Cas9/CRISPR genome editing to demonstrate the contribution of Cyp51A Gly138Ser to azole resistance in *Aspergillus fumigatus*

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Running head: Involvement of Cyp51A G138S in *A. fumigatus* azole resistance

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Abstract

Azole resistance in *Aspergillus fumigatus* is predominantly associated with increased expression of Cyp51A (lanosterol 14α-demethylase), the target enzyme of azole antifungal agents, or with single-nucleotide polymorphisms (SNPs) in *cyp51A*. Although several SNPs that may be linked to low susceptibility in azole-resistant isolates have previously been reported, few studies have been conducted to conclusively demonstrate the contribution of SNPs to decreased azole susceptibility. An *A. fumigatus* strain was isolated from the sputum of a 74-year-old male receiving long-term voriconazole treatment for chronic progressive pulmonary aspergillosis. Etest antifungal susceptibility testing showed low susceptibility to voriconazole, itraconazole, and posaconazole. Nucleotide sequencing of *cyp51A* from this isolate revealed the mutations Gly138Ser (GGC→AGC) and Asn248Lys (AAT→AAA) compared with the *cyp51A* of azole-susceptible isolates. PCR-amplified DNA fragments containing *cyp51A* with or without the mutations of interest and a hygromycin marker were simultaneously introduced along with the Cas9 protein and *in vitro*-synthesized single-guide RNA into protoplasts of the azole-resistant/susceptible strains. Etest azole susceptibility testing of recombinant strains showed an increased susceptibility via the replacement of Ser138 by glycine. In contrast, azole susceptibility was slightly decreased when a Ser138 mutation was introduced into the azole-susceptible strain AfS35, indicating that the serine at position 138 of Cyp51A contributes to low susceptibility in the azole-resistant isolate. Genetic recombination, which has been hampered thus far in clinical isolates, can now be achieved using Cas9/CRISPR genome editing. This technique could be useful to investigate the contribution of other SNPs of *cyp51A* to azole resistance.

Introduction

The filamentous fungi *Aspergillus fumigatus* is the most common opportunistic human fungal pathogen, with a wide range of clinical features including invasive pulmonary aspergillosis, chronic progressive pulmonary aspergillosis (CPPA), and allergic bronchopulmonary aspergillosis (1). Triazole antifungal drugs are the most common treatment for *A. fumigatus* infection. Itraconazole (ITC) and voriconazole (VRC) are the only oral drug treatment options for aspergillosis, which may lead to long-term administration. Since the discovery of the first ITC-resistant isolate in 1997 (2), epidemiological reports of new triazole-resistant isolates have been increasing worldwide (3). Mechanisms of acquired azole resistance may be explained by extended periods of azole exposure in the host or by environmental exposure of *A. fumigatus* to agricultural fungicides.

The primary molecular mechanisms of triazole resistance in *A. fumigatus* isolates are mutations that alter the target protein Cyp51A and prevent its interaction with the drug (4). Mutations in *cyp51A* may be classified as single-nucleotide polymorphisms (SNPs) and/or tandem repeats in the promoter region (3). The major SNPs affecting Cyp51A are positioned at Gly54, Gly138, Met220, and Gly448. The clinical isolates having these SNPs demonstrate various azole susceptibility profiles; for example, isolates with SNPs at Gly54 show resistance to ITC and varied susceptibility to posaconazole (POS) and VRC, whereas isolates with SNPs at Gly138 show resistance to pan-azoles, including ITC, POS, and VRC. Another alteration is a tandem repeat in the promoter region that results in the overexpression of *cyp51A*. Two major classes of such azole-resistant mutants are TR34/Leu98His and TR46/Tyr121Phe/Thr289Ala, which carry a 34-bp and a 46-bp sequence duplication, respectively, as well as amino acid substitutions.

Although many SNPs in *cyp51A* that may be linked to low susceptibility in azole-resistant isolates have been previously reported, few studies have been conducted to conclusively demonstrate the contribution of SNPs to decreased azole susceptibility in clinical isolates. One obstacle affecting the molecular analysis of clinical *A. fumigatus* isolates is the production of genetically manipulated mutants, as the efficiency of homologous recombination is extremely low.

