Dynamic Transcriptomic and Phosphoproteomic Analysis During Cell Wall Stress in Aspergillus nidulans

Authors
Cynthia Chelius, Walker Huso, Samantha Reese, Alexander Doan, Stephen Lincoln, Kelsi Lawson, Bao Tran, Raj Purohit, Trevor Glaros, Ranjan Srivastava, Steven D. Harris, and Mark R. Marten

Correspondence
marten@umbc.edu

In Brief
The transcriptome and phosphoproteome of the filamentous fungus Aspergillus nidulans was monitored after cell wall stress. Over 1800 genes and 700 phosphorylation sites showed dynamic expression and occupancy, respectively. These data guided selection of kinase deletion strains for testing for cell-wall related phenotypes. Kinase-substrate analysis revealed possible connections between CWIS, SIN, HOG, and calcium pathways. Fungi exposed to wall stress experience an increase in septation formation, which may function as a biological defense mechanism.

Highlights
- Dynamic transcriptomic and phosphoproteomic study of response to cell-wall stress.
- MARS model identifies significantly dynamic gene expression and phosphorylation.
- Septation formation increases during cell-wall stress (via micafungin exposure).
- Cell wall phenotype assays identified 8 putative CWIS related kinases.
Dynamic Transcriptomic and Phosphoproteomic Analysis During Cell Wall Stress in *Aspergillus nidulans*

Cynthia Chelius, Walker Huso, Samantha Reese, Alexander Doan, Stephen Lincoln, Kelsi Lawson, Bao Tran, Raj Purohit, Trevor Glaros, Ranjan Srivastava, Steven D. Harris, and Mark R. Marten.

The fungal cell-wall integrity signaling (CWIS) pathway regulates cellular response to environmental stress to enable wall repair and resumption of normal growth. This complex, interconnected, pathway has been only partially characterized in filamentous fungi. To better understand the dynamic cellular response to wall perturbation, a β-glucan synthase inhibitor (micafungin) was added to a growing *A. nidulans* shake-flask culture. From this flask, transcriptomic and phosphoproteomic data were acquired over 10 and 120 min, respectively. To differentiate statistically-significant dynamic behavior from noise, a multivariate adaptive regression splines (MARS) model was applied to both data sets. Over 1800 genes were dynamically expressed and over 700 phosphorylation sites had changing phospho-rylation levels upon micafungin exposure. Twelve kinases had altered phosphorylation and phenotypic profiling of all non-essential kinase deletion mutants revealed putative connections between PrkA, Hk-8–4, and Stk19 and the CWIS pathway. Our collective data implicate actin regulation, endocytosis, and septum formation as critical cellular processes responding to activation of the CWIS pathway, and connections between CWIS and calcium, HOG, and SIN signaling pathways.

The cellular signaling network is a complex system that controls nearly all cellular processes underlying growth, metabolism, morphogenesis, and development (1, 2). Generally, cell surface receptors are perturbed by environmental stimuli and transduce information to the nucleus via signaling proteins, which typically include G-proteins, kinases, and transcription factors. A signaling pathway of interest in fungi is the cell wall integrity signaling (CWIS) pathway. The CWIS is nominally responsible for wall maintenance and repair (3), which are critical as fungi rely on the wall for protection, shape, strength, and host invasion (4–7). The CWIS is also of interest as cell walls are present in fungi, but absent in humans, thereby making the fungal cell wall an ideal target for antifungal therapeutics (8–10).

The CWIS is activated in response to wall stress and/or damage (3) and is composed of a conserved set of proteins across a variety of fungal species including *Aspergillus nidulans* (11), *A. fumigatus* (12), *A. oryzae* (13), *Saccharomyces cerevisiae* (3), *Schizosaccharomyces pombe* (14), and *Candida albicans* (15). In the model fungus *A. nidulans*, the CWIS pathway is composed of cell surface receptors which sense cell wall stress (16). These sensors activate the guanine nucleotide exchange factor (GEF), Rom2, which acts on the GTPase RhoA to trigger activation of PkcA (16–19). This subsequently activates a MAPK cascade, which includes BckA-MkkA-MpkA (11, 20, 21). MpkA, the final kinase in this cascade, activates the transcription factor RlmA which is responsible for controlling expression of α-1,3-glucan synthase genes (11).

Notably, unlike *S. cerevisiae*, the transcription of many cell wall related genes (including β-1–3-glucan and chitin synthase genes) in *A. nidulans* is regulated in an MpkA-independent manner (11). In an effort to characterize the CWIS pathway, several studies have sought out this alternative signaling pathway to identify how β-1,3-glucan and chitin synthase genes are regulated in response to cell wall perturbation (22–27). Connections between the CWIS pathway and several other pathways have also been identified. Examples include the unfolded protein response (UPR) pathway, calcium signaling, branching regulation, iron homeostasis, the high osmolarity-glycerol (HOG) pathway, and the cyclic AMP protein kinase A (PKA) pathway (17, 28–30). These pathway networks

From the 1Department of Chemical, Biochemical, and Environmental Engineering, University of Maryland Baltimore County, Baltimore, Maryland, USA; 2Center for Plant Science Innovation and Department of Plant Pathology, University of Nebraska-Lincoln, Lincoln, Nebraska, USA; 3Department of Chemical and Biomolecular Engineering, University of Connecticut, Storrs, Connecticut, USA; 4BioScience Mass Spectrometry Facility, The U.S. Army CCDC Chemical Biological Center, BioSciences Division, Aberdeen Proving Ground, Maryland, USA; 5Department of Biological Sciences, University of Manitoba, Winnipeg, Manitoba, Canada; 6BioSciences Division, B11 Bioenergy and Biome Sciences, Los Alamos National Laboratory, Los Alamos, New Mexico, USA

Author’s Choice—Final version open access under the terms of the Creative Commons CC-BY license. This article contains supplemental data.

* For correspondence: Mark R. Marten, marten@umbc.edu.
are complex and highly interconnected, making it difficult to resolve network topography. We are interested in developing a better understanding how cell wall integrity signaling is transduced to the nucleus and identifying downstream effectors whose expression is controlled by this process.

The most common post translational modification (PTM) mediating signal transduction is phosphorylation (1). To capture the global phosphorylation-state of the cell, mass spectrometry (MS) has emerged as a powerful tool. In the past decade, major technological advances in MS have enabled researchers to accurately identify and quantify thousands of phosphorylation sites from a single run (31). However, although a single MS run provides an abundance of information, when studying signaling behavior it is important to consider the dynamics of protein phosphorylation. Phosphorylation events typically occur rapidly (within minutes), thus to capture signaling progression, multiple samples must be collected over a short time span (32). In addition, evaluating dynamic changes in phosphorylation can aid in determining which events are more likely to be direct interactions of kinases and substrates (33).

In this work, we study the dynamic response of the cell wall integrity signaling pathway to a wall perturbation. Micafungin, a β-glucan synthase inhibitor, is used as a cell wall perturbant as it is known to directly activate the CWIS pathway (11). We used quantitative, label-free, mass spectrometry (over a short period of time; 10 min) to assess dynamic changes in protein phosphorylation and transcriptomic analysis (over a longer period of time; 120 min) to assess changes in global gene-expression levels. Overall, we identified over 700 dynamic phosphorylation sites, which includes 15 sites on kinases and 16 on putative transcription factors. Over 1800 significantly dynamic genes were expressed upon micafungin treatment, 25 of which have previously been identified as cell-wall related. Our findings reveal that the coordinated response of the calcium, HOG, and Septation Initiation Network (SIN) pathways are involved in the response to cell wall stress. Furthermore, our results underscore the potential of this multi-omics approach to study signaling networks in general.

