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Cross-species discovery of syncretic drug combinations that potentiate the antifungal fluconazole

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Resistance to widely used fungistatic drugs, particularly to the ergosterol biosynthesis inhibitor fluconazole, threatens millions of immunocompromised patients susceptible to invasive fungal infections. The dense network structure of synthetic lethal genetic interactions in yeast suggests that combinatorial network inhibition may afford increased drug efficacy and specificity. We carried out systematic screens with a bioactive library enriched for off-patent drugs to identify compounds that potentiate fluconazole action in pathogenic *Candida* and *Cryptococcus* strains and the model yeast *Saccharomyces*. Many compounds exhibited species- or genus-specific synergism, and often improved fluconazole from fungistatic to fungicidal activity. Mode of action studies revealed two classes of synergistic compound, which either perturbed membrane permeability or inhibited sphingolipid biosynthesis. Synergistic drug interactions were rationalized by global genetic interaction networks and, notably, higher order drug combinations further potentiated the activity of fluconazole. Synergistic combinations were active against fluconazole-resistant clinical isolates and an *in vivo* model of *Cryptococcus* infection. The systematic repurposing of approved drugs against a spectrum of pathogens thus identifies network vulnerabilities that may be exploited to increase the activity and repertoire of antifungal agents.
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Introduction

The recent increase in fungal infection rates presents a serious clinical challenge (Arendrup *et al*, 2009; Gullo, 2009; Shorr *et al*, 2009). Immune-suppressed individuals, including transplant, cancer chemotherapy and HIV-infected patients, often succumb to opportunistic fungal pathogens from the genera *Candida, Cryptococcus, Aspergillus* and others (Groll *et al*, 1996; Baddley *et al*, 2001; Clark and Hajjeh, 2002; Richardson and Warnock, 2003). Unlike bacterial infections that can be treated with multiple antibiotic classes, therapeutic options for fungal infections are limited. The polyene amphotericin B, discovered in 1955, remains a front line fungicidal drug; however, amphotericin B non-specifically disrupts cell membrane integrity, with concomitant severe patient toxicity. Synthetic azole antifungals such as fluconazole were introduced 40 years ago and inhibit lanosterol 14α-demethylase, the gene product of *ERG11*, an essential cytochrome P450 enzyme in the ergosterol biosynthetic pathway (Groll *et al*, 1998; Revankar *et al*, 2004). Fluconazole binds to the heme Fe(III) of Erg11, resulting in depletion of ergosterol, the accumulation of C-14 methyl sterols and cell membrane disruption. The crossreactivity of azoles toward human P450 enzymes also results in toxicity and, moreover, clinical resistance is prevalent (Cannon *et al*, 2009; Marie and White, 2009). Finally, the echinocandins, which include caspofungin, micafungin and anidulafungin, were introduced 10 years ago and inhibit the cell wall biosynthesis enzyme β-(1,3)-D-glucan synthase; however, these agents have a

restricted antifungal spectrum (Sucher *et al*, 2009). The dearth of selective agents and emerging patterns of clinical resistance demand new antifungal strategies.

A primary challenge in antifungal drug discovery is the paucity of fungal-specific molecular targets that are essential for cell growth, due to the conserved biochemical and molecular biological networks of all eukaryotes. This problem is exacerbated by the observation that many essential yeast genes can provide sufficient function at a fraction of wild-type dosage (Yan *et al*, 2009). Although only ~1100 of the ~6000 genes in yeast are essential under nutrient-rich growth conditions (Winzeler *et al*, 1999), almost all genes become essential in specific genetic backgrounds in which another non-essential gene has been deleted or otherwise attenuated, an effect termed synthetic lethality (Tong *et al*, 2001). Genome-scale surveys suggest that over 200 000 binary synthetic lethal gene combinations dominate the yeast genetic landscape (Costanzo *et al*, 2010). The genetic buffering phenomenon is also manifest as a phalanx of differential chemical–genetic interactions in the presence of sublethal doses of bioactive compounds (Hillenmeyer *et al*, 2008). These observations illuminate the inherent redundancy of genetic networks, and frame the problem of interdicting network functions with single agent therapeutics (Hopkins, 2008).

This genetic network organization suggests that judicious combinations of small molecule inhibitors of both essential and non-essential targets may elicit additive or synergistic effects on cell growth (Sharom *et al*, 2004; Agoston *et al*, 2005; Fitzgerald *et al*, 2006; Lehar *et al*, 2007, 2008; Hopkins, 2008). Indeed, *ad hoc* combinations of anti-infective drugs are frequently used to treat fungal infections (Eliopoulos and Moellering, 1991; Johnson and Perfect, 2010). However, this chance approach fails to exploit richness of the chemical–genetic landscape (Sharom *et al*, 2004; Hopkins, 2008; Lehar *et al*, 2008). Instead, unbiased screens for synergistic enhancers of a specific bioactivity that are not themselves active, sometimes termed syncretic combinations, are needed to fully explore chemical space (Keith *et al*, 2005). Compounds that enhance the activity of known agents in model yeast and cancer cell line systems have been identified both by focused small molecule library screens (Borisy *et al*, 2003; Zhang *et al*, 2007; Zhai *et al*, 2010) and by computational methods (Lehar *et al*, 2007; Nelander *et al*, 2008; Jansen *et al*, 2009; Zinner *et al*, 2009). Furthermore, direct tests of synergistic compounds have successfully yielded combinations that are active against pathogenic fungi, including the combination of fluconazole with chemical inhibitors of Hsp90, calcineurin or ARF (Cowen *et al*, 2009; Singh *et al*, 2009; Epp *et al*, 2010) and the antibiotic polymyxin B (Zhai *et al*, 2010).

To extend the strategy of chemical synthetic lethality to clinically relevant fungal pathogens, we interrogated a focused bioactive library of known drugs for synergistic enhancers of the fungistatic drug fluconazole in systematic screens against *Candida albicans*, *Cryptococcus neoformans* and *Cryptococcus gattii*, as well as the genetically tractable budding yeast *Saccharomyces cerevisiae*. Compounds not previously recognized in the clinic as antifungal agents caused potent growth inhibition in conjunction with fluconazole, often in a genus- or species-specific manner. Selected combinations were characterized for mechanism of action and shown to be active against fluconazole-resistant isolates and efficacious in an *in vivo* infection model. The combinatorial redeployment of known drugs defines a powerful antifungal strategy and establishes a number of potential lead combinations for future clinical assessment.

Results

Systematic antifungal potentiation screens in model and pathogenic fungi

Cell-based high-throughput screens were performed on a panel of four fungal strains to identify small molecules that potentiate fluconazole across a range of genera and species. The human pathogens *C. neoformans* (H99), *C. gattii* (R265) and *C. albicans* (Caf2-1) as well as the model fungus *S. cerevisiae* (BY4741) were screened in duplicate against the Prestwick library, which consists of 1120 off-patent drugs and other bioactive agents (http://www.prestwickchemical.com). To identify compounds that potentiate the effect of fluconazole, yet have minimal antifungal activity on their own, each screen was performed in the presence and absence of 0.5 minimal inhibitory concentration (MIC) of fluconazole at a single compound concentration of 30 μM. Residual activity was calculated for each compound and all data were normalized for plate- and row/column-specific effects (Supplementary Figure S1; see Supplementary Table S1 for screen data). Hits were determined using median absolute deviation (MAD) statistics. By this criterion, 43 compounds were active against *S. cerevisiae*, 30 against *C. albicans*, 70 against *C. neoformans* and 91 against *C. gattii* (Figure 1A and B).

The set of 148 compounds that potentiated the antifungal action of fluconazole in one or more of the screens (Supplementary Figure S2) was structurally diverse and represented a broad range of different therapeutic activities, including antiparasitics, cardiovascular protectives, dermatologicals, genitourinary tract anti-infectives, hormone modulators and a variety of neuroleptic drugs. Notably, 15 of the 17 tricyclic phenothiazine/thioxanthene antipsychotics present in the Prestwick library exhibited strong interactions with fluconazole against *C. gattii* and *C. neoformans* (Figure 1C). Derivatives of tricyclic phenothiazines inhibit fatty acid synthesis and disrupt lipid trafficking (Li *et al*, 2008).

A striking number of hits were species or genus specific (Figure 1B). Six compounds were hits in all screens: (i) the antidepressant sertraline (Zoloft®); (ii) the monoamine oxygenase inhibitor pirlindole, also known to have antidepressant activity; (iii) the allylamine antifungal naftifine; (iv) the antibiotic prodrug pivampicillin; (v) the antinausea drug thiethylperazine (Torecan®); and (vi) the antipsychotic drug zuclopenthixol. The latter two compounds are members of the large family of phenothiazines that have antipsychotic and other central nervous system (CNS) activities.