Cas9/CRISPR (the clustered regularly interspaced short palindromic repeats) is essentially a bacterial defense system for adaptive immunity against invading nucleic acids and has been applied as a powerful genome editing tool in various organisms (5). By forming a ribonucleoprotein complex with an artificial single-guide RNAs (sgRNAs) designed to target a cellular gene, the Cas9 nuclease efficiently introduces double-stranded breaks (DSBs) at the corresponding target locus (6). The sgRNA hybridizes to its complementary DNA sequence, immediately upstream of the protospacer adjacent motif (PAM), which consists of NGG for the *Streptococcus pyrogenes* Cas9 variant (7). DSBs in the target genomic DNA can be repaired either by homology-directed repair or non-homologous end joining (NHEJ) (5, 8, 9). DNA repair via homology-directed repair requires a homologous DNA template with sequence similarity to the adjacent region of the DSB locus, whereas NHEJ ligates the DSB, leading to indels in a template-independent manner. The Cas9/CRISPR system has also been successfully applied to *A. fumigatus* (10-12).

In the present study, we investigated the antifungal mechanisms of a pan-azole-resistant strain isolated from a patient receiving long-term VRC treatment for CPPA. Although two

92 polymorphisms were found in *cyp51A* from the isolate, it was unclear which of the SNPs
93 contributed to low azole susceptibility. We genetically demonstrated that one of the SNPs
94 predominantly contributes to low susceptibility in the azole-resistant clinical isolate using a
95 Cas9/CRISPR genome editing technique.

Methods

***A. fumigatus* strains and media.** The *A. fumigatus* strains used in the present study are listed in Table 1. A clinical isolate NIID0345 was obtained in 2016 from the sputum sample of a 74-year-old male patient with CPPA, who had received VRC treatment for 3 years. *A. fumigatus* cultures were routinely grown in *Aspergillus* minimal medium (AMM: 10 g glucose, 0.516 g KCl, 0.516 g $MgSO_4 \cdot 7H_2O$, 1.516 g KH_2PO_4, 1.516 g $Mg(NO_3)_2 \cdot 6H_2O$, 1 mL trace elements (13) in 1 L distilled water), Czapek-Dox medium (CD, BD Difco Laboratories Inc., Franklin Lakes, NJ), YG medium (13), or potato dextrose agar medium (PDA, BD Difco). For solid medium, 1.5% agar was added. *A. fumigatus* conidia were obtained from mycelia cultured on AMM or PDA at 30˚C for 3–7 days, harvested with PBS containing 0.05% (v/v) Tween 20 and 20% (v/v) glycerol, and filtered through a 40-µm nylon cell strainer (Greiner Bio-One, Germany).

DNA extraction, PCR, and sequencing. Genomic DNA extractions and purifications were performed using a DNeasy Plant Mini Kit (QIAGEN, Germany). Primers for the amplification and sequencing of *cyp51A* are listed in Table 2. Identification was confirmed by sequencing of the internal transcribed spacer (ITS) and D1/D2 regions and the β-tubulin gene. PCR amplification of *cyp51A* was performed using NIID0345 genomic DNA as a template and primers Discheck5 and Discheck3 using Q5 Hot Start High-Fidelity 2× Master Mix (New England Biolabs, Ipswich, MA).

sgRNA *in vitro* synthesis. We manually searched for target sequences consisting of G(N)15(A/T)(N)3NGG near the N-terminus (for sgRNA1) and C-terminus (for sgRNA2) as

sgRNA target sequences and synthesized two oligonucleotides (T7-sgRNA1 and T7-sgRNA2, Table 2) consisting of the T7 promoter, sgRNA target sequence, and overlap sequence with Cas9 scaffold. These oligonucleotides were used for sgRNA synthesis via the EnGen® sgRNA Synthesis Kit, *S. pyogenes* (New England Biolabs). The synthesized sgRNAs were purified using an RNA clean & concentrator-25 (Zymo Research, Irvine, CA), quantified using a QuantiFluor RNA system (Promega, Madison, WI) and Quantus Fluorometer (Promega), and used for ribonucleoprotein formation with Cas9.

Repair templates. A pHph plasmid harboring a hygromycin B resistance cassette (*hph*) was generated by deletion of two *loxP* sequences and HSV1 thymidine kinase sequences from pSK397 (14). Primers for the repair template construction are listed in Table 2. A region from 825-bp upstream to 1503-bp downstream of the *cyp51A* coding region was used for repair templates. The mutations and *hph* marker were introduced via PCR sewing or overlap extension PCR. The *hph* marker for selection of transformants was inserted between nucleotides 500 and 501 downstream of the *cyp51A* stop codon. Q5 Hot Start High-Fidelity 2× Master Mix (New England Biolabs) was used for PCR amplification. Primer combinations for overlap extension PCR are listed in Table 3. Briefly, NIID0345 or AfS35 genomic DNA was used as a template to generate overlapping PCR products with the corresponding site-specific mutations or junctions between *cyp51A* and the *hph* marker. The overlapping PCR products were mixed together and used as a template in the PCR-sewing step using the primers LFH1 and LFH4. Overlapping PCR product combinations are listed in Table 4. The fused PCR products were purified using a NucleoSpin® Gel and PCR Clean-up kit (Takarabio, Japan) and used for *A. fumigatus* protoplast transformation.

