Multiplex genetic engineering exploiting pyrimidine salvage pathway-based self-encoded selectable markers

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ABSTRACT

Selectable markers are indispensable for genetic engineering, yet their number and variety is limited. Most selection procedures for prototrophic cells rely on the introduction of antibiotic resistance genes. New minimally invasive tools are needed to facilitate sophisticated genetic manipulations. Here, we characterized three endogenous genes in the human fungal pathogen *Aspergillus fumigatus* for their potential as markers for targeted genomic insertions of DNAs-of-interest (DOIs). Since these genes are involved in uptake and metabolization of pyrimidines, resistance to the toxic effects of prodrugs 5-fluorocytosine and 5-fluorouracil can be used to select successfully integrated DOIs. We show that DOI integration, resulting in the inactivation of these genes, caused no adverse effects with respect to nutrient requirements, growth or virulence. Beside the individual use of markers for site-directed integration of reporter cassettes including the 17-kb penicillin biosynthetic cluster, we demonstrate their sequential use inserting three fluorescent protein encoding genes into a single strain for simultaneous multicolor localization microscopy. In addition to *A. fumigatus*, we validated the applicability of this novel toolbox in *Penicillium chrysogenum* and *Fusarium oxysporum*.

Enabling multiple targeted insertions of DOIs without the necessity for exogenous markers, this technology has the potential to significantly advance genetic engineering.

55 **INTRODUCTION**

56 Genetic engineering commonly involves the introduction of DNA-of-interest (DOI) into a target
57 cell followed by its integration into the genome. However, the efficiency of such genetic
58 transformations is typically very limited. Therefore, selection of successfully modified cells
59 usually involves co-transformation of selectable marker genes together with the DOI to allow
60 growth under selective conditions. Widely used marker cassettes either compensate for the
61 inability to synthesize vital metabolites (auxotrophic selection markers) or confer resistance to
62 growth inhibitory compounds such as antibiotics (dominant selectable markers) (Su et al. 2012).
63 While auxotrophic markers are restricted to auxotrophic recipients, dominant selectable
64 markers can be used for virtually any prototrophic recipient cell that is susceptible to the
65 antibiotic used for selection. Hence, dominant selectable markers play a crucial role in the
66 genetic manipulation of most wild-type cells. Examples of selectable markers commonly
67 employed for the genetic engineering of fungi include hygromycin B, phleomycin, pyrithiamine,
68 kanamycin and nourseothricin resistance conferring genes (Punt and van den Hondel 1992;
69 Kubodera et al. 2002; Kück and Hoff 2006). Their expression allows growth in the presence of
70 the corresponding antibiotic, which classifies them as positive selectable markers. Negative
71 selectable markers, in contrast, inhibit growth of the target cells during selective conditions. In
72 addition to the herpes simplex virus-1 thymidine kinase gene (Wigler et al. 1977; Borrelli et al.
73 1988), genes encoding cytosine deaminase (CD) and uracil phosphoribosyltransferase (UPRT)
74 have been employed as negative selectable markers in diverse organisms (Mullen et al. 1992;
75 Fox et al. 1999; van der Geize et al. 2008; Orr et al. 2012; Shi et al. 2013). The presence of
76 functional gene copies providing CD and UPRT activity renders host cells susceptible to
77 prodrugs 5-fluorocytosine (5FC) and 5-fluorouracil (5FU). CD and UPRT, both enzymes of the
78 pyrimidine salvage pathway, convert 5FC and 5FU into 5-fluorouridine monophosphate
79 (5FUMP) (Fig. 1a). Further metabolization of 5FUMP into toxic ribo- and deoxyribonucleotides
80 blocks cellular growth (Vermes et al. 2000).

81 Here, we characterized the pyrimidine salvage pathway in the human fungal pathogen
82 *Aspergillus fumigatus* and present its application for fungal genetic engineering. In the described

technology, endogenous genes encoding 5FC uptake, CD and UPRT serve as counterselectable markers for targeted, genomic introduction of multiple DOIs. Homologous recombination-driven replacement of marker genes by DOIs results in their inactivation, which can be selected *via* 5FC/5FU resistance. In addition to the individual use (e.g. integration of reporter cassettes as well as the 17-kb penicillin biosynthetic cluster), the potential sequential use of the three loci is demonstrated by the insertion of three different fluorescent protein-encoding genes for multicolor imaging of three cellular compartments. Demonstrating its versatile applicability, the described technology was implemented in the industrial work-horse *Penicillium chrysogenum* and the plant pathogen *Fusarium oxysporum*.

