MIT Libraries | DSpace@MIT

MIT Open Access Articles

Inhibiting GPI Anchor Biosynthesis in Fungi Stresses the Endoplasmic Reticulum and Enhances Immunogenicity

Massachusetts Institute of Technology

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Inhibiting GPI Anchor Biosynthesis in Fungi Stresses the Endoplasmic Reticulum and Enhances Immunogenicity

Catherine A. McLellan [1,2], Luke Whitesell [1], Oliver D. King[3], Alex K. Lancaster [1], Ralph Mazitschek[4] and Susan Lindquist [1,2,5]

Affiliations:

1. Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, Massachusetts 02142, USA

2. Howard Hughes Medical Institute, MIT, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, USA

3. Boston Biomedical Research Institute, 64 Grove Street, Watertown, MA 02472, USA

4. Center for Systems Biology, Massachusetts General Hospital, Richard B. Simches Research Center, 185 Cambridge Street, Suite 5.210, Boston, MA 02114, USA

5. Department of Biology, MIT, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, USA

Contact Information:

Susan Lindquist
Whitehead Institute
9 Cambridge Center
Cambridge, MA 02142
Tel: 617-258-5184
Fax: 617-258-7226
Email: Lindquist_admin@wi.mit.edu

Abstract

In fungi, the anchoring of proteins to the plasma membrane via their covalent attachment to glycosylphosphatidylinositol (GPI) is essential and thus provides a valuable point of attack for the development of antifungal therapeutics. Unfortunately, studying the underlying biology of GPI-anchor synthesis is difficult, especially in medically relevant fungal pathogens because they are not genetically tractable. Compounding difficulties, many of the genes involved in coupling GPI to proteins are essential. Here, we report the discovery of a new drug-like small molecule christened gepinacin (for GPI acylation inhibitor) which selectively inhibits Gwt1, a critical acyltransferase required for the biosynthesis of fungal GPI anchors. After delineating the target specificity of gepinacin using genetic and biochemical techniques, we used it to probe key, therapeutically relevant consequences of disrupting GPI anchor metabolism in fungi. We found that unlike all three major classes of antifungals in current use, the direct antimicrobial activity of this compound results predominantly from overwhelming stress to the endoplasmic reticulum. Gepinacin did not affect the viability of mammalian cells or inhibit their orthologous acyltransferase, which enabled its use in co-culture experiments to examine its effects on host-pathogen interactions. In isolates of *Candida albicans*, the most common fungal pathogen in humans, exposure to gepinacin at sub-lethal concentrations impaired filamentation and unmasked cell wall β-glucan to stimulate a pro-inflammatory cytokine response in macrophages. These results highlight Gwt1 as a promising antifungal drug target and define a useful probe for studying how disrupting GPI-anchor synthesis impairs viability and alters host-pathogen interactions in genetically intractable fungi.

Introduction

Fungi are a prominent cause of hospital-acquired infections that are becoming increasingly difficult to control (*1*).   This disturbing trend is driven by the growing number of severely immunocompromised individuals in the population that has occurred as a result of advances in the management of cancer, organ transplantation, autoimmune disorders and HIV. Most fungus-related morbidity and mortality is caused by the pathogens *Candida albicans* and *Aspergillus fumigatus,* which remain costly to treat and extremely difficult to eradicate in the immunocompromised host. *Candida* species are currently the fourth leading cause of hospital-acquired bloodstream infection and kill up to 40% of their victims, while disseminated *Aspergillus* infections kill up to 80% of the patients they afflict (*2, 3*).

Fungal pathogens present a particular therapeutic challenge because as eukaryotes, they share many of the same basic molecular mechanisms that support the maintenance and proliferation of mammalian cells. As a consequence, the number of unique exploitable drug targets that have been identified in fungi remains very limited. Only three mechanistically distinct classes of anti-mycotic agents are in widespread clinical use for the treatment of systemic infections. The most widely deployed class, the azoles (e.g. fluconazole), inhibit the cytochrome P450 enzyme 14 α-demethylase. This blocks the conversion of lanosterol to ergosterol, the functional homolog of cholesterol in mammals. Ergosterol is an essential component of the fungal membrane and the selective fungistatic activity of the azoles results from their disruption of its biosynthesis. Ergosterol itself is the primary target of the oldest class of antifungals, the polyenes (e.g. amphotericin B) which selectively bind this sterol and directly disrupt fungal membrane integrity. The newest class of antifungals, the echinochandins (e. g. caspofungin), inhibits 1, 3 β-glucan synthase. This enzyme mediates an essential step in the production of glucan, the major structural component of the fungal cell wall. Unfortunately, high-grade resistance to

all three classes of antifungals occurs frequently in the clinical setting through molecular mechanisms that can involve both target-related mutations and increased transporter-mediated drug efflux. Clearly, to combat this mounting medical problem, effective new anti-fungal strategies are urgently needed.

Motivated by a long-standing interest in the basic biology of stress responses and how they enable diverse organisms to adapt and evolve, we identified a novel chemical structure that induces profound stress in the endoplasmic reticulum (ER) of fungi. Intrigued by its high fungal-selective activity against a broad range of medically relevant species, we sought to define its mechanism(s) of action through a combination of genetic and biochemical approaches. We found that this drug-like compound, which we here name gepinacin, specifically inhibits an essential step in the production of glycosylphosphatidylinositol (GPI)-anchors within the ER of fungi, but not mammalian cells. Guided by this insight, we used the compound as a probe to investigate how inhibiting this biosynthetic pathway disrupts protein homeostasis in fungi and alters key interactions between pathogen and host that are known to contribute to fungal virulence.

RESULTS AND DISCUSSION

In the course of high-throughput screening for small molecules that inhibit the function of heat-shock proteins, we encountered a false positive compound that possessed surprisingly broad anti-fungal activity. Under screening conditions, the compound caused swelling of the yeast, *Saccharomyces cerevisiae,* which caused it to score erroneously in a cell growth assay. Closer examination revealed that the compound was actually toxic to yeast. In contrast, no growth inhibition was seen when the compound was incubated with mammalian cells in culture. Intrigued by its marked fungal selectivity, we took advantage of the powerful genetic tools developed in the model fungal organism *S. cerevisiae* to identify the target of this small molecule and to understand the basis of its selective toxicity to fungi. Following target validation, we used this compound as a chemical probe to further characterize its mode

of action and explore the therapeutic implications of target inhibition in azole-resistant isolates of the far

less genetically tractable but more medically relevant fungal pathogen, *C. albicans*.

Broad spectrum of highly selective antifungal activity

The phenoxyacetanilide compound identified in our screen (**Fig. 1a**) inhibited the growth of

diverse yeasts and molds separated by approximately 800 million years of evolution, including *S.*

cerevisiae, *Candida glabrata, Candida albicans* and *Aspergillus terreus* (4). This suggested the

involvement of a mechanism widely conserved amongst fungi (**Fig. 1b**). Importantly, it inhibited the

growth of multiple isolates of the common fungal pathogen *C. albicans*. These had been chosen to

represent resistance to each of the three major classes of antifungals that are currently in clinical use

(**Fig. 1c**). The lack of cross-resistance and the absence of structural similarity to established antifungal

drugs suggested the compound might exert its activity through a novel mode of action. A complete lack

of cytotoxicity for mammalian cells suggested sufficient divergence of target and/or mechanism between

humans and fungi to provide a useful therapeutic index for eventual development of the chemotype as an

antimicrobial (**Fig. 1d**).

Target Identification Using Yeast Genetics

To determine the mechanism responsible for the antifungal activity of the compound, we utilized

the powerful genetic tools available in the model fungal organism *S. cerevisiae*. We screened both an

arrayed haploid over-expression library and a pooled heterozygous deletion library for enhancement or

suppression of toxicity. The over-expression library consisted of 5336 individual haploid strains arrayed

in 384-well plate format. With each strain expressing one open reading frame (ORFs), this library

covered approximately 90% of the yeast genome. (*5*). Our heterozygous deletion library consisted of a

pool of 5797 diploid strains in which one copy of approximately 95% of the ORFs in the *S. cerevisiae*

genome had been disrupted previously by targeted insertion of a bar-coded antibiotic resistance cassette

(*6*). Only one gene, *GWT1* was recovered as a hit shared by both libraries. Gwt1 is essential for growth of *S. cerevisiae* under normal conditions and has previously been characterized as an acyltransferase that is critical for GPI-anchor biosynthesis (*7*). Having identified this putative target for the compound we christened the compound gepinacin for GPI acylation inhibitor. When *GWT1* was over-expressed, it rescued gepinacin toxicity and when it was deleted, toxicity was enhanced. Furthermore, *GWT1* was not identified as a hit for 8 unrelated compounds that were tested in parallel for other projects. To confirm the effect of *GWT1* we constructed yeast strains engineered to express a wide range of *GWT1* gene copies (**Fig. 2a**). Starting with a wild type diploid (Wt) strain that carries two genomic copies of *GWT1*, we introduced a low copy (CEN) plasmid expressing *GWT1* or *GFP* (as a control). In parallel, the same plasmids were transduced into a heterozygous deletion strain (*gwt1Δ/GWT1*) which had only one genomic copy of *GWT1*. As expected, the toxicity of gepinacin inversely correlated with *GWT1* copy number. High level expression of *GWT1* driven by a two micron plasmid further decreased gepinacin activity in both genotypes (**Supplementary Fig. 1**). These genetic data establish Gwt1, or at least the pathway in which it acts, as the most functionally relevant target for the antifungal activity of gepinacin.

