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Review Article

CRISPR-Cas9, the new kid on the block of fungal molecular biology

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Abstract

Research on fungal pathogens with the aim to identify virulence determinants strictly relies on the generation of defined, recombinant strains, a task that is executed by means of a sophisticated molecular biology toolbox. Recent developments in fungal genome engineering have opened a new frontier by implementing the CRISPR-Cas9 technology, based on expression of the Cas9 endonuclease that is loaded by a single guiding RNA (sgRNA) molecule to target a defined site in the recipient genome. This novel approach has been adapted successfully to engineer fungal genomes, among them the one of the human-pathogenic mould *Aspergillus fumigatus*. Implementation of the required components was achieved by various means that differ with respect to expression of the Cas9 enzyme and sgRNA delivery. Validation of CRISPR-Cas9-mediated mutagenesis could be executed by targeting selected candidate genes of *A. fumigatus* to provide a promising perspective for screening and multiplexing approaches to scrutinize the virulome of this opportunistic fungal pathogen in a comprehensive manner, such as by analyzing genetic polymorphisms or the function of gene families.

Key words: CRISPR/Cas, *Aspergillus*, molecular biology.

Introduction

The incidence of human diseases caused by fungal pathogens is collectively underestimated, albeit its significant impact on global health systems and individual patients.[1] This shortcoming is partly based on diagnostic deficiencies as well as common ignorance about mycoses as severe threats and complications in distinct clinical settings. Besides the apparent lack of a broad spectrum of approved antifungal drugs that is accompanied by shortcomings in screening and developing novel targets and compounds for antifungal therapy,[2] the underlying mechanisms of fungal infection and disease progression in humans are poorly understood. Scrutinizing fungal pathogenicity and virulence traits strictly relies on advanced means of molecular biology, supported by suitable models of infection and host immunity. The recent decade has seen a tremendous progress in approaches to manipulate fungal pathogens in a targeted and defined manner, emerging from publicly accessible genome sequences, genetic backgrounds and markers that foster gene targeting, as well as numerous molecular

tools such as modules for conditional gene expression or reporters to monitor infectious processes.

The recent implementation of the CRISPR-Cas9 technology, which is based on a bacterial immune system,[3] in various fungal hosts, and among them prominent pathogens, promotes fungal molecular biology further. This approach of manipulating the genome of interest in a precise and defined manner holds the promise of a new era in fungal molecular biology. Accordingly, it is the aim of this short review to provide an up-to-date overview about this novel genetic tool: the system itself with its components and its implementation in various fungi with an emphasis on *Aspergillus* as a predominant human-pathogenic fungus. Emerging from this, a perspective commentary on the next steps and needs for solidly embedding this technique in fungal molecular biology is provided, highlighting the current shortcomings and future applications for this sophisticated technology.

The CRISPR-Cas9 technology is based on a bacterial adaptive immune system

The first description of what was later coined as 'clustered regularly interspaced palindromic repeats' (CRISPR) dates back to the year 1987, when the noticeable structure of the 3′ region flanking the *Escherichia coli iap* gene was described to contain direct repeats of homologous sequences 29 nt in lengths that were interrupted by 32 nt spacers but also stating that "the biological significance of these sequences is not known."[4] This was followed by various descriptions of similar modules in bacterial genomes, while any functional role of the encoded transcripts remained entirely ambiguous. Recognition that the CRISPRs harbour spacers that stem from viruses or plasmids,[5–7] accompanied by the identification of associated *cas* gene products that contain helicase and nuclease domains[8] lead to the hypothesis that this system serves as memory and defence against invading phages, which was then experimentally and conclusively validated in 2007 for the bacterial host *Streptococcus thermophilus*[3] and afterward for many others.

