Inducible Cell Fusion Permits Use of Competitive Fitness Profiling in the Human Pathogenic Fungus *Aspergillus fumigatus*

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Antifungal agents directed against novel therapeutic targets are required for treating invasive, chronic, and allergic *Aspergillus* infections. Competitive fitness profiling technologies have been used in a number of bacterial and yeast systems to identify druggable targets; however, the development of similar systems in filamentous fungi is complicated by the fact that they undergo cell fusion and heterokaryosis. Here, we demonstrate that cell fusion in *Aspergillus fumigatus* under standard culture conditions is not predominately constitutive, as with most ascomycetes, but can be induced by a range of extracellular stressors. Using this knowledge, we have developed a barcode-free genetic profiling system that permits high-throughput parallel determination of strain fitness in a collection of diploid *A. fumigatus* mutants. We show that heterozygous *cyp51A* and *arf2* null mutants have reduced fitness in the presence of itraconazole and brefeldin A, respectively, and a heterozygous *atp17* null mutant is resistant to brefeldin A.

**KEYWORDS** *Aspergillus fumigatus*, anastomosis, antifungal, chemical genomics, functional genomics, hyphal fusion, imaging

Fungal diseases are estimated to kill between 1.5 and 2 million people each year, similar to the number of deaths from tuberculosis and greatly exceeding those from malaria (1). Approximately 600,000 of these deaths are the result of invasive and chronic mold infections caused by *Aspergillus* species (1–3). The current antifungal armamentarium for the treatment of these infections is limited to four classes of agents, which all suffer pharmacological shortcomings, including toxicity, drug-drug interactions, and poor bioavailability (4, 5). With the exception of flucytosine, which is only recommended for the treatment of cryptococcal meningitis, all commercially available antifungals target and disrupt the integrity of the fungal cell (6). The azole class of antifungals is the current first-line therapy for the treatment of *Aspergillus* infections; however, the development of resistance to this antifungal class is an emerging problem, particularly, in northern Europe (7, 8). Mortality rates for invasive aspergillosis are on the order of 50% (1). However, for individuals infected with an azole-resistant isolate, mortality exceeds 80% (7–9). Novel drugs that act via different mechanisms of action are desperately needed. Since the development of the echinocandin class of antifungals in the 1990s, there has been a lack of agents against novel drug targets approaching registration. Recent high-profile and relatively late-stage antifungal development failures, such as efungumab (Mycograb) and MGCD290, have been disappointing, and currently, only four antifungal compounds with novel mechanisms of action for the treatment of systemic disease are in clinical trials (6).
Target-driven anti-infective discovery has failed to deliver on its promise to revolutionize the pathway to drug development. This was exemplified by an honest and stark review of antibacterial drug discovery by Payne et al. (10), who described how Glaxo-SmithKline, over a period of 7 years, invested more than $70 million to extensively validate 67 drug targets with high-throughput screening campaigns. They concluded that the development of antibacterials via this route was financially inviable. One key problem with this approach was that it was not possible to assess if the targets they had identified were accessible to and capable of being inhibited by the drug.

Successful strides have been made to improve the way genomics is used in drug discovery, relying on it less as a driver to identify novel targets and more to facilitate the identification of the mechanism of action of drugs identified through more traditional screening approaches. Chemical genomics technologies, such as haploinsufficiency profiling (HIP) in Saccharomyces cerevisiae (11–13) and the Candida albicans fitness test (CaFT) (14), employ potent antifungal drug-like chemicals that lack mammalian toxicity to identify targets that are druggable and selective. The principle of these approaches is based on chemically induced haploinsufficiency, whereby the deletion of a single allele of a drug target in a diploid organism renders the strain more sensitive to that drug. Cognate targets of drugs with an unknown mechanism of action can be identified by screening large genetically barcoded libraries of mutant isolates. Strains that exhibit fitness defects in a pooled culture at subinhibitory drug concentrations are potential targets of drug action. This technology greatly facilitated the determination of the mode of action of the actinomycete-derived natural product parafunginin in C. albicans (15). Subsequently, the presumptive mechanisms of action of more than 60 antifungal agents have been identified (16). Recently, these technologies have been improved further using barcode analysis by sequencing (Bar-seq), a method that employs next-generation sequencing (NGS) to directly count specific barcodes in each strain to enable a more rapid and higher throughput method for the screening of inhibitory chemical compounds (17).

While the ability to identify novel druggable targets in yeasts has been successfully addressed, the changing disease landscape, and more particularly, our understanding of the significance of mold-based infections, requires similar technologies to be developed in filamentous fungi. A number of issues have prevented this technology from being used with Aspergillus fumigatus, including the fact that it is a haploid organism and that a high-throughput gene knockout methodology was not previously available to generate a suitable library of deletion mutants. In addition, filamentous fungi, unlike yeasts, commonly undergo prolific vegetative cell fusion, which permits the exchange of cell components including genetic material (18–20) that would ultimately prevent the successful analysis of the fitness of individual genetic isolates in a pooled culture. Vegetative cell fusion is very common and occurs constitutively in diverse filamentous fungi; it has been most extensively investigated in the model Neurospora crassa (19, 21–23).

