

1 **Recyclable CRISPR/Cas9 mediated gene disruption and deletions in *Histoplasma***

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14 Running Head: Recyclable CRISPR system for *Histoplasma* gene editing

15 **Abstract**

16 Targeted gene disruption is challenging in the dimorphic fungal pathogen *Histoplasma*  
17 due to the low frequency of homologous recombination. Transformed DNA is either  
18 integrated ectopically into the genome or maintained extra chromosomally by ~~the~~ *de novo*  
19 addition of telomeric sequences. Based on a system developed in *Blastomyces*, we  
20 adapted a CRISPR/Cas9 system to facilitate targeted gene disruption in *Histoplasma* with  
21 high efficiency. We express a codon-optimized version of Cas9 as well as guide RNAs  
22 from a single ectopic vector carrying a selectable marker. Once the desired mutation is  
23 verified, one can screen for isolates that have lost the Cas9 vector by simply removing the  
24 selective pressure. Multiple mutations can then be generated in the same strain by  
25 retransforming the Cas9 vector carrying different guides. We used this system to disrupt a  
26 number of target genes including *RYP2* and *SRE1* where loss-of-function mutations could  
27 be monitored visually by colony morphology or color, respectively. Interestingly,  
28 expression of two guide RNAs targeting the 5' and 3' ends of a gene allowed isolation of  
29 deletion mutants where the sequence between the guide RNAs was removed from the  
30 genome. Whole-genome sequencing showed that the frequency of off-target mutations  
31 associated with the Cas9 nuclease was negligible. Finally, we increased the frequency of  
32 gene disruption by using an endogenous *Histoplasma* regulatory sequence to drive guide  
33 RNA expression. These tools transform our ability to generate targeted mutations in  
34 *Histoplasma*.

35 **Importance**

36 *Histoplasma* is a primary fungal pathogen with the ability to infect otherwise healthy  
37 mammalian hosts, causing systemic and sometimes life-threatening disease. Thus far,

38 molecular genetic manipulation of this organism has utilized RNA interference, random  
39 insertional mutagenesis, and a homologous recombination protocol that is highly variable  
40 and often inefficient. Targeted gene manipulations have been challenging due to poor  
41 rates of homologous recombination events in *Histoplasma*. Interrogation of the virulence  
42 strategies of this organism would be highly accelerated by a means of efficiently  
43 generating targeted mutations. We have developed a recyclable CRISPR/Cas9 system  
44 that can be used to introduce gene disruptions in *Histoplasma* with high efficiency,  
45 thereby allowing disruption of multiple genes.  
46

## 47 **Introduction**

48           The fungal order Onygenales includes multiple thermally dimorphic mammalian  
49 pathogens capable of infecting healthy hosts (1). These organisms include *Histoplasma*  
50 *spp.*, which are the most common cause of fungal respiratory infections in the US.  
51 Approximately 60-90% of individuals residing in the Ohio and Mississippi River Valleys  
52 are thought to have been exposed to *Histoplasma*, with the disease histoplasmosis  
53 reaching an incidence of up to 4.3 cases per 100,000 population in endemic regions and a  
54 mortality rate of up to 7% (2, 3). *Histoplasma* grows saprophytically with a filamentous  
55 morphology in the soil that generates asexual spores termed macro- and microconidia.  
56 These conidia, the infectious agents of *Histoplasma*, are inhaled by mammalian hosts and  
57 phagocytosed by alveolar macrophages. Upon the shift to higher body temperature,  
58 *Histoplasma* converts to a pathogenic yeast form that expresses virulence factors,  
59 enabling proliferation inside the phagolysosome and eventually leading to the lysis of the  
60 infected macrophage. Thus, the dimorphic nature of this fungus is thought to be an  
61 important pathogenicity factor and is a subject of major interest (4). Several molecular  
62 tools have been developed to study morphogenesis, such as genome wide expression  
63 studies, genetic screens based on random insertional mutagenesis, and RNA interference.  
64 These techniques led to the identification of key players of dimorphic switching such as  
65 the Ryp proteins, which are required for yeast phase growth of *Histoplasma*. However, in  
66 contrast to the majority of fungal model organisms, gene disruptions or replacements in  
67 *Histoplasma* are impeded by its low rate of homologous recombination, making gene  
68 targeting challenging (5).

69           In recent years, CRISPR/Cas9-based genome editing has become a powerful  
70 addition to the genetic tool set for molecular research of fungi. CRISPR/Cas9-based  
71 systems have been successfully adapted to genetically manipulate yeasts such as  
72 *Saccharomyces cerevisiae*, *Pichia pastoris* and *Candida albicans* as well as multiple  
73 filamentous fungal species including *Aspergilli*, *Neurospora crassa*, and *Trichoderma*  
74 *reesei* (6-11). More recently, CRISPR/Cas9 tools were developed for the dimorphic  
75 fungal pathogen *Blastomyces dermatitidis* (12), a close relative of *Histoplasma*. Notably,  
76 most fungal CRISPR/Cas9 based systems rely on one of two delivery systems: i) the  
77 transformation of *in vitro* assembled Cas9 sgRNA complexes called ribonucleoproteins  
78 (RNPs) or ii) the expression of both Cas9 nuclease and sgRNA within the fungal cell.  
79 The transformation of Cas9 sgRNA RNPs is generally simpler as it does not require  
80 laborious strain development and has the advantage of being essentially marker free (13).  
81 However, this method is not applicable to all species and in fact we could not optimize it  
82 for *Histoplasma* (data not shown). In contrast, expression of Cas9 and sgRNAs inside the  
83 fungal cell usually requires the genomic integration of the respective expression cassettes.  
84 The integration can result in unwanted side effects, such as the disruption of a random  
85 gene at the integration site. Furthermore, expression of sgRNAs is usually driven by RNA  
86 polymerase III promoters to generate functional non-modified sgRNAs. However, RNA  
87 pol III promoters are not well defined in fungi and there is only limited information about  
88 their expression kinetics. This problem was circumvented by Nødvig et al. by developing  
89 a special expression system for the sgRNA, which utilizes a well-defined constitutive  
90 RNA polymerase II promoter for expression instead of an RNA polymerase III promoter

91 (14). Here the sgRNA is embedded into a larger transcript, which is flanked by self-  
92 cleaving ribozyme sequences that will give rise to the mature sgRNA.

93       Based on this expression system and the *Blastomyces* constructs (12), we have  
94 developed a recyclable CRISPR/Cas9 system, which makes use of an inherent feature of  
95 *Histoplasma* biology, the maintenance of episomal vectors (5). We embedded expression  
96 constructs for both Cas9 nuclease and sgRNA into a larger vector that is flanked by  
97 telomeric sequences that facilitate autonomous replication in *Histoplasma* (15). Removal  
98 of the selective pressure for the vector leads to the loss of the Cas9/sgRNA plasmid,  
99 enabling multiple rounds of CRISPR/Cas9 mediated gene targeting. We have used  
100 whole-genome sequencing to further validate that this CRISPR/Cas9 system has limited  
101 potential off-target mutations. Finally, we modified the *Blastomyces* system, introducing  
102 an endogenous glyceraldehyde-3 phosphate dehydrogenase (*GAPDH*) promoter in place  
103 of the *Aspergillus*  $P_{gpd}$  regulatory sequence, thereby increasing the efficiency of targeting.  
104 Our CRISPR/Cas9 system can be used to introduce gene disruptions in *Histoplasma* at  
105 high efficiency with the option to mutate multiple genes.

106

## 107 **Results**

### 108 **Assembly of an episomal CRISPR/Cas9 vector system for gene targeting in**

109 *Histoplasma*. After several unsuccessful attempts to transform in vitro assembled  
110 Cas9/sgRNA RNP complexes into *Histoplasma*, we sought to apply the CRISPR/Cas9  
111 system originally developed by Nødvig et al. to the episomal vector system we utilize for  
112 RNAi or gene expression (11). In this case transformed DNA is carried on a linear  
113 plasmid with telomeric ends, which facilitates episomal maintenance in *Histoplasma* as

114 long as selective pressure during growth is maintained. The vector is designed to express  
115 both a selective marker gene (either *URA5* or *hph*) and Cas9 from the bidirectional *H2AB*  
116 promoter. The sgRNA cassette is essentially the same as developed by Nødvig et al. (11)  
117 and includes the well-established *gpdA* promoter and *tefl* terminator sequences from  
118 *Aspergillus nidulans*, which drive the expression of the sgRNA precursor as a standard  
119 mRNA. The sgRNA itself is composed of a protospacer sequence and tracr RNA that are  
120 responsible for binding and activating the Cas9 nuclease as well as guiding the RNP to its  
121 destination in the genome. The sgRNA is further flanked by two autocatalytic self-  
122 cleaving ribozyme sequences, which will give rise to the mature sgRNA (Fig. 1). After  
123 the introduction of a double strand break at the protospacer mediated target site and a  
124 potential indel mutation due to error-prone non-homologous end joining repair, one can  
125 screen for isolates that have lost the vector when selective pressure is removed from the  
126 media.

127 Potential protospacer sequences for target genes were identified with CRISPOR  
128 (<http://crispor.tefor.net/>) (16). The main criteria for protospacer selection for a specific  
129 target gene were a low off-target score as well as a location within the first exon or the  
130 first 100 bp of the coding sequence to avoid generating a mutant allele that encodes a  
131 partially functional truncated protein due to late frame shift mutations. Protospacer  
132 sequences were introduced into the sgRNA cassette via fusion PCR with overlapping  
133 primer pairs that contain the 20 bp protospacer sequence as well as a 6bp inverted repeat  
134 of the protospacer sequence to ensure correct folding/cleavage of the hammerhead (HH)  
135 ribozyme at the 5'-end. For final CRISPR/Cas9 vector assembly, we utilized the Gateway  
136 cloning system. We constructed two different Cas9 containing expression vectors with

137 either *URA5* or *hph* as selection markers, which both incorporated the *ccdB* containing  
138 Gateway cassette instead of the sgRNA cassette. Newly assembled sgRNA cassettes were  
139 generated in pDONR vectors, which were then recombined with the Cas9 expression  
140 vectors. This method allows the generation of a sgRNA cassette that can easily be  
141 transferred into either CRISPR/Cas9 vector depending on the selection marker needed.