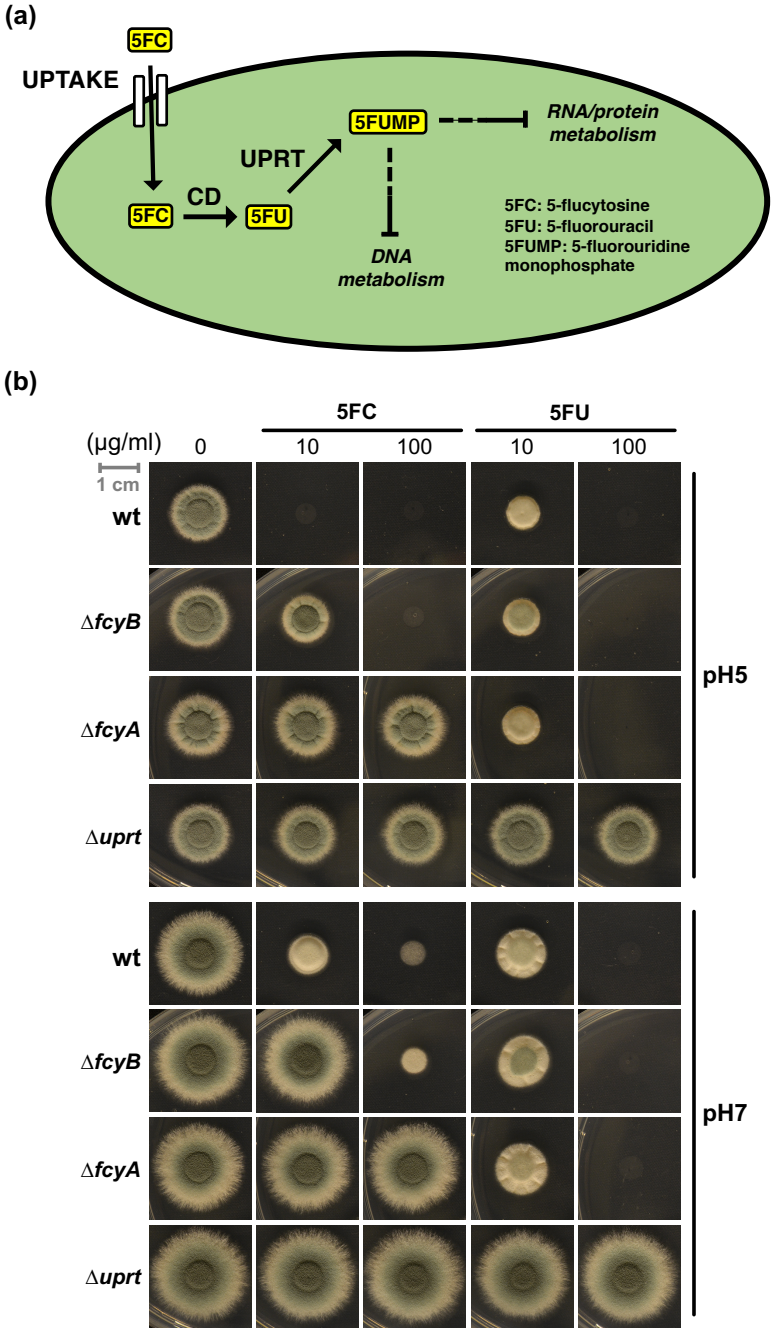

Fig 1. **Metabolization of 5FC and associated genetic factors in *A. fumigatus*.** (a) After uptake, 5FC is converted to 5FU by the enzyme cytosine deaminase (CD). Subsequently, 5FU is phosphoribosylated to 5FUMP by uracil phosphoribosyltransferase (UPRT). 5FUMP is further metabolized into RNA or DNA nucleotides that interfere with DNA, RNA as well as protein metabolism (Vermes et al. 2000). (b) Inactivation of genes encoding uptake (Δ*fcyB*), CD (Δ*fcyA*) and UPRT (Δ*uprt*) activity in *A. fumigatus* leads to different degrees of 5FC and 5FU resistance. For plate growth-based susceptibility testing, strains were point inoculated on solid AMM containing different levels of 5FC and 5FU. Images were acquired after 48 h incubation at 37 °C. Dashed lines indicate several enzymatic steps.

RESULTS

Cytosine deaminase FcyA and uracil phosphoribosyltransferase Uprt are crucial for the metabolic activation of 5FC in *Aspergillus fumigatus*

Metabolization of 5FC has been well-studied in the model yeast *Saccharomyces cerevisiae*: 5FC is converted by the CD Fcy1p to 5FU (Polak et al. 1976; Whelan 1987) and, subsequently, phosphoribosylated to 5FUMP by the UPRT Fur1p (Kern et al. 1990). Inactivation of each of these steps resulted in 5FC resistance, whereby inactivation of Fur1p also conferred 5FU resistance (Kern et al. 1990). Orthologous proteins from *S. cerevisiae* (Fcy2p), *A. fumigatus* and *Aspergillus nidulans* (FcyB), respectively, have been identified as major cellular 5FC importers (Paluszynski et al. 2006; Vlanti and Diallinas 2008; Gsaller et al. 2018).

Among other fungal species, *A. fumigatus* is susceptible to 5FC (Te Dorsthorst et al. 2004; te Dorsthorst et al. 2005) and is therefore anticipated to harbor genes encoding CD and UPRT activities in addition to 5FC uptake. BLASTP-based *in silico* predictions revealed *A. fumigatus* FcyA (AFUB_005410) and Uprt (AFUB_053020) as putative orthologs of yeast Fcy1p and Fur1p, respectively. To analyze their role in 5FC as well as 5FU metabolism, we inactivated *fcyA* and *uprt* in the *A. fumigatus* strain A1160P+ (Fraczek et al. 2013), termed wt here, using hygromycin and phleomycin resistance-based deletion cassettes. Due to the interdependency of 5FC activity and environmental pH (Te Dorsthorst et al. 2004; Gsaller et al. 2018), we investigated the contribution of both enzymes, as well as FcyB, to 5FC and 5FU activity at both pH5 and pH7.

Plate growth-based susceptibility testing revealed that, similar to previous work (Gsaller et al. 2018), 5FC levels ≥ 10 µg/ml blocked wt growth at pH5, while 100 µg/ml 5FC were

155 required at pH7 (Fig. 1b). Although FcyB is the major 5FC uptake protein, at 100 µg/ml 5FC
156 Δ*fcyB* was not able to grow at pH5 and showed severe growth inhibition at pH7. In contrast to
157 Δ*fcyB*, Δ*fcyA* and Δ*uprt* displayed full resistance to 5FC up to 100 µg/ml, regardless of the pH.
158 Furthermore, 100 µg/ml 5FU blocked growth of wt, Δ*fcyA* and Δ*fcyB* at pH5 as well as pH7, while
159 Δ*uprt* was resistant.

160 Our data confirm the role of FcyB as major 5FC cellular importer but indicate the presence
161 of additional uptake mechanisms. Similar to the orthologous proteins in *S. cerevisiae*, our
162 findings reveal the essential roles of FcyA and Uprt for 5FC activity and of Uprt for metabolic
163 activation of 5FU in *A. fumigatus*.

