CRISPR Cas9 mediated gene knock-in in *Candida glabrata*

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Abstract

Although modifications of the genome have been a standard procedure for decades, homologous recombination dependent gene knock-ins still prove to be problematic. Development of the clustered regularly interspaced short palindromic repeats CRISPR associated (CRISPR Cas9) toolbox has led to higher preciseness and efficiency by introducing a double stranded break at a specific location and recruiting the cell's homology directed repair (HDR) mechanism to the target site. None the less, many organisms prefer the non-homologous end joining (NHEJ) repair pathway resulting in low knock-in efficiency. One of these species is *Candida glabrata* (*C. glabrata*), the most common cause of candidiasis after *Candida albicans.* Increasing occurrences of resistance to antifungals stress the need for effective gene editing tools to expand the knowledge on this pathogen in order to develop new treatments. Since most CRISPR Cas9 techniques are not developed in *C. glabrata* specifically, methods from mammalian cells are the major source of inspiration. This essay first gives an overview over the CRISPR Cas9 method, deoxynucleic acid repair mechanisms and explains how the application varies in different organisms. Further, several methods of HDR enhancement are discussed and evaluated in order to propose an efficient knock-in procedure in *C. glabrata*. Finally, the essay arrives at the conclusion that knockout or knockdown of Ligase IV, which is a crucial part of the NHEJ machinery, would be able to impede NHEJ and thereby enhance HDR in *C. glabrata*.

Contents

1 Introduction

The development of the clustered regularly interspaced short palindromic repeats CRISPR associated (CRISPR Cas9) tool for sequence specific modifications of the genome has significantly broadened the scope of biotechnology. The main player in this technique is the endonuclease Cas9 that introduces a double stranded break (DSB) in a precise location of the genome. The repair mechanisms non-homologous end joining (NHEJ) and homology directed repair (HDR) that come into place to reconnect the broken deoxynucleic acid (DNA) strands facilitate the use in genetic engineering. It is relevant to the outcome and efficacy of the experiment which of these two pathways is prevalent or active. While NHEJ introduces random indel mutations HDR allows for precise modification of the DNA's sequence. Consequently, HDR offers a wider set of applications like base exchanges, knock-ins and deletions. However, in many organisms NHEJ is the favored mechanism over HDR leading to low efficiency of template incorporation. One of these organisms is *Candida glabrata* (*C. glabrata*), an opportunistic pathogen and the most common cause of yeast infections after *Candida albicans* (*C. albicans*). Resistances to accessible antifungals become increasingly more common. Finding new effective treatments would be facilitated by the availability and use of an efficiently working CRISPR Cas9 gene editing system. This essay gives an overview on the literature describing the mechanism, use and ways of enhancement of CRISPR Cas9 and discusses these strategies to accomplish efficient genome editing in *C. glabrata*.

2 Genetic engineering with CRISPR Cas9

Humans have modified the properties of living organisms for a long time, achieving e.g. more fruitful crops or domestication of animals [1], [2]. One of the biggest advances in modifying organisms came with the discovery of the genome and the central dogma of molecular biology establishing DNA as the code to all structures of life [3]. Ever since, multiple tools have been developed to manipulate the DNA sequence, each becoming more efficient and specific. Nowadays, genetic engineering in a directed and precise manner has become the central technique of research in the life sciences and biotechnology [4].

CRISPR Cas9 is a recently developed and highly accurate instrument used for genetic engineering. In short, a DSB is introduced at a specific location in the DNA. This cut is then repaired either by NHEJ or the template-based HDR allowing for gene knockouts, deletions or knock-ins. CRISPR Cas 9 applies the endonuclease Cas9 that can bind a ribonucleic acid (RNA) molecule called the guide RNA (gRNA). The protein is directed to the sequence within the genome that corresponds to the gRNA's sequence and is next to the protospacer adjacent motif (PAM). Upon binding the target site specifically Cas9 cuts the DNA and introduces a DSB (Figure 1 A) [5]–[8].