Synergy assessment and fungicidal activity

To determine whether hit compounds acted in a synergistic or additive manner with fluconazole, we selected 12 of the 148 hits (albendazole, azaperone, clofazimine, clomiphene, L-cycloserine, kawain, lynestrenol, mitoxantrone, sertraline, suloctidil, tamoxifen and trifluoperazine) for detailed studies

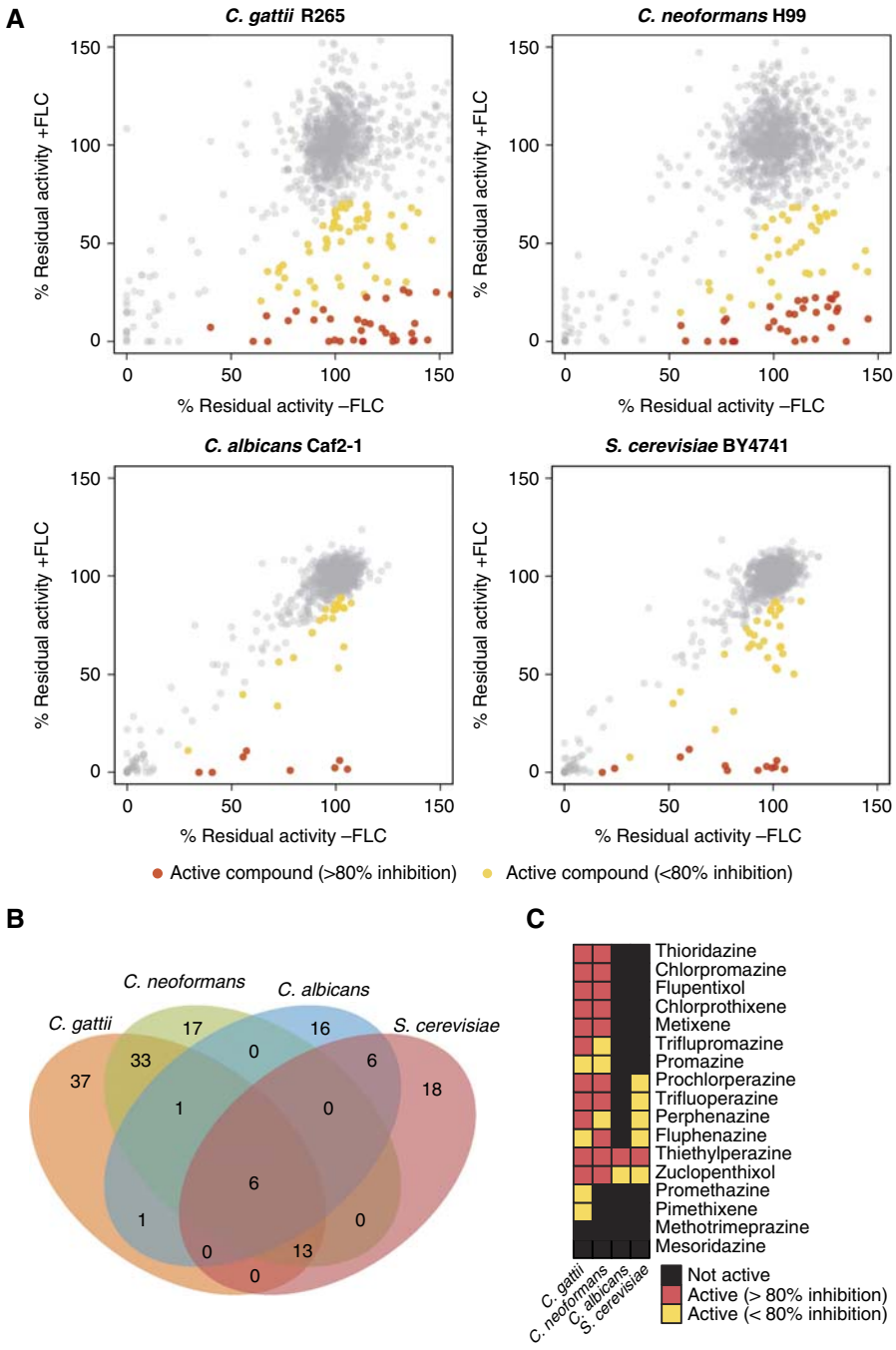

Figure 1 Unbiased screens for bioactive compounds that potentiate the antifungal activity of fluconazole. (**A**) Scatter plots for Prestwick library screens for four fungal species. Growth inhibition caused by compounds in the absence (*x* axis) and presence of fluconazole (*y* axis) is represented by residual activity after treatment. Yellow and red filled circles indicate compounds that were classified as active (2 median absolute deviations below the diagonal). Compounds that inhibited growth in the presence of fluconazole by at least 80% compared with the effect of that compound alone are highlighted in red; FLC, fluconazole. (**B**) Overlap of hits between different fungal species. (**C**) Activity of 17 phenothiazine/thioxathene compounds in different fungal species.

in all four fungal species. We based this selection on an analysis of distinct chemical class, with one or two representative structures from families of similar agents that emerged in the screen, commercial availability of compound, therapeutic importance, and known mode of action (Supplementary Figure S3). These criteria yielded a tractable number of hit compounds for detailed downstream analysis. We also tested five known antifungal drugs, both as positive controls and to explore other potential interactions with fluconazole: amphotericin B, the ergosterol biosynthesis inhibitors ketoconazole, terbinafine (an allylamine analog of naftifine used in the clinic) and fenpropidin (an agricultural fungicide), and the echinocandin caspofungin. Dose-dependent MIC values for these 17 compounds were determined for each of the four species (Supplementary Table S2). The interaction of each compound with fluconazole was assessed by standard con-

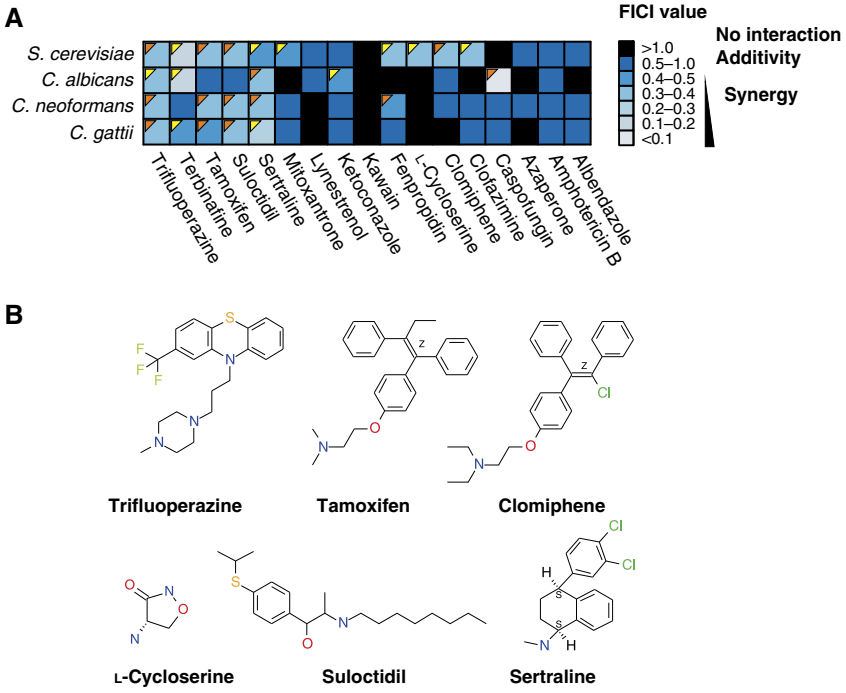

Figure 2 Synergistic drug interactions with fluconazole. (**A**) Heat map of drug interactions with fluconazole in each species. Dark blue indicates additive effects (FICI of 0.5–1); lighter shades of blue represent synergy (FICI <0.5). Orange triangles indicate fungicidal drug combinations; yellow triangles indicate fungistatic drug combinations. (**B**) Chemical structures of the six drugs chosen for detailed mode of action studies. Source data is available for this figure at www.nature.com/msb.