In this study, we show that vegetative cell fusion occurs very rarely in A. fumigatus but can be induced further by environmental stress, including nitrogen starvation and exposure to antifungal drugs. By using a nonhomologous end-joining-deficient diploid isolate of A. fumigatus and a rapid allelic replacement strategy, we generated a library of 46 heterozygous knockouts of essential genes. By exploiting the lack of significant constitutive cell fusion under our experimental conditions, we used this library to conduct competitive fitness profiling using a barcode-free sequencing strategy to confirm the mechanism of action of the antifungal agents itraconazole and brefeldin A. As a result, we identified a novel route of resistance to brefeldin A.

**RESULTS**

Cell fusion occurs infrequently in A. fumigatus under standard culture conditions. The conidia and germ tubes of N. crassa and many other filamentous ascomycetes undergo cell fusion (self fusion) during colony initiation by means of specialized cell protrusions called conidial anastomosis tubes (CATs) that permit the exchange of...
organelles and nutrients (24). However, the early onset of frequent cell fusion in *A. fumigatus* would be detrimental to the assessment of the fitness of mutant strains in a pooled culture, because it facilitates nuclear exchange between germlings (18–20).

To initially assess the prevalence of cell fusion in *A. fumigatus*, extensive low-temperature scanning electron microscopy was performed on the wild-type strain AF293 grown on oatmeal agar for 12 h following incubation at 37°C (Fig. 1A). Differential interference contrast light microscopy and fluorescence microscopy (after staining with the cell wall dye calcofluor white) were also performed on cultures grown in this way to carefully assess the occurrence of cell fusion. Neither CAT fusion during colony initiation nor the production of cell protrusions that resembled CATs formed from conidia or germ tubes were observed under these conditions. To assess if we had used suboptimal medium/culture conditions to stimulate cell fusion, we extended the incubation times to periods of up to 48 h on a range of media (oatmeal agar, Vogel’s agar, tap water agar, Czapek Dox agar, yeast-potato dextrose, *Aspergillus* minimal agar, and *Aspergillus* minimal liquid medium [AMM]) with incubation at 25°C or 37°C with two different strains (A293 or CEA10). Finally, three methods of inoculation were applied.
when using agar media: coinoculation of conidia in the center of the plate, coinoculation of conidial suspension across a whole plate, or the frontier method (25). Cell fusion was not detected in any of these circumstances (data not shown).

We hypothesized that cell fusion in *A. fumigatus* may be a rare event requiring a more sensitive microscopic method to provide compelling evidence of its occurrence between living cells under standard culture conditions. To achieve this, we generated strains in the CEA10 background expressing either the green fluorescent protein (GFP; strain identifier [ID], MFIGGFP4) or the far-red fluorescing TurboFP635 protein (strain ID, MFIGRag29) in the cytoplasm. The strains were coinoculated at a ratio of 1:1 in eight-well chambers in AMM with sodium nitrate as the sole nitrogen source (AMMNO₃ medium) and incubated as static liquid cultures at 37°C. Live-cell confocal microscopy was performed after 8 h and at 4-h intervals up to 24 h. No evidence of cell fusion was detected, as determined by the lack of GFP and TurboFP635 fluorescent signal colocalization within single cells upon manual inspection of confocal images (Fig. 1B). These results were obtained from eight independent cell culture chambers at a range of time points up to 24 h despite screening in each case an area of 0.7 mm² that contained an average of 964 germinated spores. Attempts to evaluate cell fusion between MFIGGFP4 and MFIGRag29 beyond 24 h were not possible in liquid medium, because the mycelia became too densely populated to assess isolated cells.

Having failed to observe cell fusion under liquid culture conditions, we assessed whether hyphal fusion occurs in more mature colonies on solid culture medium. Conidia from the MFIGGFP4 and MFIGRag29 strains were mixed as before and spot inoculated onto AMMNO₃ agar medium. Agar plates were incubated at 37°C for 40 to 48 h and imaged using the “inverted agar technique” (26) to check for hyphal fusion. Peripheral and subperipheral zones of the colony were assessed, but the growth in central regions was too dense to satisfactorily image isolated cells. A rigorous visual inspection for colocalized GFP/TurboFP635-expressing cells within image stacks from five independent agar cultures showed that the frequency of fused cells was very low on solid medium (see Movie S1 in the supplemental material); we detected a frequency of 1% cell fusion in this culture after manually counting 292 cells from a 1.3-mm² sample area.

**Cell fusion is enhanced by nutritional and antimicrobial stress.** Phenotypic evidence of cell fusion and the formation of diploids via the parasexual cycle were previously described in *A. fumigatus* (25). All examples of such events have occurred between auxotrophic strains with different nutritional deficiencies when placed under selective pressure that forces phenotypic complementation. To our knowledge, there are no records in the literature providing microscopic evidence of the presumed cell fusion occurring in *A. fumigatus*, and the frequency of these events is unknown. To assess if cell fusion occurred more frequently in auxotrophic strains lacking the ability to grow on nitrate as a sole nitrogen source, stable nitrate assimilation mutants lacking functional *niaD* and *cnx* genes (reversion rate, <1/10⁷ spores) were isolated from the MFIGGFP4 and MFIGRag29 strains to produce the following strains: AfGFPniaD, AfTurboFP635niaD, AfGFPcnx, and AfTurboFP635cnx.