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143 ***A. fumigatus* transformation.** *A. fumigatus* protoplasts were generated and fungal
144 transformation was performed as previously described (13), with slight modifications. Briefly,
145 conidia were incubated in YG medium for 6 h at 37°C. Following incubation, the cell walls of
146 germlings were digested with 0.2 g/mL VinoTaste Pro (Novozymes, Denmark) for 1 h at 30°C;
147 20 pmol Cas9-NLS protein (New England Biolabs) and 10 pmol each *in vitro*-synthesized
148 sgRNA1 and sgRNA2 were mixed and incubated for 25 min, generating ribonucleoproteins
149 (RNPs). Protoplasts were transformed with 2–3 μg of repair templates and RNPs and plated onto
150 CD supplemented with 1 M sucrose. Using NIID0345 clinical isolate as a host, repair templates
151 0345-mut1-S138-K248-mut2-hph, 0345-mut1-G138-K248-mut2-hph,
152 0345-mut1-S138-N248-mut2-hph, or 0345-mut1-G138-N248-mut2-hph were used to generate
153 strains NIID0345-mut1-2, NIID0345-S138G, NIID0345-K248N, or NIID0345-S138G-K248N,
154 respectively. Using AfS35 strain as a host, repair templates 35-mut1-G138-mut2-hph or
155 35-mut1-S138-mut2-hph were used to generate strains AfS35-mut1-2 or AfS35-G138S,
156 respectively. Following a 15-h incubation at 37°C, plates were overlaid with CD top agar
157 containing 400 μg/mL hygromycin. Positive colonies were confirmed by colony PCR using
158 KOD FX Neo DNA polymerase (TOYOBO, Japan) with the primers Discheck5 and Discheck3
159 (which were designed at the region outside the repair template sequence), followed by nucleotide
160 sequencing of *cyp51A*, including the promoter region.

161

162 **Antifungal susceptibility testing.** Susceptibility to VRC, ITC, and POS were evaluated with
163 Etest strips according to the manufacturer's instruction (Biomerieux, France). Strains were
164 grown at 37°C, and growth inhibition was visually evaluated after 48 h. Susceptibility tests were

165 performed in three independent Cyp51A-sequence-confirmed transformants for each strain.

Results

Gly138Ser and Asn248Lys were found in the Cyp51A sequence of an azole-resistant clinical isolate.

The susceptibilities of the clinical isolate from a patient with CPPA were determined by Etest methods. The isolate was not susceptible to VRC, ITC, or POS (Fig.1), but was susceptible to amphotericin B, micafungin, and caspofungin (data not shown). We identified NIID0345 as an *A. fumigatus* strain via sequence analysis of the ITS and D1/D2 regions and the β-tubulin gene. Comparison of *cyp51A* from the azole-resistant isolate (NIID0345) with those from azole-susceptible strains (Af293 and AfS35) revealed that NIID0345 carried two amino acid substitutions: Gly138Ser (GGC→AGC) and Asn248Lys (AAT→AAA). These results indicated that one or both of these SNPs may be responsible for azole resistance.

Cas9/CRISPR-mediated substitution of Serine at 138 to Glycine in *cyp51A* of NIID0345.

To verify which SNP is involved in azole resistance, we substituted the nucleotide sequences corresponding to amino acid Ser138 and/or Lys248 in the clinical isolate NIID0345. We attempted to replace the genomic *cyp51A* gene locus with a linear DNA fragment harboring mutations by homologous recombination. It is well known that the rate of homologous recombination is very low in a host strain having an NHEJ repair pathway, such as clinical isolates. In addition, the Cas9/CRISPR system can be used to increase the efficiency of homologous recombination in *Candida glabrata* (15). Therefore, we used Cas9/CRISPR to create double-stranded breaks close to the N- and C-terminus of the *cyp51A* coding region, and to facilitate the replacement of the genomic *cyp51A* locus with a repair template DNA fragment via homologous recombination. Off-target effects are commonly encountered when Cas9 and