centration matrix (checkerboard) analysis (Figure 2A; Supplementary Table S3). Data were quantified by calculation of the fractional inhibitory concentration index (FICI), the accepted method for drug interactions in infectious disease (Eliopoulos and Moellering, 1991; Odds, 2003). Only two compounds, sertraline and trifluoperazine, exhibited synergy with fluconazole against all four fungal species. A number of synergizers exerted effects exclusively on a particular species: *S. cerevisiae* was uniquely susceptible to four different compounds in the presence of fluconazole (clofazimine, clomiphene, L-cycloserine and mitoxantrone), while only *C. albicans* was susceptible to ketoconazole or caspofungin in combination with fluconazole. Neither *Cryptococcus* species exhibited any unique synergistic susceptibilities. Most hits from the screens were confirmed as synergistic with fluconazole, except for albendazole, azoperone and kawain in *S. cerevisiae*, and azaperone, L-cycloserine and lynestrenol in *C. albicans* (Supplementary Figure S4). Quantification of interactions at different drug concentrations revealed some additional synergies with fluconazole: trifluoperazine exhibited synergy against *C. albicans*, tamoxifen against *C. gattii* and *C. neoformans*, and suloctidil against *C. neoformans* and *S. cerevisiae* (Supplementary Figure S4). Based on the detailed analysis of these 12 compounds, the high-throughput screens proved a reliable means to identify synergistic drug interactions, with an estimated false positive rate of 0.20 and a false negative rate of 0.28. Importantly, and in contrast to the merely fungistatic effect of fluconazole alone, several combinations of fluconazole and different synergistic compounds were fungicidal, often in a species-dependent manner (Figure 2A). For example, trifluoperazine exhibited synergy with fluconazole and was fungicidal in all species with the exception of *C. albicans*.

Chemical–genetic profiles of synergistic combinations

We explored the molecular basis for the synergy of trifluoperazine, tamoxifen, clomiphene, sertraline, suloctidil and L-cycloserine with fluconazole (Figure 2B), using established genome-wide methods in *S. cerevisiae* to identify gene deletion strains that are sensitive to drug treatment (Giaever *et al*, 1999; Parsons *et al*, 2006; Hillenmeyer *et al*, 2008). Genome-wide pools of deletion strains were grown in the presence of drugs, genomic DNA isolated from drug-treated and control cultures, and barcode sequence tags amplified and hybridized to barcode microarrays (Cook *et al*, 2008).

First, we profiled compound action in haplo-insufficiency screens, in which the $\sim$1000 deletion strains heterozygous for essential genes were tested for drug sensitivity to identify candidate drug targets (Giaever *et al*, 1999). As expected, deletion of one copy of *ERG11* conferred sensitivity to fluconazole (Figure 3A; Supplementary Figure S5). Deletion of one copy of either *LCB1* or *LCB2*, which encode subunits of the enzyme serine palmitoyltransferase that catalyzes the committed step of sphingolipid biosynthesis, caused sensitivity to L-cycloserine (Figure 3A; Supplementary Figure S5). In yeast membrane extracts, high concentrations of L-cycloserine (1 mM) partially inhibit serine palmitoyltransferase (Pinto *et al*, 1992). All five remaining compounds—trifluoperazine, tamoxifen, clomiphene, sertraline and suloctidil (referred to as the membrane active group)—conferred sensitivity to loss of one copy of the *NEO1* gene (Figure 3A; Supplementary Figure S5), which encodes an essential aminophospholipid translocase required for membrane trafficking and vacuolar biogenesis. Deletion of the ortholog of *NEO1* in *C. neoformans* (*APT1*) has recently been shown to result in hypersensitivity to amphotericin B and fluconazole, as well as

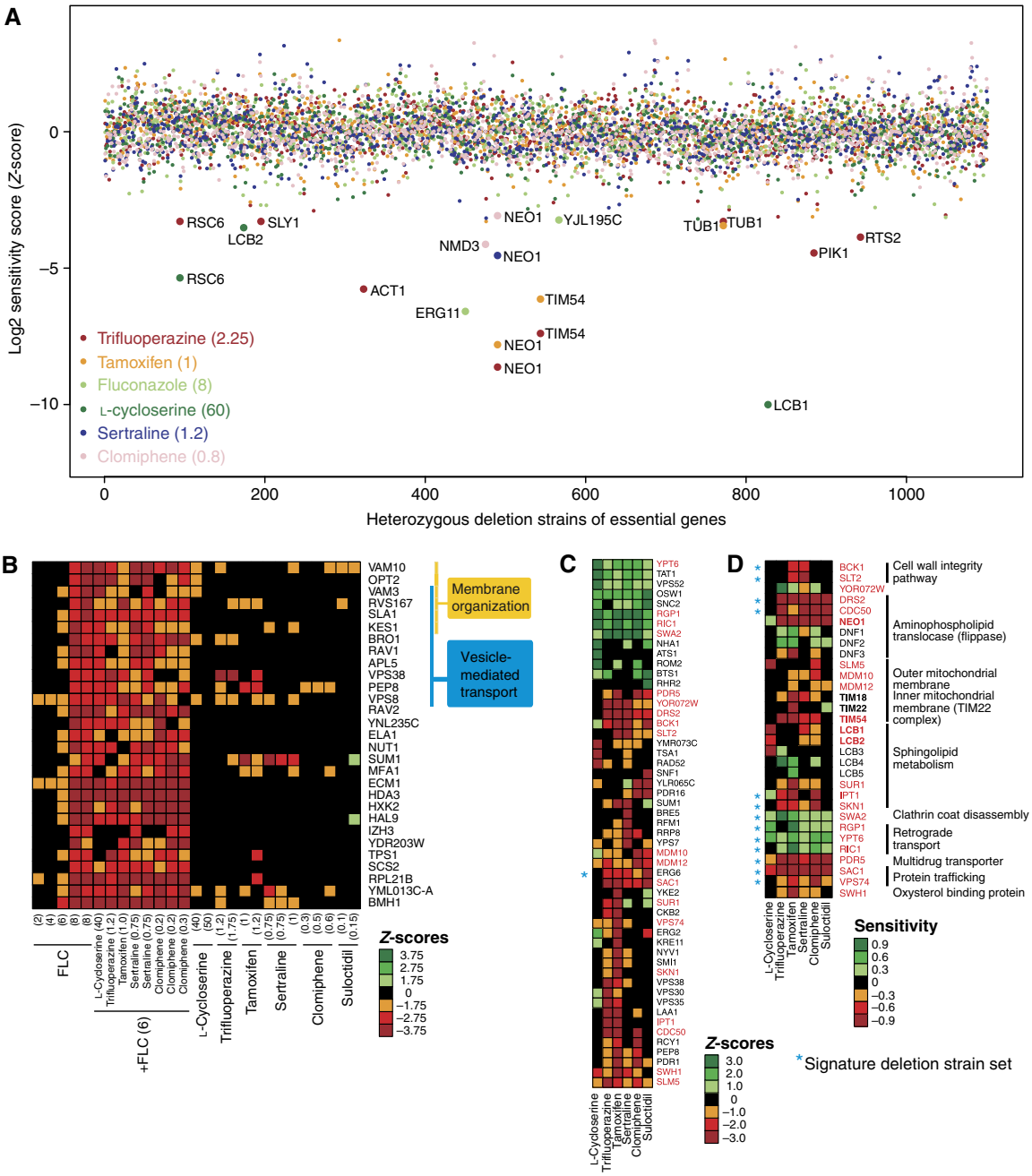

Figure 3 Chemical–genetic interactions of six syncretic synergizers. (**A**) Sensitivity of heterozygous essential deletion strains to five different syncretic drugs and fluconazole, as assessed by barcode microarray hybridization. (**B**) Core set of haploid deletion strains that are sensitive to fluconazole, as assessed by barcode microarray hybridization. Several concentrations of fluconazole were tested to correlate the signature with MIC. The effect of the six syncretic drugs on the core fluconazole profile was examined in the presence or absence of a threshold concentration of fluconazole (6 μg/ml). Genes implicated in membrane organization and vesicle-mediated transport are indicated. (**C**) Main cluster of haploid deletion strain sensitivities to the six syncretic drugs in the absence of fluconazole, as assessed by barcode microarray hybridization. Strains that have a *Z*-score more significant than ± 3 for at least one of the drugs in duplicate profiles are shown. Gene names in red indicate deletion strains that were chosen for verification by quantitative growth curve assays. (**D**) Log-ratio scores calculated from individual growth curve assays to confirm chemical–genetic interactions of the six syncretic drugs. Gene names in bold indicate heterozygous deletion strains for essential genes. Values in parentheses indicate drug concentration in μg/ml. Negative *Z*-scores and log-ratios indicate sensitivity of a strain to a given drug, whereas positive scores represent resistance. Asterisks indicate 14 deletion strains that comprise the core signature set for membrane active compounds. Source data is available for this figure at www.nature.com/msb.

attenuated virulence (Hu and Kronstad, 2010). In addition, deletion of one copy of *TIM54*, a translocase of the inner mitochondrial membrane, confers sensitivity to tamoxifen and trifluoperazine (Figure 3A), consistent with the potential membrane targets of these drugs.

