

A genome-wide screen in *Saccharomyces cerevisiae* reveals a critical role for the mitochondria in the toxicity of a trichothecene mycotoxin

John E. McLaughlin^{a,1}, Mohamed Anwar Bin-Umer^{a,1}, Andrew Tortora^a, Natasha Mendez^a, Susan McCormick^b, and Nilgun E. Tumer^{a,2}

^aBiotechnology Center for Agriculture and the Environment and the Department of Plant Biology and Pathology, School of Environmental and Biological Sciences, Rutgers University, New Brunswick, NJ, 08901-8520; and ^bMycotoxin Research Unit, National Center for Agricultural Utilization Research, United States Department of Agriculture, Agricultural Research Service, Peoria, IL, 61604

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Trichothecene mycotoxins synthesized by *Fusarium* species are potent inhibitors of eukaryotic translation. They are encountered in both the environment and in food, posing a threat to human and animal health. They have diverse roles in the cell that are not limited to the inhibition of protein synthesis. To understand the trichothecene mechanism of action, we screened the yeast knockout library to identify genes whose deletion confers resistance to trichothecin (Tcin). The largest group of resistant strains affected mitochondrial function, suggesting a role for fully active mitochondria in trichothecene toxicity. Tcin inhibited mitochondrial translation in the wild-type strain to a greater extent than in the most resistant strains, implicating mitochondrial translation as a previously unrecognized site of action. The Tcin-resistant strains were cross-resistant to anisomycin and chloramphenicol, suggesting that Tcin targets the peptidyltransferase center of mitochondrial ribosomes. Tcin-induced cell death was partially rescued by mutants that regulate mitochondrial fusion and maintenance of the tubular morphology of mitochondria. Treatment of yeast cells with Tcin led to the fragmentation of the tubular mitochondrial network, supporting a role for Tcin in disruption of mitochondrial membrane morphology. These results provide genome-wide insight into the mode of action of trichothecene mycotoxins and uncover a critical role for mitochondrial translation and membrane maintenance in their toxicity.

Fusarium head blight | ribosome | translation | deoxynivalenol | mycotoxin

The trichothecenes are toxic sesquiterpenoids produced by various *Fusarium* species. *Fusarium* head blight (FBH), also known as scab, caused by *Fusarium graminearum*, is a devastating disease that results in yield reductions and in the contamination of cereals with trichothecene mycotoxins, the most prominent being deoxynivalenol (DON) (1, 2). The trichothecenes are potent cytotoxins of eukaryotic cells and are a public health concern because contamination of human foods and animal feed with these toxins is a worldwide problem (1, 2). Toxic effects of trichothecenes include growth retardation, hemorrhagic lesions, reproductive disorders, immune dysfunction, vomiting, and reduced weight gain (3, 4). Trichothecenes all share a 12,13-epoxytrichothecene core structure and have been classified based on their substitution pattern of specific side groups. Trichothecenes that have a hydroxyl group, an ester group, or no side chain at all at C8 have been classified as Type A (T-2 toxin and diacetoxyscirpenol), while trichothecenes that have a ketone functional group at C8 are classified as Type B (DON, trichodermin, and trichothecin). Type C are characterized by a second epoxide function (crotocin), while type D trichothecenes contain a macrocyclic ring (verrucarin and satratoxins) (1, 2, 4). A mutation in the *RPL3* gene, encoding ribosomal protein L3, referred to as *tcm1* conferred resistance to trichodermin (5, 6). Other trichothecenes were shown to target L3 at the peptidyl-

transferase center in *Saccharomyces cerevisiae* and inhibit peptidyltransferase activity of eukaryotic ribosomes (7–9). Trichodermin-resistant yeast mutants were also resistant to anisomycin (9) and the trichodermin binding site on the 60S ribosomal subunit was closely related to the binding site of anisomycin (9, 10).

Trichothecenes and translation inhibitors like anisomycin that target the peptidyltransferase center are known to induce the ribotoxic stress response that leads to rapid activation of mitogen activated protein kinases (MAPKs), induction of proinflammatory responses, and cell death (11, 12). In the macrophage, DON induced mobilization of MAPKs to the ribosome (13) and promoted 28S rRNA cleavage at the peptidyltransferase center (14), suggesting that ribosome interactions of trichothecenes play an important role in their cytotoxicity.