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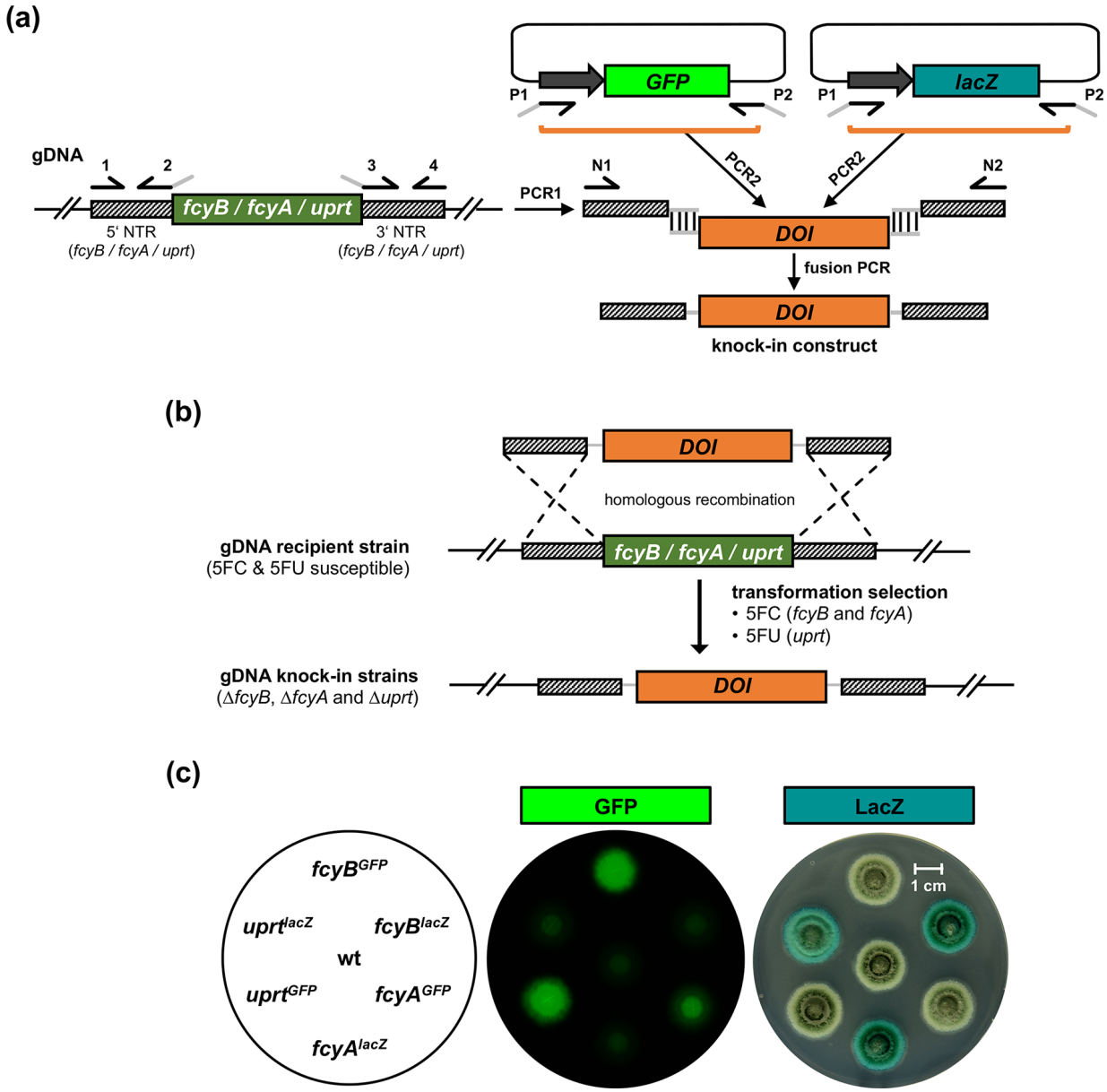
165 **Self-encoded loci *fcyB*, *fcyA* and *uprt* can be used for 5FC/5FU-based transformation**
166 **selection**

167 Lack of FcyB, FcyA (CD activity) or Uprt (UPRT activity) confers resistance to 5FC (Δ*fcyB*, Δ*fcyA*
168 and Δ*uprt*) or 5FU (Δ*uprt*) (Fig. 1b), which suggested the utilization of the encoding genes as
169 counterselectable markers for positive selection of cells with targeted integration of DOIs.
170 Moreover, the different degrees in 5FC resistance observed for Δ*fcyB* and Δ*fcyA* indicated that
171 5FC can be used for selection of loss of FcyB at low 5FC concentrations (10 µg/ml) and loss of
172 FcyA at high 5FC levels (100 µg/ml) (Fig. 1b). Selection for loss of Uprt was carried out at 100
173 µg/ml 5FU.

174 For proof-of-principle, both green fluorescent protein (GFP) and ß-galactosidase (LacZ)
175 expression cassettes were used to replace *fcyB*, *fcyA* as well as *uprt*. To achieve homologous
176 recombination-mediated replacement of these loci with the reporter cassettes, approximately 1
177 kb 5'- and 3'-non-translated regions (NTRs) of the respective gene were linked to each cassette
178 *via* fusion PCR (Fig. 2a). The yielding knock-in constructs were transformed into wt protoplasts,
179 which underwent selection for resistance to 5FC and 5FU (see above; Fig. 2b). Southern blot
180 analyses confirmed site-specific integration of the DOIs into each of the three loci (Fig. S1). In
181 agreement, all knock-in strains displayed resistance phenotypes according to the respective
182 mutation in the pyrimidine salvage pathway (compare Fig. 1b and Supplementary Fig. 2).

183 Fluorescence imaging and ß-galactosidase staining confirmed functionality of the knock-in
184 cassettes (Fig. 2c). To analyze the transformation efficiency using individual selectable marker
185 genes, we employed the corresponding LacZ knock-in constructs for each locus. For *fcyB*, *fcyA*
186 and *uprt*, 10, 27 and 13 transformants, respectively, were recovered on the corresponding
187 selective media (Supplementary Fig. 3). Out of these 10 (100%), 26 (97%) and 12 (92%)
188 displayed LacZ activity. Southern blot analysis of 10 LacZ-positive transformants for each locus
189 confirmed correct integrations.

190 Taken together, 5FC- and 5FU-mediated selection allowed replacement of each of the
191 three loci by either *GFP*- or *lacZ*-expression cassettes, which demonstrates the suitability of *fcyB*,
192 *fcyA* and *uprt* as selectable markers for targeted, integrative transformation in *A. fumigatus*.



193 Fig 2. **Replacement of self-encoded selectable markers *fcyB*, *fcyA* and *uprt* by DOIs.** (a)
194 Scheme of the generation of knock-in constructs. 5' and 3' NTR (PCR1) of the respective loci as
195 well as the DOIs (PCR2; *GFP* or *lacZ* reporter cassette) are amplified from genomic DNA (gDNA)
196 and plasmid DNA, respectively. Both NTRs and DOIs contain overlapping DNA (gray line) for
197 subsequent connection via fusion PCR, yielding the knock-in constructs. (b) Double crossover
198 homologous recombination-based replacement of *fcyB*, *fcyA* or *uprt* by DOIs. Transformation
199 selection was carried out using 5FC (*fcyB* and *fcyA* locus) or 5FU (*uprt* locus). (c) Visualization of
200 *GFP* as well as *LacZ* expression in the corresponding knock-in strains after incubation on solid
201 AMM for 48h at 37°C.
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204 ***fcyB*, *fcyA* and *uprt* can be consecutively used for multiple genomic integrations**