189 sgRNA are continuously expressed via the introduction of plasmid or DNA; however, recent
190 studies have shown that off-target effects were reduced by the direct introduction of the Cas9
191 protein and synthesized gRNA (16). Successful genome editing via the introduction of
192 Cas9-gRNA ribonucleoprotein has also been reported in filamentous fungi, such as *A. fumigatus*.
193 In the repair template DNA fragment, the nucleotide sequence responsible for amino acid
194 substitutions thought to confer azole resistance in NIID0345 was substituted for a nucleotide
195 sequence corresponding to that present in the azole-sensitive isolates. Furthermore, the construct
196 was made Cas9/CRISPR resistant by introducing nuclease-resistant silent mutations into the two
197 *cyp51A* gRNA target sites (Fig. 2). Cas9/sgRNA ribonucleoproteins and the repair template were
198 simultaneously transformed via the protoplast-polyethylene glycol method into the *A. fumigatus*
199 azole-resistant clinical isolate, and the transformants were selected with hygromycin. By
200 confirming the nucleotide sequence of colony-directed PCR fragments of *cyp51A*, four kinds of
201 recombinant strains were produced: a strain with only the nuclease-resistant mutation, those with
202 Ser138Gly substitution, those with Lys248Asn substitution, and those with both Ser138Gly and
203 Lys248Asn substitutions.
204
205 Next, we examined azole susceptibility testing using Etest strips on the constructed recombinant
206 strains. The strains in which only nuclease-resistant silent mutations were introduced
207 demonstrated a similar azole-resistant profile as the parental strain NIID0345 (Fig. 3A),
208 indicating that Cas9/CRISPR-mediated homologous recombination had no effect on azole
209 susceptibility. Both of the recombinant strains with Ser138Gly and Ser138Gly/Lys248Asn amino
210 acid substitutions showed increased susceptibilities to all azoles tested, whereas the strain with
211 only Lys248Asn substitution showed a similar azole-resistant profile to the parental clinical

isolate. These results indicate that Lys248 is not associated with azole resistance, and Ser138 is responsible for azole resistance in this clinical isolate.

Gly 138 was substituted in the azole-susceptible strain AfS35.

To verify whether Gly138 in Cyp51A is responsible for azole resistance, amino acid substitution of Gly138 to serine was introduced into the azole-susceptible strain AfS35. The method to produce the recombinant strain was the same as above; however, highly efficient homologous recombination was expected because the strain AfS35 is deficient in the NHEJ repair system. As expected, almost all transformants exhibited ideal recombination. We constructed two kinds of recombinant strains: a strain with only nuclease-resistant silent mutations and one in which Gly138 was substituted with Ser138. Azole susceptibility testing by Etest of the recombinant strains showed a slight decrease in azole susceptibility when the Ser138 mutation was introduced into the azole-susceptible strain AfS35 (Fig. 3B). The strain with only nuclease-resistant silent mutations demonstrated a similar azole susceptibility profile as the parental strain AfS35. From these results, we elucidated the direct involvement of Gly138 in Cyp51A in azole resistance, which had previously been supported only by indirect epidemiological evidence.

Discussion

The Cas9/CRISPR genome editing technique used in this study has enabled site-directed mutagenesis, altering Ser138 to glycine on the genomic Cyp51A locus in an azole-resistant clinical strain. This is, to our knowledge, the first example of site-directed mutagenesis performed in a clinical, azole-resistant fungal isolate to elucidate whether azole susceptibility is altered by mutations in the genomic Cyp51A locus. Although many azole-resistant isolates with SNPs in *cyp51A* have been identified, there have been few studies that genetically confirm their correspondence to low azole susceptibility. Although genetically recombinant strains harboring mutations such as TR34-Leu98His (17), TR46-Tyr121Phe-Thr289Ala (18), Gly54Trp (17), and Thr301Ile (19) in the genomic Cyp51A locus have been reported to date, all these strains were constructed in the *akuB*/Ku80-deficient strain as a recipient. It is well known that wild-type strains, such as clinical isolates, tend to exhibit low efficiency in homologous recombination, largely because of high NHEJ activity. To overcome this limitation, the gene encoding either KU70 or Ku80, which are the components of NHEJ machinery, was knocked out, leading to a significant increase in the frequency of homologous recombination (14, 20). In contrast, our method using Cas9/CRISPR can facilitate efficient homologous recombination without the inactivation of the NHEJ pathway, which is supported by previous studies, concluding that the frequency of homologous recombination can be increased by the Cas9/CRISPR system in *C. glabrata* (15).