**EXPERIMENTAL PROCEDURES**

**Strains and Media—**Aspergillus nidulans A1405 (Fungal Genetics Stock Center; FGSC) was used as the control strain. Frozen stocks were spread on MAGV plates (2% malt extract, 1.5% agar, 2% glucose, 2% peptone, and 1 mL/L Huter’s trace elements and vitamin solution) and incubated for 2 days at 28 °C (29). 1E7 spores were spread on MAGV plates (2% malt extract, 1.5% agar, 2% glucose, 2% peptone, and 1 mL/L Hutner’s trace elements and vitamin solution) and incubated for 2 days at 28 °C (29). 1E7 spores were spread on MAGV plates (2% malt extract, 1.5% agar, 2% glucose, 2% peptone, and 1 mL/L Hutner’s trace elements and vitamin solution). Culture was grown in a 250 ml baffled flask at 250rpm and 30 °C (29). 1E7 spores were spread on MAGV plates (2% malt extract, 1.5% agar, 2% glucose, 2% peptone, and 1 mL/L Hutner’s trace elements and vitamin solution) and incubated for 2 days at 28 °C (29). 1E7 spores were spread on MAGV plates (2% malt extract, 1.5% agar, 2% glucose, 2% peptone, and 1 mL/L Hutner’s trace elements and vitamin solution). Culture was grown in a 250 ml baffled flask at 250rpm and 30 °C (29). 1E7 spores were spread on MAGV plates (2% malt extract, 1.5% agar, 2% glucose, 2% peptone, and 1 mL/L Hutner’s trace elements and vitamin solution). Culture was grown in a 250 ml baffled flask at 250rpm and 30 °C (29).

**Dynamic Micafungin Treatment, Experimental Design, and Phosphoproteomic Sample Preparation—**Fungi were grown until mid-exponential phase (about 20 h) and 20 ng micafungin/ml culture per 1g/kg DCW was added. About 25 ml of culture was removed from the flask while the shaker was still shaking (ensuring a homogeneous sample) at 0, 30s, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 7.5, and 10 min after micafungin exposure. Immediately after removing sample, the fungal mass separated from broth, frozen in liquid nitrogen, and stored at −80 °C. This experiment was run with two biological replicates.

**LC-MS/MS and LFQ Analysis—**Stored samples were reconstituted in 20 μl of acetonitrile/water/formic acid 5/95/0.5 (v/v/v) and run in duplicate on a Dionex Ultimate 3000 nanoLC system coupled to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific, San Jose, CA). Separation of peptides was performed on EASY spray C18 75 μm × 50 cm column for a 190 min gradient at flow rate of 200 nL/min using mobile phase A of 0.1% formic acid in water and mobile phase B of acetonitrile/water/formic acid 80/20/0.1 (v/v/v). Mass spectrometry data were collected in positive ionization mode using a data dependent acquisition method with a full MS scan for m/z range 350–1500 in orbitrap at 120 K resolution. Consecutive MS/MS scans were performed in the ion trap by top-speed decision selection with a dynamic exclusion of 20 s. Precursor ions selected from the first MS scan were isolated with an isolation width of 1.6 m/z and then 500 μm wide (1 μg trypsin) overnight in a 37 °C water bath. The samples were lyophilized to dryness and phosphopeptides were collected using the Pierce High-Select TiO2 Phosphopeptide Enrichment Kit (Pierce, Rockford, IL) protocol. Samples were dried to completeness and stored at −80 °C until mass spectrometry analysis.

**Western Blotting—**After ~20 h of growth (mid-exponential growth phase), 20 ng/ml micafungin per 1g/kg dry cell weight (DCW) was added to the control strain culture. DCW was determined by removing ~25 ml of culture from the shake flask during growth. Amount of culture removed was determined by weight (i.e. “wet weight” kg culture). Fungal biomass was recovered from the liquid via vacuum filtration, and then dried at 100 °C before determining “dry weight.” Dry cell weight (DCW) is the concentration of biomass in culture, and was calculated as “dry weight” divided by the “wet weight.” Twenty-five milliliters of culture was removed after 0, 1, 3, 5, 10, 15, and 30 min of micafungin exposure and mycelia were separated from broth and immediately frozen in liquid nitrogen. Frozen mycelia were crushed with mortar and pestle into a fine powder and mixed with TNE buffer at 1:1 (v/v) (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA). Samples were spun at 10rpm at 4 °C for 10 min then centrifuged at 4 °C and 10,000g for 10 min and BCA assay (Pierce, Rockford, IL) was performed on the supernatant. One hundred microliters of protein was exposed to SDS-page electrophoresis and transferred to a nitrocellulose membrane (Life technologies, Carlsbad, CA). Western blotting preparation steps were performed as described in Chelius et al., 2019. Two biological replicates were modeled using Michaelis-Menten kinetics.
quantification of ion intensities which compares the abundance of the same peptide species across all runs. Parameters that were used include: fixed modification of carbamidomethyl (C), variable modifications of phosphorylation (STY), acetylation (N terminus), and oxidation (M), trypsin as the protease, and 2 missed cleavages. The mass tolerance for precursor ions was set to 20 ppm in the first search and 4.5 ppm in the main search, and the fragment mass tolerance was set to 20 ppm. The FDR was set to 0.01. MS/MS spectra were searched against a target database consisting of 10,555 entries from the UniprotKB database (UP000000560). Combining a high number of timepoints (13) with several replicates (2 biological replicates with 2 technical replicates) ensures a sample size large enough to provide statistical power. Peak lists from all MS/MS spectra were extracted from MaxQuant result files using MS-viewer (35) and can be accessed at http://msviewer.ucsf.edu/prospector/cgi-bin/mstable.cgi?form=msviewer (search key: yhspxctyvc).

**Experimental Design and Statistical Rationale**—Phosphoproteomic analysis was completed on two biological replicates each containing 13 timepoints. All samples were run in duplicate for a total of 52 mass spectrometry runs. The control is the first time point (0 min) which was taken immediately before micafungin addition (i.e., cell perturbation). To determine if the phosphorylation level is significantly dynamic, multivariate adaptive regression splines (MARS) model (36) was used following a method developed by Oliveira et al., 2018. This method ensures that our dynamic response is not background noise.

**Phospho-site Filtering and Statistical Assessment**—Perseus 1.6.1.1 software was used to filter phosphorylation sites (37). Sites were removed if there was a contaminant, reverse match, or the location probability was less than 0.75. Next, we applied filtering rules to the biological replicates separately. A phosphorylation must be present in both technical replicates of at least 10 of the 13 time points and present in either the initial time point (0 min) or the 30 s time point (our control) (33). Finally, these rules applied to both biological replicates resulted in a total of 925 highly confident phosphorylation sites. For the remainder of the analysis the intensity values were transformed into log 2-fold changes. A MARS model was fit to the fold changes from 26 (13 time points, 2 technical replicates) samples using ARE- SLab, a Matlab package (38). The arespredict function (with default settings) determined the quality of fit (mean square error; MSE) of the actual data and 10,000 random permutations of the fold change levels. The p value was calculated as the fraction of randomly permuted models with better fit than the actual model. If the p value was less than 0.05 the phosphorylation site was deemed significantly responsive (Class I sites are significant in both biological replicates, Class II are significant in 1 biological replicate).

**RNA Sequencing and Statistical Analysis**—Fungi were grown and treated with micafungin identically to phosphoproteomic samples. Samples were taken at 0, 5, 10, 15, 20, 25, 30, 40, 50, 60, 75, 90, and 120 min for three biological replicates. Harvested mycelia were frozen with liquid nitrogen and ground into a fine powder by mortar and pestle. RNA extraction and preparation was completed as previously described (39). The samples were sent to the University of Nebraska Medical Center’s Bioinformatics and Systems Biology Core for library preparation and RNA Sequencing (https://unmc.edu/bbsbc). The raw samples were of single end reads of high quality in 4 separate lanes in FASTQ files. The following costume RNA-Sequencing pipeline was used: HISAT2 2.1.0 (40), HTSeq-Count 0.9.1 (41), DESeq2 1.20.0.0 (42). The annotation file (Gene Transfer File) and whole genome of the fungal strain FGSC A4 FNA file was supplemented from the database Ensemble Fungi (43).

Like the phosphoproteomic data, genes were required to have reads in 2 out of 3 replicates in all 13 timepoints. The normalized reads were transformed into log 2-fold change values. Finally, the dynamic test (MARS model) described above was applied to all replicates. Significance threshold was set to p < 0.01.