Biochemical validation of Gwt1 as target

Production of GPI-anchors begins on the cytoplasmic surface of the ER but is completed on the luminal side. Gwt1 (in yeast) or PIG-W (mammalian) acts at the first step on the luminal side of the ER, the acylation of glucosamine phosphatidylinositol (GlcN-PI) (**Fig. 2b**) (*8*) (*9*). To provide direct biochemical evidence that Gwt1 is the proximal protein target of gepinacin we performed *in vitro* acylation reactions using yeast membrane preparations. UDP[^{3}H]GlcNAc was incubated with the membranes, the resulting lipid products were recovered by chemical extraction and fractionated by silica gel thin layer chromatography (TLC). The appearance of a phospholipase-C insensitive band indicated the production of the acylated product. Gepinacin inhibited the acylation of GlcN-PI at low micromolar

concentration (**Fig. 2c**) while a compound that was structurally very similar but biologically inactive did

not (**Fig. 1a**, **Supplementary Fig. 2**).

Having demonstrated that the biochemical target of gepinacin is not restricted to fungi, we were

interested in investigating the basis of its lack of toxicity to mammalian cells in culture. To determine if

gepinacin remained active under mammalian cell culture conditions we cultured fungi (either an

amphotericin B-resistant (**Fig. 3a**) or fluconazole-resistant (data not shown) isolate of *C. albicans*) and

mammalian cells together. These co-culture experiments demonstrated profound inhibition of

proliferation for both fungal strains under serum-containing cell culture conditions, with no effect on

mammalian cells in the same well.

To determine whether the basis of fungal selectivity resided in the fungal protein itself, we

constructed transgenic *S. cerevisiae* strains in which genomic deletion of the essential *GWT1* gene was

rescued by heterologous expression of either the human or yeast genes (*PIG-W* or *GWT1*, respectively)

thus confirming the highly conserved function of Gwt1/PIG-W. While gepinacin was inactive in the

PIG-W expressing strain, it potently inhibited both cell growth and acyltransferase activity in the strain

expressing the yeast gene (**Fig. 3b, 3c**). These results confirm the proximal determinant of gepinacin

toxicity as the fungal Gwt1 protein and demonstrate highly species-selective activity for the compound.

Deletion of Trafficking Adaptor Protein EMP24 Partially Suppresses Toxicity

To identify mechanisms that might confer resistance we isolated two spontaneously arising

gepinacin-resistant clones carrying mutations that partially suppressed gepinacin toxicity in *S cerevisiae*

(**Fig. 4a**). Whole genome sequencing of these strains showed that resistance was due to two different

mutations in the gene *EMP24* (**Fig. 4b**). This finding was recapitulated with targeted deletion of *EMP24*

(**Supplementary Figure 3**).

The non-essential protein Emp24 is a component of a large multi-protein complex that regulates

GPI-anchored protein transport and quality control (*10*). GPI-anchored proteins do not contain

transmembrane domains. As an integral membrane protein, Emp24 specifically interacts with the

anchor portion of GPI-anchored proteins monitoring the completion of their processing and assisting

their incorporation into ER to Golgi transport vesicles (*11*). When GPI-anchored proteins are

incompletely remodeled, Emp24 is thought to facilitate their return to the ER. The deletion or

inactivation of *EMP24* has been shown to allow GPI-anchored proteins to bypass this quality control

step and to exit the ER (*11*). Deletion of *EMP24* could suppress gepinacin toxicity in an analogous

manner by relieving a trafficking block induced by the compound.

To determine if gepinacin does indeed alter trafficking and change Emp24 distribution we

examined yeast in which *GFP* was fused in frame to the 3' end of genomic *EMP24*. Emp24-GFP

normally shows a classical ER distribution pattern consisting of well-defined circles in the interior of the

cell with a fainter cortical ring and a few bright dots consistent with some Golgi localization. After

overnight culture in gepinacin, however, Emp24-GFP dramatically re-localized into large, bright dot-

like structures (**Fig. 4c**). This pattern indicates that the compound had induced severe disorganization of

the ER, and/or a profound block in retrograde trafficking that would normally return Emp24 to the ER as

part of its physiological cycling.

GPI-specific trafficking defects caused by gepinacin induce ER stress

To further pursue the effects of gepinacin on protein trafficking in the secretory compartment,

we compared the effects of gepinacin on maturation of the sentinel proteins Gas1 and CPY. Both

proteins undergo characteristic molecular weight changes as they are processed in transit through

specific cellular compartments, a feature that has been used extensively to study protein trafficking in

the secretory system. Gas1 travels through the ER where it acquires a GPI-anchor before translocating

to the Golgi and outward to the cell surface (*12*). CPY is not GPI-anchored but undergoes sorting and

processing in the secretory pathway before ending up in the yeast vacuole (*13*).   In actively growing

wild type yeast, the mature (m), Golgi-processed form of Gas1 constitutes the predominant species,

relative to the precursor (p) form found in the ER (**Fig. 5a**). This distribution was inverted following

exposure to gepinacin. In contrast, the processing of CPY which is not GPI-anchored was identical in

control and gepinacin-treated cells. This confirms a highly restricted effect of Gwt1 inhibition on the

trafficking of GPI-anchored proteins. The control compound, tunicamycin, a natural product that blocks

production of all N-linked glycoproteins, alters the processing of both proteins as expected (*14*).

The specific effects of gepinacin on GPI-anchored proteins provided a highly selective tool to

investigate the impact of inhibition of inositol acylation on protein homeostasis within the ER. Culture

for 5 hours in gepinacin induced a massive, concentration-dependent activation of the unfolded protein

response (UPR) as monitored by the GFP reporter construct (UPRE-GFP), similar in extent to the effect

produced by tunicamycin **(Fig. 5b)**. Activation of the UPR was not seen when yeast were treated with

representative compounds from the three major classes of anti-fungals: amphotericin B, caspofungin and

fluconazole (**Supplementary Figure 4**). As expected based on its target selectivity, gepinacin did not

perturb ER function in mammalian cells as monitored by induction of BIP, an ER-resident chaperone

and classical marker of the UPR in mammalian cells (**Supplementary Figure 5**) (*15, 16*). Deletions of

HAC1 and *IRE1* which are essential components of the UPR activation pathway in yeast, both greatly

increased the toxicity of gepinacin (**Fig. 5c**). As a positive control, similar enhancement of tunicamycin

toxicity was seen in association with deletion of these genes (**Supplementary Figure 6a**). In contrast,

toxicity of the conventional antifungal fluconazole which targets ergosterol biosynthesis was completely

unaffected by these UPR-disabling deletions (**Supplementary Figure 6b**) (*17*).

Gwt1 Inhibition Blocks Filamentation

GPI-anchoring of proteins is conserved in all eukaryotes. However, major differences in the utilization of this post-translational modification between species provide an attractive point of attack in developing new antimycotics. In fungi, unlike mammalian cells, GPI-anchored proteins become covalently linked to β-1, 6 glucan following translocation to the cell surface which helps maintain integrity of the cell wall. In addition, their presence at the cell surface permits GPI-anchored proteins in fungi to play important roles in adhesion, filamentation and sensing of the environment. Also important to pathogenesis, they provide a heavily glycosylated and phosphorylated outer coat to shield fungi from recognition and attack by the immune system of the mammalian hosts they invade (*18, 19*).

Because of its role in tissue invasion, a key determinant of fungal virulence is the ability to switch between yeast and filamentous forms (*20-22*). To determine whether gepinacin impairs this important process in *C. albicans*, we used a series of three increasingly fluconazole-resistant clinical isolates that had been isolated over a two year period from an HIV patient treated with fluconazole, strains CaCi2, 8 and 17 (*23*). These strains were grown overnight with gepinacin or vehicle control in rich medium at 30° C, to maintain the cells in the yeast form. To induce morphogenic transformation they were then transferred to filamentation medium (Spider medium) at 37°C for 3 hours in the continued presence or absence of gepinacin (*24*). In the absence of gepinacin, all three drug-resistant clinical isolates underwent marked transformation to large macroscopic mats, readily visible to the eye. Because this process is so dependent on GPI-anchored proteins, however, a concentration of gepinacin that minimally reduced proliferation completely blocked the ability of all three strains to undergo such filamentation (**Fig. 6a**). The inability to form filaments was also apparent at the cellular level as demonstrated by the photomicrographs provided in **Supplementary Figure 7**. Comparable results were obtained when using serum-containing medium to induce filamentation instead of "Spider medium".

Gepinacin also blocked filamentation on solid media using the well-characterized *S. cerevisiae* strain

Sigma 1278B, which spontaneously grows as filamentous mats on agar substrates (**Fig. 6b**). Clearly the

ability of gepinacin to inhibit conversion of fungi to their invasive filamentous forms is broadly relevant

to diverse species and growth conditions with important implications for their pathogenicity in animal

hosts.