142 **Highly efficient gene disruption of *RYP2*.** We first tried to use this system to disrupt  
143 *RYP2*, a key transcriptional regulator required for yeast phase growth at 37°C (Fig. 2A)  
144 (17). Successful disruptions of *RYP2* should be identifiable by filamentous colony  
145 morphology even under yeast promoting growth conditions. Upon electroporation of the  
146 linearized *RYP2* targeting CRISPR/Cas9 vector, 97% of the primary transformants  
147 showed at least a partial filamentous phenotype whereas control transformants carrying a  
148 Cas9 expressing vector without a sgRNA appeared exclusively in the yeast morphology  
149 (Fig. 2B). From the primary transformants we isolated a pure filamentous colony by  
150 passaging the primary colony for two generations. Both primary transformants as well as  
151 first generation passages appeared as mixed yeast-filamentous colonies, indicating  
152 mosaic colonies consisting of wild-type (yeast-form) and *ryp2* mutant (filamentous-form)  
153 cells (Fig. 2C). We validated the disruption of the *RYP2* target site in the filamentous  
154 mutant by PCR amplification of a 500bp region surrounding the expected cut site  
155 followed by Sanger sequencing. We could observe a two-base pair insertion exactly at the  
156 protospacer-mediated cut site three bp upstream of the protospacer adjacent motif (PAM)  
157 (Fig. 2D). As predicted, sequencing of the same locus from Cas9 expressing control  
158 strains without sgRNA all resulted in wild-type sequences.



159 **Successive CRISPR/Cas9 disruption of multiple genes in *Histoplasma*. We**  
160 investigated whether this system could be used to disrupt multiple genes in *Histoplasma*  
161 via vector recycling. We first disrupted *SRE1*, which encodes a repressor of siderophore  
162 biosynthesis. Siderophores are small high affinity iron scavengers that serve to transport  
163 iron across membranes in bacteria and fungi. When complexed with iron, siderophores  
164 confer an orange tinge to colonies, especially when siderophores are in excess as  
165 previously observed by targeting *SRE1* with RNAi (18). We reasoned that CRISPR-Cas9-  
166 generated *sre1* mutants should be easy to identify on a plate due to increased siderophore  
167 formation and thus characteristic orange pigmentation. However, all primary  
168 transformants were indistinguishable from the wild-type or Cas9-expressing controls and  
169 appeared as creamy white colonies. Similar to primary *ryp2*<sup>-</sup> mutant transformants, which  
170 appeared as mixed colonies of yeast and filamentous phenotype, primary transformants  
171 from the *SRE1* targeting CRISPR/Cas9 vector showed a mixed population of cells with  
172 either wild-type or the disrupted allele. To identify the frequency of successfully  
173 disrupted mutant cells we applied a method commonly used for CRISPR applications in  
174 cell lines of higher eukaryotes called Tracking of Indels by Decomposition (TIDE). TIDE  
175 identifies the frequency and efficiency of Cas9-mediated frame shift mutations in a mixed  
176 pool of CRISPR/Cas9 mediated mutants from Sanger sequencing traces (19). To identify  
177 possible frame shift mutations in the *SRE1* target region, we used colony PCR to amplify  
178 a 519 bp fragment centered around the putative Cas9 cutting site from colonies of either  
179 wild-type, Cas9-expressing controls, or mixed colonies of transformants with the *SRE1*-  
180 targeting Cas9 vector. The resulting fragments were sequenced via standard Sanger  
181 sequencing and sequence traces were uploaded to the TIDE online platform

182 (<http://tide.nki.nl>). The TIDE algorithm aligns the wild-type sequence with sequence  
183 traces of potential mutant/mixed colonies and determines the frequency and alterations in  
184 the alignment following the cutting site. The number of aberrant sequences in the mixed  
185 pool compared to the wild-type gets interpreted as efficiency of the respective sgRNA to  
186 generate mutant cells. Interestingly, our initial colonies of *sreI*<sup>-</sup> mutants had relatively  
187 low disruption efficiency, between 0.5% - 8.6% (Fig. 3A). Passaging the mutants with the  
188 highest disruption efficiency successively increased the efficiency of following  
189 generations until we obtained a mutant with 96.6% efficiency in generation three, thus  
190 approaching homogeneity. The TIDE algorithm further allows the identification of the  
191 CRISPR-mediated frame shift mutation, which was an insertion of a guanine at the  
192 protospacer mediated cutting site (Fig. 3A, right panel). This insertion was further  
193 confirmed via gDNA extraction and sequencing of the respective region (Fig. 3B). The  
194 resulting mutant colony was then passaged on non-selective media for three generations  
195 and individual colonies were replica-plated on selective and non-selective media to  
196 identify mutants that had lost the *SREI* targeting Cas9 vector. Two individual colonies  
197 that carried the guanine insertion mutation and had lost the Cas9 vector were selected for  
198 further CRISPR/Cas9 experiments.

199       As mentioned above, we expected pure *sreI*<sup>-</sup> mutants to display increased  
200 siderophore production, resulting in orange-pigmented colonies especially on high-iron  
201 containing media. Phenotypic characterization revealed that the mutants indeed showed  
202 increased orange pigmentation compared to wild-type (Fig. 4A). To explore the  
203 possibility of making double mutants using CRISPR, we turned to the siderophore  
204 biosynthesis pathway. The first enzyme in this pathway, L-ornithine monooxygenase, is

205 encoded by *SIDI*, which is essential for both extra- and intracellular siderophores. *SreI*  
206 normally represses *SIDI* expression under iron-replete conditions (Fig. 4B) (20, 21). To  
207 verify that our CRISPR/Cas9 system can make multiple gene disruptions in the same  
208 strain, we disrupted *SIDI* in the *sreI*<sup>-</sup> mutants followed by Cas9-vector recycling in non-  
209 selective media. The resulting *sreI*<sup>-</sup> *sidI*<sup>-</sup> double mutants were confirmed by Sanger  
210 sequencing and compared with wild-type as well as *sreI*<sup>-</sup> and *sidI*<sup>-</sup> single mutants for  
211 their capacity to produce siderophores. The disruption of *SIDI* in the *sreI*<sup>-</sup> background  
212 led to a complete loss of pigmentation (Fig. 4A), consistent with the inability of the  
213 mutant to produce siderophores (Fig. 4C).

214       The selection marker that was originally used to select for the Cas9 vector to  
215 generate the *sreI*<sup>-</sup> mutant was re-purposed to complement the mutant. We introduced a  
216 genomic copy of the *SREI* wild-type locus as well as an overexpression construct in  
217 which the expression of *SREI* was driven by the constitutive *GAPDH* promoter into the  
218 *sreI*<sup>-</sup> mutant and analyzed siderophore production levels by chrome azurol S (CAS) assay  
219 (Fig. 4C). We observed partial complementation of repression of siderophore  
220 biosynthesis when *SREI* was expressed under its native promoter and complete  
221 complementation when it was constitutively expressed under the control of the *GAPDH*  
222 promoter. As expected, both *sidI*<sup>-</sup> and *sreI*<sup>-</sup> *sidI*<sup>-</sup> double mutants were unable to produce  
223 siderophores (Fig. 4C).

224 **Whole genome sequencing revealed slightly increased SNP accumulations in Cas9**  
225 **expressing strains.** To fully characterize the genotypes of the first round of CRISPR  
226 strains, we subjected them to deep sequencing along with several control strains derived  
227 from the same frozen WU15 stock as the *sreI*<sup>-</sup> CRISPR strains. These isolates included

228 strains transformed with a Cas9 control vector (Cas9-1 and Cas9-2), a strain passaged  
229 without transformation (G217B\_ura5\_old), and a strain grown in liquid culture for  
230 genomic DNA purification without passaging (G217B\_ura5\_new). The relationships  
231 among the sequenced strains are illustrated in Fig. 5.

232 Based on comparison among strains, we discarded 917 variant positions that could not be  
233 distinguished among our sequencing samples, including 314 positions representing clear  
234 errors in the reference. This left 36 variant positions, including the targeted CRISPR  
235 mutation in *SRE1* (shared by *sre1-1* and *sre1-21*). Strain-unique variant positions,  
236 corresponding to mutations acquired after limited passaging, are quite rare: 1 in *sre1-21*  
237 and 2 in Cas9-1. An additional 6 variants (2 insertions, 2 adjacent polyG deletions, and 2  
238 point mutations) are shared by *sre1-1* and *sre1-21*. There are 21 variants common to the  
239 WU15-derived strains, of which 18 are adjacent to a 3617 bp deletion excising 3 genes  
240 including *URA5* (Fig. S1). We attribute both the point mutations and the large deletion to  
241 the UV mutagenesis that produced the WU15 strain. Although this strain has been  
242 previously reported and is widely used, this is the first precise characterization of the  
243 genomic change responsible for the Ura<sup>-</sup> phenotype. Finally, there is one short sequence  
244 duplication shared by G217B\_ura5\_new and Cas9-2.

245 We observed no large deletions in non-repeat regions for Cas9 transformed strains, with  
246 or without CRISPR guides.