This technology was developed from part of the adaptive prokaryotic immune system. Bacteria save part of the sequence from a pathogen that has attacked them previously in the CRISPR region and use it to make the RNA that will direct the endonuclease. In case of a repeated infection the pathogenic nucleic acid sequence is recognized and cleaved. The cut strands are inactive and the infection is stopped. [9]

In comparison to earlier methods, CRISPR Cas9 comes with many advantages and fulfils multiple requirements at the same time. First, the use of an RNA dependent endonuclease allows a specific target localization leading to highly efficient placement of DSBs [10]. While the previously established use of transcription activator-like effector nucleases (TALEN) or zinc finger nucleases (ZFN) also allows for exact targeting in higher eukaryotic cells, it requires the

design of a new protein [11]. On the other hand, design for CRISPR Cas9 is simplified as it is restricted to nucleic acid sequences. At the same time, there are methods enabling directed modification in prokaryotes and yeast such as homologous recombination. However, those rely on a selection marker that is not needed in CRISPR Cas9 editing since in the latter unrepaired DSBs are lethal resulting in negative selection. Additionally, the efficiency of the Cas9 endonuclease activity facilitates its use in di- and polyploid strains enabling editing in the case of multiple alleles [12]. Furthermore, the use of several gRNAs allows for simultaneous targeting of different loci, accelerating multiplex genetic edits (Figure 1 B) [13], [14].

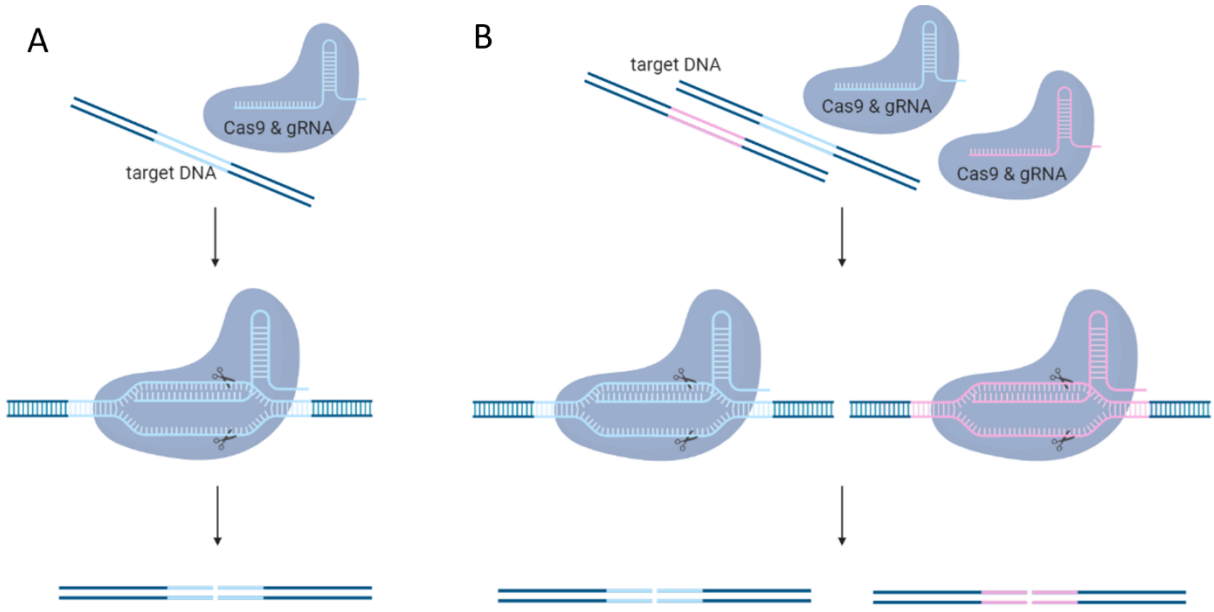

Figure 1 The activity of the gRNA directed endonuclease Cas9 is highly specific and efficient allowing for precise cuts (A) and multiplex genetic edits (B). (A)The endonuclease Cas9 recognizes its target site based on the associated gRNA. Upon binding the target, a double stranded break is introduced. (B) The use of various gRNAs allows the Cas9 to target multiple sites at the same time leading to multiplex gene edits.

2.1 Significance of repair mechanisms in CRISPR Cas9

An essential part of the CRISPR Cas9 procedure are the cellular repair pathways. While the DSBs resulting from Cas9 are lethal, there are mechanisms able to mend the DNA. The two main pathways are homology directed repair (HDR) and non-homologous end joining (NHEJ) [15], [16]. These processes are highly conserved in eukaryotic cells [17].

In HDR the break in the DNA is completed based on a homologous piece of ds DNA that serves as a repair template. This completing fragment can be provided either internally e.g. by a homologous chromosome or derive from an external source by transformation [18]. In HDR the DNA at the cut is resected mainly by the 5’ – 3’ exonuclease Exo1 upon recognition by the Mre11-Rad50-Nbs1 (MRN) complex. By resection the cell commits to proceed with HDR [19], [20]. Homologous recombination is then initiated by invasion of the homologous DNA strand facilitated by Rad51. The migration of the branch leads to formation of the Holliday junction, which is then resolved by the MUS81-MMS4 dimer [21].