Gwt1 inhibition enhances immunogenicity

We took advantage of the fungal selectivity of gepinacin to investigate another key determinant

of fungal virulence, namely the ability to escape immune recognition. The large amount of β-glucan in

the cell wall of fungi constitutes a potent pro-inflammatory stimulus, but it is normally masked by a

mannoprotein coat (*25*). We predicted that disrupting GPI-anchor synthesis through inhibition of Gwt1

would unmask β-glucan leading to enhanced recognition of *Candida* by mammalian immune cells.

Indeed, as revealed by immunostaining and fluorescence microscopy, sub-lethal concentrations of

gepinacin did dramatically increase β-glucan presentation on the cell surface of *C. albicans* (**Fig. 7a**).

Quantitation of this effect by flow cytometry confirmed a 4.5-fold increase in median channel

fluorescence for gepinacin-treated candida compared to control-treated yeast. Such β-glucan exposure

has been demonstrated previously for caspofungin, an echinochandin that perturbs cell wall synthesis. In

contrast to gepinacin, however, the effect of caspofungin is limited to only filamentous forms of *C.

albicans* (*26*). As an important functional consequence of increased β-glucan exposure, incubation of

gepinacin-treated *C. albicans* with a mouse macrophage cell line (RAW264.7) more than doubled the

secretion of the major pro-inflammatory cytokine TNFα by these professional antigen-presenting cells

(**Fig. 7b**).

Gwt1 as a target for antifungal drugs

The biosynthesis of GPI anchors in fungi was first proposed as a potential antifungal drug target by Tsukahara et al (*27*). In an extensive screening effort, they identified 1-[4-butylbenzyl] isoquinoline (BIQ) as an inhibitor of the surface expression of GPI-anchored proteins (structure in **Supplementary Figure 8a**). *GWT1* was subsequently cloned as a dosage-dependent suppressor of BIQ-induced phenotypes. Further discovery and optimization efforts by this group led to the synthesis of E1210, a potent and selective Gwt1 inhibitor with properties suitable for clinical development (structure in **Supplementary Figure 8b**). E1210 shows good activity *in vitro* and in mouse models against a broad spectrum of yeast and molds including medically relevant species of candida, aspergillus and fusarium (*28, 29*). Like gepinacin, it is non-toxic to mammalian cells at concentrations that far exceed those required for antifungal activity (*30*). The unusually high degree of selectivity for both gepinacin and E1210 arises from species-specific discrimination at the biochemical level of target inhibition and from the different roles that GPI-anchored proteins play at the biological level in fungi versus mammals.

Although the structurally distinct compounds gepinacin and E1210 were discovered through completely different screening strategies, they share key biological properties rooted in Gwt1, the molecular target they hold in common. Both compounds inhibit fungal proliferation, compromise cell wall integrity and impair the morphogenic filamentation program which is required for pathogenicity in animal hosts (*30*). Our discovery of a new chemotype that selectively inhibits the fungal protein Gwt1 highlights the suitability of this protein as a highly druggable, therapeutic target. This is noteworthy from the drug development perspective because Gwt1 and its close mammalian ortholog are multi-pass transmembrane proteins for which atomic-level structural information is not available to help guide medicinal chemistry efforts. In pursuing the work presented here, we have constructed gene-swapped yeast strains in which the sole source of essential acyltransferase activity for GPI anchor synthesis is

provided by either Gwt1 or its human ortholog PIG-W. These strains can now be used for SAR studies

to optimize the potency and selectivity of the known chemotypes and also to efficiently screen for yet

other compounds that preferentially inhibit the fungal enzyme or alternatively that inhibit the human

enzyme to study its role in mammalian biology (*9*).

Using gepinacin as a probe in medically relevant pathogens for which few genetic tools are

available, we have uncovered new consequences of Gwt1 inhibition with important clinical implications.

First, Gwt1 inhibition profoundly stresses the fungal ER leading to critical dependence on activation of

compensatory response pathways. Such dependence creates new liabilities for the organism that might

be targeted in future work to synergistically enhance the antifungal activity of Gwt1 inhibition. Second,

the cell wall compromise caused by Gwt1 inhibition in fungi not only exposes β-glucan on the cell

surface, but enhances recognition by antigen-presenting cells and activation of a pro-inflammatory

immune response. These effects would be expected to enhance clearance by host defense mechanisms

and delay if not eliminate the emergence of resistance. In concert, these new insights further advance

Gwt1 as a promising antifungal drug target and validate a useful new probe for studying the mechanisms

by which inhibition of fungal GPI-anchor synthesis directly impairs viability and indirectly disrupts the

complex process of pathogenesis.

METHODS

Materials. Gepinacin, acetamide, *N*-(4-methoxyphenyl)-2-[3-(2-methylpropoxy) phenoxy, CAS registry

304692-07-7 was purchased from Ryan Scientific, Inc. The inactive compound, CAS registry 3922235-

70-0 was purchased from Scientific Exchange, Inc.

Plasmids. Harvard Institute of Proteomics FLEXGene *Saccharomyces cerevisiae* (yeast) ORF collection

(pBY011 expression vector) (*5*) and *PIG-W* entry clone HsCD00295253 were obtained from the Dana

Farber/HCC DNA Resource Core. All other gateway vectors used are available from Addgene (*31*).

The reporter for the unfolded protein response, pRS304-4XUPRE-GFP, was a gift from Peter Walter (UCSD).

Fungal Strains. Archives of all strains were maintained in 25% glycerol at -80°C. A complete list of all strains and construction details for the humanized yeast strain are provided in the supplement.

Mammalian Cell Lines. 293T, NIH 3T3, and the RAW264.7 murine macrophage line were purchased from ATCC. Primary MEF were generated from day 13.5 mouse embryos using standard techniques.

Antifungal Susceptibility Testing. Sensitivity to gepinacin was determined in 96-well, flat bottom microtiter plates using a modification of the broth microdilution protocol NCCLS M27-a and RPMI as culture medium. Fungi were inoculated at ~ 10^3 cells per ml and incubated for 48 hours at 37° C after which plates were sealed, shaken and the optical density read at 600 nm (OD_{600}) on a Tecan Safire 2 plate reader. All samples were run in duplicate or triplicate and assays were repeated at least once. Results are expressed as fraction of growth in the absence of compound and the mean and standard deviation are plotted. For the mold *A. terreus,* inoculum was 10^4 cells per ml and plates were incubated at 35° C for 48 hours in the dark. Plates were then visually scored as specified by CLSI document M38-A2 to determine the MIC50 and MIC80.

Mammalian Cell Toxicity Testing. For co-culture experiments, confluent layers of NIH 3T3 cells were established in 6-well plates. The following day, the medium was replaced with RPMI1640+10%FBS containing the amphotericin-resistant strain CaAmphR at 10^3 colony forming units per ml and 40 µM Gepinacin or an equivalent amount of DMSO. Co-cultures were incubated for 24 hours at 37° C in a micro-isolator box. Images were acquired on a Nikon Eclipse TS100 microscope at 40X.

Genetic Screening. Flexgene plasmids were transformed in parallel into *S. cerevisiae* strain BY4741. The resulting library was arrayed in 384-well plates, grown to saturation in selective media, diluted and

replicated using a Tecan EVO robot. Gepinacin (20 µM) or an equivalent amount of DMSO in SGal-

URA-NH4 media was added to duplicate plates before incubation in a humidified chamber at 23°C in

the dark. Plates were sealed, shaken, and read at 24 hours and then 4 more times over the next 2 days.

Data analysis details are provided in the supplement. The heterozygous deletion collection was pooled

and grown at 30° C in SGal-CSM with shaking to an OD_{600} of approximately 1-2 and then diluted to an

OD_{600} of 0.05 in fresh media containing DMSO or 1.25 µM gepinacin. This was repeated 4 times with

dilutions every ~ 12 hours.   Genomic DNA preparation, PCR and chip hybridization were done as

previously described (*6, 32, 33*). All hits were re-tested by growth assay in 384-well plate format and

the relevant deletion confirmed by PCR with deletion specific primers.

In vitro **Acylation Assays.** Experiments were performed using a previously published protocol (*7*) with

the following modifications; UDP[^{3}H]GlcNAc was used instead of [^{14}C] and TLC plates were imaged

by autoradiography. Lipid extracts were treated overnight with phosphatidylinositol-specific

phospholipase C to confirm that the band identified as GlcN-(acyl)PI was resistant to cleavage (data not

shown).

Filamentation.   For liquid assays, *C. albicans* strains were grown overnight in YPD with DMSO or 5

µM gepinacin. After dilution to an OD_{600} of 0.1 in Spider media, growth was continued for an

additional 3 hours with agitation at 37°C in the presence of compound before transfer to a 24 well plate

for imaging (*34*). Assays with *S. cerevisiae* strain Sigma 1278B on solid media were performed as

previously described (*24*) except gepinacin was added directly to partially cooled plate media and

colonies were imaged after growth for 6 or 9 days at 30° C.

Protein trafficking analysis. HA-tagged *GAS1* was expressed in strain BY4741 *CPY-GFP* (Yeast GFP

collection (*35*) under control of its own promoter using plasmid pCM-*HA-GAS1*. This construct was

created as previously reported except in a gateway plasmid backbone (*36*). To assess effects on trafficking,

strains were incubated for 1 hour at 30°C with test compounds. Total cellular protein (5 μg) was

separated by electrophoresis (Invitrogen 8% gels) and transferred to nitrocellulose membranes. Blots

were hybridized with antibodies against HA (Covance), GFP (Roche) or CPY (Invitrogen). Sizes for the

various post-translationally modified products of Gas1HA2 and CPY have been previously reported

(*36*). CPY-GFP was used to facilitate discrimination of CPY processing steps because GFP is cleaved

from CPY in the yeast vacuole. The experiment, however, was also performed with untagged CPY with

the same results.