**Predictive Software - PHOSIDA, NetworkKIN, STRING, GO and Cluster Analysis**—Several software tools were used to further analyze the phosphorylation hits. PHOSIDA analyzes all phosphorylation motifs to identify statistically overrepresented motifs (http://141.61.102.18/phosida/index.aspx) (44, 45). PHOSIDA parameters were set to the following: minimum score at 10 and minimum proportion of matching sites was 5% (46). Dynamically phosphorylated sites were analyzed by NetworkKIN to predict which kinases target specific phosphorylation sites from the yeast proteome (http://networkinfo.info/) and phosphosite-kinase interactions with a score greater than 2 are considered significant (47). STRING database (http://string-db.org) was used to build a protein-protein interaction web based on both computational predictions and experimental observations (48). All Class I and Class II phospho-proteins were imported into the database and the interaction sources selected include text mining, experiments, databases, co-expression, and co-occurrences. The minimum required interaction score was 0.700 (or high confidence). The Aspergillus genome database (AspGD) Gene Ontology (GO) Term Finder tool (http://www.aspergillusgenome.org) was used to identify enriched GO terms from imported proteins (49). Here a significance threshold of p value < 0.01 was used. A heat map of significantly expressed genes was created using Perseus v 1.6.5.0 (http://www.perseus-framework.org). Average Log2Fold Change is depicted, the micafungin exposure time order was preserved, and the distance metric was set to Euclidean.

**Apparent Cell Wall Strength**—To measure apparent cell wall strength in shake flask culture mycelia were removed from shake flasks (~5 ml) during exponential growth phase and subjected to particle size analysis using a Mastersizer 3000 instrument with a Hydro SM manual sample dispersion unit following published methods (29, 50). The average mycelial size (S90) for each biological replicate was calculated from at least 3 timepoints (between 17 and 24 h growth) using duplicate technical replicates (p value set at 0.05, n=3). In parallel with size analysis, dry cell weight measurements were taken to determine the specific growth rate of these strains.

**Fungal Growth in Micafungin**—To test whether these kinases may respond to cell wall perturbation, we grew kinase deletion mutants both in submerged culture and agar plates with micafungin. For plate growth experiments, spores were harvested from fresh fungal lawns in 10 ml of sterile diH2O and the spore concentration was determined. Growth experiments, spores were harvested from fresh fungal lawns in 10 ml of sterile diH2O and the spore concentration was determined. To test whether these kinases may respond to cell wall perturbation, we grew kinase deletion mutants both in submerged culture and agar plates with micafungin. For plate growth experiments, spores were harvested from fresh fungal lawns in 10 ml of sterile diH2O and the spore concentration was determined. Growth experiments, spores were harvested from fresh fungal lawns in 10 ml of sterile diH2O and the spore concentration was determined. To test whether these kinases may respond to cell wall perturbation, we grew kinase deletion mutants both in submerged culture and agar plates with micafungin. For plate growth experiments, spores were harvested from fresh fungal lawns in 10 ml of sterile diH2O and the spore concentration was determined. Growth experiments, spores were harvested from fresh fungal lawns in 10 ml of sterile diH2O and the spore concentration was determined. To test whether these kinases may respond to cell wall perturbation, we grew kinase deletion mutants both in submerged culture and agar plates with micafungin. For plate growth experiments, spores were harvested from fresh fungal lawns in 10 ml of sterile diH2O and the spore concentration was determined. Growth experiments, spores were harvested from fresh fungal lawns in 10 ml of sterile diH2O and the spore concentration was determined. Growth experiments, spores were harvested from fresh fungal lawns in 10 ml of sterile diH2O and the spore concentration was determined. Growth experiments, spores were harvested from fresh fungal lawns in 10 ml of sterile diH2O and the spore concentration was determined. Growth experiments, spores were harvested from fresh fungal lawns in 10 ml of sterile diH2O and the spore concentration was determined. Growth experiments, spores were harvested from fresh fungal lawns in 10 ml of sterile diH2O and the spore concentration was determined. Growth experiments, spores were harvested from fresh fungal lawns in 10 ml of sterile diH2O and the spore concentration was determined. Growth experiments, spores were harvested from fresh fungal lawns in 10 ml of sterile diH2O and the spore concentration was determined. Growth experiments, spores were harvested from fresh fungal lawns in 10 ml of sterile diH2O and the spore concentration was determined. Growth experiments, spores were harvested from fresh fungal lawns in 10 ml of sterile diH2O and the spore concentration was determined. Growth experiments, spores were harvested from fresh fungal lawns in 10 ml of sterile diH2O and the spore concentration was determined. Growth experiments, spores were harvested from fresh fungal lawns in 10 ml of sterile diH2O and the spore concentration was determined. Growth experiments, spores were harvested from fresh fungal lawns in 10 ml of sterile diH2O and the spore concentration was determined. Growth experiments, spores were harvested from fresh fungal lawns in 10 ml of sterile diH2O and the spore concentration was determined. Growth experiments, spores were harvested from fresh fungal lawns in 10 ml of sterile diH2O and the spore concentration was determined. Growth experiments, spores were harvested from fresh fungal lawns in 10 ml of sterile diH2O and the spore concentration was determined.
there has been a shift from static -omic studies to dynamic recent advancements in mass spectrometry technologies, min (13 samples total).

analysis at 0, 5, 10, 15, 20, 25, 30, 40, 50, 60, 75, 90, and 120 these previous findings, we drew samples for transcriptomic involved micafungin induced expression changes. Based on

in the first 5 min after perturbation with micafungin. We thus jority of the dynamic MpkA phosphorylation response occurs

constant, $k_t$, was determined to be 2 min, meaning the ma-

Michaelis-Menten kinetics). The half-saturation $V_{max}$ was determined using Primer3 (supplemental File S8) (51). Three technical replicates and two bio-

studies (33, 55–63). However, comparison of these dynamic studies reveals significant differences in experimental design as well as data processing and analysis, such that no clear best-practice currently exists. For example, although some studies use many time points with little to no replication (33), others utilize relatively few time points, but include replicates (64). In an attempt to combine these approaches, we designed our study to include many time points (13) with multi-

results, there is some discrepancy between biological replicates (Fig. 2). This occurs because biological replicates are for samples taken only 30 s apart, such that even small differences in behavior shift values between time points. Previous studies have not manifested this behavior, as they have used multiple biological replicates over fewer time points, typically over much longer time scales (e.g. hours) (60–62).

To address this issue, we developed a data-analysis pipe-

line to identify, with high confidence, phosphosites that were significantly dynamic (i.e. show a significant change in occupancy after micafungin addition (Fig. 3)). We note that in the 52 different mass spectrometry runs carried out for this study, a total of 5106 phosphorylation sites were observed. How-

other replicates (2 technical and 2 biological) and used label-

free quantification (peak intensity) to survey phosphosite occupancy.

Although our technical replicates show good correlation, some discrepancies exist between biological replicates (Fig. 3). This occurs because biological replicates are for samples taken only 30 s apart, such that even small differences in behavior shift values between time points. Previous studies have not manifested this behavior, as they have used multiple biological replicates over fewer time points, typically over much longer time scales (e.g. hours) (60–62).

To address this issue, we developed a data-analysis pipe-

line to identify, with high confidence, phosphosites that were significantly dynamic (i.e. show a significant change in occupancy after micafungin addition (Fig. 3)). We note that in the 52 different mass spectrometry runs carried out for this study, a total of 5106 phosphorylation sites were observed. How-

other replicates (2 technical and 2 biological) and used label-

free quantification (peak intensity) to survey phosphosite occupancy.

Although our technical replicates show good correlation, some discrepancies exist between biological replicates (Fig. 3). This occurs because biological replicates are for samples taken only 30 s apart, such that even small differences in behavior shift values between time points. Previous studies have not manifested this behavior, as they have used multiple biological replicates over fewer time points, typically over much longer time scales (e.g. hours) (60–62).