In NHEJ the broken DNA strands are reconnected haphazardly and without a template. As a result, the majority of this kind of repair leads to insertions and deletions (indels). [16]. This repair mechanism depends on the complex of Ku70 and Ku80 and the DNA dependent protein kinase catalytic subunit that bind the separated strands. The ends are reconnected in the following step by the DNA Ligase IV [22], [23].

Depending on the experimental design, the researcher relies on a different repair mechanism. Gene knockouts often make use of NHEJ and selection of an appropriate site for the DSB. In case the cut is located in a coding region, the resulting indel mutation can cause a change in the reading frame or a premature stop codon. Consequently, there is a high chance that there is no functional product resulting in a knockout of the targeted gene. Genome modifications that require precise changes of the genome, such as gene knock-ins, single nucleotide exchanges or exact deletions, requires HDR and the design of a template. The template design includes homologous regions at the ends of the fragment and the final desired sequence in between the homology arms [24].

2.2 CRISPR Cas9 protocols in different organisms

Introduction of the CRISPR Cas9 method significantly facilitated genome editing. Even though the method relies to a large extent on the machinery introduced (Cas9 and gRNA), it has been shown that not all tools are easily transferrable to another organism. One of the causes of decreased efficiency can be variability in the expression of Cas9 and gRNA that can be combatted e.g. by adjustment of promoters or codon optimization of Cas9 [25]–[27]. Other issues arise from the elements that are provided within the organism like the DNA repair mechanism regulation. A major limitation of using CRISPR Cas9 for gene editing experiments is that many species favor NHEJ over HDR. Consequently, especially gene knock-ins are inefficient because they rely on the HDR mechanism.

Modifying the genome of the commonly used model organism *Saccharomyces cerevisiae* (*S. cerevisiae*) has proven to be straightforward compared to other organisms. Homologous recombination and homology directed repair are very active pathways in baker's yeast. Consequently, the HDR dependent and CRISPR Cas9 mediated deletions and insertions are highly efficient without any further optimization [28]. On the other hand, NHEJ constitutes the prevalent pathway in other fungi including *Candida glabrata,* lowering the efficiency of genetic modification strategies [26], [29], [30].This preference can be coincides with the observation of rare recombination events and the lack of a sexual cycle in *C. glabrata*, a trait shared with many other fungal pathogens [31], [32].

Pathogenic yeast species are posing a growing threat as resistances to antifungals become increasingly widespread. *C. glabrata* is the leading cause of candidiasis after *C. albicans* [33]. Despite sharing many morphological traits with the other pathological *Candida* species, it is closer related to the model organism *S. cerevisiae*. Still, many aspects of *C. glabrata's* virulence remain uncertain [34], [35]. Therefore, enhancement of powerful genome editing strategies is imperative to accelerate the research on this organism. The gained knowledge will allow for the development of new drugs and therapies to combat the growing numbers of resistances.

There have been studies looking for ways to enhance CRISPR Cas9 efficiency in *C. glabrata* and other fungi specifically [26], [27], [36]. However, because of the closer relation to humans, most reported improved CRISPR Cas9 protocols are designed in vertebrates or more specifically mammals and human cell lines [37], [38]. The proximity of the species makes them more alike. As a result, the closer related species represents a more exact model of the human organism. Similarly to the pathogenic fungi, these organisms prefer NHEJ over HDR to reconnect DSBs [39]. Since there are many conserved processes and pathways including DNA repair mechanisms [17], improved methods that are developed for mammals can also inspire new ways to enhance CRISPR Cas9 in the distant relative *C. glabrata*. Instead of yeast serving as a model for mammalian cells the opposite would take place. Consequently, the numerous methods developed for human and other mammalian species can give rise to potential solutions in *C. glabrata*.

3 Strategies to efficient CRISPR Cas 9 mediated Knock-in

A knock-in describes the process of exchanging or adding a gene or part of it. As a result, there is a gain or alteration of function that can be observed e.g. in the phenotype. One possible application is the correction of mutations that lead to genetic diseases like cystic fibrosis. On the other hand, mutations that are known to cause such dysfunctions can be introduced in other species to create an animal model that allows a more in-depth study of the illness's physiology [40]. Moreover, many aspects of biotechnology rely on knock-ins e.g. metabolic engineering [41]. These applications usually take place in unicellular model organisms that can be handled easily. Replacing part of the genome in a directed manner has been a standard procedure in the model organism *S. cerevisiae* for decades [42].

