G base pairs could be changed to A:T, with the sequence of the gRNA determining the place of exchange[40] (*Figure 5*). Optimized fusions based on this platform were developed, notably BE3, BE4, BE4-Gam which all showed increased editing efficiency in a higher sequence window with less indels formation, especially compared to HDR via dsDNA breaks[26]. Fusing an uracil-DNA glycosylase inhibitor with dCas9 in BE3 especially helped editing efficiency, by suppressing activation of the NHEJ pathway in both prokaryotes, as well as eukaryotes[43]. Directed evolution on TadA, an *E. coli* adenine RNA deaminase, yielded functional dCas9/adenine deaminase constructs which made it finally possible to mutate any DNA base in range of a PAM sequence to any other DNA base[44]. The development of other platforms than Cas9 to target different PAM sequences, as well as increased editing efficiency is also ongoing (Chapter 2).

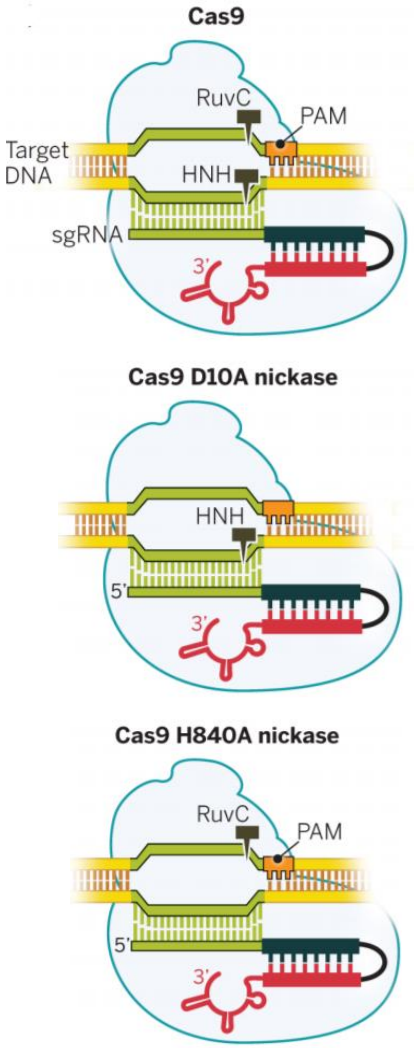

Figure 4 | (**a**) Wild type Cas9 creates a DSB using the RuvC and HNH nuclease domains. (**b**) D10A nickase eliminates RuvC activity and only cuts the targeted strand. (**c**) H840A nickase eliminates HNH activity and only cuts the complementary strand to the target. Both nickases can be combined with shifted gRNAs to create sticky overhangs. [23]

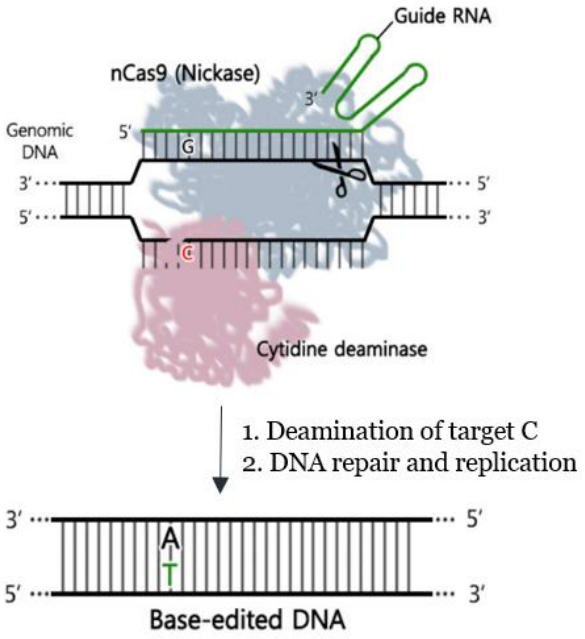

Figure 5 | *Schematic overview of a base exchange protein (in this case cytidine deaminase) coupled to a nickase Cas9, allowing for base exchange. By changing the nickase, both strands can be targeted in accordance with a PAM sequence. Fusions based on dCas9 proved more successful (adapted from [104]).*