We then generated haploid chemical–genetic profiles for the syncretic compounds individually and in combination with fluconazole (Supplementary Figure S6). This profiling method reveals genes that buffer against drug toxicity and can identify compounds with similar bioactivities based on shared chemical–

genetic interaction profiles (Hillenmeyer *et al*, 2008). Strains deleted for genes that function in vesicle-mediated transport and membrane organization were sensitive to fluconazole alone (Figure 3B). For drug combinations, we chose a concentration of fluconazole (6 μg/ml) that caused ~20% growth inhibition compared with control and thereby minimized the selection against fluconazole-sensitive strains. Importantly, the syncretic drugs alone did not impair the growth of fluconazole-sensitive deletion strains (Figure 3B; Supplementary Figure S5), but significantly sensitized cells to low doses of fluconazole (Supplementary Table S4). To explore the potential mechanism of this sensitization further, we examined the chemical–genetic profiles of single compounds (Figure 3C; Supplementary Figure S7; Supplementary Table S5). The membrane active group of trifluoperazine, tamoxifen, clomiphene, sertraline and suloctidil caused growth inhibition of a core set of deletion strains that included genes that encode the post-Golgi-associated aminophospholipid translocase (flippase) Drs2 and its activating subunit Cdc50, the ergosterol biosynthesis enzyme Erg6, the protein trafficking factors Sac1 and Vps74, the mitochondrial outer membrane import factors Mdm10 and Mdm12, and the cell wall integrity MAPK kinase Slt2 and its upstream activating kinase Bck1. A number of genes implicated in downstream steps of sphingolipid metabolism, including *IPT1*, *SUR1*, *SKN1*, *YPK1* and *SWH1*, were also required for cell survival in the presence of the membrane active compounds. Notably, strains disrupted for non-essential genes implicated in uncoating of clathrin vesicles (*SWA2*) and retrograde transport to the cis-Golgi network (*YPT6*, *RGP1*, *RIC1* and *VPS52*) were resistant to all six fluconazole synergizers, suggesting that altered vesicle trafficking may compensate for membrane perturbation and/or Erg11 inhibition.

We confirmed the chemical–genetic interactions between these haploid deletion strains and each drug using quantitative growth curve assays (Figure 3D). We also assessed strains that were heterozygous for *ERG11*, *NEO1*, *LCB1* and *LCB2* as well as *TIM18* and *TIM22*, which function in a complex with TIM54. In addition, we included haploid deletion strains for *DNF1/2/3*, the other three flippases in *S. cerevisiae*, and *LCB3/4/5*, which function downstream of *LCB1/2*. The quantitative growth curves corroborated the barcode microarray results, with the exception of the dubious ORF *YOR072W*. The range of drug concentrations tested in the growth curve assays revealed additional chemical–genetic interactions, such as *TIM18*, which were not recovered at the single drug concentrations used in the barcode profiles.

The results of these genome-wide chemical–genetic screens point to two related modes of action for the syncretic combinations tested. Trifluoperazine, tamoxifen, clomiphene, sertraline and suloctidil appear to cause general perturbation of membrane, vesicle trafficking and lipid biosynthesis functions, whereas L-cycloserine preferentially interferes with an early step in sphingolipid biosynthesis, consistent with its proposed mechanism of action (Pinto *et al*, 1992). To test the latter hypothesis, we examined the effects of myriocin, another known inhibitor of the first step of the sphingolipid biosynthesis pathway (Miyake *et al*, 1995), and found that it also potentiated the inhibition of cell growth by fluconazole (FICI=0.625).

Cell biological effects of synergistic combinations

We assessed the effects of trifluoperazine, tamoxifen, sertraline and L-cycloserine alone and in combination with fluconazole on *S. cerevisiae* cell physiology. The diagnostic fluorescent dyes Calcofluor White, FM4-64 and Mitotracker Green were used to visualize cell wall and bud scars, vacuolar membranes and mitochondria, respectively. For each reporter dye, fluconazole produced staining patterns that were similar to solvent controls. In contrast, treatment with trifluoperazine, tamoxifen, clomiphene and sertraline caused a drastic loss of localization and strong intracellular accumulation of each dye (Figure 4A; Supplementary Figure S8). In particular, the disruption of vacuolar structure revealed by FM4-64 staining suggested severe loss of cell membrane integrity. Consistent with its different genetic target profile, treatment with L-cycloserine had no observable effects on the localization of any of the dyes (Figure 4B).

Lethal perturbation of the membrane and/or cell wall can often be rescued by osmotic stabilization. Sorbitol (1 M) effectively suppressed the syncretic growth inhibitory effects of trifluoperazine, tamoxifen, clomiphene, sertraline and suloctidil, again supporting a common membrane perturbation mechanism for these compounds, but had no such protective effect on cells treated with L-cycloserine and fluconazole (Figure 4C). These cell biological results affirm the different mechanisms of action of the two compound classes.

Integration of chemical–gene interactions with genetic interaction networks

A primary challenge in the discovery of synergistic drug combinations is the vast number of possible combinations of drug pairs (Sharom *et al*, 2004; Hopkins, 2008; Lehar *et al*, 2008). Integration of drug-induced gene expression profiles (Lum *et al*, 2004) and chemical–genetic profiles (Hillenmeyer *et al*, 2008) with comprehensive genetic interaction networks (Costanzo *et al*, 2010) can allow computational prediction of synergistic drug pairs (Lehar *et al*, 2007; Nelander *et al*, 2008; Jansen *et al*, 2009). To assess whether the individual profiles of fluconazole and each of the syncretic drugs could rationalize drug interactions, we integrated the chemical–genetic profiles generated above for each syncretic compound with a global genetic interaction network composed of both high-throughput (HTP) and low-throughput (LTP) data compiled from the primary literature (Breitkreutz *et al*, 2008; Costanzo *et al*, 2010). Deletion strains that were sensitive to treatment with single drugs were used to assess the number of genetic interactions linked to the chemical–genetic space (CGS) of fluconazole and each of the synergizers. A core set of haploid deletion strains affected by the membrane active group of compounds, referred to as the signature strain set (Figure 3D), exhibited many genetic interactions with the top 50 fluconazole-sensitive strains (Figure 5A). The top 50 most sensitive deletion strains for each individual drug (*Z*-scores above ~2.0) also showed many genetic interactions with the fluconazole profile. We tested the significance of the genetic connections between the profiles using simulations of genetic interactions shared between randomly chosen gene sets of a specific size, based on the known chemical sensitivities of 1143