247 **Cas9 dual sgRNA vectors facilitate complete gene deletions.** The method described  
248 thus far generates gene disruptions rather than deletions. However, during the course of  
249 protocol optimization, we noticed that introducing two sgRNAs into the CRISPR/Cas9  
250 vector that target the same locus resulted in a deletion of the fragment between the

251 protospacer-mediated cut sites. We decided to explore this phenomenon further by using  
252 two sgRNAs with protospacer sequences targeting the start and end of a whole coding  
253 region to facilitate complete gene deletions. We designed two sgRNA cassettes that target  
254 the 5'- and the 3'- end of the coding sequence for the velvet protein *Veal*. *VEAI* RNAi  
255 studies have shown that *Veal* is required for appropriate dimorphic switching upon  
256 temperature change (22). Primary transformants were screened by PCR using a primer  
257 that would anneal to the *VEAI* 5'- and 3'- flanking regions, resulting in two different  
258 bands based on the absence or presence of the *VEAI* CDS (Fig. 6A). Selected colonies  
259 were passaged and screened by PCR until the *VEAI* wild-type band disappeared. The  
260 deletion of the *VEAI* CDS in the mutants was validated by Southern hybridization using  
261 probes in either the 5'UTR flanking region of *VEAI* (Fig. 6B) or the *VEAI* CDS (Fig.  
262 S2). Phenotypical characterization of the *veal* $\Delta$  mutants confirmed the results of  
263 Laskowski-Peak et al. (22), which showed a faster rate of filamentation in *VEAI* RNAi  
264 strains at room temperature compared to wild-type. Similarly, we observed the  
265 appearance of white, filamentous patches much earlier for the *veal* $\Delta$  strains compared to  
266 the parental strain (Fig. 6C).

### 267 **Modulating sgRNA expression alters the frequency of Cas9-mediated editing**

268 To further optimize Cas9-mediated gene editing, we assessed the effect of expressing  
269 sgRNAs under the control of a native *Histoplasma* promoter. We constructed episomal  
270 Cas9 plasmids driving the expression of an identical mCherry sgRNA under the control  
271 of either the *A. nidulans* *GAPDH* promoter ( $P_{\text{gpdA}}$ ) adapted from Nødvig *et al.* 2015 or the  
272 native *Histoplasma* glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) promoter  
273 ( $P_{\text{GAPDH}}$ ) (Fig. 7A). These plasmids were then transformed into a *Histoplasma* strain

274 (mCherry HcG217B (23)) harboring an integrated mCherry marker driven under the  
275 expression of the *CBPI* promoter. The robust mCherry expression in this strain results in  
276 yeast colonies that are bright pink by eye with levels of fluorescence that are detectable  
277 via microscopy and flow cytometry (Fig. 7B). Following transformation, we initially  
278 observed that a majority of  $P_{GAPDH}$  transformants were cream colored by eye in contrast  
279 to  $P_{gpdA}$  transformants that retained some degree of pink color. Following these  
280 preliminary observations, two  $P_{GAPDH}$  isolates and two  $P_{gpdA}$  isolates were selected at  
281 random and passaged under selection in liquid culture. These isolates were subsequently  
282 used for Sanger sequencing and flow cytometry to quantify editing efficiency and  
283 mCherry signal, respectively. Interestingly, our TIDE analysis revealed that in contrast to  
284 the average 25% editing efficiency observed in  $P_{gpdA}$  transformants,  $P_{GAPDH}$  transformants  
285 displayed an average editing efficiency of 93% (Fig. 7C). This finding was also  
286 corroborated by our flow cytometry data, where 100% of  $P_{GAPDH\_1}$  and 94.1% of  
287  $P_{GAPDH\_2}$  cells were negative for mCherry signal (Fig. 7D). These findings suggest that  
288 optimizing expression of sgRNAs through use of a native promoter can enhance editing  
289 efficiency and minimize the passaging time required to generate a pure mutant.

## 290 **Discussion**

291 Here we describe the development of a recyclable CRISPR/Cas9 system which can be  
292 used to introduce gene disruptions or deletions in *Histoplasma*, without leaving major  
293 marks in the genome other than the targeted mutations. As proof of principle, we  
294 generated disruptions of *RYP2*, *SRE1*, and *SID1*, as well as a complete deletion of *VEA1*.  
295 This method allows the rapid generation of targeted mutants with a very low risk for off-  
296 target mutations. Furthermore, multiplexing sgRNA expression cassettes in the Cas9

297 expression vector can be used to successfully delete whole genes and has the potential to  
298 accelerate gene disruptions for multiple loci.

299 The molecular toolbox for studying the basic biology of *Histoplasma* includes genome  
300 wide expression studies, genetic screens based on random insertional mutagenesis, and  
301 RNA interference. However, in contrast to other fungi, targeted gene disruptions or  
302 replacements are rare, due to the low rate of homologous recombination (24). In the  
303 present study we built on technology applied in *Blastomyces* (12) to demonstrate that  
304 CRISPR/Cas9 technology can be utilized to generate targeted gene disruptions and  
305 deletions in *Histoplasma*. We utilized an inherent feature of *Histoplasma*, the  
306 maintenance of extra chromosomal DNA, and embedded both Cas9 and sgRNA  
307 expression cassettes into an episomal vector system that can be recycled after successful  
308 gene disruption. Initial genome editing efficiency after transformation of the Cas9-  
309 sgRNA constructs was not always high in *Histoplasma*, resulting in colonies with mixed  
310 populations of wild-type and mutated cells, but successive passaging led to rapid increase  
311 in editing efficiency resulting in homogenous mutated colonies after 2-4 passages. This is  
312 in contrast to the CRISPR/Cas9 strategy applied in the dimorphic pathogen *Blastomyces*  
313 *dermatitidis* where Kujoth et al. could not detect increased genome editing upon  
314 successive passaging (12). Interestingly, expressing Cas9-sgRNA constructs under the  
315 control of the *Histoplasma GAPDH* promoter rather than the *A. nidulans GAPDH*  
316 promoter led to increased efficiency of targeting in *Histoplasma*.

317 We found that the combination of colony PCR and the TIDE algorithm simplifies and  
318 streamlines the screening process of primary transformants and passaged isolates to  
319 obtain targeted disruptions. It was most helpful to use TIDE as a tool to select

320 transformants with highest editing efficiency for further passaging to isolate  
321 homogeneous mutant colonies. Additionally, we have used TIDE to evaluate general  
322 efficiency of different sgRNAs targeting the same genomic locus. This screening is  
323 particularly helpful because the efficiency of individual sgRNAs can vary widely for  
324 unknown reasons. The G/C content of the protospacer as well as the four bases preceding  
325 the PAM sequence seem to have a huge influence on the efficiency of sgRNAs (25, 26).  
326 Choosing the most desirable sgRNA can become a compromise between location of the  
327 protospacer sequence in the target region and the predicted efficiency of the sgRNA  
328 based on its sequence. TIDE can be used after transformation to identify the most  
329 efficient sgRNA for a locus with limited protospacer availability.

330 The delivery of Cas9-sgRNA RNPs into fungal cells is usually achieved either via  
331 transformation of *in vitro* assembled RNPs or by integration of expression cassettes for  
332 Cas9 and sgRNA into the genome (9, 12, 13, 27, 28). Concerns about potential off-target  
333 effects due to irregular Cas9 activity have led to the development of transient Cas9  
334 expression systems, where Cas9 and sgRNA expression cassettes can be removed from  
335 the genome after successful gene editing (10, 11, 29). A transient expression system bears  
336 several advantages, the first being the ability to reuse selectable marker genes, especially  
337 in fungi with limited marker availability. In *Histoplasma*, there are currently three marker  
338 genes with documented use: *URA5* (orotate phosphoribosyltransferase), *hph* (hygromycin  
339 phosphotranseferase), and *Sh ble* (bleomycin/Zeoicin resistance gene). Of these, *URA5*  
340 requires the auxotrophic *ura5<sup>-</sup>* background strain, leaving just two marker genes for  
341 genetic manipulations in the wild-type strain. Another advantage of a recyclable transient  
342 expression system is the elucidation of complex genetic networks by consecutive gene



343 disruptions or deletions as exemplified here by the successive deletion of *SRE1* and *SID1*.  
344 Additionally, loss of the Cas9-sgRNA expression construct allows introduction of a  
345 complementation clone without concern that the complementation sequence will be  
346 targeted by Cas9. Recycling of the Cas9 nuclease further prevents the risk of potential  
347 off-target mutations, which could accumulate due to prolonged Cas9 expression.  
348 Interestingly, potential cytotoxic effects due to Cas9 expression in fungi are a matter of  
349 debate. Whereas it seems that some fungal groups of the genera *Aspergillus*, *Candida*,  
350 *and Cryptococcus* are not affected by prolonged Cas9 expression, cytotoxic effects have  
351 been shown in *Saccharomyces* and *Candida glabrata* (30). However, it is still unclear  
352 whether it is the Cas9 expression itself that actually results in increased off-target  
353 mutations. We investigated this by comparing the genomes of CRISPR/Cas9 generated  
354 mutants to Cas9-only expressing strains and wild-type strains. We observed a slight  
355 increase in variants in the genome of the CRISPR-generated mutants compared to wild-  
356 type. Five of these variants were strain-unique and 6 were shared among the CRISPR  
357 edited mutants. Further experimentation will determine whether these latter 6 mutations  
358 will continue to be overrepresented in strains expressing CRISPR/Cas9. Another concern  
359 that has been raised for CRISPR-based applications in cells of higher eukaryotes is the  
360 potential for large-scale genomic rearrangements in the target region (31). However, we  
361 did not observe any large deletions or rearrangements in any of the sequenced isolates.  
362 Our results confirm previous observations that CRISPR/Cas9 applications in fungi are  
363 generally a robust and suitable alternative to classical molecular biology methods, such as  
364 homologous recombination. The approach described here is likely to have a  
365 transformative effect on dissecting *Histoplasma* biology.  
366

367 **Materials and Methods**

368 ***Histoplasma* strains and culture conditions.** All experiments were carried out in the  
369 *Histoplasma* G217B *ura5<sup>-</sup>* background. A list of generated strains can be found in Table  
370 S3. Yeast cultures of *Histoplasma* strains were propagated in liquid *Histoplasma*  
371 macrophage medium (HMM) (32) supplemented with uracil (200 µg/ml), hygromycin  
372 (200 µg/ml) or Zeocin (50 µg/ml) as indicated. Liquid cultures were grown at 37°C with  
373 5% CO<sub>2</sub> on an orbital shaker with 120 rpm. For phenotypical characterization of the  
374 strains, yeast cultures were diluted to an OD<sub>600</sub> = 1 and 10 µl were spotted on solid HMM  
375 plates, which were incubated at 37°C with 5% CO<sub>2</sub>.