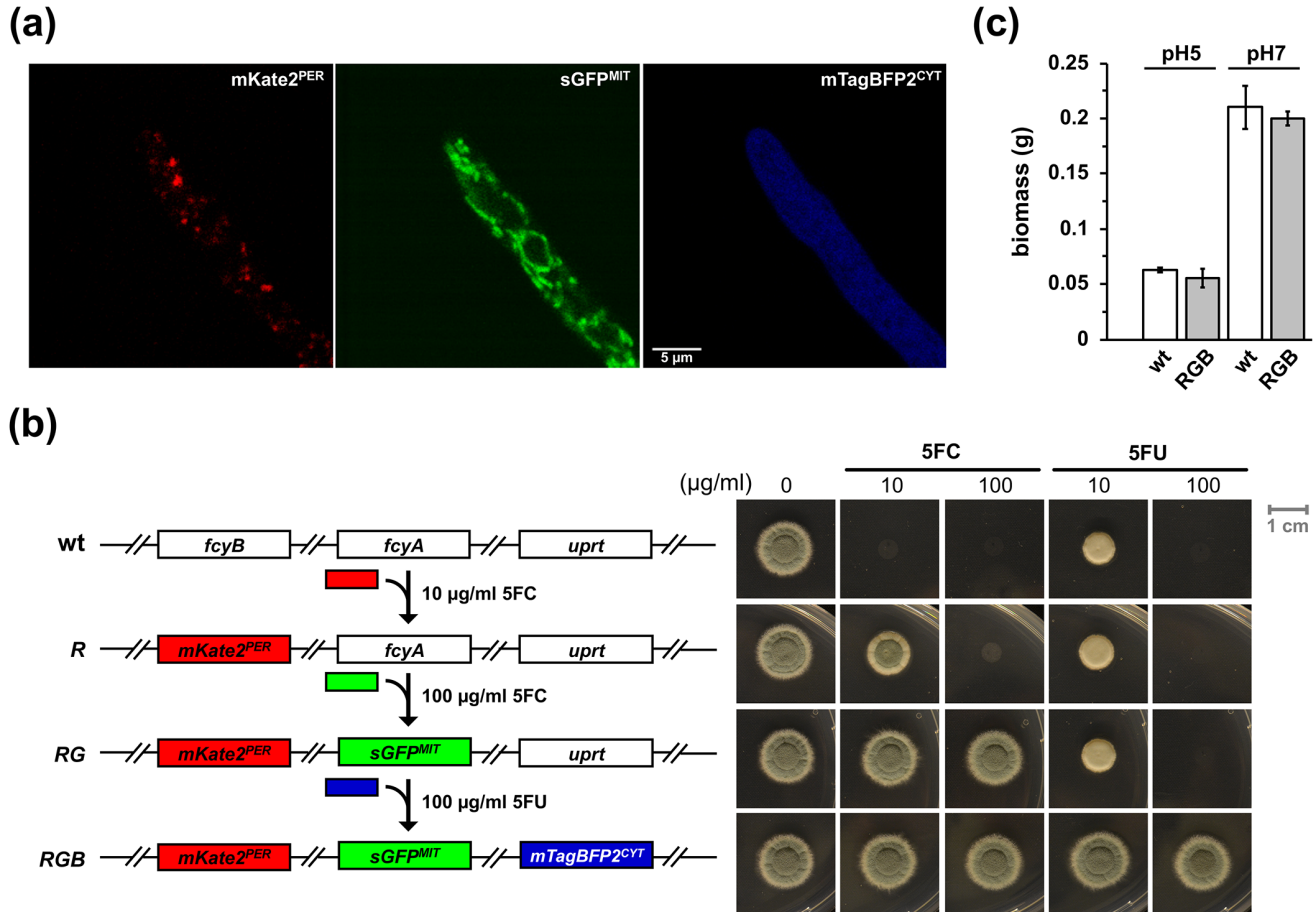
205 Due to the fact that inactivation of *fcyB* and *fcyA* led to different levels of resistance to 5FC and
206 inactivation of *uprt* caused resistance to 5FU (Fig. 1b), we investigated if these marker genes can
207 be sequentially employed for transformation selection in a single strain. As an exemplary
208 application, we aimed to generate a strain expressing three fluorescent proteins for multicolor
209 imaging: GFP (sGFP), red fluorescent protein (RFP, mKate2) and blue fluorescent protein (BFP,
210 mTagBFP2).

211 The pursued strategy and order of markers used for selection was based on the following
212 aspects (Fig. 1b): (i) in contrast to wt, Δ*fcyB* can grow in the presence of 10 µg/ml 5FC at pH5;
213 (ii) in contrast to Δ*fcyB*, Δ*fcyA* can grow at 100 µg/ml 5FC, which allows discrimination of
214 Δ*fcyB*Δ*fcyA* from Δ*fcyB*; (iii) Δ*fcyB* and Δ*fcyA* are still able to import and metabolize 5FU, which is
215 expected to allow discrimination of Δ*fcyB*Δ*fcyA* and Δ*fcyB*Δ*fcyA*Δ*uprt* in the presence of 100
216 µg/ml 5FU.

217 In the first step, we integrated an expression cassette encoding mKate2 carrying a C-
218 terminal peroxisomal targeting sequence (PTS1, tripeptide SKL) (Olivier and Krisans 2000) in
219 the *fcyB* locus, yielding strain RFP^{PER} (Δ*fcyB*::$mKate2^{PER}$). In this strain, a second expression
220 cassette encoding sGFP containing an N-terminal mitochondrial targeting sequence derived
221 from citrate synthase (Min et al. 2010) was targeted into the *fcyA* locus, yielding strain
222 $RFP^{PER}GFP^{MIT}$ (Δ*fcyB*::$mKate2^{PER}$Δ*fcyA*::$sGFP^{MIT}$). In the last step, an expression cassette encoding
223 mTagBFP2 with expected cytoplasmic localization was integrated into the *uprt* yielding strain
224 $RFP^{PER}GFP^{MIT}BFP^{CYT}$ (Δ*fcyB*::$mKate2^{PER}$Δ*fcyA*::$sGFP^{MIT}$Δ*uprt*::$mTagBFP2^{CYT}$). Multicolor laser

225 scanning confocal microscopy confirmed expression of all three fluorescent proteins in
226 *RFP*^{PER}*GFP*^{MIT}*BFP*^{CYT} and localization to distinct subcellular compartments (Fig. 3 and
227 Supplementary Fig. 4). A noteworthy finding was that the lack of FcyB, FcyA and Uprt (strain
228 *RFP*^{PER}*GFP*^{MIT}*BFP*^{CYT}) affected neither growth nor virulence of *A. fumigatus* in a murine model of
229 invasive aspergillosis (Supplementary Fig. 5).