Knock-in strategies depend on the homologous recombination pathway. In order to introduce a gene at a specific location in the genome, a template with the according sequence has to be introduce. Additionally, the ends of this piece of DNA with the required sequence consist of short homology arms that correspond to the sequence adjacent to the target site. Further, the fragment usually also includes a selection marker to facilitate the screen of positive recombinants [43]. CRISPR Cas9 is applied to avoid the use of a marker. Since this method is able to create DSBs efficiently in a precise location it can recruit the homology directed repair machinery to a specific site of the genome. As a result, the recombination at the cut locus is favored and the incorporation efficiency increased. Additionally, an unrepaired DSB is lethal to the host. Consequently, the repair fragment does not need to include a marker for screening [26], [44].

Integration or exchange of a sequence requires an active recombination pathway. Therefore, gene knock-in experiments are conducted at high efficiency in the model organism *S.* cerevisiae. Other organisms, such as mammals but also the closely related *C. glabrata,* favor NHEJ over HDR leading to low efficiencies of these experiments. For this reason, there have been many attempts to enhance knock-in effectiveness. Many of these studies focus on the repair systems and look for ways to either downregulate NHEJ or upregulate HDR. Others explore new ways to harness the CRISPR Cas9 machinery to improve gene knock-in efficiency.

3.1 Favored selection of homology directed repair

In the case of a DSB many organisms prefer the repair via NHEJ over HDR. Genetic engineering strategies often rely on homologous recombination. One possibility to increase efficiency of such an experiment in these organisms is by devising methods to change the ratio of HDR to NHEJ in favor of HDR.

Suppression of NHEJ is a possible approach to enhance the rate of HDR. One possibility to achieve this is to impede the expression of parts that are crucial to the machinery by knockout or knockdown. It has been observed in mouse and human cell lines, *Drosophila melanogaster* and different yeast species including *C. glabrata, Pichia ciferii* and *Pichia pastoris* that lower expression of DNA Ligase IV and/or Ku70 and Ku80 leads to increased homologous recombination efficiency [36], [45]–[49].

The abundance of a protein can also be lowered by its degradation. Lig IV can be degraded by the adenovirus 4 ubiquitination complex of E1B55K and E4orf6 [50]. Chu, et al. measured a 8 times higher efficiency of homologous recombination when they promoted degradation of the ligase by this complex instead of silencing it where they observed 4-5 fold increase.

Finally, another approach to disturb the NHEJ pathway is to inhibit its crucial components. Activity of Lig IV can be impeded by the inhibitor SRC7 [51]. Application of this molecule during

a HDR mediated knock-in experiment improved modification efficiency in multiple cases [46], [52], [53]. Other molecules that have been discovered to inhibit NHEJ include NU7026 [54], Trichostatin A, MLN4924, NSC 15520 [55]. Each of these have a different mechanism of action and impede one of the components necessary for functional NHEJ. In the “CRISPY” mix, multiple inhibitors are combined to achieve higher efficiency of NHEJ inhibition leading to an up to 7.2-fold increase in HDR frequency [55].

Instead of disrupting NHEJ, stimulation of homologous recombination could be achieved by the activation of HDR. RS-1 (3-((benzylamino) sulfonyl)-4-bromo-N-(4-bromophenyl) benzamide) has been shown to be an activator of the protein Rad51 that is crucial to the initiation of homologous recombination in human cells [56]. Use of this molecule was able to increase CRISPR Cas9 efficiency 2 – 5 fold in rabbit [57].

NHEJ is active all throughout the cell cycle to repair possible DSB. In contrast, HDR reaches a peak in activity during S/G2 (Figure 2) [58]. This knowledge can be used in order to time experiments correctly. One possibility to do so is the application of cell cycle progression inhibitors. It has been shown that the efficiency of HDR can be increased 2-3 fold in human cells when CDC7 is inhibited by XL413 [59]. According to the authors, XL413 increases the population of cells in early S phase, where the amount of DNA repair proteins is high and HDR is favored.

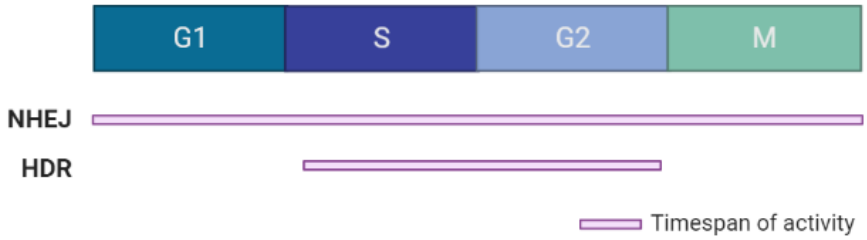

Figure 2 While NHEJ is active throughout the cell cycle HDR activity peaks in S/G2 phase.