To build the Cas9/CRISPR system in *A. fumigatus* clinical isolates, we incorporated several additional methods to improve the efficiency and accuracy of *cyp51A* gene replacement events. Improved efficiency of *cyp51A* replacement was achieved by introducing two DSBs via the

251 design of two target sequences for sgRNA at sites close to the N-terminus and C-terminus,
252 repressing homologous recombination within the *cyp51A* coding region. Additionally, to avoid
253 digestion of the repair template and re-digestion of the edited target after the homologous
254 recombination event, nuclease-resistant silent mutations were introduced in two loci of three
255 codons immediately upstream from the PAM sites of the repair template, preventing it from
256 being targeted by Cas9/CRISPR(21). To minimize off-target effects from DNA-based continuous
257 Cas9 and sgRNA expression (which should be considered whenever Cas9/CRISPR system is
258 used for genome editing), we introduced ribonucleoproteins consisting of commercially available
259 recombinant Cas9 protein and *in vitro*-synthesized sgRNAs directly into protoplasts of clinical
260 isolates. As one means of minimizing off-target effects, directly transfected Cas9 protein reduces
261 the off-target cleavage rate when compared with Cas9 expression by a plasmid or mRNA
262 transfection in mammalian cells (16). One recent study has demonstrated that direct delivery of
263 Cas9–gRNA ribonucleoprotein can facilitate genome editing in *A. fumigatus* (10). Based on these
264 improvements, we produced a simple, efficient, and accurate site-directed mutagenesis system to
265 investigate structure–phenotype relationships of the azole target Cyp51A. Since this system can
266 be applied to numerous genes other than *cyp51A*, this method will accelerate the progress of
267 many pathogenic fungal studies.
268
269 Multiple Gly138 polymorphisms in *cyp51A* have been identified in *A. fumigatus* azole-resistant
270 isolates, most of which alter Gly/GGC to Cys/TGC and Ser/AGC (22-25). Although
271 *Saccharomyces cerevisiae* expressing *cyp51A* with the Gly138Cys mutation showed reduced
272 susceptibility to all three azoles compared with the control strain, no genetic studies using *A.*
273 *fumigatus* as a host have been performed to date to investigate the function of Gly138 in

Cyp51A. We isolated an azole-resistant *A. fumigatus* strain from a CPPA patient with long-term VRC treatment and identified two amino acid substitutions—Gly138Ser and Asn248Lys—which, when compared with Cyp51A nucleotide sequences from azole-susceptible strains, may be potential polymorphisms conferring azole resistance. According to the homology model structure of the Cyp51A protein, Gly138 is located in a channel 1 helix close to the heme cofactor, and a mutation at this position could disturb the heme environment, which may lead to multiple azole resistance (26, 27). Structure modeling and epidemiology have predicted that the Gly138Ser mutation is the amino acid responsible for azole resistance, and our Cas9/CRISPR gene replacement system has molecularly confirmed that the Ser138 is responsible for the pan-azole-resistant phenotype in the clinical isolate NIID0345. However, genome-edited mutants harboring Gly138 in the NIID0345 genetic background have exhibited much lower susceptibility to all azoles tested than the strain AfS35, in which the amino acid at position 138 is intrinsically glycine. Similarly, the genome-edited mutant harboring Ser138 in AfS35 genetic background has exhibited higher susceptibility than the strain NIID0345, in which the amino acid at position 138 is intrinsically serine. These results indicate that the strain NIID0345 may have low azole susceptibility for reasons other than alteration in *cyp51A*. Additional genomic analysis is needed to identify unknown, non-Cyp51A mechanisms of azole resistance in the clinical isolate NIID0345.

In conclusion, we have developed a simple, efficient, and accurate gene replacement system using Cas9/CRISPR genome editing techniques, and applied these techniques to investigate the mechanisms of azole resistance via Cyp51A alteration. We confirm at the molecular level that the Gly138Ser mutation is one reason for azole resistance in a clinical isolate. There are many

297 *cyp51A* mutations that may result in potential, but unconfirmed amino acid changes conferring
298 azole resistance. Further investigation of Cyp51A using our Cas9/CRISPR system is required to
299 verify whether the diverse SNPs reported to date are in fact responsible for azole resistance.
300
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306 We declare the authors have no conflicts of interest.

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389

Figure Legends

Figure 1. Antifungal susceptibility testing using Etest strips for voriconazole, itraconazole, and posaconazole in the azole-susceptible *Aspergillus fumigatus* strain AfS35 and clinical azole-resistant *A. fumigatus* strain NIID0345. The number below each photo represents the MIC (μg/mL).

Figure 2. Overview of genetic modification via Cas9/CRISPR-promoted homology-directed repair. (a) Cas9 protein and *in vitro*-synthesized sgRNAs were mixed to form two RNPs. The repair template and two RNPs were transformed into *Aspergillus fumigatus* protoplasts. (b) The dual Cas9–sgRNA complex introduced two double-stranded breaks at the N-terminus and C-terminus of *cyp51A* (c and d). The cleaved *cyp51A* on the genomic DNA is replaced by the repair template, resulting in the introduction of the desired mutations and *hph* marker. The silent mutations mut1 and mut2 on the repair template and the replaced genomic DNA cannot be cleaved by RNPs nuclease.