2. Developments in CRISPR/Cas9

Other nucleases than Cas9

Although Cas9 is utilized in most existing applications of DNA engineering, developments in other Cas-proteins show promising results. Since different nucleases each depend on a different PAM sequence, developing other CRISPR based platforms allows for much greater versatility in genome engineering. Cpf1 (Cas12a) especially has gained traction for its capabilities in gene insertion[45,46]. Although a member of type II, Cpf1 creates sticky overhangs of 4/5 nt instead of blunt ends when acting upon dsDNA[47]. This can be helpful in designing gene constructs, aiding insertion efficiency. A side effect of double stranded binding of Cas12a (Cpf1) to dsDNA is the activation of catalytic cleavage on ssDNA molecules, which is not regulated by the target gRNA[48]. This indiscriminate catalytic effect makes Cpf1 overall a more experimental nuclease than Cas9. Also, although Cpf1 shows less off-targeting events compared to Cas9, developments in improved Cas9 variants might supersede this advantage in the future[49].

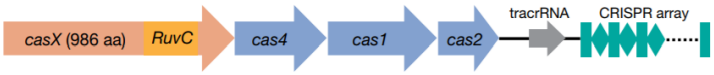

Figure 6 | *Localization of CasX on chromosome as found in the original bacteria[1]*

Very recently, genomic analysis of bacteria from groundwater revealed a new, more concise (~400aa smaller) nuclease temporarily titled CasX (*Figure 6*) [1]. The activity of this nuclease on dsDNA appears to be comparable to Cas9. A smaller footprint is an advantage in gene editing, since size is sometimes a restriction for proper utilization of Cas9 (*Chapter 3*).

Cas13

Initially only nucleases capable of restricting DNA were discovered, computational genome analysis led to the discovery of several potential Cas13 proteins[50]. Further characterization of C2c2 (Cas13a) uncovered its potential in RNA editing[51]. A member of the type IV systems, four different orthologs of Cas13 (a to d) have so far been uncovered, all showing RNAse domain activity[52]. Working very similarly to Cas9, specificity is encoded by a protospacer-derived sequence on the crRNA, while a Cas9-homologue PFS-sequence on the RNA is required for restriction activity in prokaryotes, no PFS-requirement for activity has been observed yet in mammalian cells[53].

Cas13 has been utilized in several important applications. The inherent RNA-breakage activity of the wild type enzymes creates RNA knockdown possibilities so far restricted to unfolded areas of RNA[54]. Different subtypes of Cas13 all show different activities for certain RNA transcripts, and measured knockdown efficiency has never reached levels higher than 95%, which makes it complicated to create an effective knockdown protocol, as is pointed out by *Konermann et al* [52].

Inspired by dCas9 coupling to base exchangers, RNA base exchanger fusions based on Cas13b have also been produced[53]. Denoted as the REPAIR system, an adenosine deaminase RNA (ADAR) was coupled to catalytically inactive dCas13b, making an exchange of adenosine to inosine possible. Inosine functions the same as guanine in the translation machinery, effectively creating a swap of A:U to G:C pairing in dsRNA. Although this was successfully tested in mammalian cells, no knowledge on integration of REPAIR in a prokaryotic model could be obtained.

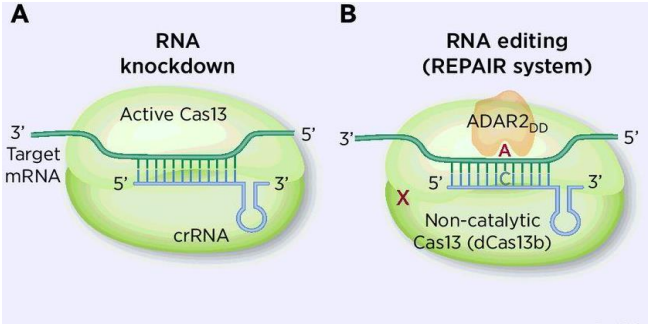

Figure 7 | ***(a)*** *RNA knockdown is partially possible using Cas13 orthologs by gRNA and PFS recognition.* ***(b)*** *The REPAIR system offers an experimental base exchange platform for RNA edits (adapted from [57]).*