Another way to enhance HDR, that has not been explored in literature so far, can be facilitated by increasing the abundance of essential HDR components. One way to achieve this could be by introducing an overexpression plasmid that encodes the needed proteins into the organism. On the other hand, the expression from the cell’s own genome could be activated. Chromatin remodeling has been used previously to express otherwise silent gene clusters in fungi [60], [61]. However, this method could lead to many off target effects as it uses non-specific histone deacetylases to change DNA packaging. Instead, the use of small activating RNAs (saRNAs) [62], [63] or a deactivated Cas9 (dCas9) that is fused to an expression activator [64]–[67] are more directed methods. Interesting targets for overexpression could be the exonucleases involved in the resection of the DNA at the cut as resection commits the cell to repair the DSB by means of homologous recombination [18]. One of those enzymes is Exo1 which is involved in the extension of the resection. Increased abundance of Exo1 could support favoring of HDR over NHEJ. However, there are no reports in literature supporting the overexpression of HDR components to achieve enhanced homologous recombination.
Further elucidation of the regulation of repair mechanisms concerning the components, timing, checkpoints or signaling pathways could propose new methods to enhance HDR.

3.2 Modification of the standard CRISPR Cas9 methodology

3.2.1 Cas9 variants

The main component of the CRISPR Cas9 machinery is Cas9 which introduces a DSB depending on the sequence of the associated gRNA. The endonuclease achieves a DSB

thanks to its two catalytic nuclease sites (Figure 3 A). Protein engineering has provided different variants of this enzyme to broaden the applications of the CRISPR Cas9 toolbox.

One of these modifications yielded nickases that cut still in a directed manner but just one of the two strands (Figure 3 B). Application of two nickases would lead to paired nicks and different overhangs depending on the way either enzyme is directed (Figure 3 C). Bothmer, et al. investigated the effect of these overhangs on the selection of the repair mechanism in human cell lines. According to the authors, paired nicks that lead to 3' overhangs prefer the NHEJ repair mechanism, similarly to DSB. On the other hand, 5' overhangs stimulate the HDR mechanism [39]. Consequently, the CRISPR Cas9 experiment could be designed to yield 5' overhangs after digestion instead of the classical blunt cut to enhance homologous recombination (Figure 3 C).