Differences have been reported in the mode of action of trichothecenes, including mechanisms of translation inhibition (3, 10) and different signaling pathways that lead to cytotoxicity and apoptosis (15, 16). These observations suggest that toxicity of trichothecenes might not be a simple function of translational arrest and that they may have multiple mechanisms of toxicity. Despite the early studies that implicated DNA synthesis (2), respiration (17), mitochondrial protein synthesis (17), and membrane structure and integrity (18) in the cytotoxicity of trichothecenes, the molecular mechanisms of their toxicity are not well understood, and the proteins targeted by trichothecenes other than L3 have not been identified. To develop a better understanding of the trichothecene mechanism of action, we used a chemical genomics approach in *S. cerevisiae* to identify genes that when deleted confer resistance to trichothecin (Tcin). Our results provide genome-wide insight into the mechanism of toxicity of a trichothecene mycotoxin and demonstrate that its toxicity is mediated by the mitochondria.

Results

High-Throughput Screen of the Yeast Deletion Library Reveals a Role for Mitochondrial Translation in Trichothecin Sensitivity. To identify the genes involved in mediating sensitivity to trichothecenes, we carried out a systematic screen of the 4,720 viable *S. cerevisiae* diploid gene deletion collection for Tcin resistance. The screen was carried out using 4 μ M Tcin, which severely inhibited the

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¹J.E.M. and M.A.B.-U. contributed equally to this work.

²To whom correspondence should be addressed. E-mail: tumer@aesop.rutgers.edu.

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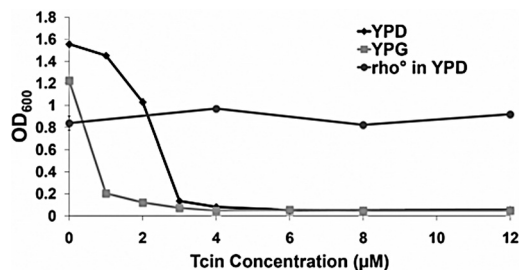


Fig. 1. Growth comparison of BY4743 in YPD or YPG media. The parental strain BY4743 was grown in liquid media supplemented with 2% dextrose (YPD) for 20 h or with 3% glycerol (YPG) for 48 h at 30 °C in the presence of different concentrations of Tcin. The ρ^0 version of BY4743 was grown for 48 h at 30 °C. The experiments were performed in triplicate and repeated twice. Growth is represented by mean OD₆₀₀ values (\pm SE).

growth of the parental strain, BY4743 (>90%) with an IC₅₀ of 2.5 μ M in liquid YPD media (Fig. 1). We identified 138 deletion strains as resistant to 4 μ M Tcin from three independent experiments (Table S1). The largest group of deletions (89/138 or 64%) that showed resistance to 4 μ M Tcin encoded proteins associated with the mitochondria or affected mitochondrial function (Fig. 2A). Strains associated with mitochondrial ribosomes constituted the largest group (43%) within the mitochondria category (Fig. 2B). Approximately 25% (35/138) of the Tcin-resistant strains associated with the mitochondria had abnormal mitochondrial morphology based on the *Saccharomyces* genome database (SGD) (<http://www.yeastgenome.org/>).

The relatively large proportion of resistant strains that had defects associated with the mitochondria were surprising in light of the reported role of trichothecenes in inhibition of cytosolic, but not mitochondrial protein synthesis (2, 4) and their lack of inhibition of bacterial growth and translation (19, 20). To determine whether mitochondria were critical for Tcin sensitivity, the parental strain, BY4743, was grown in liquid media containing glycerol (YPG), which forces the cells to depend on mitochondria for energy. The IC₅₀ of BY4743 for Tcin was significantly reduced from 2.5 to 0.75 μ M in YPG media (Fig. 1) reflecting a distinct mitochondrial role in the sensitivity to Tcin. To confirm this, we tested the sensitivity of a ρ^0 strain derived from BY4743 by ethidium bromide treatment. Unlike the parental strain, the ρ^0 strain was resistant to Tcin, confirming the role of mitochondria in Tcin sensitivity (Fig. 1).