Although Cas13 shows very specific nuclease activity, upon hairpin formation of the crRNA, it was reported that this activity transformed the Cas13 afterwards into a non-specific ribonuclease, restricting any RNA molecule on its path until the enzyme degraded[54]. The binding and subsequent hairpin formation of the crRNA induced structural changes in Cas13 causing mayhem in cells. Hypothesized to be a part of programmed cell death mechanics in a response to viral infections, no collateral activity was observed in mammalian cells[55]. As *Zhu et al* point out[56], developments of rational Cas13 orthologs might remove this important side effect in prokaryotes, but hitherto the effectivity has been minimal in prokaryotes.

RNA/DNA detection by CRISPR/Cas

Research on Cpf1 (Cas12a) uncovered when Cpf1 is bound by a target sequence (crRNA) to dsDNA, nonspecific nuclease activity for ssDNA is upregulated[58]. When the crRNA is bound to the target strand, and nuclease activity on dsDNA has been exhibited, the enzyme removes itself from the PAM sequence region and starts trans restriction activity on ssDNA[58]. In combination with a fluorescent quencher and isothermal recombinase polymerase amplification (RPA) of DNA, this method dubbed DETECTR has been successfully used to characterize different HIV-virus samples from patients.

Quantification of RNA molecules has also found its way in SHERLOCK (*Figure 8)*, an RNA detection mechanism based on the Cas13 platform[59]. Functionally almost the same as DETECTR, SHERLOCK uses amplification of DNA using an RPA mix, or amplification of RNA by reverse transcriptase RPA to transcribe the amplified dsDNA into ssRNA. When a fluorescent quenching probe is attached to the corresponding target nucleic acid sequence that is also inserted into the reaction mixture, Cas13a activity will cause cleavage of the quencher and a subsequent fluorescent signal, allowing for sensitive detection of transcript. SHERLOCKv2 was introduced as a matured continuation, including multiple Cas enzymes and fluorescent reporters to maximize amplification of detection[59].

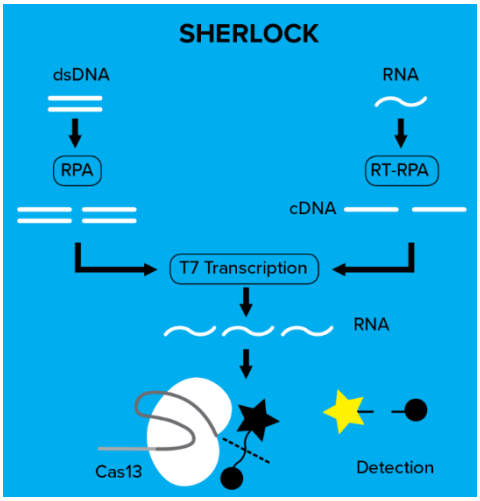

Figure 8 *| Schematic design overview of the SHERLOCK system. Still highly experimental, it showcases the possibilities of Cas13 based detection[105].*

Transcriptional repression (CRISPRi)

Development of dCas9 led to its recognition as a stable DNA binding platform. Successfully utilized for base editing purposes (*Chapter B of this thesis*), applications in transcription regulation were also produced. Endogenous repression of promoter regions (CRISPR interference/CRISPRi) by targeting of gRNAs in bacterial cells using dCas9 was proven to have potential[42], showing 99.9% downregulation in bacterial cells, while 10% activity in both human and yeast cells was still observed. Subsequent fusions with transcription repressor domains (e.g. KRAB) increased the repression capabilities in human and eukaryotic cells to 99.9% on comparable promoter regions[60]. Improved engineering effector domains also increased the amount of repression in mammalian cells to nearly silenced levels[61].

Repression using CRISPRi is generally accepted as becoming a viable replacement for RNAi (*Chapter 3)*[60,62]. CRISPRi has also been utilized in combination with different fluorescent proteins to study chromosomal movement in mammalian cells and appears to be a very versatile and safe platform for genomic engineering[63].