Complementary strains (i.e., AfGFPniaD with AfTurboFP635cnx and AfTurboFP635niaD with AfGFPcnx) were coinoculated in eight-well cell culture chambers containing liquid medium with nitrate as the sole source of nitrogen (AMMNO₃), incubated at 37°C, and observed microscopically after 24 h. Due to the lack of an optimal nitrogen source in the growth medium, these strains were under significant nitrogen starvation stress and grew slower than their prototrophic counterparts (data not shown). A manual inspection of individual confocal sections in three-dimensional (3D) image stacks of cells growing in these wells showed that <0.2% of the cells (3 of 1,275 cells) had fused in this treatment (Fig. 2A). Direct cell fusion was observed between conidia and between germ tubes. They involved short cell protrusions/hyphae that were thinner than germ tubes (Fig. 2B) and superficially resembled conidial anastomosis tubes (CATs) in other fungi (20–22). An increase in the incubation time to 32 h did not result in an increase
in the number of fusion events (data not shown). Reliable assessment of cell fusion beyond this point was not possible because of the high density of growth. These data indicate that cell fusion can occur by a process resembling CAT fusion but, under liquid culture conditions, is an exceptionally rare event, even between complementary auxotrophic strains.

To assess if cell fusion occurs more frequently between complementary auxotrophic strains cultured over a longer period on solid medium, the strains were coinoculated on AMMNO₃ agar plates and incubated at 37°C for up to 4 days. The cultures were checked for fusion after 12 h and subsequently at 8-h intervals. To speed up and manage the analysis of the large data sets that were being obtained, we employed an unbiased automated analysis macro, which counted colocalized and total fungal voxels from large-tiled 3D image volumes (see Materials and Methods). Cell fusion was observed and measured in the auxotrophs grown on solid medium after 48 h (Fig. 3A). Significantly enhanced levels of cell fusion were clear in the auxotrophic strains at this time (27% pixel colocalization) (Fig. 3A) compared to those of the parental prototrophs (4%; \( P < 0.001 \)) (Fig. 3E and Movie S1). The proportion of fused cells was further enhanced after 91 h (53% from two independent experiments) (see Fig. S1). These data indicate that cell fusion is rare in \textit{A. fumigatus} under normal laboratory culture conditions and that fusion was greatly enhanced when strains were placed under nitrogen starvation stress on solid medium.

Since cell fusion increased when complementing auxotrophic mutants were placed under nutritional stress (nitrogen starvation) on solid medium, we hypothesized that cell fusion may be induced by other types of stress, such as the exposure to sublethal doses of antimicrobial drugs. To test this, the prototrophic MFIGFP4 and MFIGRag29 strains were coinoculated on solid AMMNO₃ medium for 48 h in the presence of sublethal concentrations of cerulenin (25 \( \mu \text{g/ml} \)) (Fig. 3B), which inhibits fatty acid biosynthesis, itraconazole (0.5 \( \mu \text{g/ml} \)) (Fig. 3C), which inhibits ergosterol synthesis in fungi, and brefeldin A (6.25 \( \mu \text{g/ml} \)) (Fig. 3D), which inhibits protein transport from the endoplasmic reticulum to the Golgi. Under each condition, from at least three independent experiments, the mean pixel colocalizations within fungal cells were significantly higher upon exposure to itraconazole and cerulenin than in the untreated control (76% \( [ P < 0.01 ] \) and 30% \( [ P < 0.05 ] \), respectively) (Fig. 3E). An evaluation of brefeldin A (6.25 \( \mu \text{g/ml} \)) revealed a nonsignificant mean response of 13% pixel colo-
FIG 3 Cell fusion frequency is increased under nutritional and antimicrobial stresses under solid culture conditions. Representative images of TurboFP635- and GFP-expressing *A. fumigatus* cells grown on various solid conditions. (Continued on next page)
calization within fungal cells compared to that for the untreated control \((P = 0.32)\). The brefeldin A response was not consistent, since large variation existed between the four replicates. Clear cell fusion in one replicate is depicted in Fig. 3D, which was less pronounced or absent in the other three replicates. Overall, our data indicate that cell fusion in *A. fumigatus* is primarily an inducible phenomenon that is enhanced in response to the antifungals itraconazole and cerulenin and to nutritional stress.