230 Collectively, these data demonstrate the feasibility of sequential use of self-encoded
231 selection markers *fcyB*, *fcyA* and *uprt* for integration of up to three DOIs without adverse effects
232 on *A. fumigatus* growth and virulence.



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234 Fig 3. **Multicolor imaging and phenotypic analysis of *RFP*^{PER}*GFP*^{MIT}*BFP*^{CYT} (*RGB*) expressing**
235 **three fluorescent proteins with distinct subcellular localization.** (a) Using laser scanning
236 confocal microscopy, the expression of peroxisomal RFP, mitochondrial GFP and cytoplasmic
237 BFP was monitored in *RGB* after incubation for 20 h in liquid AMM at 30 °C. (b) Scheme
238 illustrating the sequential integration of fluorescent proteins (left) and 5FC/5FU resistance
239 profiles (right) of *RFP*^{PER} (*R*), *RFP*^{PER}*GFP*^{MIT} (*RG*) and *RGB* after incubation on solid AMM for 48 h.
240 (c) Biomass production (dry weight) of *RGB* and wt. Liquid cultures were incubated for 20 h at
241 pH5 and pH7. The data illustrate the mean of biological triplicates. Error bars indicate the
242 standard deviation. p-values were calculated by Student's T-test (two-tailed, unpaired): 0.38
243 and 0.32 for pH5 and pH7, respectively (reference: wt).

244 **Self-encoded markers can be used for the integration of biotechnologically-relevant, large**
245 **DNA fragments**

246 Fungi play important roles as cell factories in food industry as well as medicine. Pursuing a
247 potential biotechnological approach using the described selection method, we tested if we can
248 integrate the 17-kb penicillin biosynthetic cluster (PcCluster) of *P. chrysogenum* into the genome
249 of *A. fumigatus*. Therefore, a knock-in plasmid was constructed comprising the PcCluster as well
250 as 5'- and 3'-*fcyB* NTR. Linearization of the plasmid with *Pme*I allowed double crossover
251 homologous recombination-mediated replacement of *fcyB* by the PcCluster, as illustrated in Fig.
252 4a and Supplementary Fig. 6.

253 Subsequent to transformation of this construct in wt (selection: 10 µg/ml 5FC, pH5), we
254 validated its site-specific integration at the *fcyB* locus (strain $fcyB^{PENG}$, Supplementary Fig. 1).
255 Northern blot analysis confirmed expression of penicillin biosynthetic genes *pcbAB*, *pcbC* and
256 *penDE* in three transformants (Fig. 4b). Concomitant to successful expression of this
257 heterologous gene cluster in *A. fumigatus,* we detected penicillin G and its degradation product
258 penillic acid in culture supernatants using nanoLC mass spectrometry (Fig. 4c). Both substances
259 have the same molecular weight of 335.1060 g/mol but can be differentiated due to structure-
260 specific fragmentation during MS/MS (Supplementary Fig. 6b).

261 Taken together, these results demonstrate that pyrimidine salvage-based selectable
262 markers can be applied for the integration of large-size DNA fragments including whole gene
263 clusters.

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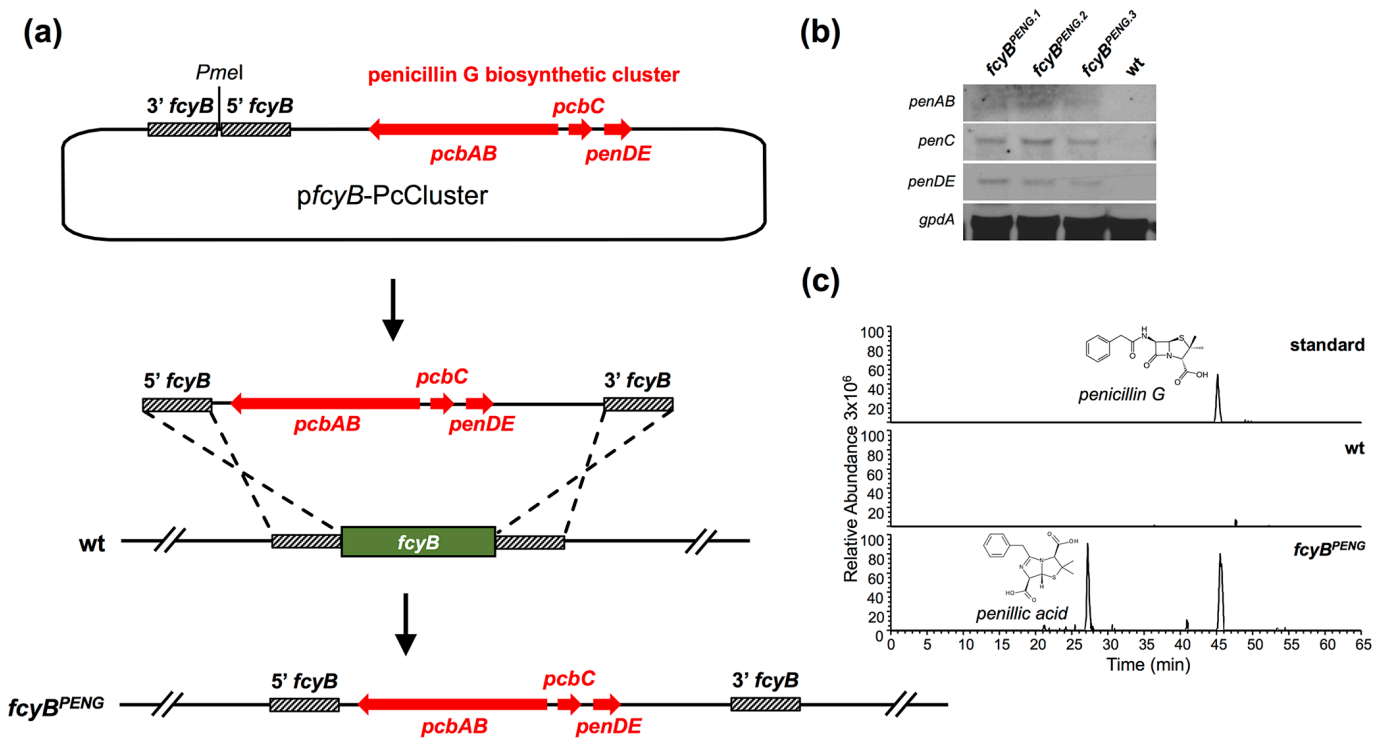