The basic concept executed by these CRISPR-Cas systems is that CRISPR-encoded RNAs that stem from viral encounters form a complex with the *cas*-encoded protein(s) to guide an endonuclease to homologous sequences within the host, where double strand breaks are induced to interfere with viral proliferation. To date, three CRISPR system types have been identified that differ in the respective *cas* operon and that can be subdivided according to an exclusive signature gene.[9] Among these, type II systems have drawn massive attention in research based on the fact that they encode one single multifunctional Cas9 protein that, in complex with the transcribed RNA from the spacer-repeat CRISPR locus (crRNA) and a trans-activating RNA (tracrRNA), eventually cleaves the target DNA site.[10] In their simplicity that one RNA-guided protein only is required for target recognition and cleavage, these type II CRISPR-Cas9 systems are straightforward and readily applicable, which contributes to the massive and unprecedented achievements that were comprehensively implemented in modern biology in such short time period.[11]

In essence, CRISPR-Cas-mediated immunity of bacterial hosts against invading viruses is sequentially executed by three distinct processes that are generally coined as the acquisition, the expression, and the interference phase, respectively.[12] During acquisition, DNA fragments from formerly encountered phages or plasmids are integrated and stored as spacer regions in the CRISPR array to expand the host memory about invading pathogens. In case of a further infection, the corresponding spacer region is readout to become part of a processed crRNA that in conjunction with a tracrRNA is incorporated in a complex with the Cas proteins. This mature complex is then guided, due to the complementary sequence homology of the crRNA, to the target site where cleavage and therefore interference with the invading element is executed.

Recognition and processing depends on complementarity of the spacer with its target site, which covers the protospacer sequence and an adjacent motif (PAM) at its 3′ end. This short nucleotide motif prevents retargeting of the system to the CRISPR array but is essential for directing the Cas9 activity to its non–self template. The commonly used Cas9 nuclease of *S. pyogenes* is associated with the canonical PAM as 5′-NGG-3′, but CRISPR-Cas systems of other bacterial origin differ from this motif in sequence and length.[13] RNA-guided cleavage of the targeted region is executed by nuclease domains of the Cas complex, in case of the multifunctional Cas9 protein an HNH and an RuvC domain that induce double strand breaks at the 20 nucleotide sequence defined by the respective spacer element.[10, 14]

The finding that the antirepeat-repeat duplex between and tracrRNA and crRNA can be transformed to a RNA single molecule by a linker loop to result in a single guide RNA (sgRNA) simplified the engineering system further and paved the road for its widespread application in modern molecular biology, making use of the DNA binding and processing capacity of the Cas complex programmed by any sequence unrelated to the spacer elements as long as it is followed by a PAM. Most prevalent are genome engineering approaches in numerous host cells of bacterial or eukaryotic origin that exploit repair mechanisms to be executed after double strand break events as induced by Cas9 at a defined position. Imprecise repair results in nucleotide insertions or deletions to introduce a mutated allele

that may result in expression of a gene product with an altered function. The loss or gain of function of such mutant alleles is the major application of CRISPR-Cas-mediated genome engineering, but several modifications of the system were developed for different purposes.[15] In the presence of DNA fragments bearing identity to the target site, replacement events based on homologous recombination result in gene insertions or corrections. Introducing inactivating mutations in the nuclease domains of Cas9 results in so-called nickase activity of the complex (nCas9) that might be exploited for staggered double strand breaks using two sgRNA molecules. Employing a nuclease inactive allele of Cas9 (dead Cas9, dCas9) and exploiting only its sgRNA-guided DNA binding activity forms the basis for labeling studies or for targeting effector domains to defined sites in the host genome where they might execute their function, may it be regulation of gene expression or recombination. The bound Cas9 complex might further serve as a scaffold by the virtue of RNA tethers. Employing more than one sgRNA at once allows multiplexing, an outstanding feature of the CRISPR-Cas-driven technology that can be further exploited for screening purposes.