Figure 3. Antifungal susceptibility testing using Etest strips for voriconazole, itraconazole, and posaconazole for the strains generated via Cas9/CRISPR-promoted gene replacement from the strains NIID0345 (A) and AfS35 (B). The number below each photo represents the MIC (μg/mL).

409 **Table 1. *Aspergillus fumigatus* strains used in this study.**

Strain	Parent	Genotype[a]	Source
NIID0345	clinical isolate		Current study
NIID0345-mut1-2	NIID0345	mut1 mut2 *cyp51A hph*	Current study
NIID0345-S138G	NIID0345	mut1 S138G mut2 *cyp51A hph*	Current study
NIID0345-K248N	NIID0345	mut1 K248N mut2 *cyp51A hph*	Current study
NIID0345-S138G-K248N	NIID0345	mut1 mut2 *cyp51A hph*	Current study
AfS35	D141	*akuA*ΔloxP	Fungal genetic stock center
AfS35-mut1-2	AfS35	mut1 mut2 *cyp51A hph*	Current study
AfS35-G138S	AfS35	mut1 G138S mut2 *cyp51A hph*	Current study

410 [a] mut1 and mut2 are silent mutations for Cas9-nuclease resistance.

411 **Table 2. Oligonucleotide primers used in this study.**

Primer	Sequence (5′-3′) [a]	Orientation	Use
Discheck5	ATGCAGTGAAAAATTCCTAGCAG	Sense	cyp51A amplification and verification of transformants
Discheck3	ATGGTGGTGGTCAAGGTTCAGCAG	Antisense	cyp51A amplification and verification of transformants
T7-sgRNA1	TTCTAATACGACTCACTATAGCTATGGCTTACGGCCT ACAGTTTTAGAGCTAGA	Sense	in vitro sgRNA synthesis
T7-sgRNA2	TTCTAATACGACTCACTATAGGGATGAATAGTCAGTT TCAGTTTTAGAGCTAGA	Antisense	in vitro sgRNA synthesis
LFH1	GCGAGCCATGCTGGGAGGAATCTC	Sense	Repair template construction and amplification
Mut1-3	CGGCCATATACGCGGTAAGCCATAGCATCGGCAC	Antisense	Repair template construction
Mut1-5	GGCTTACCGCGTATATGGCCGTTGCGGTGCTGAC	Sense	Repair template construction
G138-3	TGAGTCAAGCCGTACTTGATGAACTTTTTCTGCTC	Antisense	Repair template construction
G138-5	CATCAAGTACGGCTTGACTCAGTCTGCGTTAGAG	Sense	Repair template construction
S138-3	TGAGTCAAGCTGTACTTGATGAACTTTTTCTGCTC	Antisense	Repair template construction
S138-5	CATCAAGTACAGCTTGACTCAGTCTGCGTTAGAG	Sense	Repair template construction
N248-3	GACGGCGCTGATTGATGATGTCAACGTAGATTGAC	Antisense	Repair template construction
N248-5	ACATCATCAATCAGCGCCGTCTTGACGGTGACAAG	Sense	Repair template construction
Mut2-3	AGTCAGTCTCTGGCACTCCTTTCTTTCCATCCAC	Antisense	Repair template construction
Mut2-5	AAGGAGTGCCAGAGACTGACTATTCATCCCTC	Sense	Repair template construction
LFH6	GATATCGGCCTGAGTGGCCTCCAGGTTTTCGCACGA GCTTCTCC	Antisense	Repair template construction
LFH3	GTTGTCGACGGCCATCTAGGCCAGTTTTTGATAGTCT TCAAAAGTCAG	Sense	Repair template construction
LFH4	CGTATTGGTGAGCTGATGATCATC	Antisense	Repair template construction and amplification
Hph5	GAGGCCACTCAGGCCGATATCACC	Sense	hph cassette amplification
Hph3	CTGGCCTAGATGGCCGTCGACAAC	Antisense	hph cassette amplification
proseq3	TTAGTAATTAGGCAACTTTCATTC	Antisense	cyp51A sequencing
seq1	CCAATGGTCTTTCATTGGGTC	Sense	cyp51A sequencing
seq1r	TCCCGTAACTGATGGTACTAC	Antisense	cyp51A sequencing
seq2	TTTACCGCTGCTCGAGCCCTC	Sense	cyp51A sequencing
seq2r	GGAACGAACTTCCTGGCCTTG	Antisense	cyp51A sequencing
seq3	AACTTCCCTTCCATCAACATG	Sense	cyp51A sequencing
seq3r	CGAATAACATGTTGATGGAAG	Antisense	cyp51A sequencing

412 [a] Letters in bold indicate the mutated nucleotide. Underlining indicates an additional sequence

413 for in vitro gRNA synthesis.