**Low levels/absence of cell fusion in A. fumigatus permits competitive fitness profiling of heterozygous diploid strains that can be used to reveal antifungal drug mechanisms of action.** To replicate the growth conditions that could be used in competitive fitness experiments to determine the mechanism of action of antifungal drugs, we assessed if cell fusion occurs in shake flask cultures in RPMI liquid medium (RPMI is defined by the European Committee for Antimicrobial Susceptibility Testing [EUCAST] for antifungal susceptibility testing [27]). Strains MFIGFP4 and MFIGRag29 were coinoculated at a density of \(2.5 \times 10^6\) spores/ml in RPMI liquid medium and incubated at 37°C with shaking. Fungal pellets recovered from 48-h cultures either untreated or treated with 0.5 \(\mu\)g/ml itraconazole were examined microscopically. Ten pellets from each condition were screened for cell fusion. A very careful manual inspection of individual confocal optical planes within tiled 3D image volumes revealed no evidence of colocalization and thus cell fusion within 48 h of inoculation (see Movies S2 and S3). A manual (rather than automated) inspection of individual confocal sections was necessary, because adjacentely bundled hyphae were consistently so close to each other that the only way of unambiguously determining whether they were truly fused (i.e. adjacent cells sharing cytoplasm) was to directly examine every cell from each confocal section in a 3D image stack.

Since cell fusion occurs at a very low frequency during the first 48 h, even when strains are placed under significant stress and selection pressure in liquid medium, we concluded that this experimental setup could be used for competitive fitness profiling of multiple strains of *A. fumigatus*. To generate a library that can be used to study chemically induced haploinsufficiency and to test this hypothesis, we attempted to generate a collection of 48 heterozygous gene deletion mutants in a diploid KUB0 A. *fumigatus* isolate (AFMB3). The genes selected for this study were either known drug targets or known to be essential for viability, and each knockout cassette was transformed in duplicates (28) (see Table S1). Of the 48 gene knockouts attempted, we successfully obtained and validated duplicate clones (designated strain IDs -1 and -2) for 46, with the failures attributed to our inability to generate a knockout cassette by fusion PCR (AFUB_004340; srp68) or an inability to isolate transformants (AFUB_043760; fasB).

The 46 null mutants with the -1 designation were used in a competitive fitness study to assess if any strains showed a chemically induced haploinsufficient phenotype in response to the antifungal agents itraconazole and brefeldin A. A library of pooled spores was grown in RPMI medium supplemented with uridine and uracil for 40 h at 37°C with shaking in the presence and absence of growth inhibitory, but sublethal, levels of drug (0.05 \(\mu\)g/ml itraconazole and 12.5 \(\mu\)g/ml brefeldin A). During this time,
The relative frequency of strains that exhibit chemically induced haploinsufficiency was expected to decrease in the presence of drug. The change in the relative abundance of each strain was determined by comparing en masse sequencing of the regions flanking the boundary between the ANpyrG selectable marker and the terminator of the gene disrupted (see Fig. 4 for a schematic representation of the methodology). The changes in the number of sequence reads were then used to calculate the relative fitness using DESeq2 (see Materials and Methods). Overall, sufficient data were gathered to assess all but two of the genes in the pool for which sequence reads were too low in the overall population to allow meaningful analysis.

To assess the reproducibility of the chemical genomics assay for the remaining 44 isolates, independent biological replicates were performed (brefeldin A, n = 2; itraconazole, n = 3). The reproducibility of relative strain counts was high, with Pearson correlation coefficients of 0.97 for the brefeldin A data (Fig. 5A) and an overall relative standard deviation for the itraconazole data of 7.8% (coefficient of variation [CV] range, 0.4% to 21%) (see Table S2).

An analysis of the relative fitness of the pooled isolates on exposure to itraconazole and brefeldin A revealed a single outlying strain exhibiting a significant reduction in fitness for each drug (Fig. 5B). These mutants were heterozygous null mutants for the known drug targets, a Δarf2 mutant (AFUB_011170/+, adjusted [Adj] P < 0.001) (Table S2) in the presence of brefeldin A and a Δcyp51A mutant (ΔAFUB_063960/+, Adj P < 0.001) (Table S2) in the presence of itraconazole. Additionally, one outlier with significantly enhanced abundance in the presence of brefeldin A was identified (Δatp17 mutant; AFUB_022530/+, Adj P < 0.001) (Fig. 5B and Table S2).
To confirm the output of the competitive fitness study for brefeldin A, the quantitative abundance of 10 of the mutants was assessed by real-time PCR using the same DNA analyzed by en masse sequencing. For all 10 strains, the real-time quantification confirmed the accuracy of the NGS analysis. Specifically, the levels of the Δarf2 mutant were depleted (relative abundance of 51.2%; $P < 0.001$) compared to that of a control isolate (Δmot1 mutant [ΔAFUB_006220/1]) chosen because it was seemingly unaffected by the exposure to the drug in our NGS data, and that of the Δatp17 mutant was enriched (relative abundance, 416%; $P < 0.001$) (Fig. 5C). To further ensure the validity of the competitive fitness profiling data, an additional experiment was carried out using brefeldin A-exposed cultures inoculated with freshly prepared spore stocks. The strain abundance was assessed by real-time PCR. Again, the Δarf2 mutant ($P < 0.001$) was depleted and the Δatp17 mutant ($P = 0.08$) was enriched in the pool. Confirmation that the Δcyp51A mutant was depleted in the pool exposed to itraconazole was also shown by reverse transcription-quantitative PCR (qRT-PCR) (Fig. 5D).