376 For cloning purposes either *E. coli* DH5α or One Shot® *ccdB* Survival™ 2 T1<sup>R</sup>  
377 (Invitrogen) for plasmids containing the *ccdB* containing Gateway cassette were used.

378 **Generation of episomal CRISPR/Cas9 vectors.** All plasmids and primers used in this  
379 study are described in Table S1 and S2. Based on the work of Nødvig et al. (11), we  
380 created an episomal vector that expresses fungal codon optimized *cas9* from  
381 *Streptococcus pyogenes* as well as a hybrid sgRNA. For the vector backbone we used  
382 pBJ209, which was amplified from the RNAi vector pSB23 with primers  
383 OAS5744/OAS5745, which introduce *ApaI* and *NheI* restriction sites respectively. Next,  
384 we amplified the *ccdB* containing Gateway cassette with primers OAS5734/OAS5735  
385 from pSB23, which introduced *ClaI* restriction sites at 5'- and 3'-ends and cloned the  
386 Gateway cassette into the *ClaI* site of pBJ209, resulting in pBJ213. The codon optimized  
387 *cas9* sequence was amplified from pPTS608-Cas9-hyg (12) with primers  
388 OAS5736/OAS5737 that introduced *NheI*- and *ApaI*-sites at the 5'- and 3'-end as well as  
389 *attB*-sites for subcloning in pDONR. The *cas9* sequence was excised from its larger

390 amplicon with NheI and ApaI and cloned into NheI/ApaI digested pBJ213 to make  
391 pBJ219. Any gRNA cassette cloned into the pDONR vector can be recombined with  
392 pBJ219 via Gateway cloning to produce a final CRISPR/Cas9 targeting vector for  
393 transformation into *Histoplasma*. Details for individual targeting vectors are given in  
394 supplemental material.

395 **Evaluation of protospacer sequences.** Protospacer sequences specific for the Sp-Cas9  
396 (20bp-NGG) were designed to target the first exon of the target genes to disrupt the  
397 coding sequence as far upstream as possible. Potential candidates were evaluated for  
398 specificity and possible off-target sites with the online tool CRISPOR  
399 (<http://crispor.tefor.net>) (16).

400 **TIDE analysis of heterogeneous colonies.** To determine the editing efficiency of the  
401 respective gRNAs, we used the online tool TIDE (<http://tide.nki.nl/>) (19). Colonies of  
402 positive transformants as well as wild-type controls were subjected to colony PCR to  
403 amplify an approximately 500bp fragment of the gRNA mediated target region centered  
404 around the putative cutting site. For colony PCR, *Histoplasma* yeast colonies were lysed  
405 by boiling in 100  $\mu$ l 0.02 M NaOH for 10 min. 2  $\mu$ l of the supernatant was directly used  
406 as template for standard PCR reactions with either Phusion or Taq polymerase. The  
407 fragments were sequenced by Sanger DNA sequencing. The sequence traces of wild-type  
408 and heterogeneous mutated colonies were aligned with the TIDE online tool, which  
409 identifies mutations introduced at or near the putative cutting site and determines their  
410 approximate frequency in a heterogeneous cell population.

411 **CAS assay for siderophore quantification.** The siderophore production level of  
412 different mutants was determined using chrome azurol S (CAS) as previously described

413 (21). *Histoplasma* yeast cultures, grown in triplicate in liquid HMM, were pelleted,  
414 washed with PBS and resuspended in RPMI without phenol red to avoid potential  
415 interference with the colorimetric assay. The cultures were grown for 24 h at 37°C. The  
416 culture supernatant was mixed 1:1 with modified CAS assay solution (0.6 M HDTMA,  
417 15  $\mu$ M FeCl<sub>3</sub>, 150  $\mu$ M CAS, 0.5 M MES, 3.4 mM 5-sulfosalicylic acid) (33) in a 96 well  
418 plate and incubated for 3-4 h. OD<sub>630</sub> was measured with a plate reader and normalized to  
419 OD<sub>600</sub> of the cultures to account for cell density.

420 **Whole genome library preparation and sequencing.** Dual-indexed paired-end libraries  
421 for whole genome sequencing were prepared with the Nextera DNA Flex Library Prep  
422 Kit from Illumina. Individual libraries were barcoded with Nextera DNA CD Indexes  
423 (Illumina) and analyzed for average size distribution and concentration on a High  
424 Sensitivity DNA Bioanalyzer chip from Agilent Technologies. Fragments of individual  
425 libraries had an average size distribution of 500-550 bp and 5 ng of each library were  
426 pooled for multiplexed sequencing. All 6 strains were sequenced in a single lane on an  
427 Illumina HiSeq 4000 at the UCSF Center for Advanced Technology (UCSF CAT)  
428 yielding 101-mer paired-end reads with ~400x coverage of the genome per strain. To  
429 distinguish mutations in the WU15 parental strain from errors in the reference, we  
430 additionally analyzed reads from wild-type G217B (34) with ~200x coverage of the  
431 genome.

432 **NGS data analysis.** The raw data are available at the NCBI Sequence Read Archive  
433 (SRA) under SRA accession PRJNA971667. The reads were analyzed as illustrated in  
434 Supplemental Fig. S3. In summary, reads were aligned to the 11/30/2004 version of the  
435 G217B genome assembly from the Genome Sequencing Center (GSC) at Washington

436 University as mirrored at <https://histo.ucsf.edu/downloads/> using BWA-MEM (35) with  
437 GATK (36) indel realignment. Long repeat sequences were annotated in the reference  
438 genome using LTRHARVEST (37) and REPEATMASKER (A.F.A. Smit, R. Hubley &  
439 P. Green RepeatMasker at <http://repeatmasker.org>) queried with repeat families identified  
440 by the GSC as well as a representative full length MAGGY LTR retro transposon. Large  
441 deletions were identified as regions of at least 300 bp with fewer than 10 reads per base  
442 pair interrupted by higher coverage regions of less than 50 bp. Deletions overlapping  
443 repeat annotations were removed, and the remaining per-strain deletions were normalized  
444 by clustering on genome location to generate a consistent set of deletion coordinates  
445 across strains. To identify short sequence variations, the full set of aligned reads was run  
446 through SAMTOOLS MPILEUP (<http://www.htslib.org/doc/samtools-mpileup.html>) and  
447 BCFTOOLS CALL (<http://www.htslib.org/doc/bcftools.html>). A variant allele was  
448 assigned to a strain if it was supported by at least 85% of the reads at the variant site for  
449 sites with at least 10 aligned reads for the given strain; variant sites where no allele  
450 passed these support criteria were flagged as ambiguous. Variant sites with zero or one  
451 alleles unambiguously assigned across all strains (*e.g.*, errors in the reference sequence)  
452 were removed as were sites overlapping annotated repeat regions or large deletions.  
453 Large deletions and variant sites were then classified among strains as summarized in  
454 Fig. 5.

455 **Southern hybridization of *vea1*Δ mutants.** A 1052-bp probe to the 5' UTR of *VEA1*  
456 was amplified from G217B *ura5*<sup>-</sup> genomic DNA using primer set OAS6659/OAS6660. A  
457 second 1013-bp probe to the *VEA1* CDS was amplified from G217B *ura5*<sup>-</sup> genomic DNA  
458 using primer set OAS6663/OAS1969. In separate reactions the resulting PCR fragments

459 were coupled with an alkaline phosphatase enzyme using the Amersham Gene Images  
460 AlkPhos Direct Labelling and Detection System (GE Healthcare), according to the  
461 manufacturers protocol. Genomic DNA from the parental strain G217B *ura5<sup>-</sup>* and the  
462 mutant strains was isolated by phenol chloroform extraction. In duplicate, 15 µg of  
463 genomic DNA from both samples was digested with restriction enzyme *Bgl*III for 1 hour  
464 at 37°C. Digested DNA was size separated by gel electrophoresis on a 0.7 % agarose gel  
465 and transferred to a Hybond-N+ nylon membrane (Amersham Biosciences). Membrane  
466 bound digested genomic DNA was hybridized at 60°C overnight with either the probe to  
467 the 5' UTR or to the *VEAI* CDS. Finally, the membranes were visualized by using CDP-  
468 Star Chemifluorescent detection system (GE Healthcare).

469 **Flow cytometry.** Transformants were selected at random and grown in liquid HMM with  
470 plasmid selection. Cultures were passaged 24 hours prior to harvest to obtain mid-log  
471 phase cells with an OD<sub>600</sub> between 5-7. Approximately 1.25 E7 cells per sample were  
472 sonicated, washed, and spun down with D-PBS before being fixed on ice with BD  
473 Stabilizing Fixative (BD Biosciences). Once fixed, samples were washed and  
474 resuspended in D-PBS and analyzed using a BD LSRII Flow Cytometer in the UCSF  
475 Parnassus Flow Core. mCherry fluorescence signal in each sample was analyzed and  
476 quantified using FlowJo v. 10. mCherry positive and negative control strains were used to  
477 set gates for quantifying signal across all transformants.

478

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- 595

596

## 597 **Figure Legends**

598 **Figure 1. Schematic representation of the CRISPR/Cas9 mediated targeted gene**  
599 **disruption in *H. capsulatum*.** Under selective pressure the CRISPR/Cas9 plasmid is  
600 maintained in *Histoplasma* as an extrachromosomal vector, which carries both a codon  
601 optimized version of Cas9 and the sgRNA (based on the concept of Nødvig et al., 2015).  
602 The sgRNA is expressed as a precursor driven by a constitutive RNA polymerase II  
603 promoter (*gpdA<sup>P</sup>*), resulting in a larger mRNA-like transcript with 5'-cap and poly-(A)  
604 tail. The mature sgRNA gets excised from its larger transcript via the action of two  
605 flanking ribozyme sequences (hammerhead, HH and hepatitis delta virus, HDV). The  
606 sgRNA then recruits the Cas9 nuclease to a protospacer (PS)-mediated target site in the  
607 genome, where it cuts the gDNA. The resulting double-strand break is repaired by the

608 non-homologous end joining (NHEJ) mechanism, an error-prone process that often  
609 introduces small indel mutations. Growing the resultant mutant strain under non-selective  
610 conditions (*i.e.* in the presence of uracil) allows loss of the CRISPR/Cas9 vector, which  
611 then permits re-use of the *URA5* marker for subsequent transformations.