Unfolded protein response. The plasmid pRS304-4XUPRE-GFP was linearized and integrated into the

TRP1 locus of *S. cerevisiae* (W303) to construct a reporter strain as previously described (*37, 38*).

Reporter cells were grown to log phase and exposed to compound at 23° C for 5 hours before analysis.

GFP reporter induction was monitored on a Guava EasyCyte Plus cytometer. The average mean channel

fluorescence of duplicate samples was determined and the experiment was performed twice with similar

results.

β-glucan staining. Overnight treatment and staining of *C. albicans* strain Sc5314 was performed as

previously described for caspofungin treatment using YPD media at 30° C to maintain growth in the

yeast form (*39*). Antibody to (1-3) β-D-glucan was obtained from Biosupplies (Australia). Cells were

propidium iodide (PI) stained to assess viability and only PI -negative cells were analyzed. Microscopy

was performed on a Nikon Eclipse microscope with a 100X oil objective.

Macrophage stimulation and TNFα measurement. Cultures of *C. albicans* Sc5314 cells were drug-

treated as described for β-glucan staining. After overnight incubation, cultures were washed

extensively, counted and added to cultures of the mouse macrophage cell line RAW264.7 at a

macrophage:yeast ratio of 1:2.5 in the continued presence of drug. After 2 hours supernatants were

harvested and TNFα concentration measured by ELISA using a kit according to manufacturer's instructions (DY410, R&D Systems).

Suppressor strains. Approximately 2×10^7 W303 *MAT*a cells were spread on a YPD plate containing 20 µM gepinacin. Three colonies were recovered 5 days later. Of these, only two grew sufficiently for further experimentation. Designated strains, 20-1 and 20-2, they were mated and sporulated. The gepinacin-resistant phenotype segregated 2:2 in the progeny indicating that the mutation conferring resistance was a single trait or more than one, but closely linked trait. The sensitivity of strains to cycloheximide was the same as wild type suggesting that the resistant phenotype was target-related, not efflux pump-mediated.

Genomic Sequencing Using an Illumina HiSeq platform, we performed whole genome shotgun (WGS) sequencing of wild-type and gepinacin-resistant strains, obtaining two lanes of 76 base pair, paired-end reads and one lane of 101 base pair, paired-end reads for each genome (raw reads are available via NCBI under BioProject accession number PRJXXXXX). Depth of coverage averaged 100-fold. Details of computational analysis are provided in the supplement.

Figure Legends

Figure 1. **Gepinacin inhibits growth of a broad spectrum of fungi but does not affect mammalian cells.** (A) Structures of gepinacin and a similar but inactive compound. (B) Anti-fungal susceptibility testing for an evolutionarily diverse group of fungi treated with gepinacin. For *A. terreus* the MIC50 and MIC80 are plotted. (C) Anti-fungal susceptibility testing for wild type *C. albicans* and strains resistant to the three major classes of anti-fungals treated with gepinacin. (D) Mammalian cell toxicity testing for proliferating human cells in culture (293T) or quiescent cells (mouse embryo fibroblasts).

Cells were treated with gepinacin (20 µM) for 48 hours after which relative viable cell number was measured by standard luciferase assay (Cell Titer-Glo®, Promega).

Figure 2. **Gwt1 is the target of gepinacin**. (A) Anti-fungal susceptibility testing of *S. cerevisiae* strains with graded levels of Gwt1 expression treated with gepinacin. Wild type diploids (Wt) or diploids with one copy of *GWT1* deleted (*gwt1Δ/GWT1*) were transformed with low copy (CEN) plasmids encoding *GWT1* or *GFP* (as a control). (B) Schematic of Gwt1 protein function in cells. Glucosamine (shown as a black circle) phosphatidyl inositol (GlcN-PI) is acylated (orange zigzag) by Gwt1 to become GlcN-(acyl)PI. (C) Autoradiograph depicting the relative amount of GlcN-(acyl)PI product formed in acylation reactions supplemented with various concentrations of gepinacin or an inactive analog (10 µM). The structure of the inactive compound is shown in Figure 1.

Figure 3. **Gepinacin inhibits Gwt1 but not its human ortholog, PIG-W.** (A) Representative photomicrograph of mammalian cells (NIH3T3) co-cultured with amphotericin-resistant *C. albicans* following addition of DMSO or gepinacin. Photographs were taken after 24 hours. DMSO-treated cultures have large, dense colonies of *C. albicans* which are not present in gepinacin-treated cultures (B) Anti-fungal susceptibility testing of *S. cerevisiae* strains in which the endogenous *GWT1* gene had been replaced by plasmid-driven expression of the human gene (*PIG-W*), or the fungal gene *(GWT1)*. CEN plasmids are low copy, 2µ plasmids are high copy. (C) Autoradiograph depicting the relative amount of GlcN-(acyl)PI product formed in acylation reactions supplemented with gepinacin and using membranes prepared from the low copy plasmid strains presented in panel B. These results confirm that activity of the human enzyme is not inhibited by gepinacin.

Figure 4. *EMP24* **deletion decreases gepinacin toxicity**. (A) Anti-fungal susceptibility testing of

gepinacin using suppressor strains 20-1 and 20-2. (B) The sequence of *EMP24* in *S. cerevisiae* is shown

with the location of mutations found by whole genome sequencing in the suppressor strains 20-1 and 20-

2 indicated. (C) Micrographs showing the redistribution of Emp24-GFP after overnight incubation with

gepinacin. Green fluorescence and DIC images are merged in the lower panels. Scale bar; 5 µm.

Figure 5. **Gwt1 inhibition by gepinacin impairs GPI-anchored protein maturation and causes ER-**

related toxicity. (A) Immunoblots of lysates prepared from *S. cerevisiae* treated with gepinacin

showing GPI-anchor-selective impairment of protein maturation. The unprocessed (u), precursor (p)

and mature (m) forms of the reporter proteins are indicated. The identity of the tagged reporter proteins

(top of panel) and the antibodies used for their detection (bottom of panel) are also indicated. Treatment

conditions are DMSO (0.05%), gepinacin (GPN, 10 µM) or tunicamycin (Tun, 10 µM). The same

lysate was used for both blots. (B) Induction of the unfolded protein response in cells carrying a GFP

reporter construct showing strong induction by gepinacin. GFP expression was monitored by flow

cytometry. (C) Anti-fungal susceptibility testing of strains deleted for *IRE1* (activator of the UPR) or

HAC1 (effector of the UPR) showing their increased sensitivity to gepinacin.

Figure 6. **Gepinacin blocks filamentation in liquid and solid culture models.** (A) Fluconazole

resistant *C. albicans* strains were treated overnight with DMSO (0.025%) or gepinacin (GPN) (5 µM) in

filamentation media and then imaged macroscopically. (B) *S. cerevisiae* strain Sigma 1278B was grown

on filamentation-inducing plates containing DMSO (0.025%) or gepinacin (GPN) (5 µM) for 6 days and

then imaged microscopically.

Figure 7**. Gepinacin treatment increases β-glucan exposure on the cell surface and enhances TNFα secretion from macrophages**. (A*)* Fluorescence photomicrographs depicting the β-glucan (green) immunoreactivity of *C. albicans* after treatment with DMSO or 5 µM gepinacin (GPN). Cells were also imaged with DIC and counter-stained with a viability marker. Representative fields of live cells are shown here. Scale bar; 5 µm. (B) Measurement of TNFα concentration in macrophage supernatant by ELISA. Supernatants were harvested after co-culture of RAW264.7 murine macrophages and *C. albicans* treated with DMSO or gepinacin at the concentrations indicated.

Acknowledgements

Supporting Information Available: This material is available free of charge via the Internet.

REFERENCES

1.   Pfaller, M. A., and Diekema, D. J. (2007) Epidemiology of invasive candidiasis: a persistent public health problem, *Clin Microbiol Rev 20*, 133-163.

2.   Cowen, L. E., Anderson, J. B., and Kohn, L. M. (2002) Evolution of drug resistance in Candida albicans, *Annu Rev Microbiol 56*, 139-165.

3.   Monk, B. C., and Goffeau, A. (2008) Outwitting multidrug resistance to antifungals, *Science 321*, 367-369.

4.   Heckman, D. S., Geiser, D. M., Eidell, B. R., Stauffer, R. L., Kardos, N. L., and Hedges, S. B. (2001) Molecular evidence for the early colonization of land by fungi and plants, *Science 293*, 1129-1133.

5. Hu, Y., Rolfs, A., Bhullar, B., Murthy, T. V., Zhu, C., Berger, M. F., Camargo, A. A., Kelley, F., McCarron, S., Jepson, D., Richardson, A., Raphael, J., Moreira, D., Taycher, E., Zuo, D., Mohr, S., Kane, M. F., Williamson, J., Simpson, A., Bulyk, M. L., Harlow, E., Marsischky, G., Kolodner, R. D., and LaBaer, J. (2007) Approaching a complete repository of sequence-verified protein-encoding clones for Saccharomyces cerevisiae, *Genome Res 17*, 536-543.