414 **Table3. Combination of primers for overlapping PCR used in this study.**

Name of PCR product	Primers	Template DNA
0345-A	LFH1/Mut1-3	NIID0345 genomic DNA
0345-mut1-G138	Mut1-5/G138-3	NIID0345 genomic DNA
0345-mut1-N248	Mut1-5/N248-3	NIID0345 genomic DNA
0345-mut1-mut2	Mut1-5/Mut2-3	NIID0345 genomic DNA
0345-G138-mut2	G138-5/Mut2-3	NIID0345 genomic DNA
0345-G138-N248	G138-5/N248-3	NIID0345 genomic DNA
0345-N248-mut2	N248-3/Mut2-3	NIID0345 genomic DNA
0345-C	Mut2-5/LFH6	NIID0345 genomic DNA
0345-B	LFH3/LFH4	NIID0345 genomic DNA
35-A	LFH1/Mut1-3	AfS35 genomic DNA
35-mut1-mut2	Mut1-5/Mut2-3	AfS35 genomic DNA
35-mut1-S138	Mut1-5/S138-3	AfS35 genomic DNA
35-S138-mut2	S138-5/Mut2-3	AfS35 genomic DNA
35-C	Mut2-5/LFH6	AfS35 genomic DNA
35-B	LFH3/LFH4	AfS35 genomic DNA
Hph	Hph5/Hph3	pHph plasmid DNA

415 **Table 4. Combination of PCR products for repair template amplification used in this study.**

Name of repair template	PCR products
0345-mut1-S138-K248-mut2-hph	0345-A, 0345-mut1-mut2, 0345-C, Hph, 0345-B
0345-mut1-G138-K248-mut2-hph	0345-A, 0345-mut1-G138, 0345-G138-mut2, 0345-C, Hph, 0345-B
0345-mut1-S138-N248-mut2-hph	0345-A, 0345-mut1-N248, 0345-N248-mut2, 0345-C, Hph, 0345-B
0345-mut1-G138-N248-mut2-hph	0345-A, 0345-mut1-G138, 0345-G138-N248, 0345-N248-mut2, 0345-C, Hph, 0345-B
35-mut1-G138-mut2-hph	35-A, 35-mut1-mut2, 35-C, Hph, 35-B
35-mut1-S138-mut2-hph	35-A, 35-mut1-S138, 35-S138-mut2, 35-C, Hph, 35-B