612 **Figure 2. CRISPR/Cas9 mediated disruption of *RYP2*.** (A) Schematic representation  
613 of *RYP2*-mediated regulation of the dimorphic switch in *H. capsulatum*. At mammalian  
614 body temperature, Ryp2 is required to promote yeast-phase growth. (B) Primary  
615 transformants carrying the CRISPR/Cas9 vector targeting *RYP2*. Whereas transformants  
616 carrying a Cas9-only expressing control vector exhibited a smooth yeast-colony shape,  
617 transformants carrying the *RYP2* targeting CRISPR/Cas9 vector showed predominantly a  
618 wrinkled/filamentous phenotype. (C) Phenotypes of first-generation and second-  
619 generation *ryp2*<sup>-</sup> mutants displaying the gradual increase from a mixed yeast/filamentous  
620 colony shape to a completely filamentous phenotype. Colonies also showed increased red  
621 pigmentation indicating altered secondary metabolism associated with filamentous  
622 growth. (D) Sanger sequencing of the *RYP2* target region of the WT and the second-  
623 generation *ryp2*<sup>-</sup> mutant revealed an insertion of TG in the protospacer-mediated target  
624 region in the mutant causing a frame-shift mutation in the first exon.

625 **Figure 3. Evaluation of *sreI* disruption mutants with the online platform TIDE.**

626 Transformants with a *SRE1*-targeting CRISPR/Cas9 vector either directly after  
627 transformation or after each passaging were analyzed with TIDE  
628 (<https://tide.deskgen.com>), which detects the predominant mutations (*i.e.* deletions or  
629 insertions) as well as their frequencies. To do so, approximately 500 bp of the sgRNA  
630 mediated target sequence were amplified via colony PCR from a control strain as well as

631 mutant colonies harboring the mutation of interest. The DNA fragments were sequenced  
632 by standard Sanger sequencing and compared with the TIDE platform. This online tool  
633 aligns the first part of the sequences before the anticipated cutting site and calculates the  
634 frequency of aberrant sequences following the cutting site for the mutant colony, in this  
635 case a disruption mutant of *SREI* (96.6%) compared to the control sequence. The bottom  
636 of panel A shows the gene-editing efficiency of the mutant isolates after subsequent  
637 generations of passaging based on TIDE analysis. The gene editing efficiency increases  
638 for most colonies after each passaging, resulting in homogeneous mutant isolates after  
639 three passages. An additional passage on non-selective medium (*i.e.* in the presence of  
640 uracil) resulted in the loss of the vector. (B) Sequencing of the final mutants after Cas9  
641 vector loss confirmed a G insertion in the first exon of the *sreI*<sup>-</sup> mutants resulting in a  
642 premature stop codon after 144 bp.

643 **Figure 4. CRISPR/Cas9 vector recycling allows successive gene disruptions and**  
644 **complementation.** (A) CRISPR/Cas9 vector recycling in the *sreI*<sup>-</sup> mutants allowed re-  
645 use of the *URA5* marker to target a second gene in the *sreI*<sup>-</sup> background. The loss of  
646 *SREI* resulted in derepressed siderophore expression, which led to orange pigmentation  
647 of the colonies due to increased iron-bound siderophores, especially on high iron  
648 containing media. Disruption of *SIDI* in the *sreI*<sup>-</sup> background, which encodes the first  
649 enzyme in the siderophore biosynthesis pathway (as diagrammed in B) resulted in  
650 complete abolishment of siderophore production, indicated by the loss of orange  
651 pigmentation of the colonies of the *sreI*<sup>-</sup> *sidI*<sup>-</sup> double mutants. (C) After loss of the Cas9  
652 plasmid in the *sreI*<sup>-</sup> mutant, the *URA5* marker gene was available for selection of  
653 complementation plasmids. Siderophore production was analyzed with the CAS assay

654 and showed partial complementation when *SRE1* was expressed under its native promoter  
655 and almost complete complementation when *SRE1* was overexpressed under the control  
656 of the constitutive *GAPDH* promoter (OE). In contrast, both *sidI*<sup>-</sup> and *sreI*<sup>-</sup> *sidI*<sup>-</sup> double  
657 mutants showed no siderophore production as expected.

658 **Figure 5. Validation of the CRISPR/Cas9 system via whole genome sequencing.**

659 We compared the genomes of two CRISPR/Cas9 generated mutants (*sre1-1*, *sre1-21*)  
660 with Cas9 only expressing control strains (*Cas9-1*, *Cas9-2*) and the parental strain from  
661 which we extracted gDNA for deep sequencing either before (G217B\_ura5\_old) or after  
662 passaging along with the CRISPR mutants (G217B\_ura5\_new). The diagram shows the  
663 strain lineages and whether strains were transformed and passaged for gene-disruption or  
664 control. Strain unique variant are given in red parenthesis including the targeted  
665 CRISPR/Cas9 mutation.

666 **Figure 6. sgRNA multiplexing for complete gene deletions.** We introduced two sgRNA

667 cassettes into the Cas9 expressing vector, targeting the 5'- and 3'-end of the *VEAI* CDS  
668 respectively. Colony PCR of primary transformants (lower part of panel A) resulted in  
669 two bands for 4 of 10 isolates and only one band for two isolates, indicating partial or  
670 complete loss of the *VEAI* CDS in those mutants. WT indicates wild-type. (B) Southern  
671 hybridization of *vea1*Δ mutants confirmed the deletion of the *VEAI* CDS in two  
672 independent mutants. Location of the probe is shown in the upper part of panel B and the  
673 Southern blot is shown in the lower part of panel B. WT indicates wild-type. (C)  
674 Phenotypical characterization of the mutants grown at room temperature (RT) was  
675 performed by spotting dilutions of wild-type (G217B *ura5*<sup>-</sup>) and *vea1*Δ mutant strains on  
676 plates. The *vea1*Δ mutants displayed accelerated filamentation as compared to the

677 parental strain as indicated by the increased density of fluffy white filamentous growth.  
678 The difference between wild-type and mutant strains was most pronounced after six days  
679 of incubation.

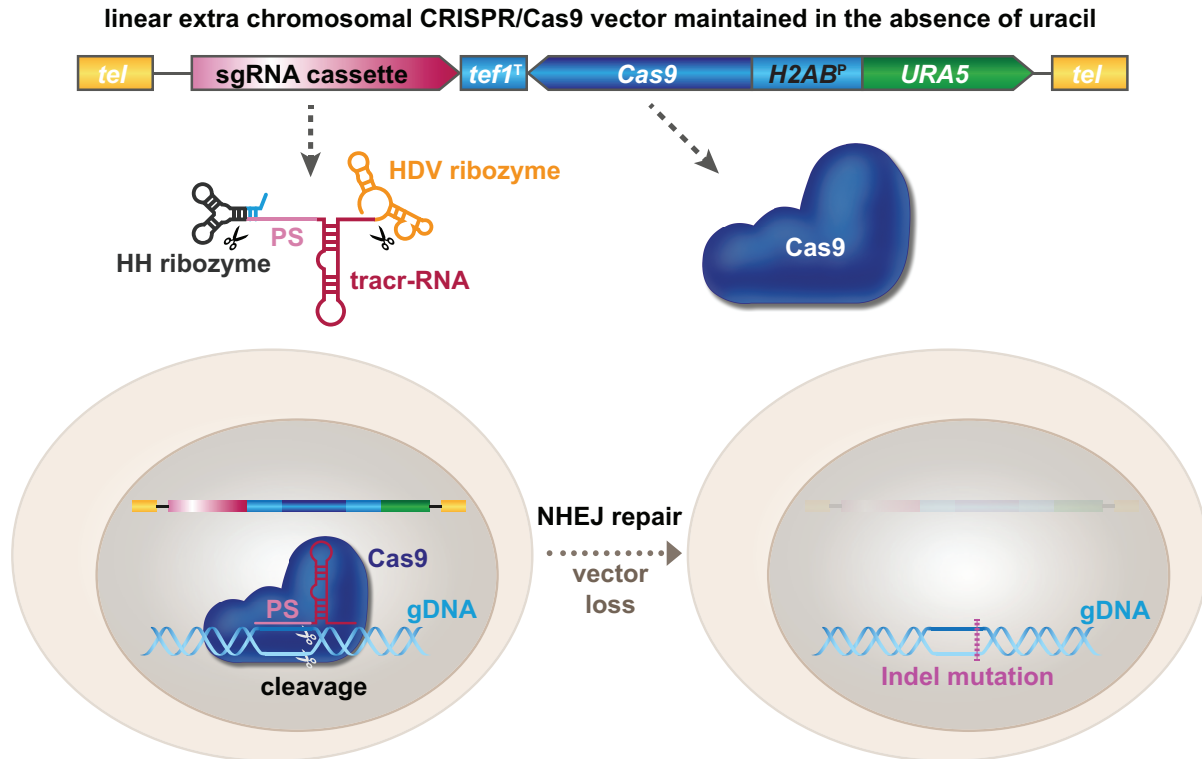
680 **Figure 7. Cas9-sgRNAs expressed under the control of the *Histoplasma* GAPDH**  
681 **promoter showed increased editing efficiency.** A) Schematic displaying experimental  
682 approach. Plasmids containing either the *A. nidulans* GAPDH promoter adapted from  
683 Nødvig et al. 2015 ( $P_{\text{gpdA}}$ ) or the native *Histoplasma* GAPDH promoter ( $P_{\text{GAPDH}}$ ) driving  
684 the expression of an identical mCherry targeting guide RNA were transformed into the  
685 mCherryHcG217B strain and used to assess differences in editing efficiencies. B) Plate  
686 images of the parental mCherryHcG217B strain alongside transformants selected from  
687 each plasmid transformation that were subsequently used for Sanger sequencing and flow  
688 cytometry. C) Indel spectrum of two individual isolates obtained from each plasmid  
689 transformation. Sanger sequencing of the mCherry locus was used to conduct TIDE  
690 analysis (<https://tide.nki.nl/>) to quantify the frequency of insertions and deletions  
691 following the predicted cut site. D) Histograms displaying measurements of mCherry  
692 signal acquired through flow cytometry. Overlay plots display normalized counts for each  
693 isolate in comparison to the starting mCherryHcG217B strain. E) Quantification of flow  
694 data displaying percentage of mCherry<sup>+</sup> cells within each isolate.