The phenotypes of the Δarf2, Δcyp51A, and Δatp17 isolates were further validated by assessing strains with the strain ID -2 designation in growth assays on RPMI agar for 72 h in the presence and absence of the drug (Fig. 6). Consistent with the results from the competitive fitness data, the Δcyp51A mutant was hypersensitive to itraconazole compared to the Δmot1 mutant. Similarly, the Δarf2 and Δatp17 mutants were hyper-
sensitive and resistant, respectively, to brefeldin A compared to the same control isolate (Δmot1 mutant).

Altogether, these data demonstrate the feasibility of assaying the fitness of multiple A. fumigatus strains in a single pooled culture.

DISCUSSION

Our study demonstrated for the first time that vegetative cell fusion in filamentous fungi can be an inducible phenomenon, and we have shown this to occur in the human pathogenic fungus, A. fumigatus. We exploited the lack of significant constitutive cell fusion in liquid media to perform competitive fitness analyses and to show chemically induced haploinsufficiency in the known molecular targets of the antifungal agents itraconazole and brefeldin A.

Filamentous fungi commonly undergo constitutive vegetative cell fusion, which permits the exchange of cell components including nutrients, water, signal molecules, and genetic material (18–21, 23). Vegetative cell fusion has been most extensively studied in the model N. crassa (22, 23). In contrast to what occurs in N. crassa, our results showed that cell fusion does not occur in A. fumigatus under normal liquid culture conditions and occurs infrequently (maximally, <6% of cells) on solid growth media. The significant appearance of fusion on solid medium may be a consequence of longer coincubation, although other factors may contribute to strain fusion. Prior to our study, the evidence for cell fusion in A. fumigatus came from research involving the use of parasexual genetics with this organism (29). The parasexual cycle is a nonsexual mechanism for nonmeiotic genetic recombination. It involves the formation of a heterokaryon by cell fusion that leads to the formation of a diploid nucleus, which is believed to be unstable and can produce genetic segregants by recombination involving mitotic crossing over and/or haploidization. The parasexual cycle was previously employed as a tool to aid the genetic and molecular characterization of A. fumigatus (25, 28, 30). Heterokaryon formation in these studies involved complementing auxotrophic mutants under selection pressure. We demonstrated that significant cell fusion occurred after mixing complementing nitrate auxotrophs expressing either GFP or TurboFP635 on solid medium. We hypothesized that the induction of cell fusion might
be related to a stress response (nitrogen starvation). In support of this hypothesis, we found that exposure to stresses from sublethal concentrations of a clinical antifungal agent (itraconazole) and another inhibitory drug (cerulenin) also induced consistently significant cell fusion. The sites of fusion between conidia and between germ tubes were identified for the first time in *A. fumigatus*. They involved short cell protrusions/hyphae that were characteristically thinner than germ tubes and superficially resembled conidial anastomosis tubes (CATs) in *N. crassa* and *Fusarium oxysporum* (20, 21).

How inducible cell fusion is regulated in *A. fumigatus* is not clear. It is also unclear why fungi such as *A. fumigatus* rarely undergo cell fusion under nonstressed conditions, while many other filamentous fungi do so constitutively. However, there must be a selective advantage for having this different mechanism of cell fusion. For example, it may be advantageous for *A. fumigatus* not to expend large amounts of energy in undergoing cell fusion when environmental conditions are favorable. However, when the environmental conditions cease to be favorable, cell fusion is induced to facilitate nonmeiotic recombination via the parasexual cycle in order to increase the genetic fitness of the fungus. Sexual reproduction of *A. fumigatus* has not been directly observed in the wild, although it has been demonstrated in culture (25). Thus, nonmeiotic recombination may be important for generating genetic variation in the predominate asexually reproducing fungus in the natural environment. Cell fusion may also play roles in the spread of antifungal drug resistance or virulence factors by means of horizontal gene transfer (19, 31) in the environment and/or the human host. Increased gene dosage resulting from horizontal gene transfer may also increase fitness against certain environmental challenges and, particularly, exposure to drugs.

The lack of efficacy, the significant toxicity, and the emergence of resistance associated with the limited antifungal agents currently in clinical use highlight the need for the development of antifungal drugs against novel targets (6). Significant advances have been made in the application of functional genomics tools to drug target discovery in the yeasts *C. albicans* and *S. cerevisiae* through the use of chemical genomics (14, 17). The power of chemical genomics comes from the fact that targets identified through this approach can be defined as both druggable and selective so long as the compounds used in the screen are potent against the pathogen of interest and lack significant toxicity against the host species. Despite its obvious usefulness, limited progress has been made in filamentous fungi, particularly because of the technical hurdles in constructing a suitable library of mutants to permit the adoption of similar technologies. This study addresses these problems and demonstrates a chemical genomics system that can be used in a cost-effective manner to rapidly and reproducibly identify the mechanism of action of antifungal compounds that are selectively active against molds (32).