To address this issue, we developed a data-analysis pipe-

line to identify, with high confidence, phosphosites that were significantly dynamic (i.e. show a significant change in occupancy after micafungin addition (Fig. 3)). We note that in the 52 different mass spectrometry runs carried out for this study, a total of 5106 phosphorylation sites were observed. However, many of these sites appeared in relatively few runs.
When stringent confidence tests are applied (Materials and Methods), 1156 and 1056 phosphosites were identified in biological replicates 1 and 2, respectively, with 925 of these phosphosites (i.e. between 80 and 90%) in common between the two replicates (supplemental File S1).

To determine whether these 925 phosphosites were significantly changing in response to micafungin perturbation, Log2 fold change of intensity was modeled with a multivariate adaptive regression splines (MARS) model (38, 56). The quality of fit (mean square error, MSE) was used to determine if individual phosphosites were significantly changing occupancy with time (56). Fig. 4 shows MARS model fits for 12 phosphosites, all on protein kinases. Although there is some discrepancy between biological replicates, it is clear that nearly all the MARS trends (i.e. lines) for biological replicates are: (1) in the same direction and (2) show similar magnitude. Thus, this approach has allowed us to overcome the challenge related to comparing biological replicates taken during periods of rapid sampling.

Using this method, we narrowed the 925 P-sites, to 431 dynamically significant phosphorylation sites in both biological replicates (Class I) and 363 in one replicate (Class II; i.e., over 700 total). From the 431 Class I sites, 51 motifs were significantly overrepresented (Fig. 5B and 5C, supplemental File S2). Further motif analysis was conducted using NetworKIN (47). Given a protein sequence, NetworKIN predicts which kinase (based on the yeast kinome) is likely to have carried out this phosphorylation. From the 431 phospho-sites, NetworKIN predicted with significant confidence that 28 different kinases phosphorylated 173 of these sites (45, 65). Of note, NetworKIN predicted that the prominent CWIS pathway kinases PkcA and MpkA phosphorylated 22 and 5 phosphosites respectively, revealing putative, new signaling connections to the CWIS pathway (Fig. 5A).

RNA-sequencing Reveals CWIS Downstream Effectors—RNA-sequencing was carried out using shake flask cultures grown identically to the phosphoproteomic set, however...
the sampling time ranged from 0 to 120 min. From the 10,687 putative ORFs in *A. nidulans* (49), transcripts were identified from 9344 and of these, transcripts were present in all 13 time points and 2 of 3 replicates for 8,188 genes (supplemental File S3). From the 8188 genes, 1810 were dynamically significant ($p < 0.01$) using the MARS method (Fig. 2).

**Fig. 4.** Dynamically changing phosphosites on protein kinases for 10 min. after micafungin exposure. Black and red circles are two different biological replicates. Lines represent MARS model fits to the data and show all phosphosites experience significant reduction in occupancy after micafungin addition.

**Fig. 5.** Overall analysis of dynamic phosphorylation sites. NetworkKin analysis shows that a majority of the phosphorylation sites are predicted to be phosphorylated by Cka1. Pkca and MpkA (CWIS signaling kinases) phosphorylate multiple proteins showing possible new direct connections to the cell wall repair pathway (A). Motif-logo showing the overall enrichment of amino acids surrounding the phospho-residue, serine, as generated by PHOSIDA (B). Top 5 enriched motifs identified from statistically dynamic phosphosites. The occurrence percent is the relative abundance of the motif in this data set with respect to the proteome and the score is a statistic equivalent to $p = 10^{-\text{score}}$ (C).
From the statistically dynamic genes, 25 were cell wall related and 3 were probed by Fujioka et al., 2007 (agsA, chsB, chsC) (Fig. 6; Table I) in their CWIS study. Expression profiles of these genes can be found in supplemental File S4. Of these, strict rules were used to parse out genes that were the most overexpressed (50 genes) and most under expressed (4 genes). Of the 54 most over and under expressed, 45 of these are uncharacterized genes (supplemental File S5). These uncharacterized genes may have yet unknown roles in cell wall maintenance and repair.

A heatmap of dynamically expressed genes reveals 6 gene expression clusters (Fig. 7). Gene ontology (GO) analysis of each cluster can be found in supplemental File S6. All dynamically expressed genes (1810) were searched for their GO biological process and only 805 had an associated GO term. The top 5 GO terms were transport, regulation of biological process, response to stress, and organelle organization (supplemental File S7). A GO term enrichment assessment revealed that the most significantly enriched group was cytoskeleton organization.

Cell Wall Strength of Kinase Deletion Mutants—In our phosphoproteomic data we observed 19 kinases and 3 phosphatases to be significantly and dynamically phosphorylated (Table II). From this group, we tested each non-essential kinase for its involvement in cell wall maintenance and repair by growing kinase deletion mutants in shake-flask culture and measuring mycelial size-distribution using laser particle-size analysis. We developed this approach previously (50) and have shown (that for similar growth rates) smaller mycelia can result from either (1) compact (i.e. highly branched) morphology, or (2) hyphal fragmentation during shaking. If compact morphology (deduced via microscopy) is absent, smaller mycelia are a result of hyphae breaking in the flask, suggesting fungal strains with smaller mycelia (when compared with a control strain) have weaker cell walls. We used this approach previously to show an A. nidulans, mpkA deletion mutant has similar growth rate and morphology to a control strain but is approximately four times smaller implying it has significantly weaker cell walls (29). We used an identical approach here, measuring growth rate and average cell size for 12 different kinase deletion mutants (Fig. 8). Compared with the control, the growth rates of all mutants were similar ($p < 0.05$). However, 8 of the 12 kinase deletion mutants had significantly smaller mycelia than the control strain ($p < 0.05$). This implies...
**TABLE I**