Fig 4. **Genomic insertion of the PcCluster transformed *A. fumigatus* into a penicillin G producer.** (a) To facilitate genomic integration of the PcCluster at the *fcyB* locus, the plasmid p*fcyB*-PcCluster comprising the respective DOI (17 kb) as well as *fcyB* 5' and 3' NTRs was generated. Linearization of this plasmid with *Pme*I allows homologous recombination-based replacement of *fcyB* coding sequence with DNA containing the PcCluster. (b) Expression of functional *pcbAB*, *pcbC* and *penDE* was monitored in three independent transformants using Northern blot analysis (*gpdA* was used as reference). (c) LC-MS/MS extracted ion chromatograms of penicillin G (peak at 45 min) and its degradation product penillic acid (peak at 27 min) in the culture supernatant of *fcyB*^{PENG} after shaking incubation for 48 h at 25 °C. wt served as negative control.

Pyrimidine salvage based-selectable markers can be utilized in *Penicillium chrysogenum* and *Fusarium oxysporum*

To identify encoded CD and UPRT activities in further fungal species, we searched for *A. fumigatus* FcyA and Uprt orthologs in biotechnologically (*Aspergillus niger*, *Aspergillus oryzae*, *P. chrysogenum*, *Komagataella phaffii* (previously *Pichia pastoris*), *S. cerevisiae* and *Trichoderma reesei*) and pathologically relevant fungal species (*Candida albicans*, *Cryptococcus neoformans* and *F. oxysporum*). *In silico* inspection of the annotated genomes of these species revealed that 8 out of the 10 species analyzed harbor a putative ortholog of *A. fumigatus* FcyA and that all species possess a putative ortholog of *A. fumigatus* Uprt with overall sequence identities ≥ 40% (Supplementary Table 1). Notably, all species encoding an FcyA ortholog were also found to

294 encode an FcyB ortholog. The genetic coupling of these two features might indicate that their
295 main function is the utilization of extracellular pyrimidines.

296 To confirm CD and UPRT activities in the species analyzed, we monitored 5FC and 5FU
297 susceptibility profiles following a broth microdilution-based method according to EUCAST
298 (Subcommittee on Antifungal Susceptibility Testing of the 2008). In agreement with our
299 homology search, species with predicted FcyA orthologs (CD activity) were susceptible to 5FC,
300 while those without were resistant to the drug (Supplementary Table 2). All strains were
301 susceptible to 5FU, which is in accordance with *in silico* predicted Uprt orthologs (UPRT
302 activity).

303 In the next step we tested the applicability of the described selection strategy in *P.*
304 *chrysogenum* and *F. oxysporum*. In agreement with the genomic data and 5FC/5FU susceptibility,
305 *P. chrysogenum* expresses both CD (Pc-FcyA, EN45_039280) and UPRT (Pc-Uprt, EN45_060980),
306 while *F. oxysporum* lacks CD but expresses UPRT (Fo-Uprt, FOXG_01418). Employing the same
307 protocol as used for *A. fumigatus* enabled the integration of GFP expression cassettes flanked by
308 5' and 3' NTR of the respective *P. chrysogenum* genes in both the *Pc-fcyA* and the *Pc-uprt* loci. In
309 *F. oxysporum*, the same strategy enabled the targeting of a GFP expression cassette at the *Fo-uprt*
310 locus. The presence and functionality of the GFP reporters was visualized as described above
311 (Fig. 5). As observed for *A. fumigatus* knock-in mutants, resistance profiles of *P. chrysogenum*
312 and *F. oxysporum* knock-in strains were in accordance with the absence of individual salvage
313 activities.

314 Taken together, these data indicate high evolutionary conservation of the described
315 pyrimidine salvage enzymes within the fungal clade and demonstrate the suitability of the
316 encoding loci as markers for transformation selection in *P. chrysogenum* and *F. oxysporum*.

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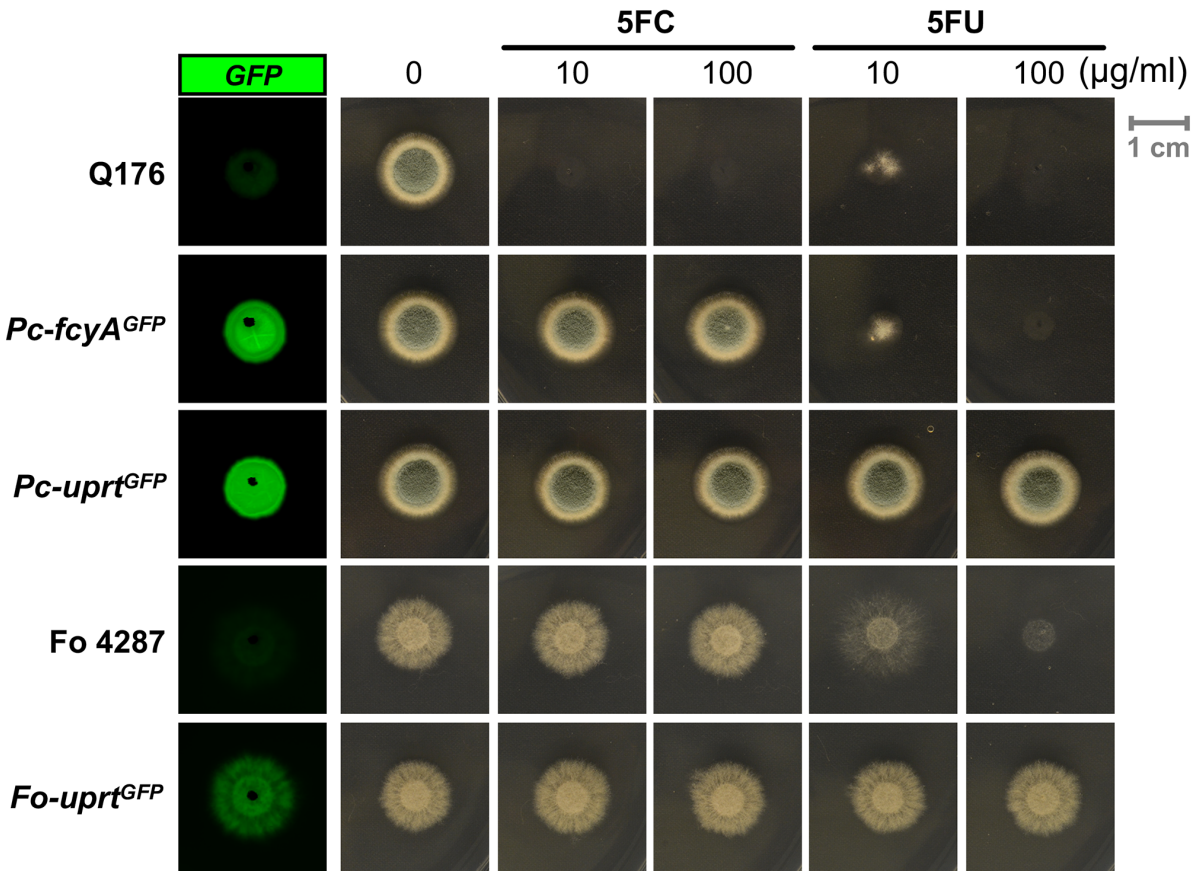