Components of CRISPR-Cas9 and their installation in fungal hosts

Various cell types and organisms have been genetically modified using CRISPR-Cas-based approaches for applications in basic research, biotechnology, or biomedicine. Among these, fungal hosts were addressed quite recently with the aim to expand the molecular toolbox of fungal biology. In essence, two components need to be functionally delivered in any host for engineering its genome, that is the Cas9 protein and the sgRNA molecule. So far, several Cas9 orthologues have been identified, but most established is the *S. pyogenes*–encoded one, for which most derivatives, for example, carrying nickase activity or being inactive to serve as effector fusions, were generated and the structure of which has been determined,[16] giving perspective on mechanistic insights and streamlining of this factor with respect to size or activity.[17] Its corresponding sgRNA consists of a spacer region that is complementary to the targeted protospacer region and fused to the guide RNA scaffold which is bound by Cas9. Both components may be expressed in the desired host cell or delivered by other means in sufficient amounts to allow Cas9-sgRNA targeting complex formation and genome engineering. Expression of Cas9 relies on common constraints, such as codon usage or promoter strengths, and might be achieved transiently or permanently in a constitutive or conditional manner. The sgRNA contains two main features: a region of approximately 20 nucleotides that defines the target site followed by a nonvariable scaffold sequence that is specific for the Cas9 protein. Delivery of the sgRNA is more sophisticated, because the fact that a defined nucleic acid molecule has to be delivered intracellular actually interdicts the common strategy of expressing a transcript from an RNA polymerase II-driven promoter, as this would result in processing like capping and polyadenylation of the pre-mRNA. Accordingly, RNA polymerase III promoters are convenient for sgRNA generation *in vivo* but might be less characterized in a given host. Alternatively, direct transformation of an *in vitro* generated molecule is possible or expression of a precursor mRNA that is processed to the desired sgRNA.

Functional CRISPR-Cas9 systems have been established in a variety of filamentous fungal hosts (Table 1), among them the moulds *Neurospora crassa*[18] and *Aspergillus nidulans*[19] that have served as powerful models for decades, or the biotechnological host *Trichoderma reesei*.[20] The technology could further be established in phyto-pathogenic fungi like *Ustilago maydis*[21] or the rice blast fungus *Pyricularia oryzae*.[22] With respect to human-pathogenic fungi, CRISPR-Cas9 genome engineering has been successfully applied to *Candida albicans*[23] and *Aspergillus* spp., among them the opportunistic pathogen *A. fumigatus*.[24,25] Several implementation strategies were followed to establish the technology in *Aspergillus* and to allow various genetic approaches for host genome manipulation, and these shall be described in more detail and juxtaposed in the following section.

CRISPR-Cas9 in *Aspergillus* – implementations and applications

Molecular biology of *Aspergillus* has made significant progress,[26,27] and establishment of the CRISPR-Cas9 technology in this host holds a lot of promise for a broad range of applications. In their seminal publication, Mortensen and coworkers were the first to describe successful implementation of the CRISPR-Cas9 technology in *Aspergillus* by using a sophisticated vector system for transient Cas9 and sgRNA expression.[19] By generating a set of plasmids carrying different genetic markers and the *AMA1* sequence, which allows for autonomous replication of the vector, a versatile delivery system for different fungal hosts was generated. Moreover, an expression cassette for a codon-optimized *S. pyogenes cas9*-encoding gene was incorporated, by which strong and constitutive transcription from the *A. nidulans tef1* promoter is executed. To ensure proper localization of the Cas9 protein, an SV40 nuclear localization signal had been added to the Cas9 coding sequence. Generation of the sgRNA molecule in the *Aspergillus* host is achieved by expression of an RNA polymerase II-generated transcript from the *A. nidulans gpdA* promoter that is further

Table 1. Overview on CRISPR-Cas9 systems established in filamentous fungi.