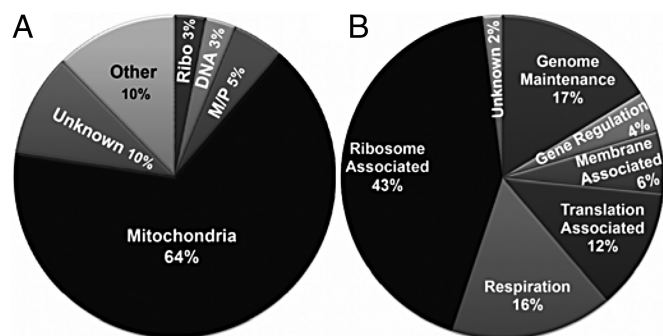


Fig. 2. Functional classification of yeast deletion mutants that conferred resistance to 4 μ M Tcin. (A) Yeast deletion mutants exhibiting resistance to 4 μ M Tcin. The figure shows the GO terms (www.yeastgenome.org) most associated with the genes identified. Genes associated with heat shock protein, signal transduction, proteasome/ubiquitination, transcription and RNA metabolism constitute the “Other” category. MIP and Ribo refer to membrane/phospholipid and cytosolic ribosomes. (B) Classification of the mitochondria-associated deletion mutants.

To determine whether any of the strains that showed resistance to 4 μ M Tcin had mitochondrial defects, the 138 resistant strains were grown on YPDG plates containing 3% glycerol and 0.2% dextrose. A large fraction (62%) of the strains that showed resistance to 4 μ M Tcin was confirmed to be *petite*, consistent with their classification in the SGD (Table S1). Only four (*ndi1* Δ , *ups1* Δ , *ycd1* Δ , and *cir2* Δ) deletion strains resistant to 4 μ M Tcin and associated with the mitochondria displayed the wild-type phenotype when grown on the YPDG media. When the Tcin-resistant *petite* mutants were grouped and analyzed using FUN-Spec (<http://funspec.med.utoronto.ca/>), 32% (27/85) encoded mitochondrial ribosomal proteins. In addition, six mitochondrial aminoacyl-tRNA-synthetases (*SLM5*, *AIM10*, *MSR1*, *MSK1*, *MSD1*, and *MSF1*), one mitochondrial translation initiation (*IFM1*), one elongation (*MEF2*), one termination (*RRF1*) factor and two translational control proteins (*CBP6* and *AEPI*) were included in the resistant group, providing evidence that mitochondrial translation may be a target of Tcin.

The genome-wide screen was repeated with 8, 12, and 24 μ M Tcin to identify the most resistant strains. Twenty-seven out of the 138 strains resistant to 4 μ M Tcin grew better than the wild-type on 8 μ M Tcin, and 24 of these strains grew well on 12 μ M Tcin. Only six of these strains (*spo13* Δ , *ups1* Δ , *tma7* Δ , *tof2* Δ , *ict1* Δ , and *ynl011c* Δ) were able to grow on 24 μ M Tcin. Table S2 shows the scores for the 15 strains that had the highest level of resistance based on their growth ratios on 4–24 μ M Tcin. These results were confirmed by examining growth of these strains on different concentrations of Tcin in liquid (Fig. S1) or solid YPD media (Fig. S2). The differences in the viability of these strains were apparent at the higher concentrations of Tcin (Fig. S2), indicating a dose-dependent response.

Tcin Affects Mitochondria at Low Doses. Because a large fraction of the resistant strains were associated with the mitochondria, we characterized the effects of Tcin on yeast growth by growing the parental strain and the top 15 deletion mutants (all ρ^+) in liquid media containing glycerol (YPG). Growth of BY4743 was inhibited by 2 μ M Tcin on YPG media, but not YPD media, indicating that the wild-type strain was more sensitive to Tcin when it depended on mitochondria for energy (Fig. S3). When the concentration of Tcin was doubled to 4 μ M, growth of BY4743 was inhibited on both the YPG and the YPD media (Fig. S3). The most resistant strains grew well on YPG media, even at 12 μ M Tcin (Fig. 3A), suggesting that the resistance in these strains was due to the mitochondria.