| ANID#   | Gene Name | p value | Cell Wall Component | Up/Down | Description                                                                                           | Reference                      |
|---------|-----------|---------|---------------------|---------|--------------------------------------------------------------------------------------------------------|--------------------------------|
| AN1604  | agnE      | 0       | alpha glucan        | Up      | Putative 1,3-a-glucanase                                                                                 | deGroot et al., 2009          |
| AN5885  | agsA      | 0       | alpha glucan        | Up      | Catalytic subunit of alpha-1,3 glucan synthase complex                                                  | Fujioka et al., 2007          |
| AN3307  | agsB      | 0.0103  | alpha glucan        | Down    | Catalytic subunit of the major alpha-1,3 glucan synthase complex                                        | Fujioka et al., 2007          |
| AN3388  | amyF      | 0.0146  | alpha glucan        | Up      | Amylase-like family                                                                                     | Nakamura et al., 2006         |
| AN3309  | amyG      | 0.0013  | alpha glucan        | Down    | Amylase-like family                                                                                     | Nakamura et al., 2006; He et al., 2014 |
| AN1551  | btgE      | 0       | beta glucan         | Down    | Putative 1,3-b-transglucosylases involved in connecting the emerging 1,3-b-glucan chains to the existing b-glucan network through 1,6-b-linkages | deGroot et al., 2009          |
| AN0933  | crh1      | 0.0053  | beta glucan         | Down    | Putative transglycosidases; involved in crosslinking b-glucan and chitin                                | deGroot et al., 2009          |
| AN3914  | crhA      | 0       | beta glucan         | Down    | Putative transglycosidases; involved in crosslinking b-glucan and chitin                                | deGroot et al., 2009          |
| AN4515  | crhB      | 0.0279  | beta glucan         | Down    | Putative transglycosidases; involved in crosslinking b-glucan and chitin                                | deGroot et al., 2009          |
| AN0472  | engA      | 0.0132  | beta glucan         | Down    | Endo-1,3-b-glucanase                                                                                     | deGroot et al., 2009          |
| AN3777  | exgB      | 0       | beta glucan         | Down    | Putative exo-1,3-b-glucanase                                                                            | de Vries et al., 2005         |
| AN7533  | exgD      | 0.0013  | beta glucan         | Down    | Putative exo-1,3-b-glucanase                                                                            | de Vries et al., 2005         |
| AN7657  | gelA      | 0.0121  | beta glucan         | Down    | 1,3-beta-glucanosyltransferase                                                                          | Fujioka et al., 2007          |
| AN0558  | gelB      | 0.0404  | beta glucan         | Down    | 1,3-beta-glucanosyltransferase                                                                          | Fujioka et al., 2007          |
| AN7511  | gelE      | 0.0045  | beta glucan         | Down    | Putative 1,3-b-transglucosylases involved in connecting the emerging 1,3-b-glucan chains to the existing b-glucan network | deGroot et al., 2009          |
| AN6697  | sunA      | 0.0001  | beta glucan         | Down    | Sun family, involved in septation, possibly b-glucosidase activity                                      | deGroot et al., 2009          |
| AN0726  | sunB      | 0.0154  | beta glucan         | Down    | Sun family, involved in septation, possibly b-glucosidase activity                                      | deGroot et al., 2009          |
| AN0779  | 0         | beta glucan | Down    | Putative exo-1,3-b-glucanase family                                                                     | deGroot et al., 2009          |
| AN2385  | 0.0002   | beta glucan | Down    | Mixed-linked glucanases, hydrolyze 1,3-b/1,4-b-glucans                                                  | deGroot et al., 2009          |
| AN4852  | 0.0024   | beta glucan | Down    | Putative exo-1,3-b-glucanase family                                                                     | deGroot et al., 2009          |
| AN8241  | chiA      | 0.0286  | chitin              | Down    | Chitinase; Class III                                                                                     | Yamazakei et al., 2008        |
| AN4871  | chiB      | 0.0008  | chitin              | Down    | Chitinase; Class V                                                                                      | deGroot et al., 2009          |
| AN2523  | chsB      | 0.0041  | chitin              | Down    | Chitin synthase B (Chitin-UDP acetyl-glucosamin transferase B); Class III                               | Fujioka et al., 2007; Yanai et al., 1994 |
| AN8481  | dfgC      | 0.0372  | chitin              | Up      | Chitinase; Class V                                                                                      | deGroot et al., 2009          |
| AN1502  | nagA      | 0.0061  | chitin              | Up      | N-Acetyl-b-D-glucosaminidase                                                                            | Kim et al., 2002              |
| AN4234  | pcmA      | 0.0127  | chitin              | Down    | UDP-N-acetylgalactosamine synthesis; Phosphoacetylglucosamine mutase                                     | deGroot et al., 2009          |
| AN9094  | ungA      | 0       | chitin              | Down    | UDP-N-acetylgalactosamine synthesis                                                                       | deGroot et al., 2009          |
| AN0221  | 0.0021   | chitin   | Up                  | Chitinase; Class V                                                                                      | deGroot et al., 2009          |
| AN0299  | 0.0218   | chitin   | Up                  | Chitinase; Class V                                                                                      | deGroot et al., 2009          |
| AN0509  | 0.0321   | chitin   | Down                | Chitinase; Class V                                                                                      | deGroot et al., 2009          |
| AN1554  | 0.002    | chitin   | Down                | Regulation of chitin synthase activity                                                                   | deGroot et al., 2009          |
| AN3122  | 0.0372   | chitin   | Down                | Regulation of chitin synthase activity                                                                   | deGroot et al., 2009          |
| AN4367  | 0.0018   | chitin   | Up                  | Chitin synthase; Class III                                                                              | deGroot et al., 2009          |
| AN4566  | 0.0006   | chitin   | Down                | Chitin synthase C (Chitin-UDP acetyl-glucosamin transferase C); Class I                                  | Fujioka et al., 2007; Motoyama et al., 1994 |
| AN5077  | 0        | chitin   | Up                  | Chitinase; Class V                                                                                      | deGroot et al., 2009          |
| AN8710  | 0.0002   | chitin   | Down                | Regulation of chitin synthase activity                                                                   | deGroot et al., 2009          |
| AN3112  | ugmA      | 0.0172   | galacto-furanose     | Down    | UDP-galactopyranose mutase; involved in cell wall biogenesis                                            | Afroz et al., 2010            |
these deletion strains have weaker cell walls and are fragmenting more frequently during culture, and that the deleted kinases (PrkA, Hk-8–4, Stk19, NpkA, Nrc2, SrrB, and Kin1) are possibly involved in mediation of cell wall maintenance and repair.

Susceptibility of Deletion Mutants to Micafungin—Following shake flask experiments, the same 12 kinase deletion mutants were grown on agar plates with added micafungin to test their susceptibility to a cell wall perturbant. As a member of the CWIS pathway, MpkA plays a role in regulating cell wall biosynthesis gene expression and the ΔmpkA strain is known to be highly susceptible to micafungin (11). To test the relative impact of micafungin on the other deletion mutants we used a highly quantifiable plate assay to determine the amount of colonial growth on micafungin agar plates. Compared with traditional spot assays, this method enables us to accurately measure small differences between strains and use statistics to confidently report susceptibility. We found that ΔmpkA, ΔprkA, Δyak1, and Δnrc2 mutants had a significantly lower number of colonies as compared with the control (Fig. 9, Mol Cell Proteomics (2020) 19(8) 1310–1329).
Table II. Work here suggests PrkA, Yak1, and Nrc2 may be involved in regulating wall-repair related genes.

*Gene Expression of Mutants Exposed to Micafungin*—Four kinase deletion mutants which showed reduced cell wall strength (Fig. 8; Hk-8–4, Stk19, PrkA, MpkA) were grown in shake flasks in the presence and absence of micafungin. Micafungin was added at the same concentration as in the -omic experiments presented above. The growth rate before and after micafungin addition was measured, and Hk-8–4, PrkA, and Stk19 all showed significantly slower growth in micafungin compared with the control (Fig. 10). For the Hk-8–4 mutant, growth rate in micafungin appears to be negative. This is likely because of a reduction in the culture dry cell weight as this strain has relatively weak walls (29) and mycelia fragment over the extended period in the flask. The other kinases (Hk-8–4, Stk19, and PrkA) have a non-negative growth rate, but grow more slowly than the control suggesting these gene products play a role in mediating cell wall repair, but are not critical to fungal survival in this environment.

To further assess the impact of these kinases (PrkA, Stk19, and Hk-8–4) on CWIS signaling and wall repair, and at the same time explore their impact on as yet unidentified CWIS effectors, we used quantitative reverse transcription PCR (qRT-PCR). During culture of the kinase deletion mutants we measured the expression of four “fingerprint” genes (agnE, brlA, AN3339, and AN2116) during growth of the kinase deletions in the presence of micafungin (Fig. 11, supplemental File S8). We selected agnE, brlA, AN3339, and AN2116 as “fingerprint” genes as they were among the 50 most over-expressed genes in our transcriptomic data (AgnE (alpha-1,3-glucanase), BrlA (zinc finger transcription factor), AN3339 (uncharacterized), and AN2116 (uncharacterized)). Each of these genes showed a significant increase in expression during growth of the control strain in micafungin. For each gene, its expression reached a maximum at 60 min and then decreased. We note two phenomena regarding expression in the kinase mutants. First, agnE appears to be regulated in an MpkA-independent manner (i.e. ΔmpkA pro-