Organism	Cas9 expression module: Promoter	Cas9 expression module: Gene	Cas9 expression module: Terminator	sgRNA generation	Features, remarks	ref.
Aspergillus spp.	*tef1*	*cas9-NLS*, codon-optimized for *A. niger*	*tef1*	*gpdA* promoter, *trpC* terminator of *A. nidulans*, release from HH & HDV ribozymes	autonomously replicating plasmid, OPTiMuS script for targeting of orthologues multiple species	19
A. fumigatus	*TEF1*	*cas9*, codon optimized for human expression	*CYC1*	*SNR52* promoter, *SUP4* 3′ region from *S. cerevisiae*	two component system: gRNA transformation in recipient strain expressing Cas9	24
	gpdA	*3xFLAG-NLS-hcas9-NLS*, codon optimized for human expression	*trpC*	U6-1/2/3 promoters or *in vitro* transcription	MMEJ-mediated mutagenesis, *in situ* tag insertion, simultaneous mutagenesis of multiple genes	25
A. oryzae	*amyB*	*FLAG-NLS-cas9-NLS*, codon-optimized	*amyB*	U6 promoter and terminator	one-plasmid system	29
Neurospora crassa	*trpC*	*cas9-NLS*	*trpC*	*SNR52* promoter, *SUP4* 3′ region from *S. cerevisiae*	transient transfection with donor vector/plasmid	18
Pyricularia oryzae	*tef*	*cas9*, codon optimized for fungal expression	*gla*	U6-1, U6-2 promoters, *trpC* promoter and terminator	gene replacement by co-transfection of CRISPR-Cas cassette and targeting vector	22
Trichoderma reesei	*pdc* (const.) *cbh1* (ind.)	*toCas9*, codon-optimzed	*pdc*	*in vitro* transcription	homologous recombination, multiplexing	20
Ustilago maydis	*otef*	*NLS-cas9-HA-NLS*, codon-optimized	*nos*	U6 promoter, human U6 terminator	autonomously replicating plasmid	21

processed by the action of two ribozymes that flank the desired sgRNA sequence. This approach obviates the need for RNA polymerase III promoter-driven expression and ensures strong and robust release of the sgRNA. Moreover, the according CRISPR-Cas9 vectors were designed in a user-friendly fashion to allow rapid and straight-forward insertion of the target-specific protospacer sequence in one step by the USER cloning approach.[28] Moreover, a Perl script was generated (OPTiMuS—One Protospacer for Targets in Multiple Species) that allows for cross-species targeting of orthologous sequences by identification of multi-species protospacers. When introducing the CRISPR-Cas9 vector to manipulate genes involved in conidial pigmentation, successful implementation in six *Aspergillus* species—*A. nidulans*, *A. aculeatus*, *A. niger*, *A. carbonarius*, *A. luchuensis*, and *A. brasiliensis*—could readily be seen from primary transformation plates. In-depth characterization of the introduced mutations revealed a wide spectrum comprising short as well as larger deletions, sometimes accompanied by an insertion. Mutagenesis was not restricted to spore color genes, as demonstrated by targeting the counter-selectable *pyrG* locus in *A. aculeatus* successfully.

For genome editing purposes in the biotechnologically relevant species *A. oryzae*, a CRISPR-Cas9 vector containing a codon-optimized version of the *Streptococcus cas9* gene fused to SV40 nuclear localization sequences and a FLAG-tag between the *amyB* promoter and terminator was constructed to achieve high and constitutive expression of the Cas9 protein.[29] Expression of the according sgRNA was executed by the same plasmid from a promoter/terminator module derived from the endogenous *U6* small nuclear RNA gene. Functionality of the system could be validated by targeting three genes, *yA*, *wA*, and *pyrG*, the gene products of which are involved in spore pigmentation and pyrimidine metabolism, respectively, using various protospacer and PAM sequences. After phenotypic screening, insertions as well as deletions could be confirmed at relatively high mutational rates. Most importantly, the CRISPR-Cas9 vector was successfully implemented in industrial *A. oryzae* strains as well to obtain *wA*-mutated isolates, demonstrating the general application potential of this system.