Tcin-Resistant Strains Are Cross-Resistant to Anisomycin, but Not to Other Translation Inhibitors. To test the specificity of the resistance, we screened the 15 most resistant deletion strains against other translation inhibitors. BY4743 and the resistant mutants failed to grow on 0.5 or 1 μ g/mL cycloheximide (Fig. S4A) and were sensitive to hygromycin B (100 or 200 μ g/mL) on YPD media, while 10 μ g/mL hygromycin B inhibited their growth on YPG media (Fig. S4B). These results indicated that the observed resistance in the deletion strains did not extend to protein inhibitors in general. The Tcin-resistant mutants were, however, resistant to 10 μ g/mL anisomycin on YPD media (Fig. 3B). We examined growth on YPG media to determine if anisomycin resistance was due to a possible effect on mitochondrial translation. As shown in Fig. S5, 2.5 μ g/mL anisomycin inhibited growth of BY4743 by 52% on YPG media relative to 12% inhibition on YPD, suggesting that anisomycin targets mitochondrial translation at low doses. The Tcin-resistant strains were resistant to 5 μ g/mL anisomycin on YPG media when growth of BY4743 was inhibited by 94% (Fig. S5). Several Tcin-resistant strains (*ups1* Δ , *yap1802* Δ , *izh4* Δ , *cue3* Δ , *pcl8* Δ , *fkc3* Δ , and *ydl173w* Δ) were more sensitive to 10 μ g/mL anisomycin on YPG media than on YPD media (Fig. 3B), suggesting that anisomycin

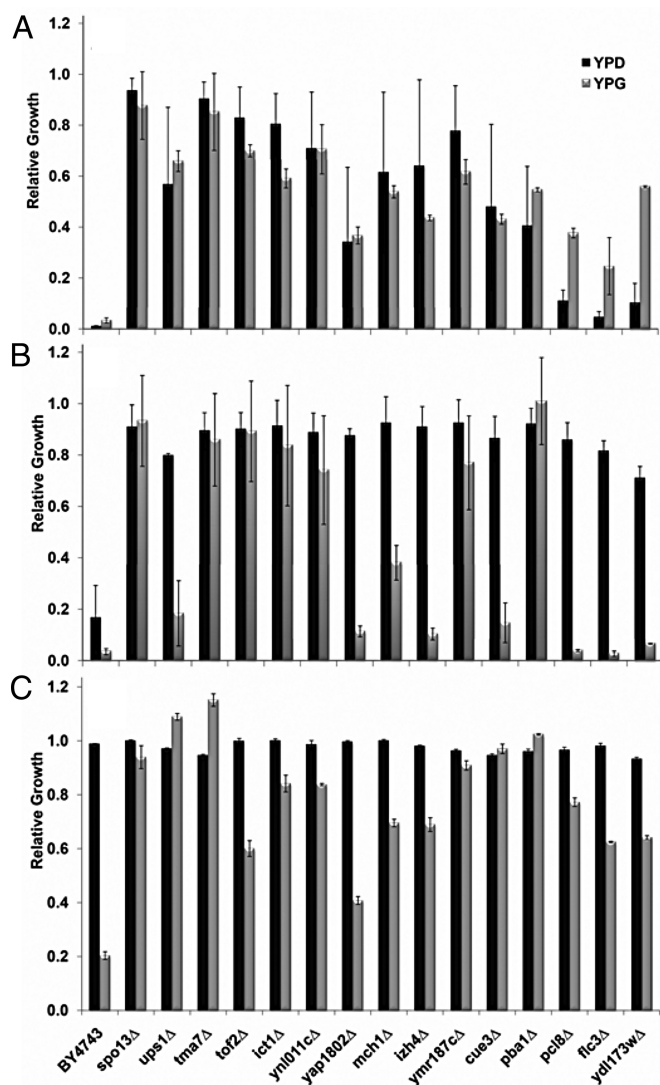


Fig. 3. Growth of the most resistant 15 deletion strains in the presence of different antibiotics in YPD or YPG media. The Tcin-resistant strains were grown in liquid YPD and YPG media containing (A) 12 μ M Tcin, (B) 10 μ g/mL anisomycin, or (C) 1 mg/mL chloramphenicol. Relative growth was calculated as the ratio between growth of treated cells to that of untreated cells with a ratio of 1.0, indicating no effect on growth. The experiments were performed in triplicate and repeated twice.

and Tcin have overlapping, but not identical effects on the mitochondria.