| Gene       | Residue | NetworKIN Prediction | Class | Direction | Cell Wall Strength | MF Plates | MF Flask |
|------------|---------|---------------------|-------|-----------|--------------------|-----------|---------|
| Kinases    |         |                     |       |           |                    |           |         |
| BckA       | S 719   | MpkA               | I     | Down      | NA                 | NA        | NA      |
| S 1003     | MpkA   | II                  | Down  |           | NA                 | NA        | NA      |
| ChkC       | S 587   | MpkA               | I     | Down      | +                  | +         | NA      |
| CmkC       | S 34    | N/A                 | I     | Up-Down   | +                  | +         | NA      |
| S 475      | CmkC   | II                  | Down  |           |                     |           |         |
| Hk-8–4     | S 314   | Ste20              | I     | Down      | -                  | +         | NA      |
| Kin1       | S 257   | Kin1               | II    | Down      | NA                 | NA        | NA      |
| MkkA       | S 169   | PkcA               | II    | Down      | NA                 | NA        | NA      |
| NimX       | Y 15    | N/A                 | I     | Down      | NA                 | NA        | NA      |
| NpkA       | S 74    | Sft1               | II    | Down      | -                  | +         | NA      |
| Nrc2       | S 433   | Nrc2               | II    | Down      | -                  | -         | NA      |
| PfkA       | S 789   | PkaB               | II    | Down      | NA                 | NA        | NA      |
| T 786      | N/A     | II                  | Down  |           |                     |           |         |
| PkiA       | S 525   | PkcA               | I     | Down      | NA                 | NA        | NA      |
| Pkcb       | S 633   | PkcB               | I     | Down      | NA                 | NA        | NA      |
| PrkA       | S 957   | N/A                 | I     | Down      | -                  | -         | -       |
| SchA       | S 827   | TorA               | I     | Down      | +                  | +         | NA      |
| S 298      | PtoA    | I                   | Down  |           |                    |           |         |
| S 285      | SteA    | II                  | Down  |           |                     |           |         |
| SidB       | S 453   | N/A                 | I     | Up-Down   | NA                 | NA        | NA      |
| S 44       | MpkA    | I                   | Down  |           |                     |           |         |
| S 47       | N/A     | I                   | Up-Down |         |                     |           |         |
| SrrB       | S 641   | PkaB               | II    | Down      | -                  | +         | NA      |
| SteD       | S 11    | Cka1               | II    | Down      | NA                 | NA        | NA      |
| Stk19      | S 875   | N/A                 | I     | Up-Down   | -                  | +         | -       |
| Yak1       | Y 502   | N/A                 | I     | Up-Down   | +                  | -         | NA      |
| PtcA       | S 41    | MpkA               | I     | Down      | NA                 | NA        | NA      |
| AN1358     | T 390   | HogA               | I     | Down      | NA                 | NA        | NA      |
| PodH       | S 818   | I                   | Down  |           | NA                 | NA        | NA      |

Phosphatases

| Phosphatases | S 41 | MpkA | I | Down | NA | NA | NA |
|--------------|------|------|---|------|----|----|----|
| AN1358       | T 390| HogA | I | Down | NA | NA | NA |
| PodH         | S 818| I    | Down | NA  | NA | NA | NA |

Last three column contain phenotype data, MF is micafungin. NA: not applicable because experiment was not run on that strain (usually because kinase is essential); –: statistically significantly lower value than the control strain, +: either the same as or slightly higher than the control strain.
file looks same as control). In contrast, it appears expression of brlA, AN3339 and AN2116 is mediated by MpkA, as they all show relatively flat profiles in the MpkA deletion mutant. Second, in the other kinase deletion strains (Hk-8–4, stk19, and prkA) gene expression levels take longer to reach a maximum (90–120 min). This suggests the kinases Hk-8–4, Stk19, and PrkA are involved in the temporal regulation of wall-related genes, but they are not required for their de facto expression.

Phosphorylation of GAPs, GEFs, and Transcription Factors—Phosphoproteomic analysis also shows thirteen putative transcription factors, with 16 phosphorylation sites, are significantly changing in response to micafungin addition (Table III). Those with known function in A. nidulans include HapC, Hsf1, RtfA, SrrA, and StuA. StuA regulates conidioaphore development (66), SrrA is a stress response regulator with ties to cell wall integrity signaling (67, 68), RtfA is associated with secondary metabolism, morphological development (69), and minor effects on cell wall integrity in A. fumigatus (70), Hsf1 appears to regulate oxidative stress response (71), and HapC is a member of the AnCP/AnCF CCAAT-binding complex (72).

Phosphorylation Network—To deduce possible protein-protein interactions, we used STRING v11 to generate an interaction map (supplemental Fig. S3, Supplemental File. S9) showing connections between differentially phosphorylated

![Fig. 8. Apparent cell wall strength of kinase deletion mutants.](image)

Growth rates of strains in rich medium were calculated from the exponential growth phase using dry cell weight. No significant differences between the control and mutants are observed (A). Particle size analysis of shake flask samples over the growth period reveal that kinase deletion mutants, ΔmpkA, ΔnpkA, Δhk-8–4, ΔsnrB, Δnrc2, Δstt19, Δkin1, ΔprkA, are significantly smaller than the control (B). A student’s t test of mutants against the control was used with \( p < 0.05 \) for significance and \( n \geq 3 \).

![Fig. 9. Micafungin susceptibility of kinase deletion mutants.](image)

100 fresh spores of the control strain were plated on MAGV plates with various concentrations of micafungin (0–0.1 \( \mu \)g/ml) in order to find the minimum inhibitory concentration (0.007 \( \mu \)g/ml) (A). 100 spores of each kinase deletion mutant were grown on MAGV plates with (0.007 \( \mu \)g/ml) and without micafungin and the number of colonies was counted. The percent growth is displayed and ΔmpkA, Δyak1, Δnrc2, and ΔprkA grew significantly less than the control strain (\( * p < 0.05, n = 3 \)) (B).
proteins (48). We see three clusters form including ribosome biogenesis, calcium and cell cycle signaling, and septation and actin regulation. Of these, the septation and actin regulation cluster included several kinases (PrkA and SidB) and phosphatases (PtcA and PtcB). Proteins with altered phosphorylation related to septation initiation and formation are shown in Table IV.

**DISCUSSION**

Our dynamic, multi-omic analysis provides insight into the signaling mechanisms and the downstream effectors involved with addressing a cell wall perturbation. Our data confidently identify statistically significant phosphorylation site occupancy changes and altered gene expression trends. We identify three main signaling pathways (septation and actin regu-
Multi-omic CWIS Study in A. nidulans

TABLE III
Phosphorylated transcription factors (TF), GAPs, and GEFs

| Gene   | Residue | Putative function | NetworKIN prediction | Yeast homolog | Direction |
|--------|---------|-------------------|----------------------|---------------|-----------|
| AN0463 | S 1880  | GEF               | N/A                  | DCK1          | Down      |
| AN2130 | S 80    | GEF               | N/A                  | CDC25         | Down      |
| AN5592 | S 760   | GEF               | AtmA                 | CDC24         | Down      |
|        | S 764   |                   | NimX                 |               | Down      |
| AN5677 | S 870   | GAP               | PckA                 | RGD2          | Up-Down   |
| AN6033 | S 320   | GAP               | N/A                  | GLO3          | Down      |
| AN757E | S 773   | GAP               | PckA                 | LRG1          | Down      |
| HycB   | S 1219  | GEF               | Ksg1                 | SEC7          | Down      |
|        | S 781   |                   | N/A                  |               | Down      |
| RicA   | S 463   | GEF               | N/A                  | N/A           | Down      |
|        | S 465   |                   | N/A                  |               | Down      |
| AN0794 | S 229   | TF                | Cka1                 | TAF9          | Down      |
| AN1500 | S 427   | TF                | N/A                  | ACE2          | Down      |
| AN1944 | S 490   | TF                | N/A                  | AIR2          | Down      |
| AN1984 | S 3      | TF                | N/A                  | BDF1          | Down      |
| AN4694 | S 316   | TF                | SrpkA                | CTI6          | Down      |
|        | S 320   |                   | Cka1                 |               | Down      |
| AN4894 | S 451   | TF                | Cka1                 | SPT7          | Down      |
| AN5898 | S 255   | TF                | N/A                  | CDC36         | Down      |
| AN9358 | S 771   | TF                | Cka1                 | NCBI          | Down      |
| HycC   | S 817   | TF                | Cka1                 | HAP3          | Up-Down   |
| Hsp1   | S 451   | TF                | Yak1                 | HSF1          | Up-Down   |
|        | S 485   |                   | NimX                 |               | Down      |
| RtfA   | S 257   | TF                | N/A                  | RTF1          | Up-Down   |
|        | T 259   |                   | N/A                  |               | Up-Down   |
| SrrA   | S 243   | TF                | N/A                  | SKN7          | Down      |
| StuA   | S 421   | TF                | PkaA                 | SOK2          | Down      |

Phosphorylation of the cyclin-dependent kinase (CDK) NimX. Stimulating the SIN, cell wall perturbation caused by micafungin, and our data imply potential mechanisms by which this could occur (Table IV). For example, exposure to micafungin results in reduced phosphorylation of the serine/threonine kinase SidB, which is a component of the septation initiation network (SIN) required for septation in A. nidulans (77, 79). Last, AN4693 is a homologue of S. cerevisiae Hof1 and S. pombe cdc15, both of which are key regulators of septation. Our results demonstrate that AN4693 is down-phosphorylated at multiple sites upon exposure to micafungin. In S. pombe, extensive dephosphorylation of Cdc15 enables interaction with multiple partner proteins that promote septum formation (80), including the SepA homologue Cdc12. Taken together, these observations suggest CWIS activation functions through the SIN and AN4693 to trigger septation. We propose that this is part of a stress response that maintains the structural integrity of damaged hyphae by confining the damage to specific regions of the mycelium.