The group of Dunlap and co-workers were the first to establish CRISPR-Cas9-mediated genome engineering in the human-pathogenic species *A. fumigatus* using a three part module that was integrated in the genome of the reference isolate Af293.[24] This construct implements Cas9 expression by a formerly validated cassette designed for *S. cerevisiae* from which transcription of a human codon optimized *cas9* gene is driven by the *tef1* promoter. Generation of the sgRNA is supported by RNA polymerase III transcription between the *SNR52* promoter and the *SUP4* terminator, and selection for the integrative event is mediated by a genetic hygromycin resistance marker. By using a *pksP*-specific sgRNA that would interfere with DHN melanin biosynthesis to yield white conidia, 53% of primary transformants displayed the expected *albino* phenotype. To assess any alteration of cellular physiology that might result from constitutive high level expression of the Cas9 factor in the fungal host, extensive phenotyping of two established *A. fumigatus* strains, Af293 and CEA10, that were transformed with the ^{p}*tef1-cas9* construct only was carried out to reveal no significant differences with respect to growth, conidiation, nor stress resistance. Most importantly, virulence of each strain was unaffected in a non-neutropenic murine infection model of pulmonary aspergillosis. Using these Cas9 expressing strains as recipients, targeting of the *pksP* locus by separate sgRNA transformation and expression was successful and resulted in significant rates (46% and 25%, respectively) of *albino* transformants. Molecular inspection of the targeted gene revealed not only single insertions and deletions proximal to the PAM site but also larger insertions, among them the transformed DNA fragment itself. Further analyses revealed that this phenomenon was apparently the dominant mechanism by which *pksP* targeting had occurred. In essence, the strains and constructs established in this study will serve as valuable research community resource for gene targeting purposes in the context of fungal virulence.

The most advanced study on implementing the CRISPR-Cas9 mutagenesis approach in *Aspergillus*, more specifically in *A. fumigatus*, was most recently published to demonstrate precise and efficient genome engineering assisted by microhomology-mediated end joining (MMEJ).[25] Starting from a two plasmid system for expression of the human codon-optimized version of the Cas9 factor and generation of the sgRNA molecule driven by endogenous U6 snRNA sequences, *pksP* targeting was apparently implemented. However, molecular characterisation of the *albino* progeny implied 'unpredicted indels' to have happened after co-transformation of the duet CRISPR-Cas9 system at high frequency, indicating low accuracy of the system for genome engineering purposes. Combining both components in an autonomously replicating plasmid containing the *AMA1* replicator in order to create a solo CRISPR-Cas9 system only marginally improved accuracy and efficiency. When testing the system in the context of homologous recombination by introducing microhomology arms of 39 bp that were identical to the PAM site-flanking sequences and fused to a selectable resistance marker gene, as significant improvement became evident: co-transformation of the selection marker construct together with a linear sgRNA expression fragment or even an *in vitro* generated sgRNA into a Cas9 expressing recipient resulted in high editing rates

for two selected gene targets, the *pksP* and the *cnaA* gene, irrespectively of the nonhomologous end-joining (NHEJ) pathway. Multiplexing could further be demonstrated by targeting both loci simultaneously to result in *albino* transformants that had a compacted colony appearance at high frequency after co-transformation of two corresponding sgRNAs. In a most sophisticated approach, an N-terminal fusion of GFP to the catalytic calcineurin subunit could be generated by using a suitable sgRNA together with a GFP template flanked by 39 bp arms identical to the respective PAM in the *cnaA* target gene. Gene editing resulted in tag insertion at the predicted site *in situ* without selection marker integration, yielding a marker-free GFP-tagged isolate.

All these studies demonstrate impressively the rapid and successful implementation of the CRISPR-Cas9 technology in *Aspergillus* research but also reflect an evident heterogeneity with respect to the established components for Cas9 expression and sgRNA delivery.