Tcin-Resistant Strains Are Cross-Resistant to Chloramphenicol. To further investigate the role of the mitochondria in resistance to Tcin, we examined the sensitivity of the Tcin-resistant strains to chloramphenicol, a known inhibitor of mitochondrial translation in eukaryotes, which binds to the A site and occupies the same position as the aminoacyl-tRNA (aa-tRNA), preventing peptide bond formation (21). One mg/mL chloramphenicol reduced the growth of BY4743 and the top 15 resistant mutants by less than 10% in YPD media (Fig. 3C), but was sufficient to inhibit BY4743 growth by 80% in YPG media, consistent with inhibition of mitochondrial translation. In contrast, growth of the top 15 mutants was either not inhibited at all or was markedly better than the parental strain on 1 mg/ml chloramphenicol (Fig. 3C). These results provided evidence that mitochondrial translation was critical for the toxicity of Tcin.

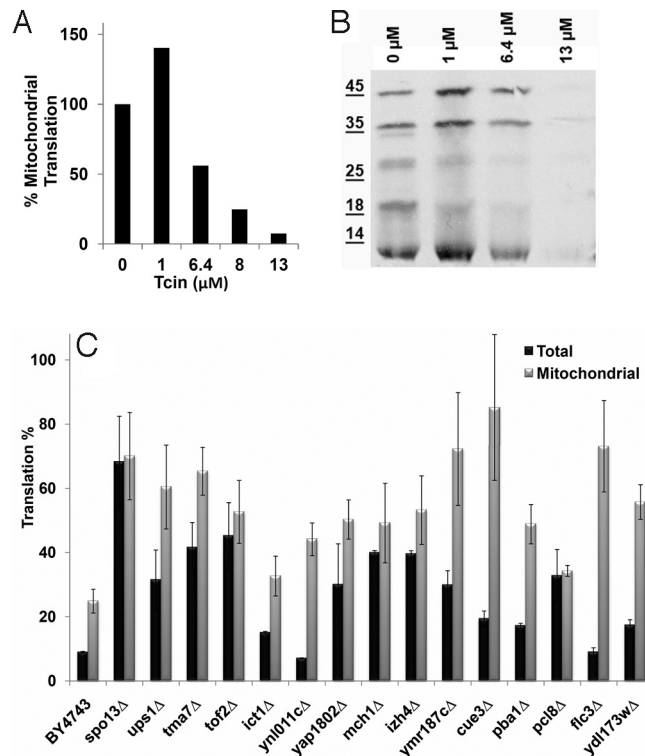


Fig. 4. Effect of Tcin on mitochondrial translation. (A) Mitochondrial translation rate of the BY4743 cells was determined by measuring the rate of [35 S]-methionine incorporation in vivo at increasing concentrations of Tcin. All treatments were performed for 1 h and the rate of translation in the absence of Tcin was normalized to 100%. (B) Autoradiogram of labeled mitochondrial translation products separated on a 15% SDS-polyacrylamide gel. (C) The ratio of translation between Tcin-treated and untreated samples for both total and mitochondrial translation is represented as percent of the untreated control. Each graph represents the mean of four independent experiments with error bars representing the standard deviation.

Tcin Inhibits Mitochondrial Translation. The wild-type cells were exposed to different concentrations of Tcin for 1 h and the mitochondrial translation rate was determined by measuring [35 S]-Met incorporation into mitochondrial protein (22). At 6.4 μ M Tcin, the mitochondrial translation efficiency was reduced by >50% and at 13 μ M Tcin, nearly complete inhibition of mitochondrial protein synthesis was observed (Fig. 4A). A general decrease in mitochondrial protein synthesis, which was not specific to any one particular protein, was observed by SDS/PAGE analysis followed by autoradiography (Fig. 4B). These results demonstrated that mitochondrial translation in the wild-type strain is sensitive to Tcin.