695

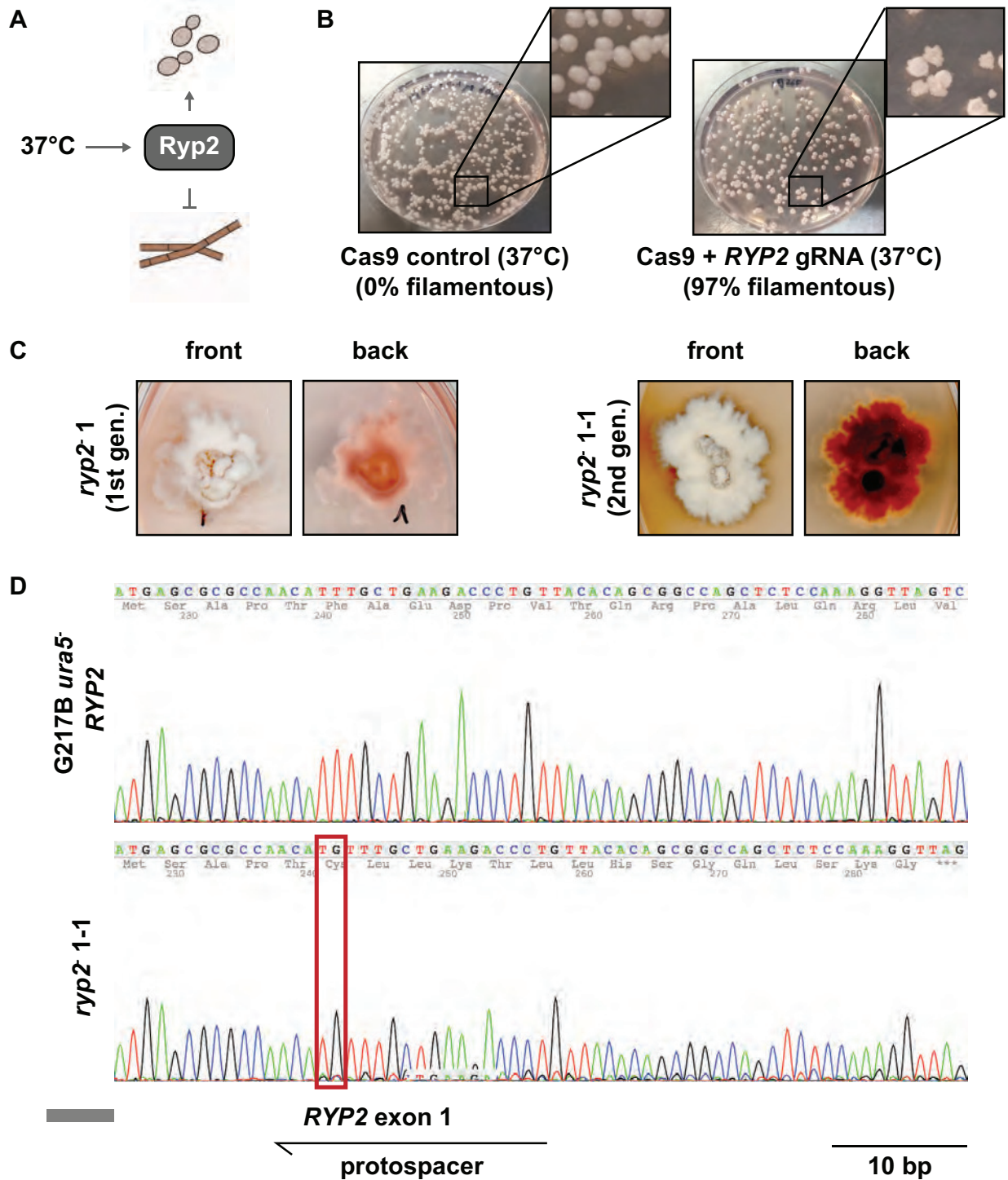
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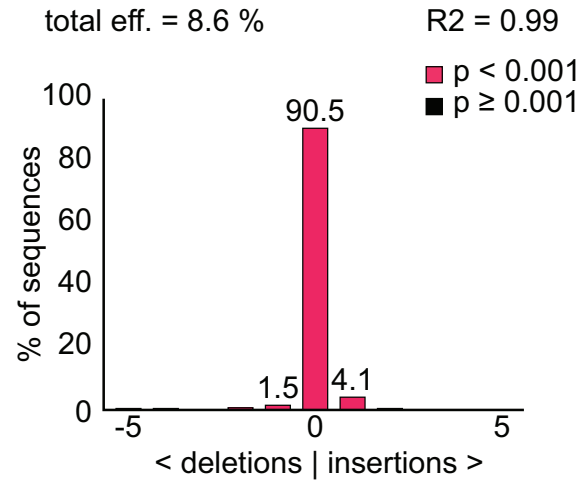


**Figure 2. CRISPR/Cas9 mediated disruption of *RYP2*.** (A) Schematic representation of *RYP2*-mediated regulation of the dimorphic switch in *H. capsulatum*. At mammalian body temperature, Ryp2 is required to promote yeast-phase growth. (B) Primary transformants carrying the CRISPR/Cas9 vector targeting *RYP2*. Whereas transformants carrying a Cas9-only expressing control vector exhibited a smooth yeast-colony shape, transformants carrying the *RYP2* targeting

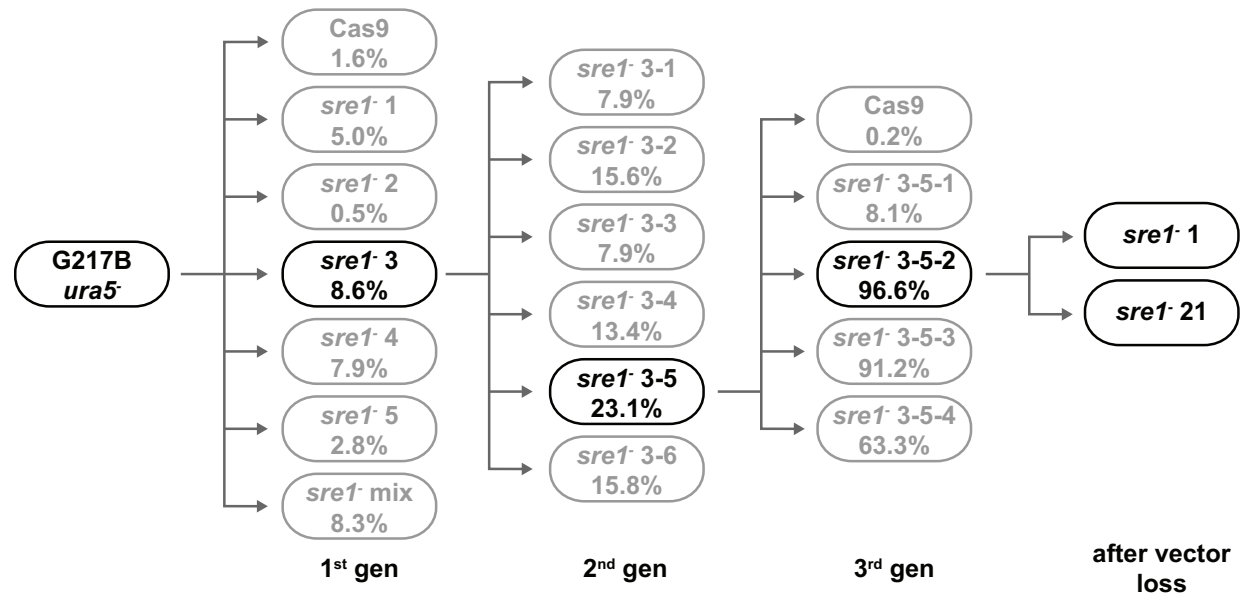
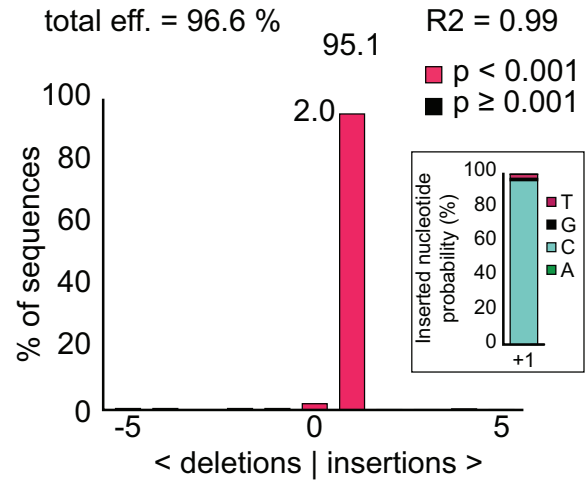


CRISPR/Cas9 vector showed predominantly a wrinkled/filamentous phenotype. (C) Phenotypes of first-generation and second-generation *ryp2<sup>-</sup>* mutants displaying the gradual increase from a mixed yeast/filamentous colony shape to a completely filamentous phenotype. Colonies also showed increased red pigmentation indicating altered secondary metabolism associated with filamentous growth. (D) Sanger sequencing of the *RYP2* target region of the WT and the second-generation *ryp2<sup>-</sup>* mutant revealed an insertion of TG in the protospacer-mediated target region in the mutant causing a frame-shift mutation in the first exon.