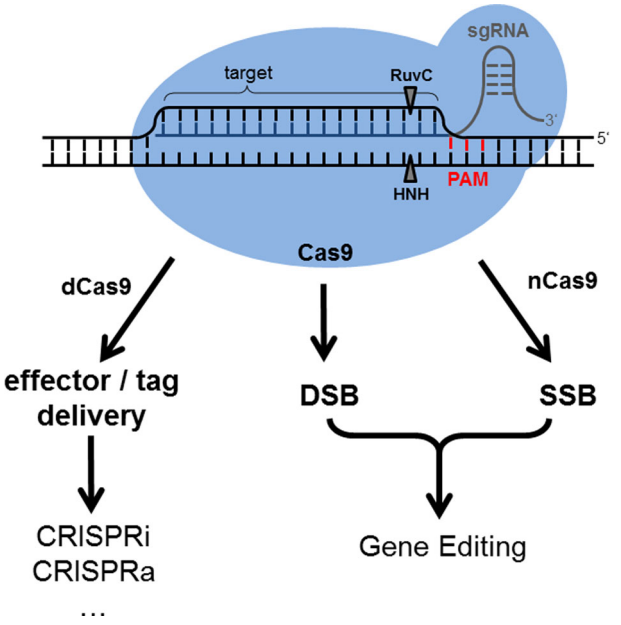

Figure 1. Schematic outline of CRISPR-Cas9-based genome targeting. The single guide (sg)RNA-loaded Cas9 protein is directed to the target site upstream of the protospacer adjacent motif (PAM) and executes its endonucleolytic function by the virtue of two nuclease domains (HNH and RuvC-like) to introduce blunt double strand breaks (DSB) or, as nickase (nCas9), single strand breaks (SSB). Error-prone cellular repair mechanisms result in mutations, while homology-based recombination may result in defined gene corrections. A nuclease dead Cas9 (dCas9) is still guided to the target site to deliver fused effector domains or protein tags, which forms the basis for functional screening approaches, such as CRISPR interference (CRISPRi) or activation (CRISPRa).

The pros and cons of CRISPR-Cas9 technology in fungal molecular biology

The described examples of CRISPR-Cas9-mediated engineering of fungal genomes demonstrate various beneficial aspects of this novel approach but also reveal several issues that have not been addressed so far. The beauty of the system clearly lies in its simplicity, with a single enzymatic activity being guided by a defined nucleic acid molecule to a target site in the (fungal) genome of interest (Fig. 1). Manipulating the Cas9 enzyme allows for variations of the theme, such as using nickases that introduce single-stranded DNA breaks or even inactive proteins that then serve as vehicles for effector domains or fluorescent tags being delivered to specific genome sites. Given the various applications that may be executed by the CRISPR-Cas9 approach,[30] the fungal field undeniably will benefit from this revolutionary technology. Scientific questions such as modifications of virulence determinants, the role of polymorphic gene variants, the variability of transcription factor binding sites, or targeting of microRNA-encoding genes are among the most obvious ones that may be addressed in the future by the help of CRISPR-Cas9. Yet several points that may hamper CRISPR-Cas9-driven manipulations of fungal genomes exist. For instance, given that the above described studies focused on *A. fumigatus* genes that result in obvious phenotypes when being inactivated (*pksP*, *cnaA*) raises the question about the actual efficiency of CRISPR-Cas9-mediated gene manipulation, as the frequency of silent nucleotide substitutions was not comprehensively assessed. Accordingly without such macroscopic indicators, the identification of isolates that carry a mutated gene allele may be tedious. Furthermore, off-target mutations have not been assessed at all in any of the studies describing fungal genome editing by CRISPR-Cas9, giving the approach a preliminary touch that might prompt researchers in the field to hold back in establishing this technique. The fact the CRISPR-Cas9-introduced mutations are less defined than, for instance, gene replacement approaches includes the possibility that truncated mRNAs or small peptides derived from the manipulated gene locus are expressed in the fungal host. The resulting effects of such alleles and their products are hard to foresee and may hamper purposes of precise gene targeting. A further obstacle that has to be considered is the evident sequence heterogeneity of fungal genomes within a species. Single nucleotide polymorphism (SNPs) are rather common when comparing different isolates of environmental or clinical origin. Such sequence variants may interfere with targeting efficiency when sgRNA design is based on a common reference template sequence. Accordingly, gene targeting by CRISPR-Cas9 may be less efficient, for example, for clinical *A. fumigatus* isolates, for which whole genome sequences are not readily available. Also the occurrence of respective PAM sites in the target fungal genome may limit comprehensive applicability of the CRISPR-Cas9