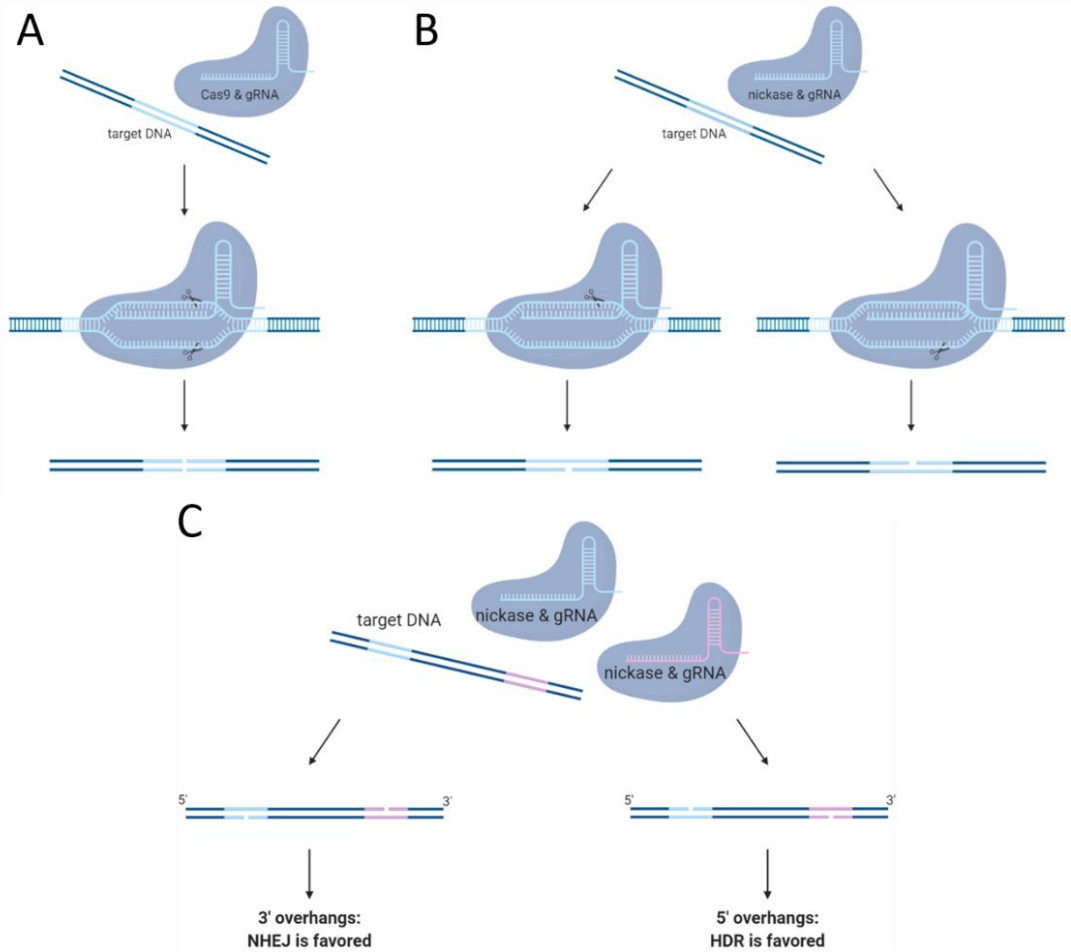

Figure 3 Engineering of the RNA guided endonuclease Cas9. (A) The native Cas9 enzyme has two cut catalytical sites and is able to introduce double stranded breaks. (B) When either one of them is mutated and becomes inactive only one of the strands can be cut and the protein is transformed into a nickase. (C) The application of two nickases can lead to either 5' or 3' overhangs, depending on the targeting strategy. The cut DNA is then repaired by the cells repair mechanisms. The choice of the process is influenced by the nature of the overhangs. While 3' overhangs are usually reconnected by NHEJ, 5' overhangs increase HDR.

Howden, et al. followed the approach to reduce NHEJ by selecting the optimal time for CRISPR Cas9 to be active. By fusing the endonuclease with the Geminin protein [68] the complex is degraded in the late M and G1 phases. As a result, the abundance of Cas9 is highest during S phase, when also HDR is the most active. Application of the Cas9-Geminin fusion has been observed to reduce NHEJ mediated indel mutations at the target site, while leading to a similar amount of HDR in human pluripotent stem cells [69]. Similar use of a Cas9 – Geminin fusion in HEK293T cells did increase the efficiency of HDR by up to 87 %, while maintaining unchanged levels of unwanted indel mutations [70].

3.2.2 Repair based on a single stranded template

Single stranded annealing is another pathway to reconnect cut DNA. Just like in HDR a template is needed for the repair, but instead of the ds DNA a single stranded oligonucleotide is used. This process has been shown to be more efficient than canonical HDR, while relying on quite short homology arms of approximately 100 bp [71]–[73].

Efficient additions with ssDNA inserts (Easi)-CRISPR is a method developed in mice relying on such a single stranded donor. [73]. The authors report an upper limit for the single stranded template of around 2 kb [74]. This template needs to be synthetized from double stranded DNA by transcribing it into RNA first that is then reverse transcribed into the single strand using a reverse transcriptase [75].

In order to further increase the efficiency of this repair mechanism, Aird, et al. fused the single stranded repair fragment to the Cas9 enzyme. As a result, the homologous template is in proximity of the cut site and can be utilized immediately to reconnect the cut DNA accordingly. This procedure yielded an up to 100-fold increase in recombination frequency in human cell lines [76].

3.2.3 Short insertions and single nucleotide exchanges

Efforts over the past years have recognized that especially minor changes to the genome can be mediated without a DSB in the genome. Such approaches minimize possible DNA damage and eliminate the need for a separate DNA template. One example is base editing, in which a deactivated Cas9 is fused with a deaminase [77], [78] typically leading to the specific conversion of cytosine to thymine [79] or adenine to guanine [80]. However, there are currently no methods to do the base transversion reactions. A method that tackles this problem is prime editing [81]. Prime editing uses a modified Cas9 that introduces a single stranded cut fused to a reverse transcriptase. The transcriptase uses the modified prime editing gRNA as a template to insert or delete sequences or introduce point mutations. The authors were able to achieve insertions of up to 44 base pair, deletions of up to 80 base pairs and numerous single nucleotide exchanges including transversions that are impossible with base editing. Both methods have the advantage of being independent from the repair mechanisms. At the same time, all components of the machinery are provided externally suggesting transferability between organisms.