In A. nidulans, polarized hyphal growth requires actin cables and actin patches; the former mediate localized exocytic delivery of vesicles at the immediate hyphal tip, whereas the latter are required for endocytosis at sub-apical sites (81–83). We identified several proteins implicated in actin patch formation that are dynamically phosphorylated because of CWIS activation (Table IV). These include regulators of the Arp2/3 complex such as PrkA, Sla1, Las17, Pan1, and Crn1. Because Arp2/3 catalyzes the formation of the branched actin filaments that form patches, these results suggest that activation of the CWIS pathway alters actin formation with subsequent effects on morphogenesis. For example, disassembly of actin patches could conceivably free up actin monomers that could be diverted toward formation of contractile actin rings to support increased septation (84). Similarly, our observation that the actin filament severing protein AN0837 is dynamically phosphorylated implies another mechanism for increasing the actin monomer pool. In addition, altered actin patch formation should impact endocytosis with resulting effects on hyphal extension. Indeed, SlaB, which localizes to actin patches and is required for endocytosis, also shows dynamic phosphorylation in response to CWIS activation.

Moreover, we observed multi-branched hyphae during micafungin exposure (Fig. 12) that most likely occurred because the extension rate of the tip slowed. The combined effect of cell wall damage and disorganization of the actin cytoskeleton may account for this. For example, the loss of actin filaments could disrupt the tip growth apparatus (85) and therefore trigger increase apical branching. We also know that a mutant (sepA) in which actin filament formation is severely reduced also displays apical branching (86).

A gene ontology (GO) analysis of the 1810 genes dynamically expressed in response to micafungin shows 7 of the top 10 GO terms are related to the cytoskeleton and actin organization (all categories p < 0.025). These include: “cytoskeleton organization,” “actin-filament based process,” “regulation of actin filament length,” and “regulation of actin polymerization,” for example (supplemental File S7). Of the 43 putative “cytoskeleton organization” genes, 18 of these have been verified (Table V). The genes budA, fimA, and actA all have implications in septum formation as its presence is transient at septation sites (82, 87). Both ampA and fimA are involved in endocytosis (82, 88). The Ras GTPase, gapA, has cortical localization and is believed to be involved in Ras
Fig. 12. **Growth in micafungin leads to changes in septation.** Control strain was grown in rich medium for 12 h on coverslips. Micafungin was added to media (10 ng/ml) and images were taken every hour thereafter. Fungi grown in micafungin show more septa and branches (A) than fungi grown in rich medium (B). Both images were taken at 17 h total growth, white arrows point to septa. Images from this experiment were used to calculate the growth rates and branching rates for 3 biological replicates with and without micafungin (*p < 0.05) (C). When you plot septation per area, it is clear that fungi in micafungin continue to septate regardless of fungal size (D).

### Table IV

**Phosphorylated proteins related to septation initiation and formation**

| Gene             | Residue | Protein description                                      | Direction  |
|------------------|---------|----------------------------------------------------------|------------|
| **Septation initiation signaling** |
| NimX/CDC28/Cdc2  | Y 15    | Cyclin-dependent kinase                                   | Down       |
| S4d/B/DBF20/Sid2 | S 453   | Kinase of the septation initiation network (SIN)          | Up-Down    |
|                 | S 44    |                                                          | Down       |
|                 | S 47    |                                                          | Up-Down    |
| AspA/CDC11/Spn3 | S 370   | Septin                                                   | Down       |
| **Arp2/3 Regulation** |
| PrkA/PRK1/Ppk30  | S 957   | Serine/threonine protein kinase                           | Down       |
| Pan1/PAN1/Pan1   | S 1226  | Predicted role in actin patch assembly                     | Down       |
| AN1462/SLA1/Shd1 | S 230   | Predicted role in actin patch assembly                     | Down       |
| AN1104/LAS17/Wsp1| T 315   | Predicted role in actin nucleation                        | Down       |
|                 | S 414   |                                                          | Down       |
| AN6341/CRN1/Crn1 | S 524   | Predicted role in actin patch assembly                     | Down       |
|                 | S 529   |                                                          | Up-Down    |
| AN4919/ARC15/Arc5| S 139   | Predicted role in Arp2/3 complex-mediated actin nucleation| Down       |
|                 | S 144   |                                                          | Down       |
| SlaB/SLA2/End4   | S 213   | Predicted actin binding protein                            | Down       |
| TeaA/TEA1/Tea3   | S 1379  | Cell-end marker protein                                   | Down       |
|                 | S 1382  |                                                          | Down       |
| AN4963/HOF1/Cdc15| S 555   | Predicted Arp2/3                                          | Down       |
|                 | S 710   |                                                          | Down       |
|                 | S 719   |                                                          | Down       |
|                 | S 744   |                                                          | Down       |
|                 | S 793   |                                                          | Down       |
|                 | S 822   |                                                          | Down       |
|                 | S 838   |                                                          | Down       |
|                 | S 902   |                                                          | Down       |
|                 | S 920   |                                                          | Down       |
| **Actin Related Proteins** |
| AN0837/NA/NA     | S 663   | Predicted role in actin filament severing                 | Down       |
|                 | S 635   |                                                          | Down       |
|                 | S 261   |                                                          | Up-Down    |
| AN5677/RGD2/Rga8 | S 870   | Predicted GTPase activator activity                       | Up-Down    |
| HypB/SEC7/Sec72  | S 1219  | Sec7-domain protein                                       | Down       |
|                 | S 781   |                                                          | Down       |

*Multi-omic CWIS Study in *A. nidulans*  
*Mol Cell Proteomics* (2020) 19(8) 1310–1329*
signaling that polarizes the actin cytoskeleton (89). Microtubule (MT) dynamics and organization genes include migA, alpA, and clipA (90–92).

Calcium and Calcineurin Signaling Aids CWIS Pathway—In our data, calmodulin (CaM) and its associated regulatory protein CmkC were dynamically phosphorylated in response to micafungin. CaM reversibly binds Ca\(^{2+}\) and is required for polarized growth and septation in \textit{A. nidulans} (93, 94). Transient CaM localization occurs at sites of hyphal growth, branch emergence, and septation, which implies that it performs a critical function in localized cell wall deposition (94). CaM has also been implicated in the activation of the CDK NimX via CmkB, which is phosphorylated and activated by CmkC (Fig. 13) (95). This provides another mechanism by which CaM could impact morphogenesis in response to cell wall stress.

Key effectors of calcium signaling that regulate cell growth and cell wall integrity in \textit{A. nidulans} are the CaM-dependent phosphatase calcineurin (CnaA) and the transcription factor CrzA (96, 97). Among proteins with roles related to calcium signaling, the genes encoding CnaA and CrzA were differentially expressed in response to micafungin (both increase expression for the first 20 min and then decrease). Accordingly, modulation of calcium signaling is likely to be an important output of CWIS activation that has broad effects on growth and morphogenesis. This is consistent with the observation that inhibition of calcineurin function exacerbates the effects of caspofungin exposure (98), and that CrzA regulates the expression of chitin synthases that are thought to reinforce damaged cell walls (99).