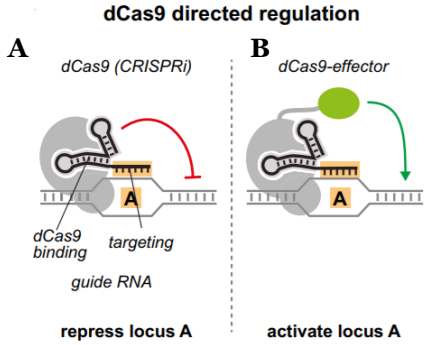

Figure 9 *| (**a**) Simple binding of dCas9 can repress transcription when bound to a promoter region (or nearby). (**b**) Fusions with repressor domains or transcription activators can enrich the toolkit of dCas9 based DNA binding[62].*

Transcriptional activation (CRISPRa)

Although repression by CRISPR/Cas can be achieved directly, efforts to induce gene activation using dCas9 proved to be tougher. A fusion of dCas9 with VP16, a viral transcription domain recruiting DNA polymerase II, as well as VP64 (a tetramer of VP16) was initially produced[60,64] showing plausible applications of a CRISPR activation (CRISPRa) platform, but activation *in vivo* was lower than pre-existing gene activation mechanisms[64]. Subsequent developments included the fusion of multiple transcription activators to dCas9 [65], using multiple, different gRNAs to target different areas around and on the promoter (Synergistic Activation Mechanism)[37], or using SunTag fusions with dCas9 [67]. SunTag is a synthetic recruitment protein that on fusions with dCas9 can recruit intracellular transcription factors with high efficiency.

Most of these methods to achieve endogenous activation are still highly experimental and show low efficiency/are on par with existing methods of transcriptional activation[68]. Recently, a system was published that used a different approach of transcriptional activation. By recruiting an intracellular transcriptional activation complex, epigenetic histone marks on promoter boxes could be remodeled with much higher efficiency to induce activation[2].

Epigenetic engineering by employment of dCas9 is not a completely new phenomenon. Existing knowledge about techniques used to control histone modifications led to fusions of dCas9 with acetyltransferase p300[69], already showing potential for transcriptional activation purposes. To analyze heterochromatin formation, demethylase fusions[70] and synthetic deacetylase fusions[71] have led to a large toolkit in mammalian genome analysis. KRAB-box repression also utilizes histone modifications and is thus important in epigenetic applications using CRISPR[60].

DNA screening using multiple vectors

The innate CRISPR libraries of pathogens itself have been used to characterize epidemic strains[72] and overall infection capabilities of certain micro-organisms. However, by using multiple gRNAs coupled to Cas-nucleases, different targets can be impacted at the same time. This technique is called multiplexing and it allows for in depth research on gene function and regulation when screening on specific phenotypes[73]. Computer models have been developed that can cross-reference loss-of-function mutations with wild type phenotypes and also propose gRNA sequences given a certain genome[74]. Commercial solutions that offer CRISPRi/CRISPRa toolkits to study regulation pathways on a larger scale are also growing. Multiplexing with Cas9 in itself is not very efficient (*Chapter 3*) yet and this technique is certainly still in its infancy, but the theoretical possibilities for simplified disease screening in the future make it very promising.

3. Problems with CRISPR/Cas and possible solutions

A lot of utility and promising applications in genomic engineering can be extracted from CRISPR/Cas. However, there are two main problems preventing wide scale adaptation. As was mentioned in *Chapter A*, recombination of double stranded DNA breakage (DSB) is vital for successful Cas9 activity, without any side effects. An overactivation of NHEJ and MMEJ pathways, generating indels still impedes editing efficiency and can cause undesirable side effects. Attempts to profile indel formation in mammalian cells showcased the importance of gRNA sequence in causing indels[75]. Synthetic gRNAs produced to prevent degradation by cellular mechanisms appear to be very important in preventing indel formation in mammalian cells[75]. Further research has shown that indel formation in human cells is predictable[76] and dependent on the gRNA sequence, the location of the PAM sequence (on the targeted strand, or the complementary strand), but the largest contribution is given by the exact sequence of the protospacer motif. Characterization of repair efficiency by NHEJ and MMEJ as a response to DSB in budding yeast has demonstrated a comparable predictability, highly dependent on the same factors[77]. Other analytic research shows that the rate of indel formation and the type of frameshift is highly dependent on the sequence, as well as the chromatin structure of the target[78]. Even in presence of a perfectly homologous template, NHEJ/MMEJ outcomes are too dominant to completely mitigate indel formation in different model organisms[79].