4 Discussion

Ever since its discovery, the CRISPR Cas9 machinery has turned out to be a very useful and practical tool for genetic engineering. Thanks to the gRNA directed endonuclease target sites can be selected easily by editing the gRNA coding sequence on DNA level. In comparison to the previous methods TALEN or ZFN no protein engineering is required. At the same time, the highly specific endonuclease can introduce DSBs to evoke repair mechanisms at virtually any location of the genome. The repair mechanisms NHEJ or HDR can be utilized for genetic engineering either by random indel mutations or based on a homologous repair fragment respectively. Additionally, modifications to the enzyme have broadened the applications of this method changing the activity from an endonuclease to a nickase or completely abolishing the endonuclease activity.

Most gene editing strategies, including CRISPR Cas9, depend on the homology directed repair pathway, which is highly active in some organisms such as the model organism *S. cerevisiae*. However, other organisms including mammals and pathogenic fungi favor NHEJ. For this reason, many enhancements have been developed to yield a higher efficiency homologous recombination in these systems.

4.1 Mammalian cells as model for enhanced CRISPR Cas9 methods

Despite the distant relation yeast has been established as a model organism to learn about processes in human cells. This is possible because of the conservation of many pathways and components. The degree of especially functional conservation of many proteins is astonishing and also includes the DNA repair mechanisms and most components crucial to NHEJ and HDR. As a consequence, it is likely that the results observed in one case can be also expected in a different setting allowing for the use of model organisms.

Research on the unicellular *S. cerevisiae* provided a lot of insights about the physiology of eukaryotes. The main advantages of using yeast are its ease of use and handling and the high degree of characterization. Since there are many labs working in this organism, many protocols have been established and optimized for it. None the less, working with this model system comes with certain limitations as there are decisive differences between yeast and mammalian or human cells like the cell wall around yeast or the size. Use of more elaborate and closer related systems like mouse models or human cell lines are needed to provide a more similar model of humans and to verify insights from yeast.

The clear advantage of a more similar system led to the development of many methods, e.g. the enhancement of CRISPR Cas9, especially for mammalian cells. Similarly to yeast being an established model for mammalian cells because of the conservation, knowledge gained from mammalian cells can provide insights to the work with yeast or other fungi. Consequently, when looking for experimental strategies in fungi, protocols from mammalian cells can be a source of inspiration, especially since both systems have lower efficiency or homologous recombination because NHEJ is the preferred repair mechanism. It should be kept in mind though that most of the time minor differences can affect the outcome and efficiency of experiments. For that reason, approaches that have been tried only in mammalian cells should be tested in fungi before final conclusions can be drawn. None the less, there is a high chance that after some optimization most protocols will work similarly across species.

4.2 Methods to improve CRISPR Cas9 efficiency

Most of the optimized CRISPR Cas9 editing protocols focus on the repair mechanism bias. These methods influence the cell to increase their efficiency of homology directed repair. First, downregulation of NHEJ by silencing or knocking out key components of the machinery forces the cell to repair a DSB using HDR in order to survive this disruption of their genome. Many studies across species have shown that lower abundance, deletion or inhibition of Ku70, Ku80 or Lig IV lead to higher efficiency of homologous recombination. The permanent knockout of the central NHEJ machinery can have side effects, especially if the target protein's function is not specific to one process. For this reason, the target selection should consider the amount of knowledge that has been gathered on the compound and what functionalities have been reported to date. Consequently, the knockout of the exclusive Lig IV should be favored over Ku70 and Ku80 proteins which are associated with functions outside of NHEJ [23], [47], [82]. To decrease the possibility of undesired consequences from missing genes, transient methods like knockdown, inhibition or degradation could be more appropriate. At the same time, it should be kept in mind that an insufficient decrease in abundance or activity could not enhance the efficiency to the same extent as a full knockout would. Additionally, reported inhibitors for NHEJ components could have other effects in another species like cytotoxicity. A strong argument for non-permanent methods is given by Chu, et al. who observed the strongest improvement of homologous recombination in mammalian cells using the specific ubiquitination complex from adenovirus 4 to degrade the ligase. The application of the E1B55K and E4orf6 complex should be tested in *C. glabrata* to confirm its efficacy in a different organism.

Another aspect that can be modified to yield higher efficiency is the CRISPR Cas9 method. The introduction of different engineered Cas9 enzymes such as nickases or fusion proteins have broadened the applications of this technique. The use of two nickases allows for breaks with overhangs instead the usual blunt DSB. The nature of these overhangs influences the repair pathway selection and 5' overhangs have been reported to enhance HDR in mammalian cells. While many components of the repair systems are conserved, there could be differences in the regulation meaning that the different overhangs do not lead to the same effect in *C. glabrata*. By fusing Cas9 with geminin researchers yielded an enzyme that would be degraded at the times of HDR being inactive. Depending on the methodic details and human cell line either a reduction of NHEJ or an increased number or homologous recombination events was observed. This variability of results within one species suggests that there could be many differences in the processing of geminin in species as distantly related as yeast and mammals. Both methods would need to be implemented in the studied organism to draw final conclusions about their effect on efficiency.