A key factor in chemical genomics technologies is the requirement for assessing the fitness of multiple strains in parallel. Whereas the competitive fitness of yeast strains has been the cornerstone of functional genomics technologies, many and possibly the majority of filamentous fungi undergo vegetative cell fusion, which enables the exchange of genetic material between cells (22). In the context of pooled fitness profiling, such exchanges would clearly be detrimental, resulting in the masking of fitness deficiencies. The lack of cell fusion in *A. fumigatus*, particularly in liquid shake culture, indicated that a parallel assessment of fitness using multiple strains should be possible in *A. fumigatus*. We demonstrated this by using chemical genomics to confirm the target of action of the well-characterized antifungal agent brefeldin A as the ARF GTPase ARF2 and that of itraconazole as lanosterol 14 alpha demethylase cyp51A.

To maximize the usefulness of this technology, a library will need to be generated that includes strains representing heterozygous mutants in the majority of the essential genes from *A. fumigatus*. Several studies have been carried out to directly identify the essential gene set in *A. fumigatus*. Hu et al. (33) identified 35 genes critical for survival by using a conditional promoter replacement strategy, and we previously identified 96 essential loci by using a transposon mutagenesis approach (28). However, with an estimated 1,000 to 2,000 essential genes in *A. fumigatus*, we are far short of a...
comprehensive understanding in this area. The whole genome knockout (KO) project in *N. crassa* has identified 1,765 genes that can only be isolated as heterokaryons; presumably, most are essential for viability (see [https://geiselmed.dartmouth.edu/dunlaploros/genome/](https://geiselmed.dartmouth.edu/dunlaploros/genome/)). This set represents a useful starting point to investigate the essential set in *A. fumigatus* using the high-throughput gene knockout approach described here. However, the finding that only around 60% of the essential genes are common between the yeasts *S. cerevisiae* and *C. albicans* (34) suggests that the differences between *N. crassa* and *A. fumigatus* may be significant. We are currently leading an *A. fumigatus* genome-wide knockout project. Once completed, we should be able to classify the essential gene set of *A. fumigatus*.

In this study, we identified that a heterozygous Δatp17 isolate displayed increased tolerance against brefeldin A. *atp17* encodes the f-subunit of the mitochondrial F1F0 ATP synthase and is essential for ATP synthase (complex V) activity (35) and mitochondrial maintenance. In the comparable HIP assay in *S. cerevisiae* (36), an ATP17 heterozygous null isolate was assessed in two independent screens with brefeldin A. No conclusive fitness result was obtained, although one screen indicated that ATP17 may provide a modest but not statistically significant increase in fitness. The mechanistic link between *atp17* haploinsufficiency and brefeldin A resistance is unclear. The mechanism of action of brefeldin A is to inhibit vesicle formation by preventing guanine nucleotide exchange factor 1 (GEF1)-mediated conversion of GDP-Arf to GTP-Arf and the subsequent assembly of the COP-1 complex (37). A disruption of respiratory function, i.e., complex V activity, is likely to lead to a reduction in ATP/ADP ratios (38) and consequently ATP/AMP ratios (39). This in turn may affect cellular metabolic flux, promoting the formation of guanine nucleotides and ultimately GTP, the substrate for Arf2, at the expense of adenosine nucleotides. Indeed deficiencies in adenylosuccinate synthase, the enzyme that catalyzes the first step in the formation of ADP from IMP (also the precursor for guanine nucleotides) has been shown to lead to significant increases in GTP (40).

This study takes on further significance because of the potential additional applications of the competitive fitness methodologies. Haploinsufficiency profiling has been used recently in an attempt to assign functional relevance to genes that have not been functionally characterized. Following the screen, a functional role for at least 97% of the genome was found. To date, around 45% of the *A. fumigatus* genome is annotated on the *Aspergillus* genome database (ASPGD) (41) as having no known molecular function, and where annotation has been given, the majority of proposed functions have come from data acquired from other organisms. This study provides the proof of concept for assessing, in a high-throughput manner, the comparative fitness of large numbers of strains in parallel for *A. fumigatus*.

In the Gram-positive bacterium *Streptococcus pneumoniae*, competitive fitness profiling of a library of 10 to 25,000 transposon mutants enabled the identification of the fitness defect caused by the loss of each gene in the genome and multiple networks of interacting genes (42). In a similar study in the pathogenic Gram-negative bacterium *Haemophilus influenzae*, fitness profiling was carried out in a murine infection model, resulting in the identification, in a quantitative manner, of 136 genes that were essential to maintain full virulence (43). Given the exceptionally low number of genes that have been associated with a reduction of virulence in *A. fumigatus*, this technology presents the opportunity to evaluate the effects of mutations in a massively parallel way in an infection model.

**MATERIALS AND METHODS**

**Strain construction.** The strains used in this study are given in Table S3 in the supplemental material. *A. fumigatus* strains were generated that constitutively expressed GFP (strain ID, MFIGFP4) or TurboFP635 (Katushka) (strain ID, MFIGRag29) in the cytoplasm. These fluorescent proteins were expressed under the control of the β-tubulin gene promoter (AFUB_086810) or the glyceraldehyde-3-phosphate dehydrogenase (gpdA) gene promoter (AFUB_030490), respectively. Fluorescent protein expression cassettes, which harbored *A. fumigatus* pyrG (AFUB_024310) as a selectable marker, were generated via fusion PCR (described elsewhere [44]) and transformed into a stable pyrG<sup>-</sup> derivative of A1163 (45). The selection of MFIGFP4 and MFIGRag29 on chlorate-containing medium permitted the isolation of strains.
unable to use nitrate as the sole source of nitrogen. Mutations in the molybdenum cofactor (cnx) and nitrate reductase gene (niaD) were confirmed by growth on minimal medium supplemented with different nitrogen sources (46). These strains were designated AKGFPcnx, AKGFPniaD, AFTurboFP635cnx, and AFTurboFP635niaD.