Because the top 15 mutants were resistant to 8 μ M or higher concentrations of Tcin in YPD media (Fig. S3), we examined the total and the mitochondrial translation efficiencies of the top 15 resistant strains in the presence of a final concentration of 8 μ M Tcin/OD₆₀₀ cells. To ensure accurate estimation of the translation rate, the [35 S]-Met incorporation was normalized against the protein concentrations. Tcin significantly inhibited total translation in the wild-type (91%) and in all of the resistant strains (55–93%) except *spo13* Δ , which showed minimal inhibition of total translation (32%) (Fig. S6). The *ynl011c* Δ and *ict1* Δ , in which total translation was 93 and 85% affected respectively, were resistant to the highest concentration of Tcin tested (24 μ M), (Table S2) indicating that Tcin resistance can be separated from the inhibition of total translation.

Analysis of mitochondrial translation by [35 S]-Met incorporation indicated that 8 μ M Tcin/OD₆₀₀ significantly inhibited

The largest group of the resistant strains associated with mitochondria encoded ribosomal proteins and translation factors, suggesting that Tcin affected mitochondrial translation. Although trichothecenes do not inhibit bacterial translation (19, 20), Tcin inhibited mitochondrial translation in the wild-type yeast. Mitochondrial translation was only minimally affected by Tcin in a majority of the most resistant 15 strains in comparison with the isogenic wild-type strain (Fig. 4C). The Tcin-resistant mutants were cross-resistant to anisomycin, but not to cycloheximide or hygromycin B. Chloramphenicol, an inhibitor of mitochondrial translation, did not inhibit growth of the Tcin-resistant mutants at a concentration sufficient to inhibit growth of the wild-type cells. Chloramphenicol and anisomycin bind at the active site crevice of the ribosome and block peptidyltransferase activity by preventing binding of aminoacyl tRNA (aa-tRNA) to the A site (26), while hygromycin and cycloheximide inhibit translocation (27, 28). The inhibitory effect of anisomycin on mitochondria has not been previously reported. At low doses, anisomycin inhibited growth of the wild-type strain when glycerol was used as the carbon source, indicating that it inhibited mitochondrial translation. Taken together, these results suggest that Tcin and anisomycin target the peptidyltransferase center of mitochondrial ribosomes and inhibit an A site associated function. Consistent with this, deletion of *MRPL9*, encoding mitochondrial ribosomal protein L3 led to Tcin resistance (Table S1), suggesting that Tcin may inhibit mitochondrial peptidyltransferase activity by targeting the mitochondrial L3. The deletion mutant of the cytosolic ribosomal protein L3 was not included in the library, because it is an essential gene. The Tcin resistant mutants identified from this screen were either resistant to both Tcin and anisomycin or resistant solely to Tcin when glycerol was used as the carbon source, indicating that Tcin and anisomycin have common, as well as unique actions on the mitochondria.

Deletion of *TMA7*, associated with the 40S ribosomal subunit, conferred a high level of resistance to anisomycin, consistent with previous findings (29). The *tma7Δ* was highly resistant to Tcin. However, we did not observe a decrease in the total protein synthesis in *tma7Δ* in the absence of Tcin (29). Total translation and mitochondrial translation were inhibited less by Tcin in *tma7Δ* than in the wild-type strain. Similarly, *spo13Δ* and *tof2Δ* also showed a high level of resistance to Tcin and anisomycin and minimal inhibition of total and mitochondrial translation. The *spo13Δ* and *tof2Δ* were identified as highly resistant to the antimicrobial dermaseptins (30). Dermaseptin-induced nuclear DNA fragmentation, reactive oxygen species (ROS) production, and caspase independent cell death were minimized in these strains (30). Tof2 is required for ribosomal DNA (rDNA) silencing and mitotic rDNA condensation, while Spo13 is a meiosis specific protein. Both are involved in cell cycle progression and their deletion increases levels of Rad52 foci, a marker for homologous recombination, which functions to maintain the integrity of genome through repair of DNA double-strand breaks (31). Additional genes involved in recombination and repair of double stranded DNA breaks (*RAD57*, *IRC19*, and *MHR1*), and cell cycle progression (*MBP1*, *RAD57*, and *HFMI*) were identified in our screen, suggesting that DNA damage, a process known to trigger apoptosis, may be a consequence of the effects of Tcin and anisomycin on mitochondrial ribosomes. The absence of *SPO13* and *TOF2* might prevent the Tcin and anisomycin-induced cell death by enhancing the DNA damage repair response.