Finally, methods that do not depend on the homology directed repair pathway in the first place show higher efficiency. They either use enzymes that are fused to Cas9 to modify the DNA sequence immediately upon Cas9 binding or apply the single stranded annealing process. While base editing is applied to change single nucleotides with deaminases, prime editing uses a modified gRNA as template allowing for insertions up to 44 base pairs. Longer inserts, up to 2 kb, can be generated using the SSA pathway and a single stranded template. However, the manual generation of a single stranded 2 kb long DNA fragment adds a lot of experimental steps and could lead to higher inaccuracy and variability in outcome. While these methods should be transferable between organisms as all components needed for editing are supplied externally, the limitations in insert length and extra steps limit their applications considerably.

While many methods have been explored, there are no reports in literature on increasing the abundance of HDR components. While there are multiple ways to achieve higher levels of a protein using saRNAs or dCas9, many of them are hardly optimized for application in yeast or not tested at all in the organism. On the other hand, techniques used in yeast such as chromatin modelling with histone deacetylases are not directed or sufficiently specific to reach the desired outcome.

4.3 Combination of methods

In order to yield even higher efficiency different approaches could be applied at the same time. The frequency of HDR could be increased further e.g. by simultaneous knockdown of the NHEJ machinery and activation of HDR components. Another example could be the use of paired nickases and NHEJ inhibitors. While, the additive effect of numerous agents could in fact lead to an increased productivity of the experiment, different techniques can also influence each other negatively. Moreover, the additional protocols performed usually lead to a higher workload and more stress to the cell. For that reason, combined use of multiple enhancement approaches should be tested quantitively to properly decide, whether it is beneficial or not.

One example from literature is a combination of multiple NHEJ inhibitors tested by Riesenberg, et al.. The "CRISPY" mix increases efficiency compared to the use of a single inhibitor and makes the procedure more robust toward changes of the studied organism as different inhibitors are compatible with different cell lines. While the application of multiple inhibitors instead of one does not lead to additional steps in the protocol, the increased load on the cell could cause higher cytotoxicity.

4.4 Conclusions

Homologous recombination is not a frequent mechanism in multiple organisms including mammals and *C. glabrata.* As a result, knock-in experiments can be problematic because of inefficiency. An efficient CRISPR Cas9 tool for gene editing in *C. glabrata* would facilitate the research in this pathogenic fungus. However, many methods of CRISPR Cas9 enhancement have been developed in mammalian cells. These techniques can provide helpful insights for improved *C. glabrata* protocols especially since the reasons for lower efficiency are the same in both systems (preferred NHEJ) and there is a general conservation of mechanisms across species.

Most techniques focus on the repair mechanism bias and force the cell to favor HDR over NHEJ. This can be achieved either by impeding NHEJ or activation of HDR i.e. with NHEJ inhibitors, HDR activators, knockdown or knockout of NHEJ machinery or correct timing. Other approaches seek to modify the CRISPR Cas9 machinery, e.g. by applying Cas9 fusion proteins that lead to higher HDR efficiency. Also, the use of the single stranded annealing repair pathway increases the incorporation. However, the template is single stranded posing restrictions on the length of the insert. Similarly, methods that perform genetic modifications without introducing any cuts are very restricted considering the length of the insert.

Because of numerous studies across different organisms including *C. glabrata* showing increased HDR efficiency upon knockdown or knockout of the NHEJ enzyme Lig IV, there is a high chance of successful application of this approach. Since a knockdown is transient as there are no permanent modifications of the genome, the physiology of the organism is not affected in the long term. In the case of a knockout the function has to be reinstated in order to exclude any secondary effects. To use inhibitors that are not designed for *C. glabrata* instead would be simpler because it does not require the establishment of a knockdown or knockout method. However, it could show undesired effects in the different organism or result in cytotoxicity. While both these approaches are influencing the cell's physiology, Cas9 fusions or paired nickases could portray more orthogonal techniques. However, since there are no reports on applying those methods in fungi, there is a high possibility that the components would need optimization.

In conclusion, impairment of Lig IV preferably by knockdown is the most likely to yield the desired outcome. In case the knockdown is not efficient enough to reduce the enzyme's level sufficiently, a knockout with subsequent reintegration should lead to efficient CRISPR Cas9 mediated knock-ins in *Candida glabrata*.

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