A stable diploid strain was engineered as the parent for mutant construction. Briefly, cnx and niaD mutants of strain A1160 (pyrG−ΔkuBmox) were isolated by the method described above. Heterokaryons/diploids of pyrG−ΔkuBmox cnx and pyrG−ΔkuBmox niaD were isolated by coculturing the strains on AMMNO3 agar containing uridine and uracil (UU) and identifying avidly sporulating colonies. As spores of *A. fumigatus* are typically uninucleate, diploids were differentiated from heterokaryons by plating spores from these colonies at low density on AMMNO3 UU agar. All colonies from diploid isolates appear as avidly sporulating colonies, whereas spores from heterokaryons grow poorly. Diploids were confirmed by haploidizing on medium containing benomyl and confirming the presence of nitrate-nonutilizing derivatives on AMMNO3 UU agar (28). All nitrate-nonutilizing derivatives of strain AFMB3 carried either niaD− or cnx− markers (46) in a ratio of ca. 1:1, indicating that the strain was not triploid. The stability of one diploid isolate termed AFMB3 (cnx+/−niaD ΔkuBmoxΔkuBmox pyrG/pyrG) was assessed by plating 300 spores on AMMNO3 UU agar from a culture passaged for 5 × 2 days on Sabouraud UU agar. No poorly growing colonies, indicative of diploid breakdown, were identified. Forty-eight targeted gene deletion cassettes were generated by fusion PCR and transformed into AFMB3 (see Tables S1 and S5 for a list of targeted genes and primers). Duplicate clones of these heterozygote strains were validated by PCR using primers flanking upstream and downstream boundaries of the deletion cassette to confirm that one copy of the gene of interest had been replaced. The presence of the second copy of the gene of interest was confirmed by amplification of a ca. 1-Kb fragment within the coding sequence. The strains were given designations -1 and -2.

Culture conditions. Strains were regularly maintained on Sabouraud agar. For strains deficient in pyrimidine biosynthesis (pyrG−), including all diploid isolates, the media were supplemented with 10 mM uridine and 1 mM uracil. Strains deficient in nitrate assimilation (cnx− and niaD−) were maintained on Sabouraud agar containing 600 mM KClO3.

The assessment of cell fusion in *A. fumigatus* (AF293) was initially tested on oatmeal agar incubated for 12 h at 25°C as previously described by Roca et al. (21). The subsequent evaluation of cell fusion (AF293 and CEA10) was performed for periods up to 48 h at 25°C or 37°C on oatmeal, Vogel’s, tap water, Czapek Dox, yeast-potato dextrose, or *Aspergillus* minimal agar medium and in liquid *Aspergillus* minimal medium (AMM) (46).

For the evaluation of cell fusion in the fluorescent strains (AKGFP; AFTurboFP635 and derivatives), conidia of different paired strains were mixed in a 1:1 ratio (final concentration, 1 × 106 spores/ml) and used to inoculate assay media (liquid and solid AMMNO3) (46) or liquid RPMI 1640 medium (27). All cultures were incubated at 37°C. Static liquid cultures were performed in 200 to 400 μl of culture medium within the individual 8-wells of IbiTreat cell culture slides (Ibidi, Martinsried, Germany). Shake flask cultures, including competitive fitness profiling experiments (50 ml), were inoculated with 2.5 × 106 spores/ml in 250-ml conical flasks at 200 rpm in RPMI 1640 and supplemented as described in Results. For the routine assessment of cell fusion on solid medium, the center of an agar plate (as defined in Results) was spot inoculated with 1 μl of a mixed 1 × 105 spores/ml suspension and incubated for 40 to 91 h.

LTSEM. Germinated conidia for examination by low-temperature scanning electron microscopy (LTSEM) were inoculated and cultured on cellophane (uncoated Rayopaque) over oatmeal agar to maintain them on the surface of the medium for imaging. The cellophane was adhered to the sample stub with Tissue-Tek (Agar Scientific, Stanstead, UK), and specimen preparation was, as described previously (47), by using a Hitachi S-4700 cold field emission scanning electron microscope fitted with a Gatan Alto 2500 cryospecimen system. The specimens were partially freeze-dried in the microscope by bringing the temperature of the microscope cold stage up to approximately −95°C and then sputter coated with 600:40% gold-palladium alloy in the cryopreparation unit.