6. Giaever, G., Chu, A. M., Ni, L., Connelly, C., Riles, L., Veronneau, S., Dow, S., Lucau-Danila, A., Anderson, K., Andre, B., Arkin, A. P., Astromoff, A., El-Bakkoury, M., Bangham, R., Benito, R., Brachat, S., Campanaro, S., Curtiss, M., Davis, K., Deutschbauer, A., Entian, K. D., Flaherty, P., Foury, F., Garfinkel, D. J., Gerstein, M., Gotte, D., Guldener, U., Hegemann, J. H., Hempel, S., Herman, Z., Jaramillo, D. F., Kelly, D. E., Kelly, S. L., Kotter, P., LaBonte, D., Lamb, D. C., Lan, N., Liang, H., Liao, H., Liu, L., Luo, C., Lussier, M., Mao, R., Menard, P., Ooi, S. L., Revuelta, J. L., Roberts, C. J., Rose, M., Ross-Macdonald, P., Scherens, B., Schimmack, G., Shafer, B., Shoemaker, D. D., Sookhai-Mahadeo, S., Storms, R. K., Strathern, J. N., Valle, G., Voet, M., Volckaert, G., Wang, C. Y., Ward, T. R., Wilhelmy, J., Winzeler, E. A., Yang, Y., Yen, G., Youngman, E., Yu, K., Bussey, H., Boeke, J. D., Snyder, M., Philippsen, P., Davis, R. W., and Johnston, M. (2002) Functional profiling of the Saccharomyces cerevisiae genome, *Nature 418*, 387-391.

7. Umemura, M., Okamoto, M., Nakayama, K., Sagane, K., Tsukahara, K., Hata, K., and Jigami, Y. (2003) GWT1 gene is required for inositol acylation of glycosylphosphatidylinositol anchors in yeast, *J Biol Chem 278*, 23639-23647.

8. Sagane, K., Umemura, M., Ogawa-Mitsuhashi, K., Tsukahara, K., Yoko-o, T., and Jigami, Y. (2011) Analysis of membrane topology and identification of essential residues for the yeast endoplasmic reticulum inositol acyltransferase Gwt1p, *J Biol Chem 286*, 14649-14658.

9.   Murakami, Y., Siripanyapinyo, U., Hong, Y., Kang, J. Y., Ishihara, S., Nakakuma, H., Maeda,

        Y., and Kinoshita, T. (2003) PIG-W is critical for inositol acylation but not for flipping of

        glycosylphosphatidylinositol-anchor, *Mol Biol Cell 14*, 4285-4295.

10.   Strating, J. R., and Martens, G. J. (2009) The p24 family and selective transport processes at the

        ER-Golgi interface, *Biol Cell 101*, 495-509.

11.   Castillon, G. A., Aguilera-Romero, A., Manzano-Lopez, J., Epstein, S., Kajiwara, K., Funato, K.,

        Watanabe, R., Riezman, H., and Muniz, M. (2011) The yeast p24 complex regulates GPI-

        anchored protein transport and quality control by monitoring anchor remodeling, *Mol Biol Cell

        22*, 2924-2936.

12.   Fankhauser, C., and Conzelmann, A. (1991) Purification, biosynthesis and cellular localization

        of a major 125-kDa glycophosphatidylinositol-anchored membrane glycoprotein of

        Saccharomyces cerevisiae, *Eur J Biochem 195*, 439-448.

13.   Bryant, N. J., and Stevens, T. H. (1998) Vacuole biogenesis in Saccharomyces cerevisiae:

        protein transport pathways to the yeast vacuole, *Microbiol Mol Biol Rev 62*, 230-247.

14.   Heifetz, A., Keenan, R. W., and Elbein, A. D. (1979) Mechanism of action of tunicamycin on the

        UDP-GlcNAc:dolichyl-phosphate Glc-NAc-1-phosphate transferase, *Biochemistry 18*, 2186-

        2192.

15.   Walter, P., and Ron, D. (2011) The unfolded protein response: from stress pathway to

        homeostatic regulation, *Science 334*, 1081-1086.

16.   Ma, Y., and Hendershot, L. M. (2001) The unfolding tale of the unfolded protein response, *Cell

        107*, 827-830.

17.   Ostrosky-Zeichner, L., Casadevall, A., Galgiani, J. N., Odds, F. C., and Rex, J. H. (2010) An

insight into the antifungal pipeline: selected new molecules and beyond, *Nat Rev Drug Discov 9*,

719-727.

18.   Orlean, P., and Menon, A. K. (2007) Thematic review series: lipid posttranslational

modifications. GPI anchoring of protein in yeast and mammalian cells, or: how we learned to

stop worrying and love glycophospholipids, *J Lipid Res 48*, 993-1011.

19.   Klis, F. M., Sosinska, G. J., de Groot, P. W., and Brul, S. (2009) Covalently linked cell wall

proteins of Candida albicans and their role in fitness and virulence, *FEMS Yeast Res 9*, 1013-

1028.

20.   Shapiro, R. S., Robbins, N., and Cowen, L. E. (2011) Regulatory circuitry governing fungal

development, drug resistance, and disease, *Microbiol Mol Biol Rev 75*, 213-267.

21.   Sudbery, P. E. (2011) Growth of Candida albicans hyphae, *Nat Rev Microbiol 9*, 737-748.

22.   Noble, S. M., French, S., Kohn, L. A., Chen, V., and Johnson, A. D. (2010) Systematic screens

of a Candida albicans homozygous deletion library decouple morphogenetic switching and

pathogenicity, *Nat Genet 42*, 590-598.

23.   White, T. C., Pfaller, M. A., Rinaldi, M. G., Smith, J., and Redding, S. W. (1997) Stable azole

drug resistance associated with a substrain of Candida albicans from an HIV-infected patient,

Oral Dis 3 Suppl 1, S102-109.

24.   Liu, H., Kohler, J., and Fink, G. R. (1994) Suppression of hyphal formation in Candida albicans

by mutation of a STE12 homolog, *Science 266*, 1723-1726.

25.   Poulain, D., and Jouault, T. (2004) Candida albicans cell wall glycans, host receptors and

responses: elements for a decisive crosstalk, *Curr Opin Microbiol 7*, 342-349.

26.   Wheeler, R. T., Kombe, D., Agarwala, S. D., and Fink, G. R. (2008) Dynamic, morphotype-specific Candida albicans beta-glucan exposure during infection and drug treatment, *PLoS Pathog 4*, e1000227.

27.   Tsukahara, K., Hata, K., Nakamoto, K., Sagane, K., Watanabe, N. A., Kuromitsu, J., Kai, J., Tsuchiya, M., Ohba, F., Jigami, Y., Yoshimatsu, K., and Nagasu, T. (2003) Medicinal genetics approach towards identifying the molecular target of a novel inhibitor of fungal cell wall assembly, *Mol Microbiol 48*, 1029-1042.

28.   Miyazaki, M., Horii, T., Hata, K., Watanabe, N. A., Nakamoto, K., Tanaka, K., Shirotori, S., Murai, N., Inoue, S., Matsukura, M., Abe, S., Yoshimatsu, K., and Asada, M. (2011) In vitro activity of E1210, a novel antifungal, against clinically important yeasts and molds, *Antimicrob Agents Chemother 55*, 4652-4658.

29.   Hata, K., Horii, T., Miyazaki, M., Watanabe, N. A., Okubo, M., Sonoda, J., Nakamoto, K., Tanaka, K., Shirotori, S., Murai, N., Inoue, S., Matsukura, M., Abe, S., Yoshimatsu, K., and Asada, M. (2011) Efficacy of oral E1210, a new broad-spectrum antifungal with a novel mechanism of action, in murine models of candidiasis, aspergillosis, and fusariosis, *Antimicrob Agents Chemother 55*, 4543-4551.

30.   Watanabe, N. A., Miyazaki, M., Horii, T., Sagane, K., Tsukahara, K., and Hata, K. (2012) E1210, a New Broad-Spectrum Antifungal, Suppresses Candida albicans Hyphal Growth through Inhibition of Glycosylphosphatidylinositol Biosynthesis, *Antimicrob Agents Chemother 56*, 960-971.

31.   Alberti, S., Gitler, A. D., and Lindquist, S. (2007) A suite of Gateway cloning vectors for high-throughput genetic analysis in Saccharomyces cerevisiae, *Yeast 24*, 913-919.

32. Hoon, S., Smith, A. M., Wallace, I. M., Suresh, S., Miranda, M., Fung, E., Proctor, M., Shokat, K. M., Zhang, C., Davis, R. W., Giaever, G., St Onge, R. P., and Nislow, C. (2008) An integrated platform of genomic assays reveals small-molecule bioactivities, *Nat Chem Biol 4*, 498-506.

33. Pierce, S. E., Fung, E. L., Jaramillo, D. F., Chu, A. M., Davis, R. W., Nislow, C., and Giaever, G. (2006) A unique and universal molecular barcode array, *Nat Methods 3*, 601-603.

34. Shen, J., Cowen, L. E., Griffin, A. M., Chan, L., and Kohler, J. R. (2008) The Candida albicans pescadillo homolog is required for normal hypha-to-yeast morphogenesis and yeast proliferation, *Proc Natl Acad Sci U S A 105*, 20918-20923.