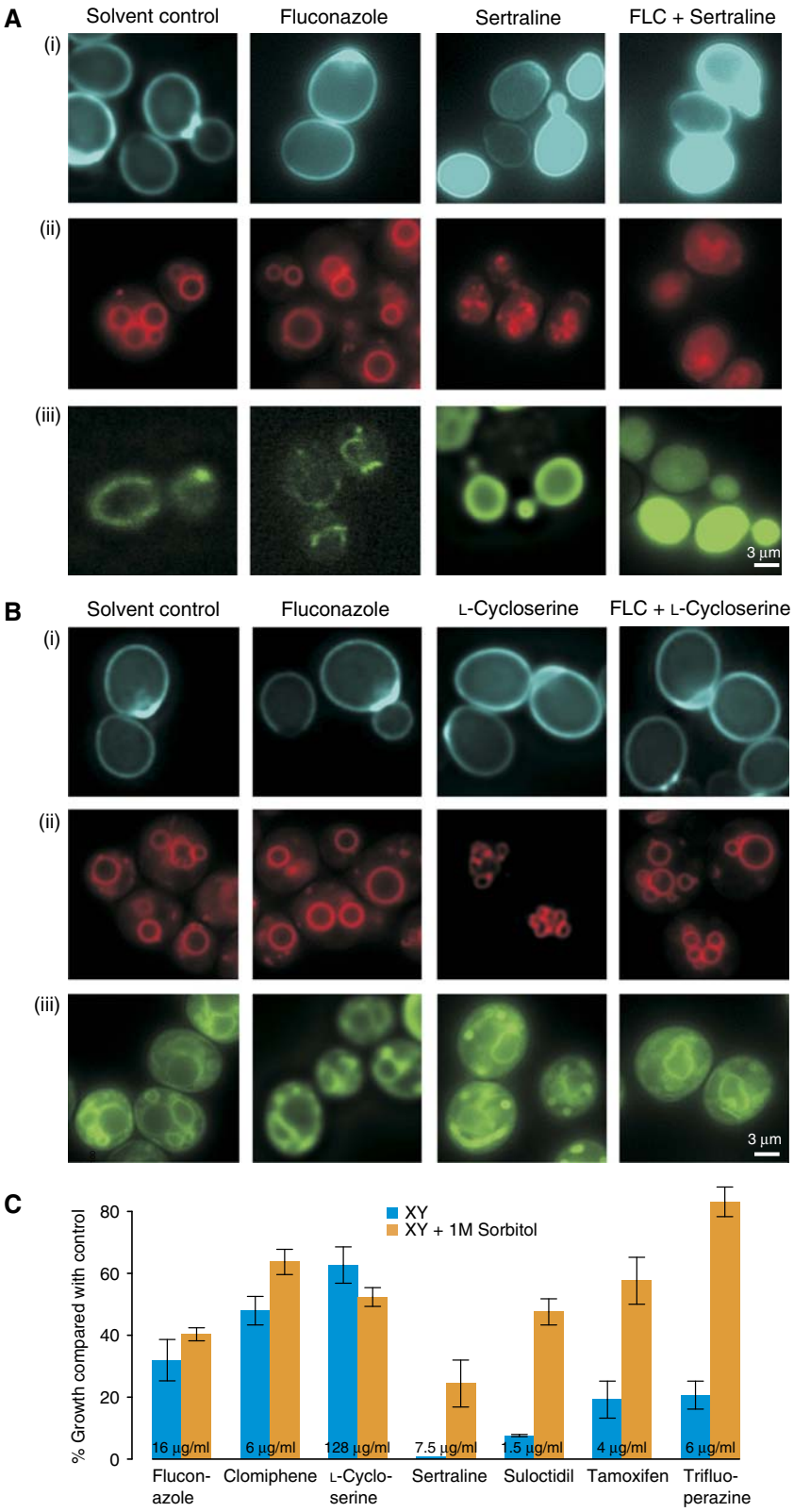

Figure 4 Effects of syncretic drugs on membrane integrity. A wild-type *S. cerevisiae* strain was grown in the presence of the indicated drugs and stained with (i) Calcofluor White M2R, (ii) FM4-64 and (iii) Mitotracker Green FM, and imaged by fluorescence microscopy. (**A**) Sertraline (128 μg/ml) in the presence and absence of fluconazole (64 μg/ml). (**B**) L-Cycloserine (128 μg/ml) in the presence and absence of fluconazole (128 μg/ml). (**C**) Growth of wild-type *S. cerevisiae* compared with control wells in the presence of the indicated drugs with and without 1 M sorbitol. The mean of four independent measurements is shown; error bars represent standard error. Source data is available for this figure at www.nature.com/msb.

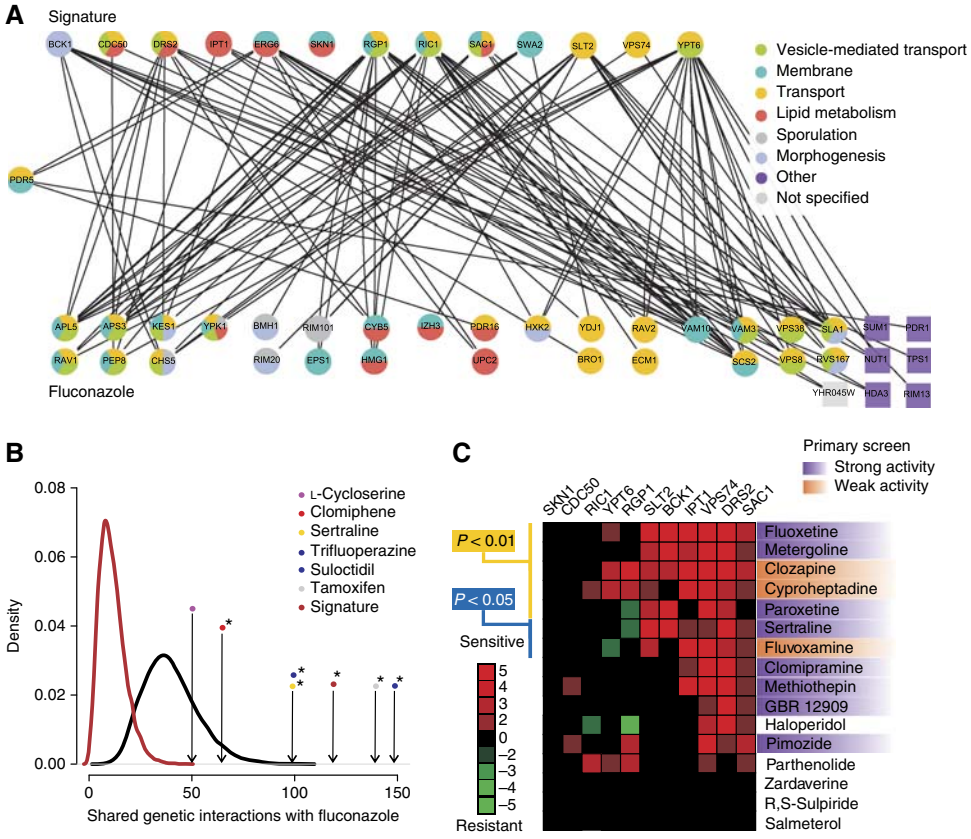

Figure 5 Rationalization of synergistic interactions by integration of chemical–genetic and genetic interaction networks. (**A**) Bipartite graph of genetic interactions between top 50 chemical–genetic interactors of fluconazole and the signature deletion strains sensitive to the five membrane active compounds. As *PDR5* was a member of both sets, it is positioned midway between the two sets. Enriched Gene Ontology (GO) SLIM biological processes are indicated (adjusted *P*-value <0.05). GO enrichment was calculated and visualized using GOlorize (Garcia *et al*, 2007). (**B**) Chemical–genetic space (CGS) simulation with the 50 most sensitive deletion strains for each of the synergistic drugs as well as the signature deletion strain set. Arrows indicate the number of actual genetic interactions for the different drugs; black curve represents the background distribution of genetic interactions between two random sample sets of 50 non-essential deletion strains chosen from 1143 strains that respond to a variety of different chemicals and drugs (Hillenmeyer *et al*, 2008); dark red curve depicts the same background distribution except that the second sample set size was chosen to match the size of signature deletion set. Asterisks indicate a *P*-value <0.05. (**C**) Drug sensitivity of 11 of the 14 signature deletion strains identified in this study for 16 previously profiled psychiatric drugs present in the Prestwick library (Ericson *et al*, 2008). Strong activity refers to compounds that were hits in the primary screens, that is, at least 2 MAD away from the diagonal, whereas weak activity refers to compounds that showed at least 20% growth inhibition and were >1 MAD away from the diagonal. Source data is available for this figure at www.nature.com/msb.

non-essential deletion strains that respond to various drug treatments (Hillenmeyer *et al*, 2008). The signature deletion set shared by the membrane active group was significantly enriched for genetic interactions with fluconazole-sensitive deletion strains (*P*-value $<10^{-7}$). The individual profiles for tamoxifen, trifluoperazine, clomiphene, sertraline and suloctidil also showed a significant enrichment of genetic interactions with the fluconazole-sensitive strain profile (all *P*-values <0.05), whereas L-cycloserine did not (Figure 5B; Supplementary Table S6). As a more conservative measure of pathway separation (Kelley and Ideker, 2005), we applied a parallel pathway permutation (PPP) test, in which the chemical–genetic interactors of fluconazole and each of the synergistic drugs were pooled and randomly assigned to two groups (Supplementary Figure S9). By this stringent method, the signature deletion set and the top 50 most sensitive deletion strains from the trifluoperazine profile also exhibited significant enrichment (both *P*-values <0.05; Supplementary Figure S9; Supplementary Table S6). The genetic interactions that link the chemogenomic profiles of synergistic compound pairs thus provide a rational basis for synergism.

To assess the predictive power of the signature deletion set derived from the membrane active compounds, we retrospectively analyzed chemical–genetic profiles for 81 psychoactive drugs known to impair yeast growth (Ericson *et al*, 2008). Of this set, 16 compounds were represented in the Prestwick library, 7 of which were predicted to synergize with fluconazole based on their effect on deletion strains in the signature set. In our primary screens against the four fungal species, four of these seven compounds were indeed hits in our screen, while the other three compounds showed weak activity (Figure 5C; Supplementary Table S1). These results demonstrate that chemical–genetic interaction profiles can predict synergistic drug combinations.