Live-cell confocal imaging of cell fusion. Live-cell confocal imaging was performed using a Leica TCS SP8x inverted confocal microscope equipped with a tuneable white light laser (WLL), a 63× (1.2 numerical aperture [NA]) water immersion objective, and HyD hybrid detectors in gating mode (0.3 to 6 ns) to eliminate laser reflection from the glass. GFP was excited at 481 nm with 20% WLL power, and the fluorescence was detected at 493 to 559 nm. TurboFP635 was excited at 592 nm with 28% WLL (1.2 numerical aperture [NA]) water immersion objective, and HyD hybrid detectors in gating mode detected either niaD− or cnx− markers (46) in a ratio of ca. 1:1, indicating that the strain was not triploid. The stability of one diploid isolate termed AFMB3 (cnx+/−niaD ΔkuBmoxΔkuBmox pyrG/pyrG) was assessed by plating 300 spores on AMMNO3 UU agar from a culture passaged for 5 × 2 days on Sabouraud UU agar. No poorly growing colonies, indicative of diploid breakdown, were identified. Forty-eight targeted gene deletion cassettes were generated by fusion PCR and transformed into AFMB3 (see Tables S1 and S5 for a list of targeted genes and primers). Duplicate clones of these heterozygote strains were validated by PCR using primers flanking upstream and downstream boundaries of the deletion cassette to confirm that one copy of the gene of interest had been replaced. The presence of the second copy of the gene of interest was confirmed by amplification of a ca. 1-Kb fragment within the coding sequence. The strains were given designations -1 and -2.

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Automated quantification of cell fusion. To automate and provide an unbiased and comparative analysis of cell fusion on solid medium, a novel pixel-based colocalization image analysis macro called “3D Pixel CoLoc” (see File S1) was developed for FIJI (v.1.52b) (48) that calculated fusion frequency by enumerating GFP, TurboFP635, and colocalized pixels in each optical section of large tile-scanned confocal image volumes (File S1). An in-house FIJI macro, “Pixel CoLoc Overlay” (File S1), the FIJI plugin Figures, IMARIS (v.8.2.0, Bitplane), and Adobe Illustrator (v.16.0.0) were used to generate representative sum projected images and 3D movies of the absence and presence of cell fusion.

Competitive fitness profiling. Equal numbers of spores of each heterozygote strain designated -1 were pooled and grown in 50-ml cultures (final inoculum of 2.5 × 106 spores/ml) at 200 rpm for 40 h in the presence and absence of drug in triplicates, after which, DNA was extracted (45).
To generate the NGS library, ~30 μg DNA was sheered by sonication to generate fragment sizes of 100 to 400 bp. Adaptors were appended to the sheered DNA as previously described (43). The 3’ sequences flanking the KO-cassette (pyrG) were enriched from the adapter-ligated DNA fragment by real-time PCR using primers PE2.0 (5’-CAACGAGAACGGCACTAGGTTCCTGTCGATGGAAC CGCTCTTCCATCT-3’) and PE1ANpyrG (5’-AATGATACGCGACACCACCGAGATCTACACCTTTCTCTACACGA CGCTCTTCGGATNNNNNNCATGATCGATGTGTTAACCGG3’), where NNNNN denotes the unique index that identifies the sample, and 1× Absolute qPCR SYBR green mix (Thermo Scientific). The PCR conditions were as follows: denaturing at 95°C for 15 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s. The reaction was stopped before the amplification curve reached the plateau stage, typically 25 to 28 cycles. Equal amounts of the PCR amplicons were pooled, and this library was run on a single lane of an Illumina MiSeq using the standard Illumina sequencing primer (5’-ACACTCTTTCCTATACACGAGGC TCTTTCCATCT-3’).

Sequenced data was filtered for quality using the FASTQ quality filter in FASTX-toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) and trimmed to remove sequence corresponding to the pyrG cassette using Cutadapt (https://cutadapt.readthedocs.io/en/stable/index.html). The resulting fastq file was mapped to an index file containing sequences corresponding to those regions immediately flanking the pyrG cassette in each heterozygous knockout strain (Table S4) using Bowtie2 (49). The counts were generated in a human readable format using a pipeline comprising SAMtools (50) and a bespoke Perl script. The fitness ratio was calculated using DEseq2 (51) to calculate the differential abundance of each strain, and then the untreated pool was compared to that of the treated pool.

Real-time PCR. Real-time PCR primers were designed to amplify ~200 bp from the Aspergillus nidulans pyrG selectable marker (using common primer AnidpyrGRT) into the 5’ genomic flanking region, generating unique sequences for each heterozygote analyzed (see Table S5). The qPCR reactions were carried out using Absolute qPCR SYBR green mix (Thermo Scientific) as follows: after denaturation, 30 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 s. For each pooled culture, each unique sequence was amplified in triplicates, and relative strain fitness was determined using the comparative threshold cycle (CT) method (ΔΔCT method) by employing Relative Expression Software Tool (REST) (52). The radial growth rates of those strains shown to be deficient in the pooled model were calculated (n = 3 replicates) on AMMNO3 agar.

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01615-18.

SUPPLEMENTAL FILE 1, PDF file, 1.9 MB.
SUPPLEMENTAL FILE 2, MP4 file, 14.6 MB.
SUPPLEMENTAL FILE 3, MP4 file, 12.5 MB.
SUPPLEMENTAL FILE 4, MP4 file, 13.5 MB.

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