Alternative repair strategies to homologous recombination can thus cause frameshifts in response to DSB breakage. However, indel screening methods have emerged over the last few years that can detect frameshifts reliably and inexpensively, like TIDE[80] and IDAA[81], as well as many commercial approaches often used in combination with NGS (Next Generation Sequencing) detection to do high turnover analysis on targeting sites. Indel formation in itself is not restricted to CRISPR/Cas applications, other DSB inducing methods like zinc fingers and TALENs also have this issue.

The other important side effect in most applications of CRISPR/Cas is that of off-targeting: activity of the Cas-nuclease on an undesired part of the DNA that shows homology with the sequence encoded on the gRNA[82]. Roughly ~50% of all[83] cutting events with Cas9 result in off-target mutations, caused by NHEJ/MMEJ activation. However, off-target events are highly dependent on gRNA sequence and the target sequence of choice. A 3-5nt mismatch at the 3' end of the target sequence is generally attributed to a high measure of off-targeting events[39]. Later research established the important role for the final ~8nt of the target sequence that lie closest to the PAM sequence in annealing with the target DNA[84] for Cas9. Informally this is referenced to as the "seed" sequence of the target. Crucial roles for different regions +4 -7nt upstream of the PAM sequence[85], as well as other regions downstream of the PAM sequence were found, in combination with secondary structure and G:C ratio of the target sequence mostly determining target specificity[84]. gRNA sequences that promote formation of guanine quadruplexes have also been recorded to create high on target specificity[87]. The importance of restricting off-target events by gRNA engineering has motivated library analyses, generating overall guidelines for gRNA design[87].

These strategies in aiding gRNA design have helped decrease overall off-target activity in applications of particularly Cas9, but have not completely abolished it.

Parallel to research into the influence of guide RNAs ran the characterization and kinetics by which Cas9 associates with DNA and enacts its restriction activity. Crystal structure information of Cas9/gRNA constructs[22] showed a potential role in DNA stabilization for positively charged amino acids in between the restriction domains. By substituting some of these amino acids for alanine, stabilization of the Cas9 complex would solely be governed by gRNA/target strand homology, reducing off-target events[88].

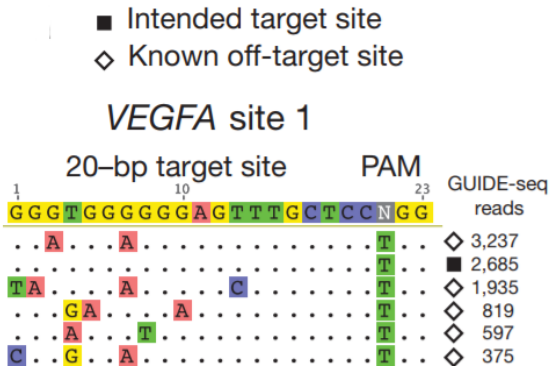

Figure 8 | *Example of analytics showcasing the presence of off targets when targeting a certain sequence, caused by a certain ratio of homology between the gRNA and undesired target sites[106].*

This rational design resulted in SpCas9 and eSpCas9, both displaying substantially decreased off-targets. Another approach was to destabilize interactions with the phosphate backbone of DNA in Cas9, focusing on DNA/gRNA interactions for affinity[89], which resulted in SpCas9-HF1, a comparable editor to eSpCas9. Knowledge surrounding these mutants and subsequent resonance transfer experiments led to a more precise understanding of Cas9/DNA interactions and the formation of hyper-accurate Cas9 (HypaCas9) which displayed far more proofreading in target annealing and thus reduced off-target activity drastically[58].