Fig 5. ***P. chrysogenum* and *F. oxysporum* strains with replaced pyrimidine salvage pathway encoding loci.** GFP expression was visualized in the corresponding knock-in mutants in the absence of drugs. 5FC/5FU resistance phenotypes of GFP reporter strains lacking CD (*Pc-fcyA*^{GFP}) or UPRT (*Pc-uprt*^{GFP}, *Fo-uprt*^{GFP}) activities. For both experiments, strains were grown on solid AMM (*P. chrysogenum*) and PDA (*F. oxysporum*) followed by 72 h incubation at 25 °C.

DISCUSSION

In this study, we report the characterization and exploitation of multiple, endogenously-encoded selectable markers for targeted genetic engineering. The marker genes encode activities mediating 5FC uptake (*fcyB*) and metabolization (*fcyA* and *uprt*) of 5FC or 5FU into cell-toxic nucleotides (Fig. 1a) (Vermes et al. 2000). Genomic replacement of these genes by DOIs results in loss of the corresponding activities and can therefore be selected *via* 5FC or 5FU resistance. We validated the applicability of orthologous marker genes encoding pyrimidine salvage activities for their use as selectable markers in three fungal species (*A. fumigatus*, *P. chrysogenum* and *F. oxysporum*) by targeted insertion of various fluorescent or enzymatic reporter genes (Fig. 2, 3 and 5). Mimicking a biotechnological application, we further introduced the 17-kb penicillin biosynthetic gene cluster from *P. chrysogenum* to *A. fumigatus*, which

338 naturally is not capable of producing this secondary metabolite. In addition to their single use,
339 we demonstrate the possibility of consecutive use of these markers in a single strain by
340 generating an *A. fumigatus* mutant expressing three fluorescent proteins (mKate2, sGFP and
341 mTagBFP2) targeted to different subcellular compartments (Fig. 3a and Supplementary Fig. 4).
342 Importantly, these endogenous genes can be used in addition to traditional selectable markers
343 and the absence of all three genes (Δ*fcyB*Δ*fcyA*Δ*uprt*) affected neither growth nor virulence (Fig.
344 3b and Supplementary Fig. 5) in *A. fumigatus*, which represents a prerequisite for downstream
345 use of engineered strains. Inspection of genomic sequences combined with 5FC/5FU
346 susceptibility profiling of different fungal species playing important roles in biotechnology,
347 medicine or agriculture revealed the presence of *uprt* orthologs in 10 out of 10 species, and *fcyB*
348 as well as *fcyA* orthologs in 8 out of 10 species (Supplementary Table 1), indicating the broad
349 applicability of the proposed selection method. Most likely, the number of self-encoded
350 selectable markers for DOI integration allows expansion beyond the here characterized genes.
351 For instance, genes coding for 5FU uptake, such as orthologs of *A. nidulans furD* or *S. cerevisiae*
352 *FUR4* (Jund and Lacroute 1970; Hamari et al. 2009), represent potential candidates.

353 Employing pyrimidine salvage pathway-based endogenous markers, virtually any DOI can
354 be site-specifically inserted into the target genome, demonstrating their huge potential for
355 diverse applications in basic as well as applied research. The sequential use of marker genes
356 enables the establishment of modular toolboxes, e.g. allowing site-directed insertion of multiple
357 reporter genes for co-localization studies. Furthermore, the possibility to equip strains with
358 multiple DNA building blocks makes this technology particularly attractive for synthetic biology.
359 Notably, the fact that marker genes have to be inactivated to allow selection based on 5FC/5FU
360 resistance enforces targeted integration of DOIs. As the described strategy avoids the use of
361 foreign selection markers, the described genetic toolset further enables so called 'self-cloning'.
362 Strains engineered this way are not considered genetically modified organisms (GMO) in some
363 countries (Steensels et al. 2014; Borner et al. 2019). For this, only endogenously encoded DOIs
364 can be used; examples include the increase of gene dosage or engineering of sequences such as
365 promoter swapping. A further advantage of self-encoded markers is the dispensability of

366 antibiotic resistance genes, the use of which is generally discouraged in the manufacturing of
367 medical- or food-related products (Yau and Stewart 2013; Mignon et al. 2015) as it may promote
368 horizontal transfer of resistance genes.

369 In summary, we characterized a powerful genetic toolbox enabling multiple, targeted
370 genomic insertions of DOIs. Evolutionary conservation of the pyrimidine salvage pathway
371 suggests broad applicability of the described marker genes. Thus, we anticipate that this
372 technology will significantly advance genetic and metabolic engineering of diverse organisms.

373

374 **MATERIALS AND METHODS**

375 **Growth conditions and fungal transformation**

376 Plate growth assays analyzing *A. fumigatus* and *P. chrysogenum* were carried out using solid
377 *Aspergillus* minimal medium (AMM; ammonium tartrate was used as nitrogen source, glucose as
378 carbon source)(Pontecorvo et al. 1953), and for *F. oxysporum* solid PDA was employed.
379 Therefore, 10^4 spores of each strain were point inoculated on agar plates. Low pH medium
380 contained 100 mM citrate buffer (pH5), and neutral pH medium 100 mM MOPS buffer (pH7). If
381 not stated in the text or images, plate growth assays were performed at pH5. For strains carrying
382 *xylP* promoter (*PxylP*) driven reporter genes (*sGFP*, *mKate2*^{PER}, *sGFP*^{MIT}, *lacZ*), the medium was
383 supplemented with 0.5% xylose to induce gene expression.