35. Huh, W. K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weissman, J. S., and O'Shea, E. K. (2003) Global analysis of protein localization in budding yeast, *Nature 425*, 686-691.

36. Fujita, M., Yoko, O. T., and Jigami, Y. (2006) Inositol deacylation by Bst1p is required for the quality control of glycosylphosphatidylinositol-anchored proteins, *Mol Biol Cell 17*, 834-850.

37. Cox, J. S., Shamu, C. E., and Walter, P. (1993) Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase, *Cell 73*, 1197-1206.

38. Pollard, M. G., Travers, K. J., and Weissman, J. S. (1998) Ero1p: a novel and ubiquitous protein with an essential role in oxidative protein folding in the endoplasmic reticulum, *Mol Cell 1*, 171-182.

39. Wheeler, R. T., and Fink, G. R. (2006) A drug-sensitive genetic network masks fungi from the immune system, *PLoS Pathog 2*, e35.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
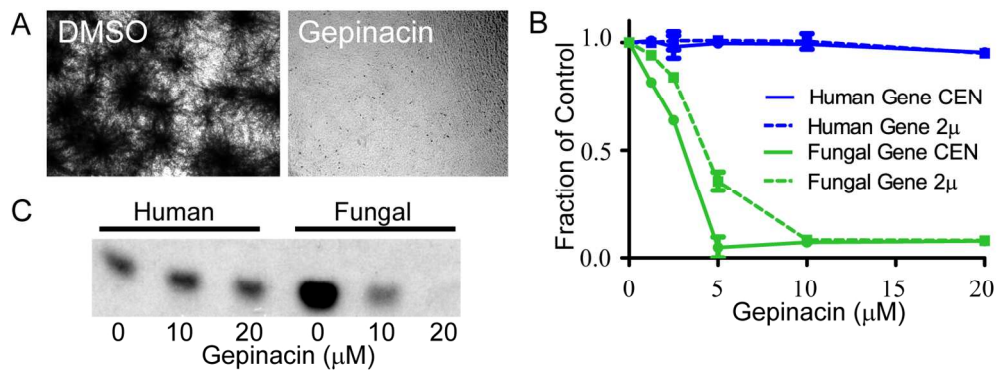35
36
37
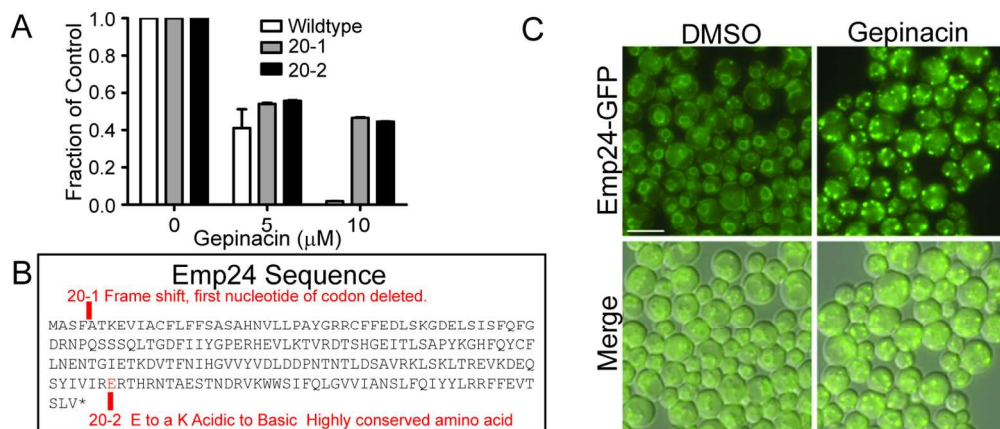38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
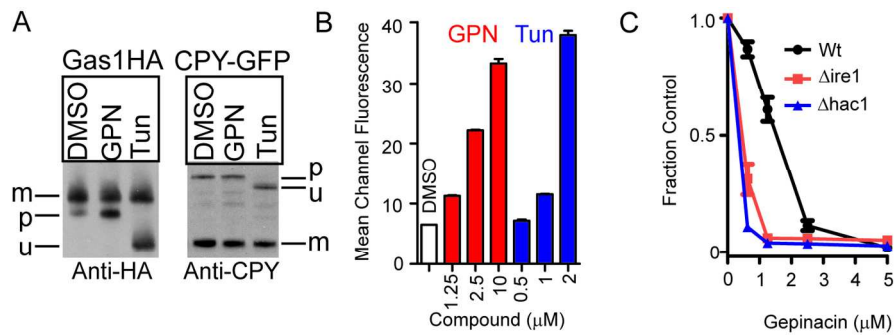58
59
60
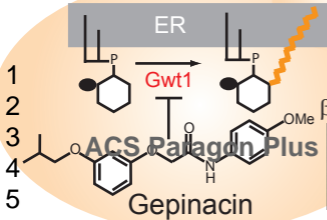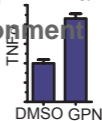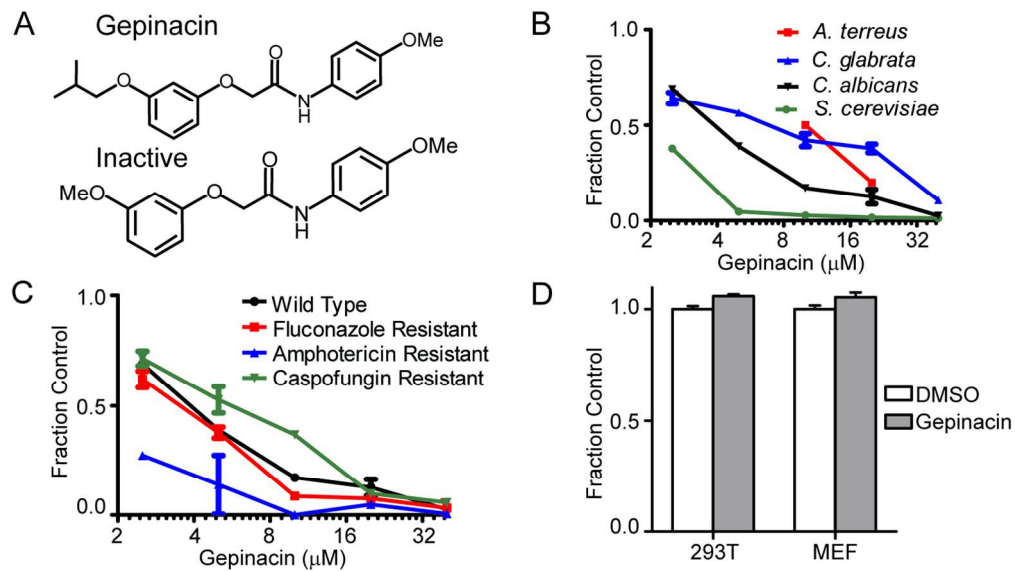
131x74mm (300 x 300 DPI)

A
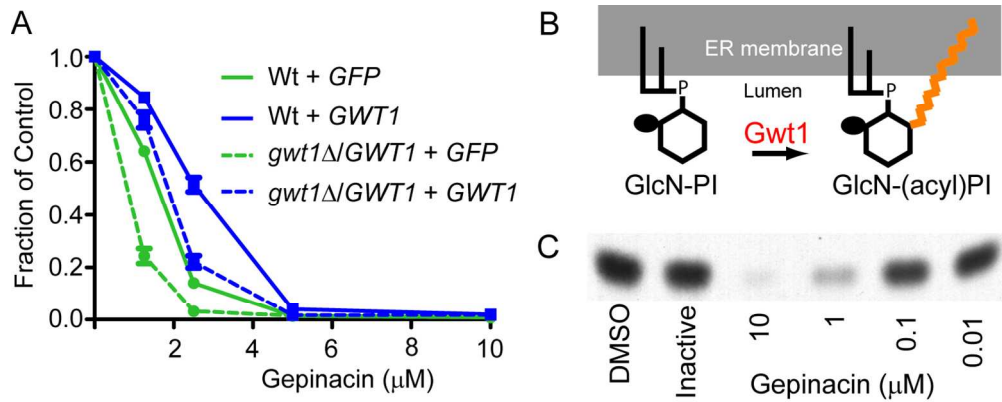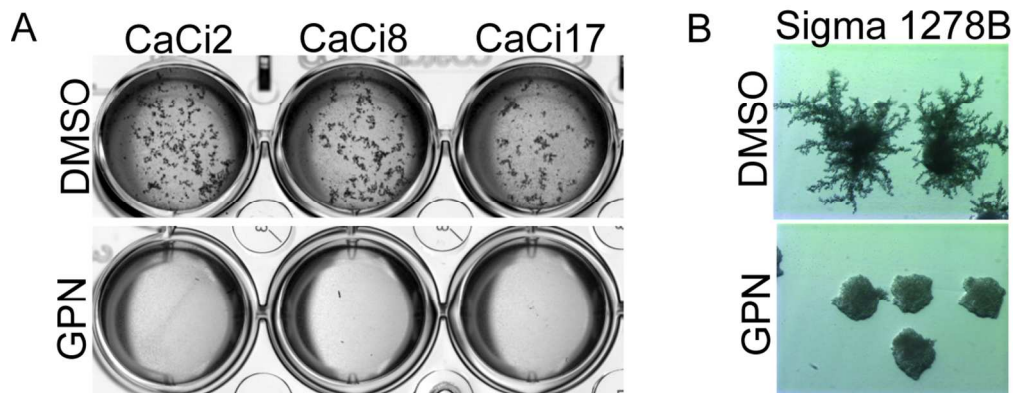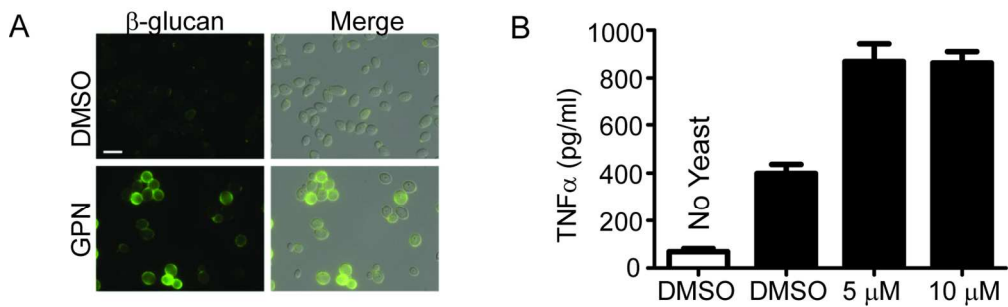