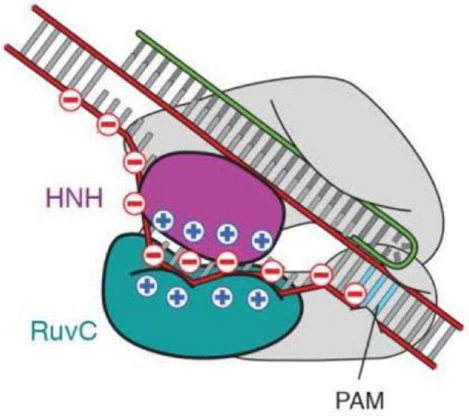

Figure 9 | *Schematic overview of Cas9 bound to dsDNA. Cationic residues in the binding pocket of Cas9 cause a too specific contact, playing a role in off-targeting[88].*

A limitation to CRISPR/Cas applicability is the requirement for a unique PAM sequence. Attempts to increase the application range of SpCas9 resulted in structurally engineered, as well as directed evolution based Cas9 mutants that target other PAM sequences than the wild type does[90]. SpCas9 wild type has a canonical PAM of 3'-NGG, but is also able to recognize alternative sequences to an extent (3' NAG/NGA)[91]. When other PAM specific mutants were produced, this alternative specificity was completely removed, restricting off-target occurrences up to ~30% [90].

Directed evolution experiments in *S. Cerevisiae* led to the engineering of evoCas9. Containing four mutations in different domains associated with both target binding, as well as PAM recognition this variant showed a four-fold improvement in off-targeting events compared to HypaCas9 and retained roughly 90% of on-site activity compared to wild type SpCas9[92].

Most methods of Cas9 expression utilize plasmid-based expression, which causes constant expression of Cas9 maximizing the chance of off-targets. When purified Cas9 together with bound gRNA was delivered directly into the cell by electroporation in the form of a ribonucleoprotein, off-target effects were also quite lower due to fast degradation of the Cas9 complex after initial catalytic effect[68,93]. However, this method is much more technically difficult than using plasmid-based expression.

CRISPR/Cas is not unique in its ability to generate DSBs in DNA by nuclease activity. Zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) are engineered proteins with DNA binding activity. Their specificity for a certain sequence of DNA is governed by the approach they are structured. Upon binding, both techniques are also able to induce a DSB and recruit cellular DNA repair pathways using a FokI restriction domain, comparable to Cas9. Both techniques also suffer from some manner of off-targeting effects and are generally expressed using plasmid-based expression. However, designing ZFNs and TALENs is time consuming and not as straightforward as designing a gRNA sequence[94]. Both techniques also utilize a protein domain to recognize DNA, which complexes cloning. However, especially ZFNs are much smaller than traditional Cas9 and TALEN methods which can sometimes be an advantage, especially for multiplexing purposes. The request for efficient multiplexing has led to development of hybrid plasmids though, able to express up to 7 gRNAs[95]. Multiplexing efficiency with Cas9 is rising, especially with developments in enhanced Cas9 variants. Also, efforts have been made to minimize the footprint of Cas9 by combining RNA-guided functionality with FokI restriction domains of ZFNs and TALENs. With the rapid advancements in CRISPR/Cas-derived RNA-guided engineered nucleases, it looks like most

shared functionality will be dominated by orthologues and chimeric based on CRISPR/Cas systems.

The reversible nature of CRISPRi techniques and lack of intricate design also shows advantages to established techniques like RNA interference for suppressing gene functions[5]. Multiplexing by the use of multiple gRNAs is an important advantage, and RNAi experiments require much more design. RNAi suppression also shows quite a lot of baseline gene activity due to their targeting of transcript sources, while CRISPRi can target expression at the DNA level, which is easily reversible[42].