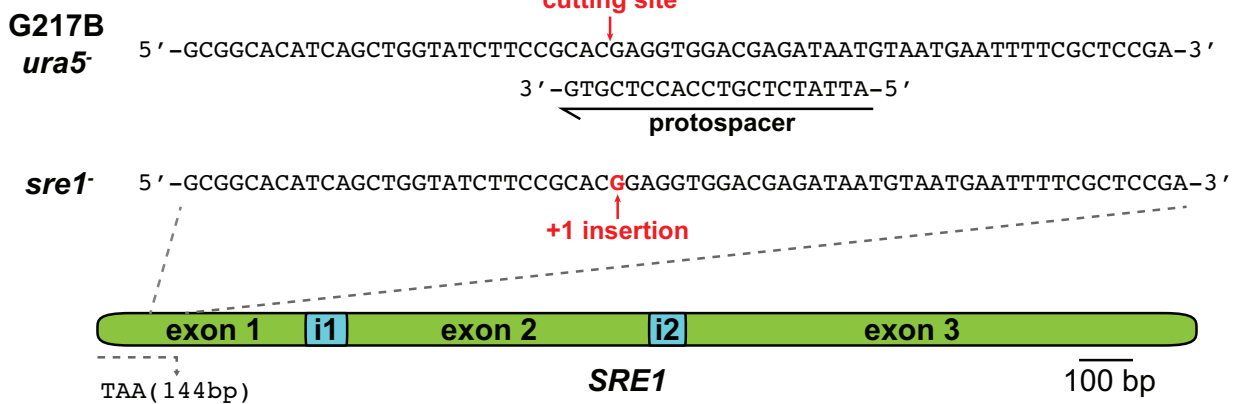
### A Indel Spectrum for *sre1*-3



### Indel Spectrum for *sre1*-3-5-2

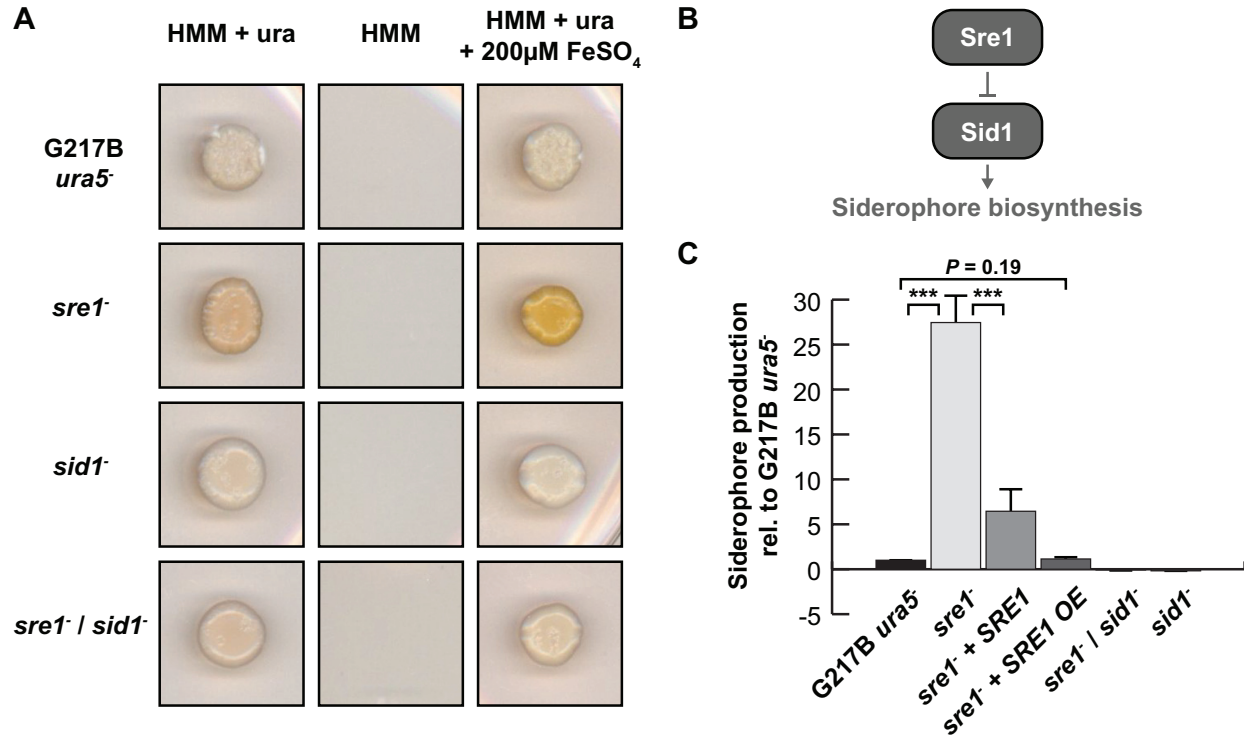


### B

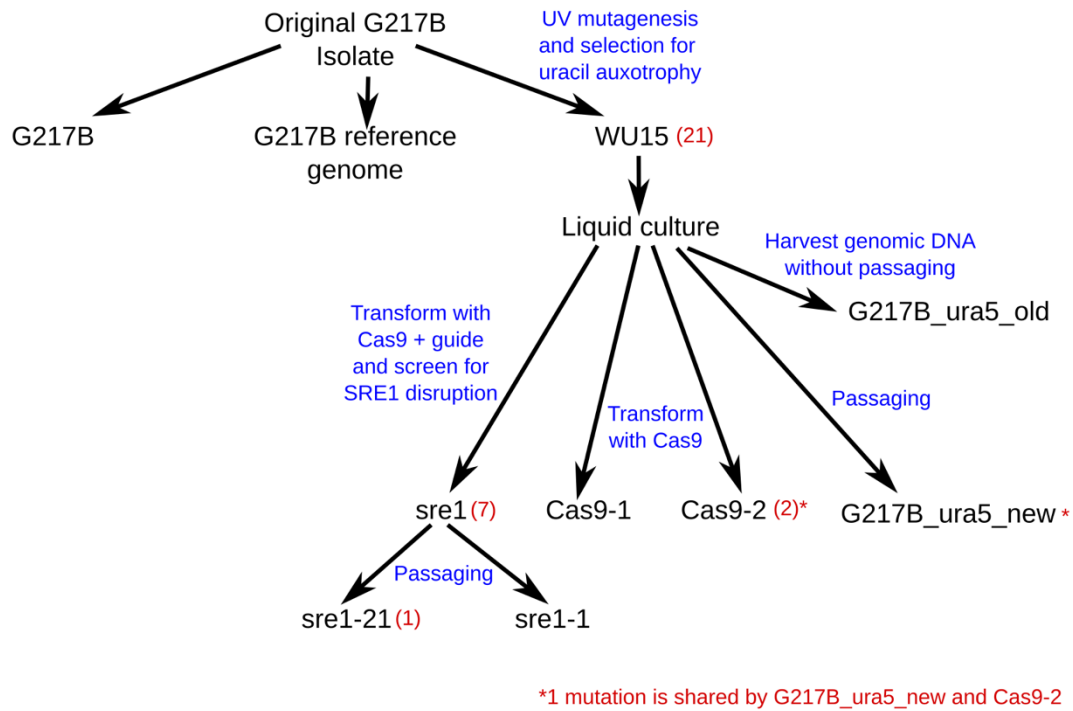


**Figure 3. Evaluation of *sreI*<sup>-</sup> disruption mutants with the online platform TIDE.**

Transformants with a *SREI*-targeting CRISPR/Cas9 vector either directly after transformation or after each passaging were analyzed with TIDE (<https://tide.deskgen.com>), which detects the predominant mutations (i.e. deletions or insertions) as well as their frequencies. To do so, approximately 500 bp of the sgRNA mediated target sequence were amplified via colony PCR from a control strain as well as mutant colonies harboring the mutation of interest. The DNA fragments were sequenced by standard Sanger sequencing and compared with the TIDE platform. This online tool aligns the first part of the sequences before the anticipated cutting site and calculates the frequency of aberrant sequences following the cutting site for the mutant colony, in this case a disruption mutant of *SREI* (96.6%) compared to the control sequence. The bottom of panel A shows the gene-editing efficiency of the mutant isolates after subsequent generations of passaging based on TIDE analysis. The gene editing efficiency increases for most colonies after each passaging, resulting in homogeneous mutant isolates after three passages. An additional passage on non-selective medium (i.e. in the presence of uracil) resulted in the loss of the vector. (B) Sequencing of the final mutants after Cas9 vector loss confirmed a G insertion in the first exon of the *sreI*<sup>-</sup> mutants resulting in a premature stop codon after 144 bp.