Species-specific effects of ergosterol pathway inhibition

As the psychiatric drugs trifluoperazine and sertraline exhibited synergy with fluconazole against each fungal species, we tested whether other ergosterol biosynthesis

Table I Combinations of syncretic drugs exhibit species-specific synergism and higher order interactions with fluconazole

	Terbinafine Trifluoperazine	Terbinafine Sertraline	Ketoconazole Trifluoperazine	Ketoconazole Sertraline
(A) FICI values for drug combinations in different fungal species				
C. neoformans (H99)	2	0.75	0.25	0.38
C. albicans (Caf2-1)	2	0.5	0.38	0.16
S. cerevisiae (BY4741)	0.38	0.52	0.38	0.31

	Sert (32)	Sert (64)	Tri	Tri + FLC (4)	Tam	Tam + FLC (4)	Suloc	Suloc + FLC (4)
(B) FICI values for double and triple drug combinations in S. cerevisiae								
Trifluoperazine	0.38	0.50						
Tamoxifen	0.50	0.63	0.50	0.75				
Suloctidil	1.25	1.50	2.00	0.56	1.00	0.75		
L-Cycloserine	1.25	0.63	1.00	2.00	1.00	0.52	2.00	0.31

(A) FICIs from combination matrix analysis of sertraline, trifluoperazine, with the ergosterol inhibitors terbinafine and ketoconazole in different species. (B) FICIs from combination matrix analysis of syncretic drugs and higher order combinations. Trifluoperazine (Tri), tamoxifen (Tam), suloctidil (Suloc) and L-cycloserine were combined as indicated, in the presence or absence of 1/8 MIC fluconazole (4 μg/ml) and assayed for growth inhibition of a *S. cerevisiae* strain (BY4741). FICI values <0.5 indicate synergy, values between 0.5 and 1 indicate additivity and values >1 indicate no interaction. Drug concentrations are given in μg/ml.

inhibitors might exhibit synergy with these compounds. We assessed interactions with ketoconazole, an imidazole inhibitor of *ERG11*, and terbinafine, an inhibitor of the Erg1 squalene epoxidase in *Cryptococcus, Candida* and *Saccharomyces*. Ketoconazole was synergistic with both psychiatric drugs in all fungal species, whereas terbinafine showed synergies with sertraline in *Candida* and *Saccharomyces* but not in *Cryptococcus*, and synergized with trifluoperazine only in *Saccharomyces* (Table IA). These findings suggest that while mechanisms of synergy are conserved between different inhibitors of the same enzymes/pathways, species-specific differences readily emerge with different compounds, likely due to subtle differences in genetic network structure (Kuo *et al*, 2010).

Higher order combinations of synergizers

Compounds that act in an identical manner are in principle expected not to exhibit synergy but instead should show only additive dosage effects. We examined pairwise combinations within four members of the membrane active group (sertraline, trifluoperazine, suloctidil and tamoxifen), as well as with the sphingolipid-selective synergizer L-cycloserine (Table IB). Despite their partially overlapping genetic profiles, synergistic interactions in *S. cerevisiae* were observed between tamoxifen and trifluoperazine (FICI=0.5), sertraline and trifluoperazine (FICI=0.4) and sertraline and tamoxifen (FICI=0.5). These synergies suggested that in addition to the core effects on membrane permeability, each compound likely elicits one or more specific effects that contribute to overall mechanism of action, and that combining these effects results in further synergism. This observation predicted that higher order combinations between synergizers might lead to even stronger growth inhibition. When compound pairs were tested in the presence of fluconazole in three-way combinations, fungal growth was often potently inhibited (Table IB). Titration of fluconazole concentrations revealed exquisite sensitivity to the suloctidil/trifluoperazine, L-cycloserine/suloctidil and L-cycloserine/tamoxifen combinations in the presence of just 1/8 MIC fluconazole (Table IB). The L-cycloserine/suloctidil pair exhibited the most potent synergy with fluconazole with an FICI of 0.31. These results demonstrate that it is possible to incrementally build higher order synergistic combinations based on the subtly different properties of individual synergizers, even within in the same class.

In vivo synergy in an insect model of infection

The caterpillar of the greater wax moth *Galleria mellonella* is a validated *in vivo* infection model for study of Cryptococcal virulence, host immune responses to infection and the effects of antifungal compounds (Mylonakis *et al*, 2005; Scully and Bidochka, 2006; Cowen *et al*, 2009). We assessed the effect of a synergistic drug combination on the survival rate of *G. mellonella* infected with *C. neoformans* because of the prevalence of this virulent pathogen in immune-suppressed individuals. *G. mellonella* was inoculated with *C. neoformans* H99 and subsequently treated with fluconazole and sertraline, individually and in combination. The synergistic action of fluconazole and sertraline was evident in *G. mellonella* survival rates when compared with either drug alone (Figure 6A and B). Survival increased by 40% when treated with fluconazole and sertraline in combination, with an overall 60% survival rate after seven days of infection ($P<0.02$ for fluconazole versus fluconazole/sertraline). These results demonstrate that potentiators of fluconazole activity identified *in vitro* exhibit comparable activity in an animal model of infection.

Synergistic activity against fluconazole-resistant *Candida* isolates

To address whether syncretic compounds can act on clinically resistant strains, we investigated whether the combination of fluconazole and sertraline is effective against fluconazole-resistant clinical isolates of *C. albicans* (F-07-2007, F-01-2008), *C. glabrata* and a resistant control strain *C. parapsilosis* (ATCC 22019). Sertraline increased the susceptibility of resistant strains to fluconazole by up to 32-fold (Figure 6C and D; Supplementary Table S7). In the presence of sertraline, fluconazole MIC values ranged from 2 to 8 μg/ml, comparable to wild-type (Caf2-1, MIC=8 μg/ml) and drug pump-deficient