131x52mm (300 x 300 DPI)
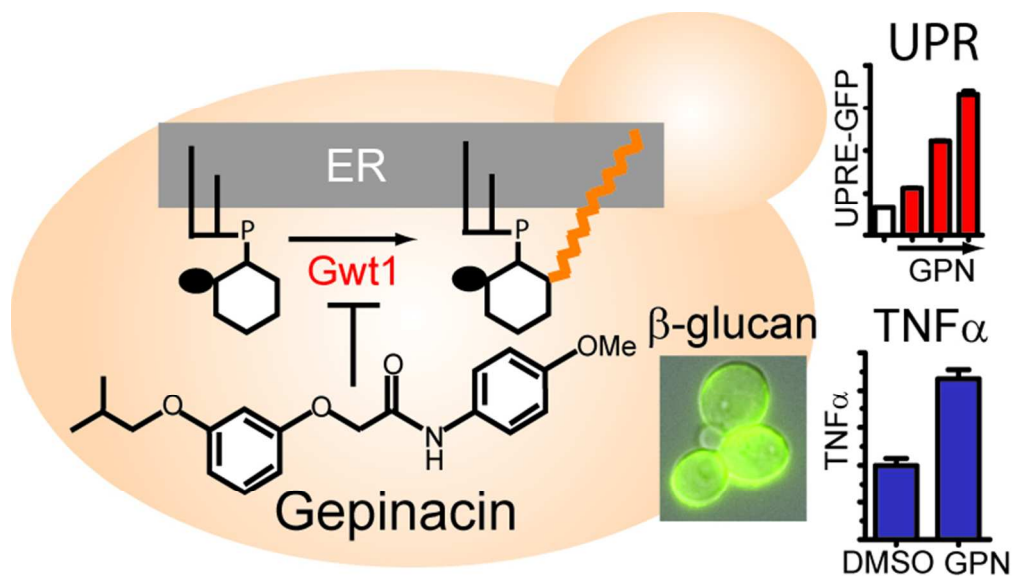
139x50mm (300 x 300 DPI)

130x55mm (300 x 300 DPI)

150x49mm (300 x 300 DPI)

107x42mm (300 x 300 DPI)

135x40mm (300 x 300 DPI)

68x39mm (300 x 300 DPI)

SUPPORTING INFORMATION

Material and Methods

Fungal strains. Standard *S. cerevisiae* lab strains W303, BY4741, BY4742 and BY4743 were used for experiments. The *C. albicans* wild type strain in Figure 1 was CaI4, a standard laboratory strain (*1*). The *C. albicans* fluconazole-resistant strains CaCi-2, CaCi-8 and CaCi17 were generously provided by Ted White and originally collected by Spencer Redding and colleagues (*2-4*). The sequenced *C. albicans* wild type strain, Sc5314 (*5*) used in β-glucan experiments was a gift from John Perfect's lab. CaAmphR, an amphotericin B-resistant strain is from ATCC (#20095). Caspofungin-resistant strain Cfr1 was derived by plating Sc5314 on caspofungin plates and isolating resistant colonies. *A. terreus* was purchased from ATCC (MYA-3633). The *C. glabrata* strain CgL5c was obtained from John Bennett's lab. The YKO heterozygous diploid strain collection is from Open Biosystems and was generated in BY4743. Haploid deletion strains were from the Yeast Knock-out Deletion Collection and purchased from Invitrogen. The over-expression library was made by transforming BY4741 with the Harvard Institute of Proteomics Flexgene library.

Humanized yeast strain. Because *GWT1* deletion is lethal in *S. cerevisiae* strain BY4741, a haploid shuttle strain was constructed in which the genomic copy of *GWT1* was deleted and replaced by *GWT1* on a plasmid that also encoded the *URA3* gene and a G418- resistance gene. To construct this shuttle strain a *gwt1Δ/GWT1* heterozygous deletion mutant was transformed with pAGGPD416-*GWT1*, sporulated and dissected. A colony was selected that grew on both SD–URA and G418-containing plates. PCR with the deletion specific primers (http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html) was performed to confirm the *GWT1* deletion. To change the source of *GWT,* this shuttle strain was transformed with plasmids containing *GWT1* (as a control) or *PIG-W* and the *LEU2* auxotrophy gene followed by selection for growth on SD-LEU plates. Plasmids were constructed by conventional Gateway technology using a *GWT1*encoding entry vector and either pAG415GPDccdB or pAG425GPDccdB as the destination vectors. Positive colonies were selected and grown on 5-Fluoroorotic acid (5-FOA)-containing plates to

promote loss of the pAGGPD416–*GWT1* plasmid. The colonies from this plate were then tested for return of uridine auxotrophy to confirm pAGGPD416-*GWT1* loss. The *PIG-W* expression vector was constructed by recombination in yeast as follows. The pAG415GPDccdB vector was cut with Xho1 and Xba1 and the larger piece was gel and column purified. The coding sequence of *PIG-W* was PCR amplified from the entry clone so that it would have ends facilitating recombination with the cut vector. The shuttle strain was then transformed with these two DNA fragments and plated on SD-LEU plates to select for recombinants. *PIG-W* plasmid was isolated from the final strain and sequence verified.

Protein lysis procedure. Yeast were spun down, washed two times with H_2O and lysed in ethanol containing phenylmethylsulfonyl fluoride. An equivalent volume of glass beads were added and after beating, samples were transferred to a -80°C freezer for an hour. The samples were then evaporated to dryness in a Savant SpeedVac concentrator. Solubilization buffer (200 ul of 2% SDS in 20 mM Tris HCl pH 6.8) was added to the dry beads which were vortexed and then boiled 5 min. Protein concentrations were determined by BCA assay (Pierce) and equal amounts of total protein were diluted into 5X reducing loading buffer (0.5 M DTT, 20 mM Tris HCl, 50 % Glycerol) for subsequent analysis by SDS-PAGE.

Data analysis of over-expression screen. Raw data for each gepinacin-treated 384-well plate was quantile normalized to achieve the same distribution as one of the DMSO replicates at the corresponding reading time. The R package *limma* (*6*) was then used to compute empirical Bayes moderated t-statistics for each gene. Genes were flagged as hits if the mean difference in normalized optical density scores between DMSO and gepinacin was at least 0.1 at any reading time, and if the p-value for this difference had an associated FDR (false discovery rate) of at most 0.2. Hits were re-tested and plasmids sequenced to confirm their identity.

Analysis of sequencing data- After quality control was performed on raw reads for each genome, we aligned the filtered reads against the *S. cerevisiae* reference sequence sacCer2, (June 2008 assembly, downloaded from UCSC on April 1, 2011:

http://hgdownload.cse.ucsc.edu/goldenPath/sacCer2/chromosomes/) using the BWA aligner (*7*). For each of the strains, we called SNPs and indels with respect to the reference using *mpileup* from the *SAMtools*

package (*8*). To identify SNPs and indels unique to each of the resistant strains, we compared the parental strain to individual resistant lines. We used a combination of custom code and the *Genome Analysis Toolkit* (GATK) (*9*) to locate, and then rank by quality, the SNPs and indels detected in open reading frames that were present only in the suppressor strains. Alignments of the reads for the top ranked SNPs and indels were then visually inspected.

Supplementary Figure Legends

Supplementary Figure 1

 ***GWT1* copy number determines gepinacin sensitivity.** Anti-fungal susceptibility testing of wild type diploids (Wt) or diploids with one copy of GWT1 deleted (gwt1Δ/*GWT1*) transformed with high copy (2 micron) plasmids containing *GWT1* or *GFP* (as a control). The strains were treated with gepinacin for 48 hours and the growth was monitored by measuring the optical density at 600 nm (OD_{600}). The results are expressed as a fraction of the OD_{600} measured in the absence of gepinacin.

Supplementary Figure 2

 A close structural analog of gepinacin does not inhibit growth. Anti-fungal susceptibility testing of a fluconazole-resistant *C. albicans* strain under standard conditions. Yeast were incubated at 30° C for 24 hours with serial dilutions of the indicated compounds. Growth was monitored by OD_{600} and is expressed as fraction of the growth observed in the absence of compound.