Several mutants (*ups1Δ*, *yap1802Δ*, *izh4Δ*, *cue3Δ*, *pcl8Δ*, *flc3Δ*, and *ydl173wΔ*) were uniquely resistant to Tcin, but not to anisomycin when mitochondria were used as the source of energy (Fig. 3B). These mutants were involved in maintaining the integrity of the mitochondrial membrane (*ups1Δ*), clathrin cage assembly (*yap1802Δ*), sterol metabolism (*izh4Δ*), mono-ubiquitination (*cue3Δ*), generation of energy (*pcl8Δ*) or FAD transport (*flc3Δ*). Additional genes identified (*CEM1*, *FAB1*,

ICT1, *UPS1*, *LCB5*, *SURI*, and *YDC1*) encoded proteins involved in phospholipid metabolism (Table S1). Trichothecenes were shown to interfere with sphingolipid metabolism by inducing accumulation of glucosylceramide in neural cells (32). *YDC1*, *LCB5*, and *SURI* identified in our screen encode proteins involved in the sphingolipid metabolism (33). Yeast mutants lacking *YDC1*, an alkaline dihydroceramidase had increased chronological life span, while overexpression of *YDC1* led to fragmentation of mitochondria, reduced life span, and increased apoptotic cell death (34). These results implicate sphingolipid metabolism in the toxicity of Tcin and suggest that membrane sphingolipids are either directly required for Tcin mediated growth inhibition or are indirectly affected.

In contrast to the wild-type cells, which contain tubular mitochondria, in Tcin-treated cells the tubular structure of the mitochondria was largely broken. Deletion of genes involved in mitochondrial fusion (*FZO1*, *PCP1*, and *AFG3*) and maintenance of the tubular morphology of mitochondria (*MMM1*, *MDM10*, and *MDM12*) conferred resistance to Tcin, suggesting a possible effect on the mitochondrial membrane dynamics. Fzo1 is an outer mitochondrial membrane GTPase essential for mitochondrial fusion. In *fzo1Δ* yeast cells, mitochondria are highly fragmented due to a deficiency in mitochondrial fusion, a defect that in turn leads to loss of mitochondrial DNA and therefore respiratory activity (35). Pcp1 and Ups1 are associated with the inner membrane and their deletion results in loss of fusion activity (35). Ups1 regulates cardiolipin metabolism, a mitochondria specific phospholipid required for the integrity of several protein complexes in the inner membrane and is essential for respiration (36). The Mmm1-Mdm10-Mdm12-Mdm34 complex has recently been shown to connect the endoplasmic reticulum (ER) and mitochondria (37). Mutations in this complex reduce cardiolipin levels and impair phospholipid biosynthesis (37). These observations imply a possible inhibitory effect of Tcin on the membranes that connect the ER and mitochondria, which would lead to impairment of the interorganelar phospholipid exchange. The mechanistic details by which trichothecene mycotoxins kill target cells are not yet fully delineated. Here, we identified genes in a number of cellular pathways previously unknown to play a role in trichothecene toxicity and demonstrated that mitochondrial membrane maintenance and translation are critical for sensitivity to a trichothecene mycotoxin. Orthologs of these genes may be involved in the toxicity of trichothecene mycotoxins in plants. Our work draws attention to the similarities between the toxic effects of trichothecenes on mammalian cells and toxin induced cell death in the yeast model.

Materials and Methods

Tcin Isolation. Tcin was extracted from liquid yeast extract-peptone-dextrose shake cultures of *Trichothecium roseum* (no. A27955 or NRRL3704), purified on silica gel columns, and eluted with hexane:ethyl acetate (3:1).