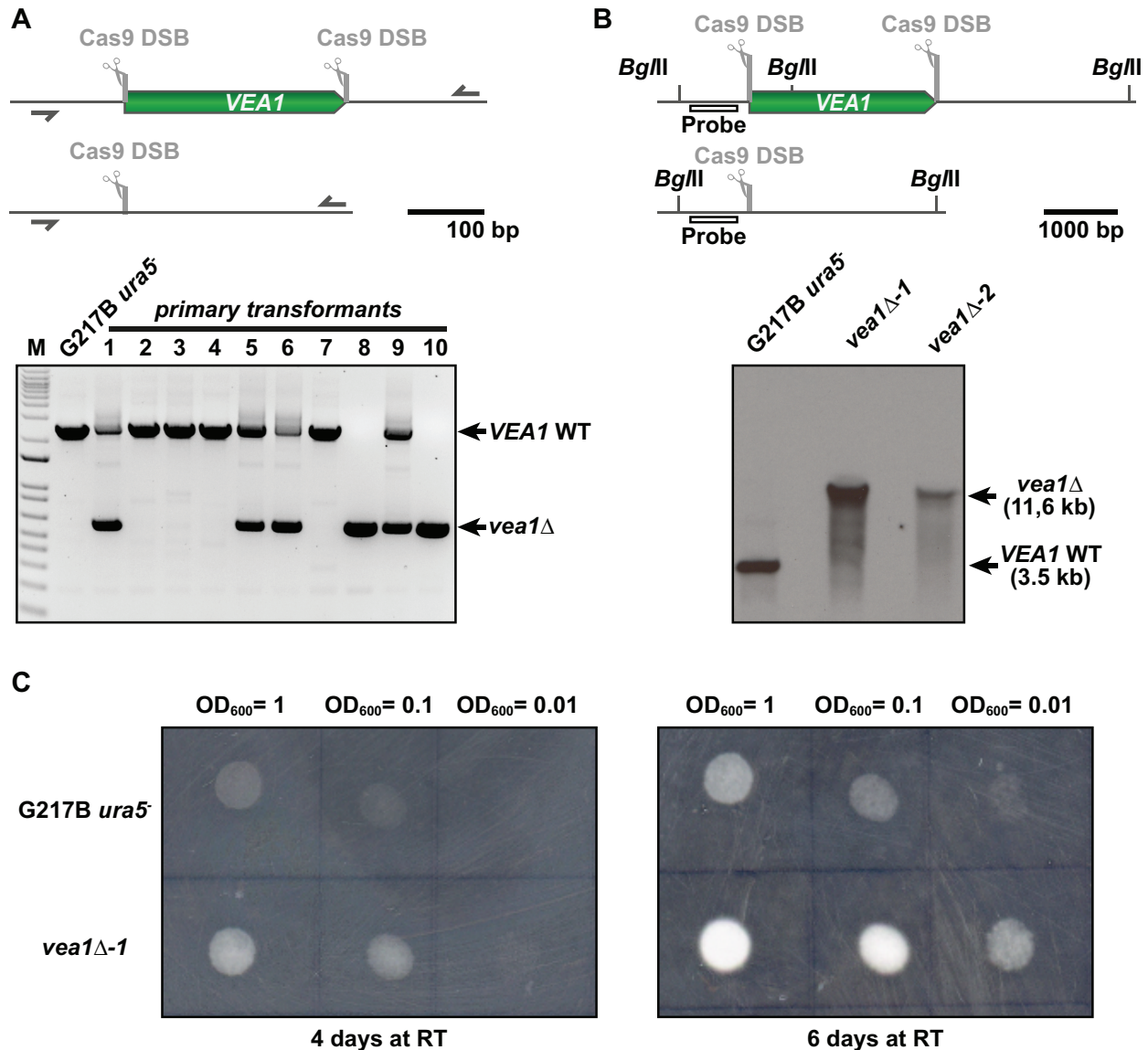


**Figure 4. CRISPR/Cas9 vector recycling allows successive gene disruptions and complementation.** (A) CRISPR/Cas9 vector recycling in the *sre1*<sup>-</sup> mutants allowed re-use of the *URA5* marker to target a second gene in the *sre1*<sup>-</sup> background. The loss of *SRE1* resulted in derepressed siderophore expression, which led to orange pigmentation of the colonies due to increased iron-bound siderophores, especially on high iron containing media. Disruption of *SID1* in the *sre1*<sup>-</sup> background, which encodes the first enzyme in the siderophore biosynthesis pathway (as diagrammed in B) resulted in complete abolishment of siderophore production, indicated by the loss of orange pigmentation of the colonies of the *sre1*<sup>-</sup> *sid1*<sup>-</sup> double mutants. (C) After loss of the Cas9 plasmid in the *sre1*<sup>-</sup> mutant, the *URA5* marker gene was available for selection of complementation plasmids. Siderophore production was analyzed with the CAS assay and showed partial complementation when *SRE1* was expressed under its native promoter and almost complete complementation when *SRE1* was overexpressed under the control of the constitutive *GAPDH* promoter (OE). In contrast, both *sid1*<sup>-</sup> and *sre1*<sup>-</sup> *sid1*<sup>-</sup> double mutants showed no siderophore production as expected.



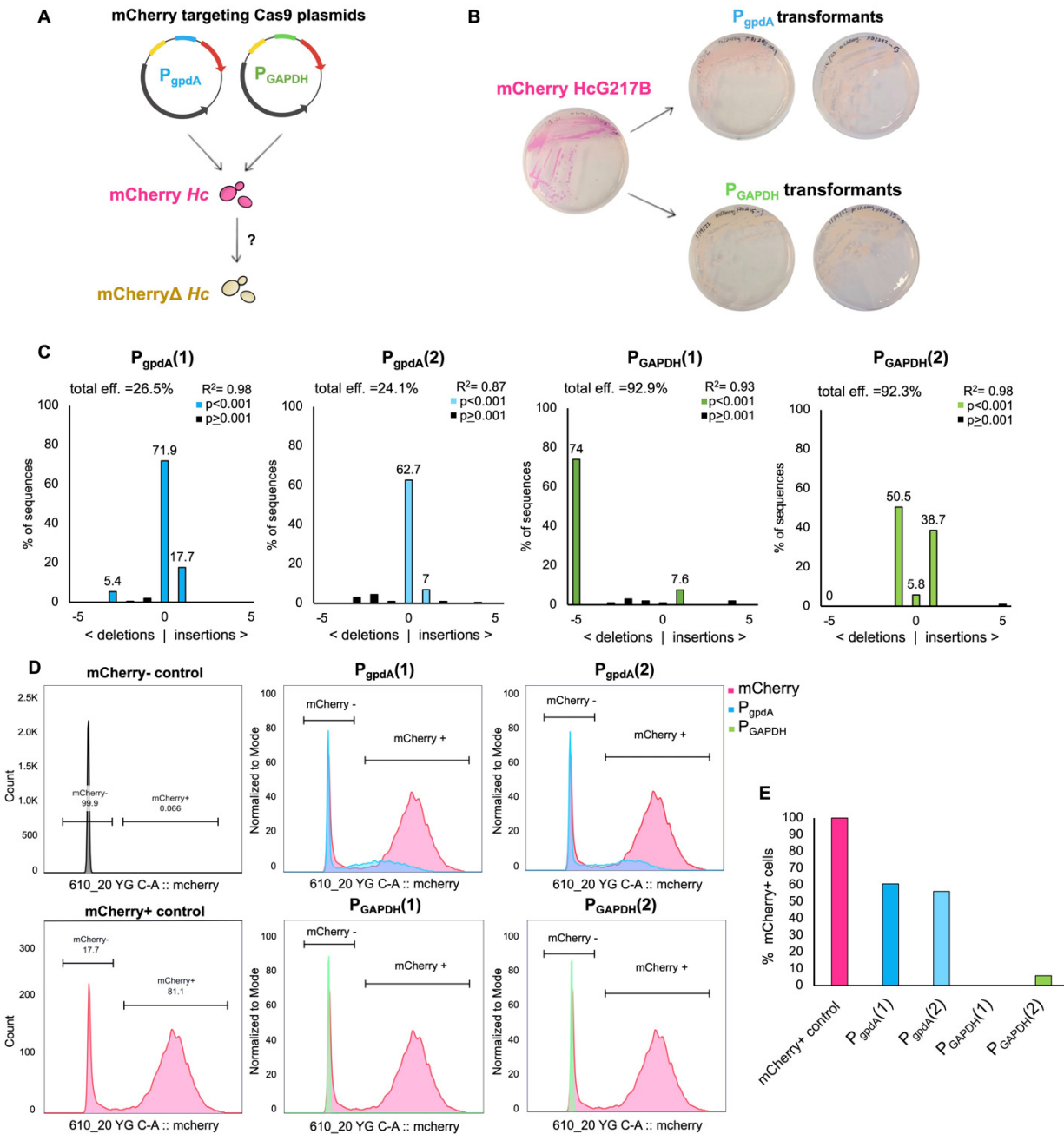
### Figure 5. Validation of the CRISPR/Cas9 system via whole genome sequencing.

We compared the genomes of two CRISPR/Cas9 generated mutants (*sre1-1*, *sre1-21*) with Cas9 only expressing control strains (*Cas9-1*, *Cas9-2*) and the parental strain from which we extracted gDNA for deep sequencing either before (*G217B\_ura5\_old*) or after passaging along with the CRISPR mutants (*G217B\_ura5\_new*). The diagram shows the strain lineages and whether strains were transformed and passaged for gene-disruption or control. Strain unique variant are given in red parenthesis including the targeted CRISPR/Cas9 mutation.



**Figure 6. sgRNA multiplexing for complete gene deletions.** We introduced two sgRNA cassettes into the Cas9 expressing vector, targeting the 5'- and 3'-end of the *VEA1* CDS respectively. Colony PCR of primary transformants (lower part of panel A) resulted in two bands for 4 of 10 isolates and only one band for two isolates, indicating partial or complete loss of the *VEA1* CDS in those mutants. WT indicates wild-type. (B) Southern hybridization of *vea1*Δ mutants confirmed the deletion of the *VEA1* CDS in two independent mutants. Location of the probe is shown in the upper part of panel B and the Southern blot is shown in the lower part of panel B. WT indicates wild-type. (C) Phenotypical characterization of the mutants grown at room temperature (RT) was performed by spotting dilutions of wild-type (*G217B ura5*) and *vea1*Δ mutant strains on plates. The *vea1*Δ mutants displayed accelerated filamentation as compared to the parental strain as indicated by the increased density of fluffy white filamentous

growth. The difference between wild-type and mutant strains was most pronounced after six days of incubation.



**Figure 7. Cas9-sgRNAs expressed under the control of the *Histoplasma* GAPDH promoter showed increased editing efficiency.** A) Schematic displaying experimental approach. Plasmids containing either the *A. nidulans* GAPDH promoter adapted from Nødvig et al. 2015 ( $P_{gpdA}$ ) or the native *Histoplasma* GAPDH promoter ( $P_{GAPDH}$ ) driving the expression of an identical mCherry targeting guide RNA were transformed into the mCherryHcG217B strain and used to assess differences in editing efficiencies. B) Plate images of the parental mCherryHcG217B strain alongside transformants selected from each plasmid transformation that were subsequently



used for Sanger sequencing and flow cytometry. C) Indel spectrum of two individual isolates obtained from each plasmid transformation. Sanger sequencing of the mCherry locus was used to conduct TIDE analysis (<https://tide.nki.nl/>) to quantify the frequency of insertions and deletions following the predicted cut site. D) Histograms displaying measurements of mCherry signal acquired through flow cytometry. Overlay plots display normalized counts for each isolate in comparison to the starting mCherryHcG217B strain. E) Quantification of flow data displaying percentage of mCherry<sup>+</sup> cells within each isolate.