416

Fig. 1

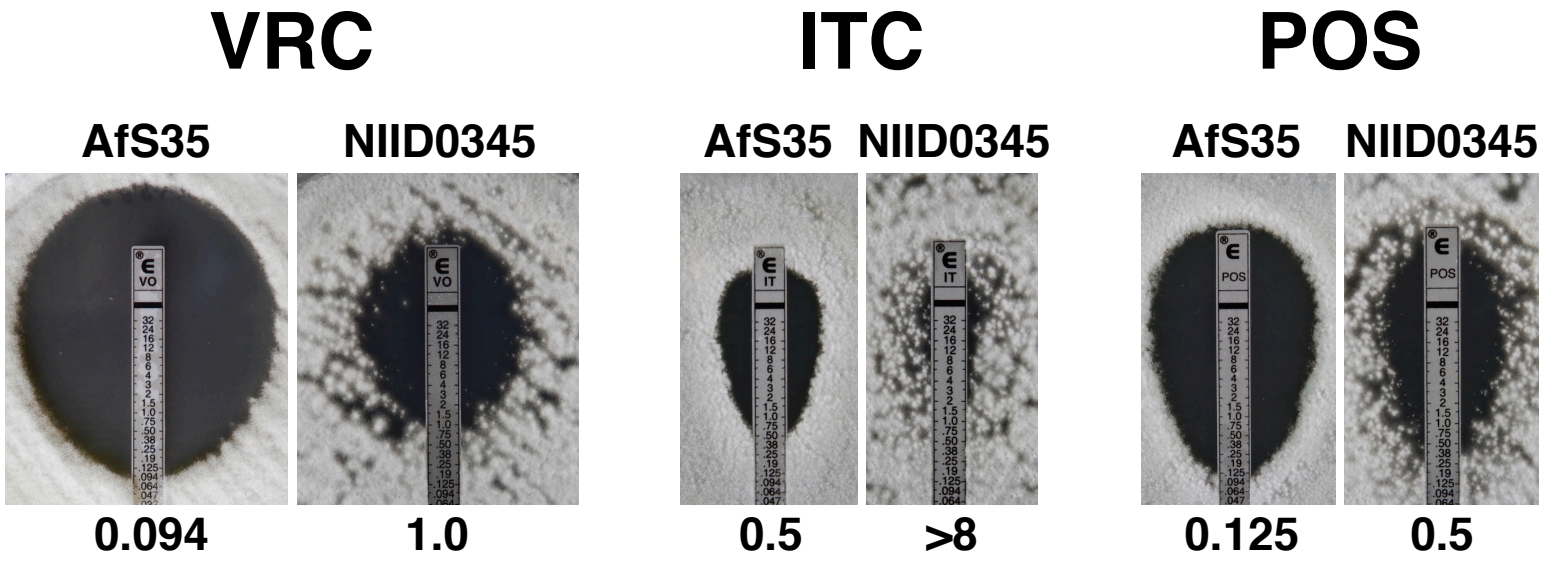

Fig. 2

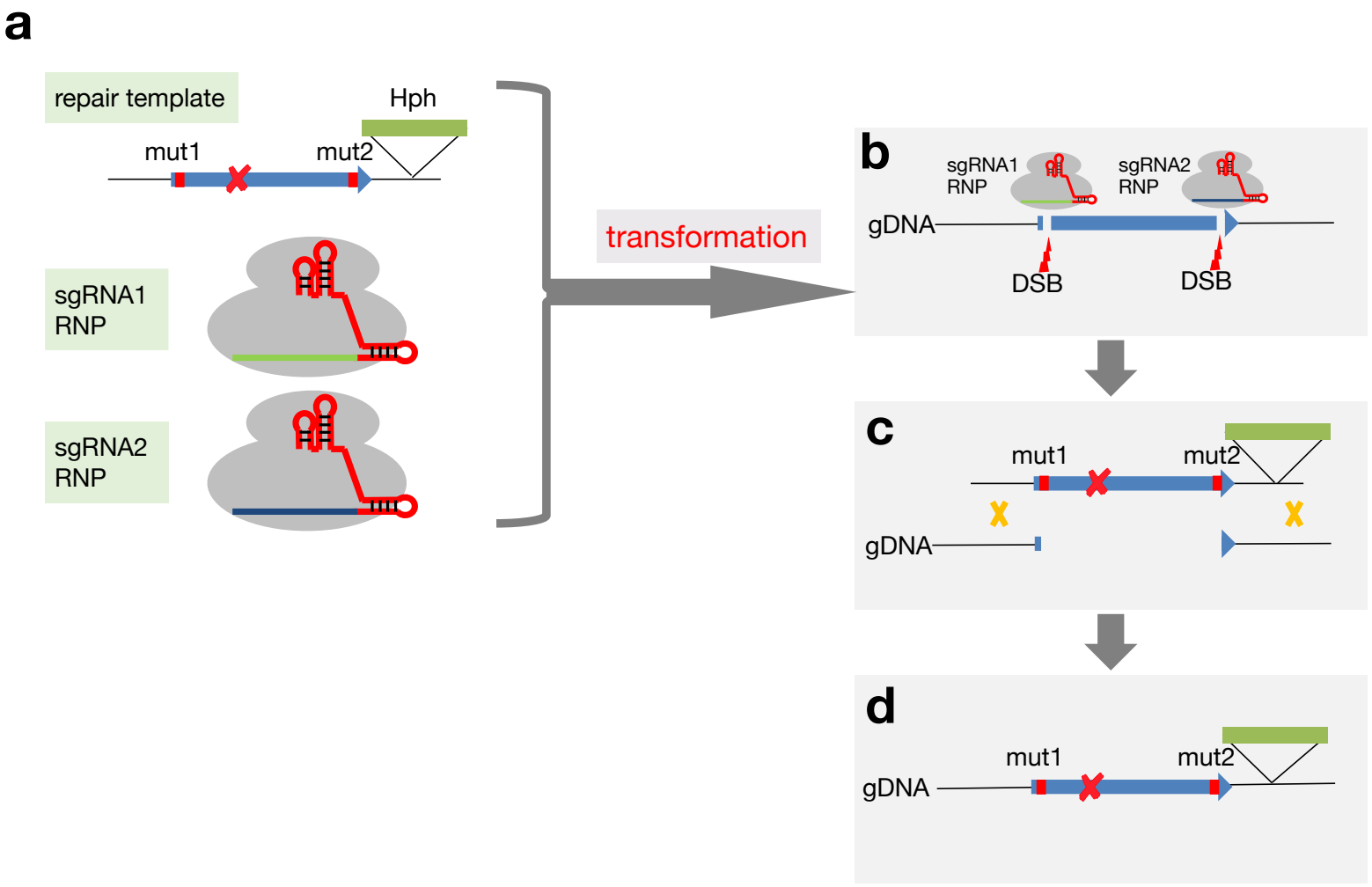

Fig. 3