Supplementary Figure 3

EMP24 **deletion partially suppresses inhibition of growth by gepinacin**. Anti-fungal susceptibility testing of *S. cerevisiae* strains that are wild type or deleted for *EMP24*. Growth was assessed by measuring the OD_{600} following gepinacin treatment for 48 hours at 30° C in 96-well plate format. Mean and standard deviation of triplicate determinations are shown.

Supplementary Figure 4

Conventional anti-fungal drugs do not induce the UPR. Induction of the unfolded protein response in cells carrying a GFP reporter construct was monitored by flow cytometry after exposure to serial dilutions

of the indicated compounds for 5 hrs. Strong induction by gepinacin and tunicamycin but not by conventional anti-fungal drugs is evident.

Supplementary Figure 5

BIP level, an indicator of the UPR in mammalian cells, is not increased after gepinacin treatment. Immunoblot of lysates prepared from human leukemia cells (K562) using an antibody to BIP after 28 hour exposure to tunicamycin (1 μg ml^{-1}), gepinacin (20 μM) or solvent vehicle (DMSO, 0.1%). Beta-actin was blotted as a loading control.

Supplementary Figure 6

Compromising the UPR enhances tunicamycin toxicity but does not alter the toxicity of fluconazole. Anti-fungal susceptibility testing of strains bearing deletion of *IRE1* (activator of the UPR) or *HAC1* (effector of the UPR) using either tunicamycin (A) or fluconazole (B). Growth was measured by OD$_{600}$ after 48 hour exposure to serial dilutions of the compounds as indicated. Results are expressed as a fraction of the OD$_{600}$ measured in the absence of gepinacin. The mean and standard deviation of triplicate determinations are shown.

Supplementary Figure 7

Gepinacin blocks morphogenic switching in *C. albicans*. Three drug-resistant strains of *C. albicans* were treated with DMSO or gepinacin (5 μM) and induced to filament for 3 hours. DIC images were acquired at 60X.

Supplementary Figure 8

Previously reported Gwt1 inhibitors are structurally distinct from gepinacin. (A) BIQ (*10*) and (B) E1210 (*11*).

References

1. Fonzi, W. A., and Irwin, M. Y. (1993) Isogenic strain construction and gene mapping in Candida albicans, *Genetics 134*, 717-728.

2.   White, T. C. (1997) The presence of an R467K amino acid substitution and loss of allelic variation correlate with an azole-resistant lanosterol 14alpha demethylase in Candida albicans, *Antimicrob Agents Chemother 41*, 1488-1494.

3.   White, T. C. (1997) Increased mRNA levels of ERG16, CDR, and MDR1 correlate with increases in azole resistance in Candida albicans isolates from a patient infected with human immunodeficiency virus, *Antimicrob Agents Chemother 41*, 1482-1487.

4.   White, T. C., Pfaller, M. A., Rinaldi, M. G., Smith, J., and Redding, S. W. (1997) Stable azole drug resistance associated with a substrain of Candida albicans from an HIV-infected patient, *Oral Dis 3 Suppl 1*, S102-109.

5.   Gillum, A. M., Tsay, E. Y., and Kirsch, D. R. (1984) Isolation of the Candida albicans gene for orotidine-5'-phosphate decarboxylase by complementation of S. cerevisiae ura3 and E. coli pyrF mutations, *Mol Gen Genet 198*, 179-182.

6.   Smyth, G. K. (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments, *Stat Appl Genet Mol Biol 3*, Article3.

7.   Li, H., and Durbin, R. (2009) Fast and accurate short read alignment with Burrows-Wheeler transform, *Bioinformatics 25*, 1754-1760.

8.   Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., and Durbin, R. (2009) The Sequence Alignment/Map format and SAMtools, *Bioinformatics 25*, 2078-2079.

9.   DePristo, M. A., Banks, E., Poplin, R., Garimella, K. V., Maguire, J. R., Hartl, C., Philippakis, A. A., del Angel, G., Rivas, M. A., Hanna, M., McKenna, A., Fennell, T. J., Kernytsky, A. M., Sivachenko, A. Y., Cibulskis, K., Gabriel, S. B., Altshuler, D., and

Daly, M. J. (2011) A framework for variation discovery and genotyping using next-generation DNA sequencing data, *Nat Genet 43*, 491-498.

10. Tsukahara, K., Hata, K., Nakamoto, K., Sagane, K., Watanabe, N. A., Kuromitsu, J., Kai, J., Tsuchiya, M., Ohba, F., Jigami, Y., Yoshimatsu, K., and Nagasu, T. (2003) Medicinal genetics approach towards identifying the molecular target of a novel inhibitor of fungal cell wall assembly, *Mol Microbiol 48*, 1029-1042.

11. Hata, K., Horii, T., Miyazaki, M., Watanabe, N. A., Okubo, M., Sonoda, J., Nakamoto, K., Tanaka, K., Shirotori, S., Murai, N., Inoue, S., Matsukura, M., Abe, S., Yoshimatsu, K., and Asada, M. (2011) Efficacy of oral E1210, a new broad-spectrum antifungal with a novel mechanism of action, in murine models of candidiasis, aspergillosis, and fusariosis, *Antimicrob Agents Chemother 55*, 4543-4551.
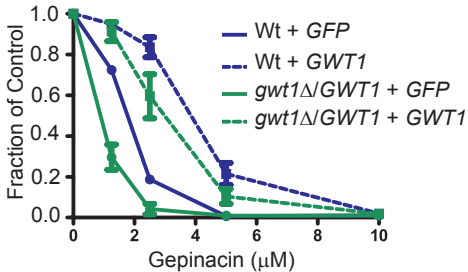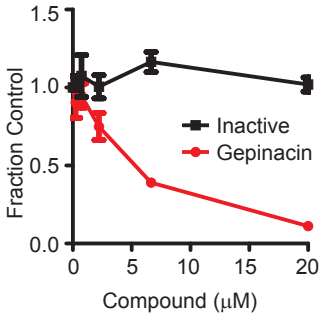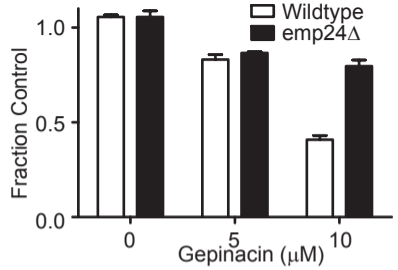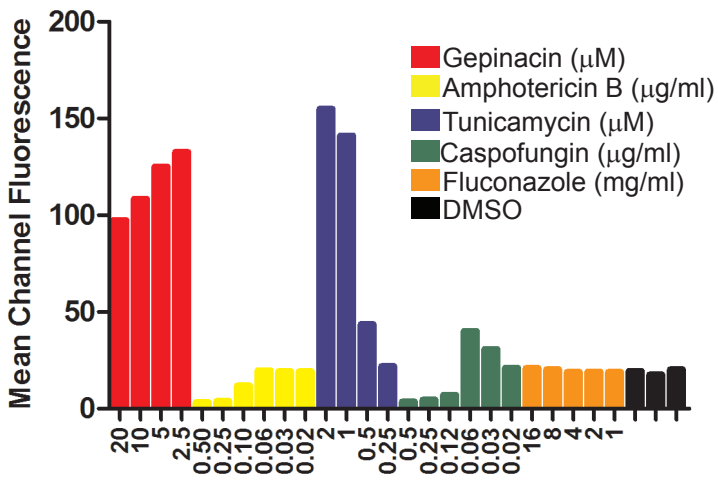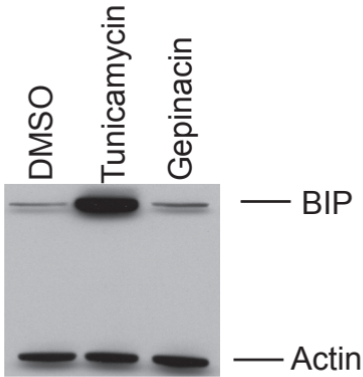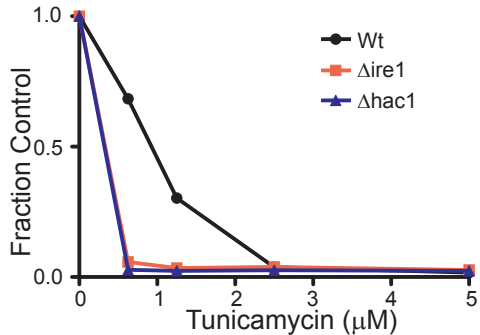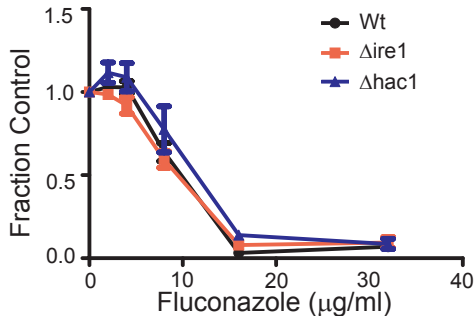
	CaCi2	CaCi8	CaCi17
DMSO			
Gepinacin			

A

BIQ

B

E1210