approach, which may be overcome by the implementation of alternative Cas9 enzymes recognizing other motifs.

When considering to target a specific gene in a fungal genome of interest, alternative approaches such as gene replacement may therefore be still the method of choice, given the nowadays rapid assembly of replacement cassettes and high rates of homologous recombination (HR) when using NHEJ-deficient recipients.[31] Yet, such HR-based approaches become tedious and less feasible when targeting numerous genes for screening purposes. Here the CRISPR-Cas9 technology unfolds its potential as it may allow for multiplex targeting using an sgRNA that spans a conserved region in several, conserved target genes or by employing pools of sgRNAs that mediate manipulation of multiple sites in one and the same genome. The most potent application of the CRISPR-Cas9 technology in the fungal field lies in its exploitation for screening purposes.[32,33] Scrutinizing gene functions on a comprehensive scale by interfering with their coding sequence or by manipulating their expression is possible by using libraries of sgRNAs for gene editing or guiding catalytically inactive Cas9 (dCas9) that is fused to an effector domain to promoter regions, where transcription may be repressed or induced. The latter approach is commonly referred to as CRISPR-based interference (CRISPRi) or CRISPR gene activation (CRISPRa), respectively, and bears several advantages, such as reversibility when using inducible dCas9 expression systems. Again, the fungal field will surely benefit from the rapid progress that is made in the molecular biology of various organisms and systems, but the careful development of a fungal-adapted implementation appears strictly obligatory in this course.

Conclusions and perspectives

Given the outstanding success of the CRISPR-Cas9 technology in numerous fields of molecular biology, it is highly likely that fungal research will massively benefit from recent advances and developments that have been validated so far. Yet, several uncharacterized issues remain and need to be resolved before considering this approach as novel state-of-the-art in *Aspergillus* molecular biology. The several studies that have implemented the system so far in various *Aspergillus* species indicate a wide and sometimes unpredictable range of mutations that are introduced in the host genome by the loaded Cas9 enzyme, and there is an obvious need for extending such efforts in order to extract common patterns of mutagenesis. Off-target effects have not been addressed so far, which requires comprehensive genotyping, respectively whole genome sequencing, of the engineered host strain after sgRNA-loaded Cas9 delivery. Comparative analyses of the different implementation systems from several research groups will aid in identifying the most straightforward and robust approaches for expressing the crucial components and provide guidance in streamlining them. As structural data on the commonly used Cas9 factor are available, tweaking this component becomes an obvious next step that will undoubtedly translate into the fungal field. Moreover, CRISPR systems from alternative bacterial hosts will allow for alternate implementations based on different Cas9 enzymes and varying components of the sgRNA molecule. In the light of these exciting perspectives, it seems natural to think of the CRIPR-Cas9 technology as a promising tool to exploit for *Aspergillus* research, may it be to gain fundamental insights in basic fungal biology, in the field of biotechnology, or in medical mycology. Especially utilization of the system for screening purposes and multiplexing approaches hold the promise for a more rapid and comprehensive characterisation of fungal virulence determinants that might serve as valid target candidates for antifungal therapy, representing a highly urgent and global need.

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Declaration of interest

The author reports no conflicts of interest. The author alone is responsible for the content and the writing of the paper.

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