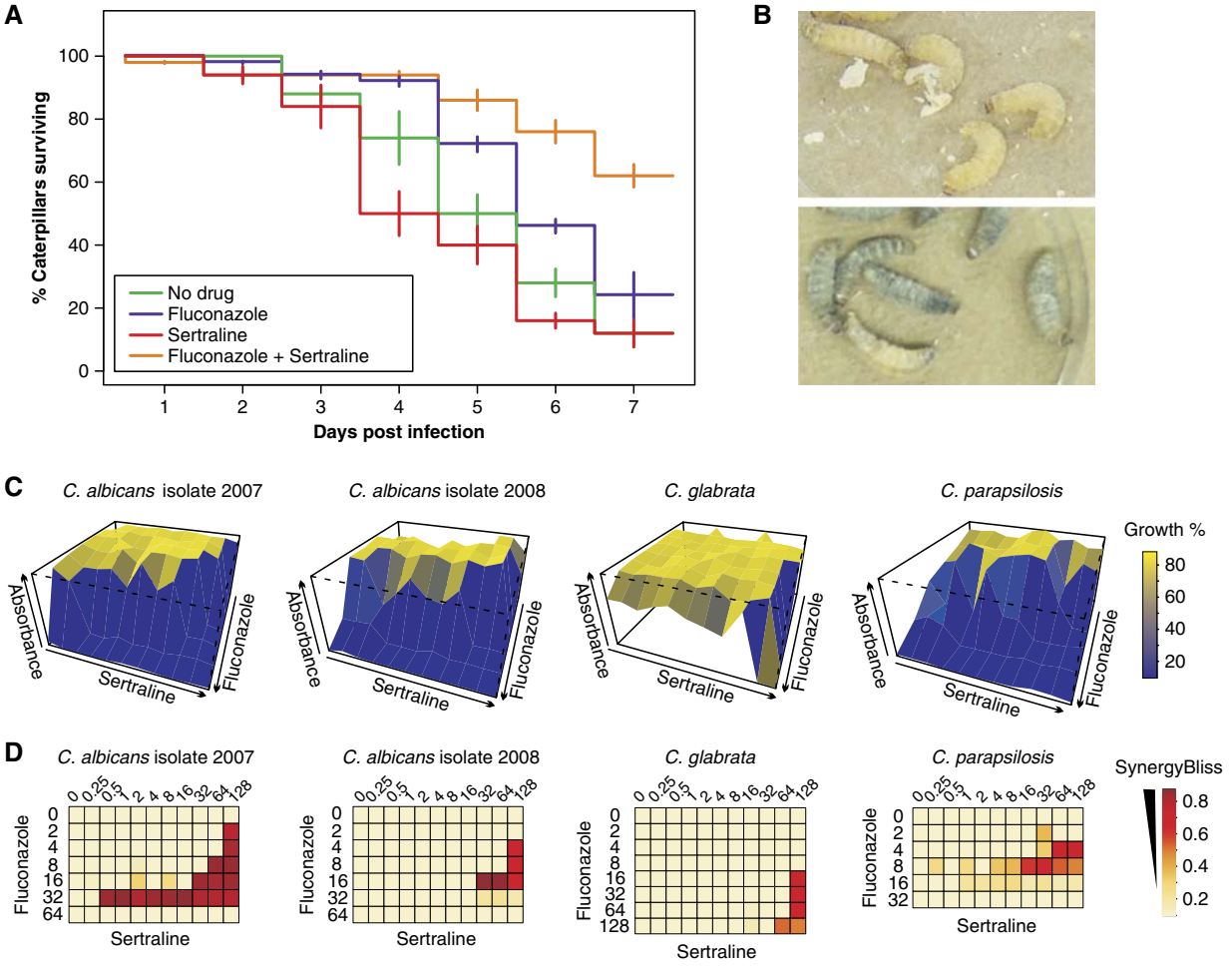

Figure 6 Synergistic activity of sertraline and fluconazole in an *in vivo* infection model and against clinical isolates. (**A**) *G. mellonella* caterpillars were injected with 8×10^3 cfu *C. neoformans* H99 on day 0 and drugs alone or in combination (1 μg fluconazole; 26 μg sertraline) on the first day and incubated for 1 week at 37°C. Values are mean of three independent experiments; error bars indicate standard deviation of the mean. (**B**) Uninfected *G. mellonella* caterpillars (top); melanization of infected *G. mellonella* caterpillars without drug treatment (bottom). (**C**) Combination matrix assays against drug-resistant *Candida* strains. Residual growth was plotted as a function of combinations of two-fold dilutions of each drug. (**D**) Bliss synergy analysis for combination assays shown in panel (**C**). The apparent absence of synergy at the highest fluconazole concentrations for *C. albicans* and *C. parapsilosis* is due to growth inhibition caused by fluconazole alone. Drug concentrations are in μg/ml. Source data is available for this figure at www.nature.com/msb.

(MIC=2 μg/ml) strains of *C. albicans*. The sertraline/fluconazole combination was synergistic in both *C. albicans* clinical isolates as well as the *C. parapsilosis* reference strain, but not in the *C. glabrata* strain. As noted above, this differential sensitivity may indicate strain-specific drug effects, or different mechanisms of drug resistance (Kuo *et al*, 2010). We conclude that syncretic activities in reference laboratory strains can be transposed to drug-resistant pathogens derived from clinical environments.

Discussion

Systematic screens for syncretic combinations reveal new antifungal chemical space

The combination of known antifungal agents is an established therapeutic tactic in infectious disease control (Johnson and Perfect, 2010). Here, we show that antifungal chemical space can be systematically expanded through the combination of a known antifungal drug with other bioactive compounds that do not have antifungal activity *per se*, including off-patent drugs previously approved for other indications. Novel syncretic drug combinations were readily identified in systematic screens against different fungal pathogens in the presence of subtherapeutic concentrations of fluconazole. These chemically diverse drug hits derive from a broad spectrum of human therapeutic areas that otherwise would not have been explored by infectious disease clinicians. Although hits from screens for fluconazole potentiation in the *S. cerevisiae* model system can be transposed to pathogenic fungi (Borisy *et al*, 2003; Zhang *et al*, 2007; Jansen *et al*, 2009; Epp *et al*, 2010), our primary screen data reveals considerable species specificity that by definition cannot be predicted from model organism drug-gene interactions. Indeed, in our primary screen, while 58% (25/43) of hits against *S. cerevisiae* exhibited synergistic activity against one or more fungal pathogens, only 19% (25/130) of the total hits against

pathogenic species were detected in *S. cerevisiae*. This observation underscores the need to undertake primary screens in the pathogen of interest.

Many syncretic combinations exhibited fungicidal activity, a highly desirable feature for neutropenic or otherwise immune-compromised patients. The fungicidal combination of fluconazole and the antidepressant sertraline (Zoloft®) was effective against all species tested, including drug-resistant clinical isolates of *Candida*, and in an *in vivo* insect model of *C. neoformans* infection. Therapeutic intervention for fungal infections of the CNS is a particular clinical challenge because of the stringent requirement to breach the blood brain barrier. The fact that sertraline targets serotonin receptors in the CNS suggests that the sertraline-fluconazole combination may be effective in the treatment of fungal meningitis.

Molecular mechanisms and prediction of drug synergism

Genome-wide chemical–genetic profiles of a selected set of six fungicidal synergizers revealed two different patterns of synergy. Five compounds—trifluoperazine, tamoxifen, clomiphene, sertraline and suloctidil—elicited genetic sensitivities and cell biological phenotypes associated with a loss of membrane integrity. The membrane perturbation caused by these compounds may increase susceptibility to accumulation of ergosterol pathway intermediates, impair fluconazole export by drug efflux pumps and/or impair import of exogenous ergosterol (Kuo *et al*, 2010). It is also possible that the synergizers affect active import of azoles through altered localization of drug transporters or general membrane perturbation (Mansfield *et al*, 2010). Notably, all five membrane active compounds are cationic amphiphilic drugs (CADs) that intercalate preferentially into one side of the lipid bilayer, thereby causing membrane expansion and cell wall stress (Sheetz and Singer, 1974), consistent with the observed chemical–genetic interactions with *NEO1*, *DRS2*, *SLT2* and *BCK1*. Moreover, genetic resistance to CADs is conferred by perturbation of vesicular membrane biogenesis and/or trafficking (Rainey *et al*, 2010). The synthetic lethal genetic interactions that occur between strains in the fluconazole and membrane active chemical–genetic profiles retrospectively predicted the synergistic effects of other hits in our primary screens. Moreover, when combined with another source of chemical–genetic interaction data (Ericson *et al*, 2008), the membrane active signature strain set correctly identified further synergistic hits in our primary screen data. In addition, the genetic interaction profile of L-cycloserine correctly predicted a novel synergistic interaction between the sphingolipid biosynthesis inhibitor myriocin and fluconazole. The potentiation of fluconazole activity by CADs and/or inhibition of sphingolipid biosynthesis may allow new general approaches to antifungal therapy in the clinic. As genetic and chemical–genetic space is elaborated, mechanism-based predictive approaches should become a powerful means of identifying new synergistic combinations.

Species-specific syncretic effects

We observed many genus- and species-specific syncretic interactions, which reflects differences in the genetic networks that dictate cellular responses to each compound (Perlstein *et al*, 2007). Since divergence from a common ancestor over 100 million years ago, different pathogenic species have adapted to particular host environments. For example, genetic plasticity of the fungal mating-type locus affects survival in mammalian hosts (Nielsen and Heitman, 2007). Developmental system drift (True and Haag, 2001) can also affect drug susceptibility, as shown by the differential effects of nikkomycin Z on chitin synthase paralogs in *Saccharomyces* and *Candida* (Gaughran *et al*, 1994; Sudoh *et al*, 2000). Marked differences in the transcriptional response of *Saccharomyces*, *Candida* and *Kluyveromyces* to fluconazole treatment underscore the quite distinct mechanisms whereby different species can respond to the same drug (Kuo *et al*, 2010). More generally, species differences in the response to chemical perturbation may reflect the evolutionary plasticity of genetic interaction networks (Kapitzky *et al*, 2010). Species-selective antifungal combinations may afford a means to both increase efficacy and decrease host toxicity. Systematic analysis of drug–drug interactions may also provide a means to classify and predict drug mechanism of action (Yeh *et al*, 2006; Hopkins, 2008).

Higher order drug–drug interactions

The densely connected structure of genetic networks predicts that it should be possible to devise higher order drug combinations with greater selectivity and potency (Sharom *et al*, 2004; Agoston *et al*, 2005; Lehar *et al*, 2007). That is, compounds that target multiple genetically redundant parallel pathways may exhibit *n*-way synergies. In an initial elaboration of this concept, we found that the combination of a non-synergistic pair (suloctidil and L-cycloserine, drawn from the membrane active and sphingolipid target classes, respectively) with a low dose of fluconazole resulted in a highly potent three-way synergism. Somewhat unexpectedly given their shared core genetic profiles, pairwise tests of four compounds in the membrane active class also revealed synergistic interactions in the absence of fluconazole. This type of drug–drug interaction, which has been observed previously with bacteria and yeast (Yeh *et al*, 2006; Jansen *et al*, 2009), suggests that, aside from the common core profile, each drug must have additional specific targets that contribute to overall synergism. The complex genetic profiles of each drug reflect effects on primary and secondary targets in the cell, drug metabolism and detoxification, and genetic feedback between different network elements (Sharom *et al*, 2004; Kitano, 2007; Lehar *et al*, 2008). Other documented interactions between fluconazole, reactive oxygen species, Hsp90, calcium metabolism and vesicle trafficking may contribute to these complex interactions (Cowen *et al*, 2009; Xu *et al*, 2009; Epp *et al*, 2010; Gamarra *et al*, 2010). We note that although shared drug profiles have been suggested to be predictive of synergistic interactions (Jansen *et al*, 2009), in many instances this is not the case (Yeh *et al*, 2006). Even drugs with well-documented mechanisms of action can have substantially different genetic interaction profiles compared with their presumptive targets. For example, although the genetic interaction profiles of fluconazole and its known target *ERG11* exhibit significant overlap, more than half of the interactions are not shared (Parsons *et al*, 2004). Recently, it has been shown that drug combinations can exhibit remarkably selective but unpredict-

able effects on the abundance of many different proteins (Geva-Zatorsky *et al*, 2010).

