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Unraveling the Biology of a Fungal Meningitis Pathogen Using Chemical Genetics

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SUMMARY

The fungal meningitis pathogen *Cryptococcus neoformans* is a central driver of mortality in HIV/AIDS. We report a genome-scale chemical genetic data map for this pathogen that quantifies the impact of 439 small-molecule challenges on 1,448 gene knockouts. We identified chemical phenotypes for 83% of mutants screened and at least one genetic response for each compound. *C. neoformans* chemical-genetic responses are largely distinct from orthologous published profiles of *Saccharomyces cerevisiae*, demonstrating the importance of pathogen-centered studies. We used the chemical-genetic matrix to predict novel pathogenicity genes, infer compound mode of action, and to develop an algorithm, O2M, that predicts antifungal synergies. These predictions were experimentally validated, thereby identifying virulence genes, a molecule that triggers G2/M arrest and inhibits the Cdc25 phosphatase, and many compounds that synergize with the antifungal drug fluconazole. Our work establishes a chemical-genetic foundation for approaching an infection responsible for greater than one-third of AIDS-related deaths.

INTRODUCTION

Invasive fungal infections are notoriously difficult to diagnose and treat, resulting in high mortality rates, even with state-of-the art treatments. The three most common pathogenic agents are *Cryptococcus neoformans, Candida albicans*, and *Aspergillus fumigatus* (Mandell et al., 2010). These organisms are opportunistic fungi that prey on individuals with varying degrees of immune deficiency. Susceptible patient populations include premature infants, diabetics, individuals with liver disease, chemotherapy patients, organ transplant recipients, and those infected with HIV (Mandell et al., 2010). Compounding the clinical challenge is the slow pace of antifungal drug development: only a single new class of drugs (the echinocandins) has been approved for use in the United States in the last 30 years (Butts and Krysan, 2012; Mandell et al., 2010; Roemer et al., 2011).

Fungal infections are estimated to cause 50% of deaths related to AIDS and have been termed a "neglected epidemic" (Armstrong-James et al., 2014). The fungus chiefly responsible for deaths in this population is *C. neoformans* (Armstrong-James et al., 2014). *C. neoformans* is an encapsulated basidiomycetous haploid yeast distantly related to *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. A 2009 CDC study estimated that ~1 million infections and ~600,000 deaths annually are caused by *C. neoformans*, exceeding the estimated worldwide death toll from breast cancer (Lozano et al., 2012; Park et al., 2009). *C. neoformans* is widespread in the environment and exposure occurs through inhalation of desiccated yeast or spores (Heitman et al., 2011). In immunocompromised patients, *C. neoformans* replicates and disseminates, causing meningoencephalitis that is lethal without treatment (Heitman et al., 2011). Induction therapy involves flucytosine and intravenous infusions of amphotericin B (Loyse et al., 2013). Both drugs are highly toxic, difficult to administer, and neither is readily available in the areas with the highest rates of disease. The current recommendation for Cryptococcosis treatment is at least a year of therapy, which is difficult to accomplish in resource-limited settings (WHO, 2011). Thus, as is the case with infections caused by other fungal pathogens, effective treatment of cryptococcal infections is limited by the efficacy, toxicity, and availability of current pharmaceuticals.

We implemented chemogenomic profiling to approach the challenges of therapeutic development in *C. neoformans*. This method involves the systematic measurement of the impact of compounds on the growth of defined null mutants to produce a chemical-genetic map. Such a map represents a quantitative description composed of numerical scores indicative of the growth behavior of each knockout mutant under each chemical condition. Cluster analysis of the growth scores for large

numbers of mutants under many chemical conditions can reveal genes that function in the same pathway and even those whose products are part of the same protein complex (Collins et al., 2007; Parsons et al., 2004; Parsons et al., 2006). In addition, the identity of genes whose mutation produce resistance or sensitivity is useful for uncovering compound mode of action (MOA) (Hillenmeyer et al., 2008; Jiang et al., 2008; Nichols et al., 2011; Parsons et al., 2006; Xu et al., 2007; Xu et al., 2009). Large-scale studies have been restricted to model organisms for which gene deletion collections have been constructed, namely *S. cerevisiae*, *S. pombe*, and *Escherichia coli* K12 (Hillenmeyer et al., 2008; Nichols et al., 2011; Parsons et al., 2006). However, as none of these are pathogens, the extent to which the resulting insights translate to pathogenic organisms is unknown. A variation on chemogenomic profiling, chemically-induced haploinsufficiency, was first developed using a diploid heterozygote gene deletion library *S. cerevisiae* to identify compound MOA. This method, which identifies genes that impact compound sensitivity based on a two-fold gene dosage change, is suited for diploid organisms and has been used in the pathogen *C. albicans* (Jiang et al., 2008; Xu et al., 2007; Xu et al., 2009).