Discussion

CRISPR/Cas based genome engineering has led to transformational techniques that have definitely expanded cost efficiency, functionality and ease of use in a wide range of applications. However, the ability of Cas9 to separate between the target sequence and closely homologous sequences is essential for high fidelity genetic engineering[97]. Research in mice has shown that Cas9 restriction events can degrade DNA on long term exposure, leading to brain damage and developmental errors, especially in mitotically active cell lines[3,34]. Also, mosaicism in which activity of Cas9 is different in between cells is an important hurdle in (therapeutic) applications in higher eukaryotes, or even humans[98]. Considering that the full extent of off-targeting events and long-term DNA damage caused by CRISPR/Cas systems is not known, caution (in mammals) is advised.

Clinical trials based on CRISPR/Cas engineering of human immune cell lines[99] have been launched, looking very promising in curing some auto-immune diseases. Tumor cell targeting based on CRISPR/Cas is also researched, but still in a very early stage of development[26, 100]. Research on human embryonal stem cell editing has sparked a lot of debate in the ethical considerations of applying genome editing tools in intelligent creatures[101]. With some arguing that the current side effects of CRISPR/Cas based (and other genome editing tools) cannot be eliminated completely[34] and that long-term effects are not studied well enough, others argue that it is too important of a tool to restrict from patients[103].

To balance this concern, advancements in characterization of side effects happens rapidly. In the past few years much has been published about off-targeting effects, especially in prokaryotes and other model organisms like yeasts[77,87]. Combined with the advancement in engineered Cas9 variants[58,92] and other nucleases[1,46,49] it remains to be seen whether off-targeting really is a substantial risk. The advance of techniques based on nickases is especially interesting. These enzymes and dCas9 based platforms show a much lower range of off-targeting than WT Cas9. In combination with advanced sequencing methods and other analytic tools, researchers are able to keep a tight control on undesired mutations[83]. The impact of CRISPR/Cas based systems has thus been tremendous in many fields. Since prokaryotes and less complex eukaryotes offer an intrinsically safer range of application due to a less complex genome and regulatory pathways, CRISPR/Cas appears to be an accurate genomic engineering tool in these organisms, provided care is taken in preventing and monitoring off-target activity. Whether CRISPR/Cas should be used to preventively edit pathogenic DNA from human embryos[103] remains to be the question.

Glossary

CRISPR - Clustered Regularly Interspaced Short Palindromic Repeats. The name of the immune system memory. Researchers often prefer the term "RGEN" for Cas-proteins, functioning using a gRNA.

crRNA – CRISPR RNA. The transcript of the CRISPR array.

tracrRNA – trans-activating crRNA. Shows homology with crRNA and is necessary for Cas9 recruitment

PAM – Protospacer Adjacent Motif. A 3-8nt recognition sequence upstream of the Cas9 crRNA complementary sequence, required for Cas9 (and other nucleases) to bind to its target sequence.

PFS – Protospacer Flanking Sequence. The same thing as a PAM sequence, but present on RNA and is reguired for Cas13 recognition.

gRNA/sgRNA – guide/single guide RNA. Interchangeable terms denoting the engineering of a synthetic tracrRNA:crRNA hybrid that can be used to direct Cas-nucleases to their target.

DSB – double stranded break. When wild type nucleases restrict their target sequence, they create a blunt, double stranded DNA break.

dsDNA – double stranded DNA

ssDNA – single stranded DNA

HDR – Homology directed repair. As a reaction to DSBs, intracellular pathways will try to find a homologous piece of DNA to smoothly repair the damage. This can be manipulated to force insertion of a certain fragment, although efficiency is not a hundred percent.

NHEJ – Non-homologous end joining. If a repair template is not present, other pathways will cause manual base insertion, often the cause of indel formation.

MMEJ – an alternative repair pathway to NHEJ (MMEJ is sometimes even referred to as alt-NHEJ), MMEJ is active in some organisms (including humans), dependent on cell cycle or the position of damage. Causes even larger frameshifts than NHEJ.

Indels – as a response to DNA damage, certain repair pathways can cause insertion, or deletion of nucleotides from the genome. These frameshifts are colloquially referred to as indels.

RGEN – RNA guided endonucleases. A collective term often used to describe manipulated CRISPR/Cas systems that function by gRNA sequence.

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