Therapeutic implications

The benefits of combinatorial anti-infective therapies include a decrease in the rate of selection of resistant strains, a lower required dosage of individual drugs, a decrease in host toxicity and enhanced antimicrobial activity (Sharom *et al*, 2004; Hopkins, 2008; Lehar *et al*, 2008). As shown in this study and elsewhere (Zhang *et al*, 2007; Jansen *et al*, 2009; Epp *et al*, 2010), syncretic combinations of drugs with improved antifungal properties can be readily identified in both model fungal species and highly pathogenic clinical isolates. Importantly, while it is a potential concern that undesirable side effects may arise from drug combinations, as occurs for example with known contraindicated drugs, it has recently been shown that synergistic combinations usually yield enhanced selectivity without adverse side effects (Lehar *et al*, 2009). As noted above, these benefits may include improved activity in therapeutically recalcitrant tissues, such as the CNS. These combinatorial principles apply equally to viral and bacterial pathogens, cancer and other genetic diseases (Borisy *et al*, 2003; Fitzgerald *et al*, 2006; Hopkins, 2008; Lehar *et al*, 2009).

Materials and methods

Chemicals, high-throughput screens and MIC determination

Fluconazole was purchased from Sandoz (Quebec, Canada). All other compounds were obtained from Sigma (St Louis) or Prestwick Chemicals (Illkirch, France). The Prestwick Chemical library was screened in duplicate in the presence and absence of 1/2 MIC fluconazole at a final concentration of 30 μM in 384-well flat bottom microtitre plates. OD_{600} was determined after 48 h at 30 or 37°C. MIC determinations were based on Clinical and Laboratory Standards Institute (CLSI) protocols (Eliopoulos and Moellering, 1991; Odds, 2003), with the exception that yeast SC medium was used instead of mammalian cell RPMI 1640 medium. Overnight cultures in synthetic complete media (SC: 0.67% Difco™ yeast nitrogen base w/o amino acids, 0.08% amino acid add back and 2% glucose) were diluted in 0.85% NaCl to an OD_{530} of 0.11, followed by a 1:100 dilution in 0.85% NaCl, and a final 1:20 dilution in SC media. Two-fold dilution series (0–128 μg/ml) of fluconazole and other antifungal drugs were added to 200 μl of diluted culture in 96-well plates and OD_{600} determined after 48 h at 30 or 37°C. For fluconazole, MIC was set at the lowest concentration that caused 80% reduction in growth, corresponding to two on the azole MIC numerical scale. For other drugs, MIC was set as the lowest concentration that yielded no growth.

Time kill MIC assays were performed at six different concentrations of compound and fluconazole (fluconazole at MIC, fluconazole at 1/4 MIC, compound at MIC, compound at 1/4 MIC, both fluconazole and compound at MIC, and both fluconazole and compound at 1/4 MIC). At 0, 24 and 48 h dilutions from each well were spotted on an SC agar plate, incubated for 48 h and colony counts determined. A fungicidal effect was defined as >3log10 (99.9% killing) reduction in CFU/ml at synergistic concentrations after 24 h incubation.

Synergy matrix assays

Fluconazole and syncretic compounds were two-fold serially diluted across the rows and columns of a 96-well plate (0–128 μg/ml; for daunorubicin HCl, terbinafine, trifluoroperazine dihydrochloride and ellipticine dilutions were from 0–64 μg/ml), incubated with fungal cultures and OD_{600} determined after 48 h. The FIC index of each drug combination was determined by adding the individual FIC values, as calculated by standard CLSI protocols (Eliopoulos and Moellering, 1991; Odds, 2003). To probe chemical interactions between sertraline, trifluoperazine, L-cycloserine, suloctidil and tamoxifen, checkerboard assays were carried out between these five compounds in the absence and presence of 1/2 and 1/8 MIC fluconazole (16 and 4 μg/ml, respectively).

Chemical–genetic profiles and secondary assays

S. cerevisiae deletion collections (*MAT***a** haploid and heterozygous essential deletion strains) were obtained from Research Genetics (Germany). Compounds were screened at a concentration that caused ~30% growth inhibition at a final DMSO concentration of 0.2% (Giaever *et al*, 1999). Deletion pools were grown for 10 generations, gDNA extracted and barcode tags amplified with fluorescently labeled UP and DN primers, followed by hybridization of PCR products to spotted barcode microarrays (Cook *et al*, 2008). Arrays were scanned on a GenePix 4200AL and analyzed with GenePix Pro 6.0 software. Data sets are available at ArrayExpress (E-MTAB-394). Chemical–genetic interactions were confirmed in quantitative growth assays at 30°C with continuous shaking at 564 r.p.m. on a Sunrise shaker/reader (Tecan); OD_{600} readings were taken every 15 min and values at the end of logarithmic phase used to calculate the log ratio between deletion and wild-type strains. For sorbitol rescue, wild-type strains were grown in the presence of indicated compounds and 1 M sorbitol. For microscopy, cells were embedded in 1% low melt agarose and stained with Calcofluor White M2R (Sigma), Mitotracker Green FM (Molecular Probes) or FM4-64 (Molecular Probes) and imaged at × 100 on a Leica DMI 6000 B microscope with a Hamamatsu Orca ER-AG camera and Volocity 4 software. Images were deconvolved using AutoDeblur Gold CWF using 2-D blind deconvolution and 10 iterations per image.

Computational analysis of gene-drug network interactions

The 50 most sensitive deletion strains from duplicate chemical–genetic profiles for clomiphene, L-cycloserine, sertraline, suloctidil, tamoxifen and trifluoperazine were tested against the top 50 fluconazole-sensitive deletion strains (from replicate arrays at 8 μM). Shared genetic interactions between the sets of deletion strains were determined based on genetic interaction data obtained from BioGRID (Breitkreutz *et al*, 2008; BIOGRID release 2.62, http://www.thebiogrid.org). Visualization of bipartite graphs and simulations was performed with an online tool available at http://tyerslab.bio.ed.ac.uk/tools/genelookup_bipartite.php. Simulations based on CGS were derived from 1143 non-essential deletion strains that respond to various drug treatments (Hillenmeyer *et al*, 2008). For each drug pair, control gene sets of the same size were picked at random and the number of genetic interactions counted to generate a background distribution of the number of interactions that would occur by chance, based on the compiled genetic interaction data. This distribution was used to calculate the *P*-value for each drug pair. For PPP, the chemical–genetic interactors of fluconazole and each of the synergistic drugs were pooled, randomly assigned to two groups and genetic interactions counted to obtain a background distribution for each drug pair. The definition of the signature deletion strain set was based on confirmatory quantitative growth curve assays (Figure 3D). For both the CGS and PPP methods, 10 000 simulations were conducted for each drug pair. To predict potential synergistic candidates based on overlap with published chemical–genetic profiles (Ericson *et al*, 2008), we used a binary data matrix based on a *Z*-score cutoff of ±3. The significance of enrichment was calculated based on the number of genes that overlapped with the signature strain set; a subset of 4 out of 11 genes was significant with a *P*-value <0.05.

Insect larvae assays

Ten weight matched (250–400 mg/worm) *G. mellonella* caterpillars per dish were inoculated with *C. neoformans* H99 and subsequently

injected with different combinations of compound, fluconazole and/or control solutions (Mylonakis *et al*, 2005). Over a 7-day period, caterpillars were examined visually for discoloration due to melanization and for failure to respond to touch as an inviability end point.

Supplementary information

Supplementary information is available at the *Molecular Systems Biology* website (www.nature.com/msb).

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Conflict of interest

The authors declare that they have no conflict of interest.

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