Yeast Deletion Library Screen. The yeast genome homozygous diploid gene deletion set (Open Biosystems) was screened for resistance to 4 μ M Tcin. The strains from the deletion library were replica plated into 96-deep-well plates containing YPD (2% glucose) liquid media with or without Tcin. Experiments were performed in triplicate. Cells were grown for 20 h in the HiGro shaker (GeneMachines) at 250 rpm at 30 °C. The OD₆₀₀ for treated and untreated samples was obtained and the relative growth determined as the growth ratio of toxin treatment to the control. A ratio of 1 indicated that the treatment had no effect on the strain. The coefficient of variation (CV) calculated by taking the standard deviation of the ratio scores and dividing by the mean, was used as a measure of variability. The resistance level of a particular strain was based jointly on the mean ratio score and the CV. The threshold for 4 μ M Tcin resistance was defined as strains which had a ratio of 0.25 or higher (at least 25% growth relative to the nontoxin treatment of that same strain) and a CV of <0.5.

Antibiotic Assays. Overnight cultures were transferred into 96-well plates containing Tcin or the different antibiotics in liquid YPG (3% glycerol) or YPD. Antibiotics were used at the following concentrations: Tcin (1, 2, 4, 8, 12, 16,

and 24 μM), cycloheximide (0.5, 1, and 10 $\mu\text{g/mL}$), anisomycin (2.5, 5, 7.5, and 10 $\mu\text{g/mL}$), hygromycin B (10, 50, 100, and 200 $\mu\text{g/mL}$) and chloramphenicol at 1 mg/mL. Cells were grown for 20 h in YPD and for 48 h in YPG media. The relative growth was measured as described above. All experiments were performed in triplicate and repeated twice.

Strain Verification. Yeast strain verification was carried out by PCR using knockout cassette specific primers for each of the 15 Tcin-resistant deletion mutants (yeastdeletion.stanford.edu) (Fig. S7A).

rho⁰ Strains. rho⁰ versions of BY4743 were generated as described in ref. 38. The respiratory deficiency of the rho⁰ strains was confirmed by the complete lack of growth on YPG media. The absence of mitochondrial DNA was confirmed by staining paraformaldehyde-fixed cells with DAPI and by Southern blot analysis (Fig. S7B) using a mitochondrial COX1 probe (39).

Total Translation Assay. Cultures of BY4743 and the top 15 resistant mutants were grown in SD-Met containing MSG as the nitrogen source and 2% glucose. Each culture, grown to an OD₆₀₀ of 0.5–0.6 ($4.5\text{--}5.4 \times 10^7$ cells/mL), was then split into two halves. One-half was treated with 8 μM Tcin/OD₆₀₀ cells and the other half with an equivalent amount of ethanol (0.03%) for 1 h, shaking at 30 °C. Cold methionine (50 μM) and 1 μL [³⁵S]-Met (Perkin-Elmer, NEG-009A, >1,000 Ci/mmol) was added. The reaction was stopped after 60 min, and [³⁵S]-Met incorporation was measured as described in ref. 40. The rate of incorporation of [³⁵S]-Met was linear up to 60 min.

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Mitochondrial Translation Assay. Mitochondrial translation was measured using a modified assay developed by Fox et al. (22). Fresh YPR (2% raffinose) media was added to an overnight yeast culture, grown in YPR, and incubated for 2–3 h. An equivalent of 10 OD₆₀₀ cells were pelleted, washed twice and resuspended in SD-Met media supplemented with 2% raffinose and treated with 8 μM Tcin/OD₆₀₀ or an equivalent amount of ethanol (control) for 1 h at 30 °C. Cycloheximide was immediately added to each sample to stop cytosolic translation followed by addition of 25 μCi [³⁵S]-Met. Mitochondria were isolated, and [³⁵S]-Met incorporation was determined as described in ref. 22.

Microscopy. Mitochondrial morphology was examined by epifluorescence microscopy (Olympus BX41) using cells transformed with pVT100U-mtGFP, which contains green fluorescent protein (GFP) targeted to the mitochondria with the presequence from the subunit 9 of the F₀-ATPase of *Neurospora crassa* (24).

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