384 For fungal manipulations, 2 µg DNA of each construct was transformed into protoplasts of
385 the respective recipient. For the regeneration of transformants, solid AMM (*A. fumigatus* and *P.*
386 *chrysogenum*) or PDA (*F. oxysporum*) supplemented with 342 g/l or 200 g/l sucrose,
387 respectively, were used. Selection procedures using conventional selectable marker genes (*hph*,
388 *ble*) were carried out as described previously for *A. fumigatus* (Gsaller et al. 2018).

389

390 **Deletion of *A. fumigatus fcyA* and *uprt***

391 Strains and primers used in this study are listed in Supplementary Table 3 and 4. Coding
392 sequences of *fcyA* and *uprt* were disrupted in wt (A1160P+) using hygromycin B and zeocin
393 resistance cassettes, respectively. Therefore, deletion constructs comprising approximately 1 kb

394 of 5' and 3' NTR linked to the central antibiotic resistance cassette were generated using fusion
395 PCR as previously described (Fraczek et al. 2013). Correct integration of constructs was
396 confirmed by Southern analyses (Supplementary Fig. 1).
397
398 **Generation of *A. fumigatus* knock-in strains**
399 Knock-in constructs for *A. fumigatus* loci *fcyB*, *fcyA* and *uprt, P. chrysogenum* loci *Pc-fcyA* and *Pc-*
400 *uprt*, as well as *F. oxysporum Fo-uprt* were generated similarly to the gene deletion fragments
401 described above using fusion PCR. Here, instead of the antibiotic resistance cassettes, DOIs (for
402 reporter templates, see also Supplementary Fig. 7) were connected to approximately 1 kb 5' and
403 3' NTR of the respective locus (Fig. 2a).
404
405 **LacZ-based colorimetric assay and fluorescence imaging**
406 For the detection of LacZ activity (conversion of X-Gal into the blue compound 5,5'-dibromo-
407 4,4'-dichlor-indigo) (Horwitz et al. 1964), a 5 ml layer of a 1 mM X-Gal/1% agar/1% N-
408 lauroylsarcosin solution was poured over fungal colonies. GFP expression of fungal colonies was
409 visualized using the laser scanner Typhoon FLA9500 (Ex 473 nm; Em ≥ 510 nm).
410 Images of $RFP^{PER}GFP^{MIT}BFP^{CYT}$ were taken using an HC PL APO CS2 63x/1.30 glycerol
411 objective on an SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with
412 a 80 Mhz pulsed white light laser (WLL) and a 405 nm CW diode laser (405 nm diode) according
413 to Nyquist sampling. Gating of the red signal only was used in order to remove unspecific red
414 autofluorescence. Images of $mKate2^{PER}$ (Ex: 588 nm WLL, Em: 598- 750 nm, Gating: 0.2. to 8 ns),
415 $sGFP^{MIT}$ (Ex: 489 nm WLL, Em: 499 -578 nm) and $mTagBFP^{CYT}$ (Ex: 405 nm Diode, Em: 415 – 479
416 nm) were processed using ImageJ (Fig. 3a). For the generation of 3D reconstructions
417 (Supplementary Fig. 4), z-stacks, acquired under the same imaging conditions using a z-interval
418 of 180 nm, were deconvolved using the CMLE algorithm of Huygens Professional version 18.10
419 (Scientific Volume Imaging, Amsterdam, The Netherlands) and further processed with Imaris
420 9.3.0 (Bitplane, Zurich, Switzerland).
421

422 **Expression analysis of penicillin biosynthetic genes and detection of penicillin G in**
423 **culture supernatants**
424 Northern analysis was carried out as described previously, using digoxigenin-labeled probes
425 (Gsaller et al. 2012).
426 To detect the potential production of penicillin G, strains were grown in AMM for 48 h at
427 25 °C and 2 ml of culture supernatant were extracted with 1 volume of butyl acetate. The organic
428 phase was collected in a new reaction tube and dried using a centrifugal vacuum concentrator
429 (speed-vac). NanoLC-MS-based detection of penicillin G was carried out using an UltiMate 3000
430 nano-HPLC system coupled to a Q Exactive HF mass spectrometer (Thermo Scientific, Bremen,
431 Germany). The samples were separated on a homemade fritless fused-silica microcapillary
432 column (100 µm i.d. x 280 µm o.d. x 19 cm length) packed with 2,4 µm reversed-phase C18
433 material (Reprosil). Solvents for HPLC were 0.1% formic acid (solvent A) and 0.1% formic acid
434 in 85% acetonitrile (solvent B). The gradient profile was as follows: 0-4 min, 4% B; 4-57 min, 4-
435 35% B; 57-62 min, 35-100% B, and 62-67 min, 100% B. The flow rate was 300 nL/min.
436 Mass spectra were acquired in positive ion mode applying a precursor scan over the m / z
437 range 50 to 500 in the FT analyzer. The ions at m / z = 335.1060 were selected from this
438 precursor scan for MS/MS fragmentation in the linear ion trap.
439
440 **Murine infection model**
441 Specific-pathogen-free female outbreed CD-1 mice (18–20 g, 6–8 weeks old, Charles River,
442 Germany) were housed under standard conditions in individually ventilated cages and supplied
443 with normal mouse chow and water *ad libitum*. All animals were cared for in accordance with
444 the European animal welfare regulations, and experiments were approved by the responsible
445 federal/state authority and ethics committee in accordance with the German animal welfare act
446 (permit no. 03-027/16). Mice were immunosuppressed with cortisone acetate and intranasally
447 infected with 2 x 10^5 conidia in 20 µl PBS as described before (McCormick et al. 2012). Infected
448 animals were monitored twice daily and humanely sacrificed if moribund (defined by a score
449 including weight loss, piloerection, behavior and respiratory symptoms).

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453

454 **AUTHOR CONTRIBUTIONS**

455 LB, AD, MS L-B, MO, MS, BS, OS, IB, BA and FG performed experiments; LB, AD, MS L-B, IDJ, MS,
456 MO, HL, BS, HH and FG analyzed data; LB, IDJ, HL, HH and FG designed experiments; LB, HH and
457 FG wrote the manuscript; FG conceptualized the study; all authors edited the manuscript.

458

459 **DISCLOSURE DECLARATION**

460 A patent related to this work has been filed. The authors declare no competing non-financial
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462

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