We report here the generation of a large-scale chemogenomic map for *C. neoformans* using defined, commonly available knockout mutants, assessments of data quality, and extensive experimental verification. Comparisons of the *C. neoformans* profile with two large-scale published profiles from *S. cerevisiae* revealed that for most types of compounds, the chemical-genetic interactions are distinct even among orthologous genes, emphasizing the importance of pathogen-focused investigation. We used nearest-neighbor analysis to predict new genes involved in polysaccharide capsule formation and infectivity, which we validated through experiment. We also utilized genetic responses to predict the G2/M phase of the cell cycle and the Cdc25 phosphatase as targets of a thiazolidone-2,4-dione derivative, which we confirmed in vivo and in vitro. Finally, because of the unmet need for improved antifungal drug efficacy, we developed a new algorithm, O2M, to predict new compound synergies based on the profiles of pairs known to be synergistic. Experimental tests demonstrate that the method performs vastly better than random expectation, thereby enabling the identification of synergistic compound combinations. Our studies establish a chemical-genetic foundation to approach the biology and treatments of *C. neoformans* infections, which are responsible for more than one-third of HIV/AIDS deaths worldwide.

RESULTS

A Chemical-Genetic Map of *C. neoformans*

We assembled 1,448 *C. neoformans* gene deletion strains (Chun et al., 2011; Liu et al., 2008) (Table S1 available online), corresponding to a substantial fraction of 6,967 predicted *C. neoformans* genes (Janbon et al., 2014), and a collection of compounds for screening (Table 1). Compounds were selected based on cost and literature evidence that they could inhibit the growth of fungi. Where feasible, compounds were chosen that are known to target specific biological processes. For each small molecule, we determined an approximate minimum inhibitory concentration (MIC) in agar, then measured growth of the knockout collection on each small molecule at 50%, 25%, and 12.5% MIC using high density agar plate colony arrays and a robotic replicator. We then measured the size of each colony using flatbed scanning and colony measurement software (Dittmar et al., 2010). We performed a minimum of four replicate colony measurements for each mutant-condition pair. Plate-based assays are subject to known nonbiological effects, such as spatial patterns. To mitigate these errors, a series of corrective measures were implemented using approaches described previously, including manual filtration of noisy data, spatial effect normalization and machine learning-based batch correction (Baryshnikova et al., 2010). In addition, the data for each deletion mutant and compound was centered and normalized. Each mutant-small molecule combination was assigned a score with positive scores representing relative resistance and negative scores representing compound sensitivity (Table S2). A global summary of the processed data organized by hierarchical clustering is shown in Figure 1A.

The importance and validity of the computational corrections is shown in Figures 1B and S1. We estimated how reproducible the chemical-genetic profiles were by calculating the correlation scores for data obtained for different concentrations of the same small molecule (purple). This measures the degree of overlap between the overall chemical-genetic profiles, which are themselves each composed of a score for each mutant-small molecule combination. We found significant correlation ($p = 2.67 \times 10^{-176}$) between data obtained for different concentrations of the same small molecule compared to those between profiles generated by data set randomization, suggesting significant reproducibility. Moreover, correlation scores between chemical-genetic profiles of different concentrations of different compounds (gray) are centered at approximately 0 (Figure 1B). This difference in correlation scores is apparent even when comparing experiments performed on the same day, when spurious batch signal can contribute to false positives (Baryshnikova et al., 2010). Our batch-correction algorithms resulted in same-batch screening data with strong positive correlation scores for the same compounds but correlation scores close to zero for different compounds (Figure S1), demonstrating successful removal of spurious signal (Baryshnikova et al., 2010). We compared chemical-genetic profiles between compounds in the azole family (Figure 1C). Despite the fact that the azoles tested include those of diverse uses, from agricultural pesticides to FDA-approved drugs (Table 1), many exhibit a significant profile correlation ($p = 2.82 \times 10^{-6}$), further indicating significant signal in the data. As a final assessment, we performed hypergeometric testing across all compounds to determine whether the same sensitive gene knockouts (defined by $Z < -2.5$) are identified at different concentrations of the same compounds. Using a Bonferonni-corrected p value cutoff, nearly all compounds display significant overlap of responsive genes at different concentrations (Figure 1D).

We assigned at least one phenotype (sensitivity or resistance to a compound) to 1,198 of 1,448 mutants (Figure 1E, Tables S2, S3, and S4). Of these, 855 exhibit one to ten phenotypes, while remaining 343 displayed from 11 to 146 phenotypes. Gene