High-osmolarity Glycerol (HOG) Pathway Crosstalk—Several putative high-osmolarity glycerol (HOG) signaling proteins showed changing phosphorylation levels in response to micafungin. For example, SrrA underwent dynamic changes in phosphorylation, which is notable given that it has been previously implicated in cell wall assembly because \textit{sr}r\textit{A} deletion mutants show significant resistance to micafungin (68). In addition, PtcB, which is yet uncharacterized in \textit{A. nidulans}, was down-phosphorylated in the presence of micafungin and is a predicted target of HogA. In \textit{A. fumigatus}, a \textit{\Delta ptcB} strain is more susceptible to cell wall perturbing agents, has increased levels of chitin and \(\beta\)-1,3-glucan, and has higher
MpkA phosphorylation levels (100). Additional connections between components of the HOG and CWIS signaling pathways have been described in both *S. cerevisiae* and *S. pombe* (Fig. 13) (101–104), thereby reinforcing the notion that pathway cross-talk might be an integral feature of the response to cell wall perturbation. In *A. nidulans*, Zhou *et al.* recently reported that hyperosmotic stress could trigger septation in a HogA- and SIN-dependent manner (105). This raises the intriguing possibility that a general function of stress-induced MAP kinase signaling in filamentous fungi might be activation of septum formation.

**Kinases with Cell Wall Abnormalities**—Eight kinase deletion strains, Δhk-8–4, Δstk19, ΔprkA, Δkin1, Δnrc2, ΔnpkA, Δyak1, and ΔsrrB exhibited phenotypes consistent with cell wall integrity signaling activity (Table II). These kinases are not members of the conserved CWIS BckA-MkkA-MpkA MAPK module (11) of which only the MAPKKK, BckA (S719, S1003) and MAPKK, MkkA (S169) were identified from phosphotyrosinomics. Both PrkA and Kin1 have been discussed above in regards to their roles in Arp2/3 regulation. As many of these kinases have not been characterized in *A. nidulans*, here we have identified their putative function in the CWIS network for the first time by combining phenotype and phosphotyrosinome data.

Hk-8−4 is a histidine kinase (HK), had weaker cell walls than the control strain (Fig. 8B), was phosphorylated at S314, apparently by Ste20 (based on NetworKIN analysis) which has been implicated in the SHO1 branch of the HOG pathway (106), and its deletion mutant had altered putative cell wall regulated gene expression trends from the control (Fig. 11).

We hypothesize Hk-8−4 is connected with cell wall stress mediation possibly through its interaction with Ste20 and HOG. Similarly, Stk19 had decreased cell wall strength, altered gene expression (qPCR), and was sensitive to micafungin in shake flask culture. Stk19 (S875) is uncharacterized in *A. nidulans*, but has orthologues in *S. pombe* (ppk16), *S. cerevisiae* (YPL150W), and *N. crassa* (stk-19) (79) all of which have diverse cellular functions (107–109).

The nrc2 deletion mutant was more susceptible to micafungin on solid media, had weaker cell walls, and was predicted to be auto phosphorylated at S433. Nrc2 is uncharacterized in *A. nidulans*, however its yeast homologue, Fpk1, is known to be involved in cell wall integrity signaling as it contributes (directly or indirectly) to Mpk1 (MpkA homologue) activation (110). Nrc2 (S74) is predicted to be phosphorylated by Snf1 and its deletion mutant (Δnrc2ΔmkA) had a weaker cell wall (this work) and showed aberrant septa formation (111).

The yak1 mutant showed a significant decrease in colony growth in the presence of micafungin (Fig. 9B). We found Yak1 was dynamically phosphorylated at Y502, a tyrosine predicted to control full kinase activity (112). SrrB (S645) was predicted to be phosphorylated by PkaB (NetworKIN). Not only this, but ΔsrrB shows sensitivity to osmotic stress (79) and had significantly smaller mycelia in shake flask culture (this work) suggesting it plays a role in CWIS (Fig. 8B).

**Putative, Currently Unknown Genes Downstream of Micafungin Perturbation**—Previous work identified two genes, *agsA* and *agsB*, that are downstream of the conserved MAPK signaling cascade, BckA-MkkA-MpkA (11). However, all other cell wall related genes were not dependent on this cascade.

### Table V

| Gene   | ANID   | Description                                      | Cluster | Avg FC | *S. cerevisiae* | Reference                |
|--------|--------|--------------------------------------------------|---------|--------|-----------------|--------------------------|
| actA   | AN6542 | Gamma-actin                                      | Green   | -      | ACT1            | Fidel et al., 1988       |
| alpA   | AN5521 | Microtubule stabilizing, plus end-binding protein | Green   | -      | STU2            | Enke et al., 2007        |
| ampA   | AN2516 | Component of the endocytic internalization machinery | Green   | +      | RVS167         | Araujo-Bazan et al., 2008 |
| ampB   | AN8831 | Role in contractile ring assembly                | Green   | +      | RVS161         | Araujo-Bazan et al., 2008 |
| benA   | AN1182 | Beta-tubulin, highly conserved component of microtubules | Green   | -      | TUB2           | May et al., 1987         |
| budA   | AN1324 | Putative actin-monomer binding protein           | Green   | -      | BUD6           | Virag and Harris, 2006   |
| cdcA   | AN5057 | Putative phosphoprotein phosphatase              | Green   | -      | CDC14          | Brown et al., 2013       |
| cipA   | AN1475 | Role in microtubule dynamics                     | Green   | -      | BIK1           | Harris et al., 2009      |
| fimA   | AN5803 | Predicted timbrin protein                        | Green   | +      | SAC6           | Upadhyay and Shaw, 2008  |
| gapA   | AN4998 | Putative Ras GTPase-activating protein            | Purple  | -      | IRA2           | Harispe et al., 2008     |
| gcpC   | AN4867 | Gamma-tubulin complex protein 3                  | Green   | -      | SPC98          | Xiong and Oakley, 2009   |
| gcpE   | AN8120 | Gamma-tubulin complex protein 5                  | Green   | +      | N/A            | Xiong and Oakley, 2009   |
| kinA   | AN5343 | Kinesin-family protein; involved in microtubule destabilization | Green   | -      | SMY1           | Requena et al., 2001     |
| migA   | AN2101 | Required for spindle and microtubule positioning | Green   | -      | KAR9           | Manck et al., 2015       |
| nce102 | AN7683 | Eiosomal protein; regulates sphingolipid biosynthesis | Purple  | -      | NCE102         | Athanasopoulos et al., 2015 |
| pphA   | AN6391 | Protein phosphatase                               | Green   | -      | PPH21          | Son and Osmani, 2009     |
| tubB   | AN7570 | Alpha-tubulin, promotes microtubule assembly      | Green   | -      | TUB1           | Kirk and Morris, 1991    |

---

**TABLE V**

Dynamic expression of actin related genes

| Gene   | ANID   | Description                                               | Cluster | Avg FC | *S. cerevisiae* | Reference                |
|--------|--------|-----------------------------------------------------------|---------|--------|-----------------|--------------------------|
| actA   | AN6542 | Gamma-actin                                               | Green   | -      | ACT1            | Fidel et al., 1988       |
| alpA   | AN5521 | Microtubule stabilizing, plus end-binding protein          | Green   | -      | STU2            | Enke et al., 2007        |
| ampA   | AN2516 | Component of the endocytic internalization machinery       | Green   | +      | RVS167         | Araujo-Bazan et al., 2008 |
| ampB   | AN8831 | Role in contractile ring assembly                          | Green   | +      | RVS161         | Araujo-Bazan et al., 2008 |
| benA   | AN1182 | Beta-tubulin, highly conserved component of microtubules   | Green   | -      | TUB2           | May et al., 1987         |
| budA   | AN1324 | Putative actin-monomer binding protein                     | Green   | -      | BUD6           | Virag and Harris, 2006   |
| cdcA   | AN5057 | Putative phosphoprotein phosphatase                        | Green   | -      | CDC14          | Brown et al., 2013       |
| cipA   | AN1475 | Role in microtubule dynamics                               | Green   | -      | BIK1           | Harris et al., 2009      |
| fimA   | AN5803 | Predicted timbrin protein                                  | Green   | +      | SAC6           | Upadhyay and Shaw, 2008  |
| gapA   | AN4998 | Putative Ras GTPase-activating protein                     | Purple  | -      | IRA2           | Harispe et al., 2008     |
| gcpC   | AN4867 | Gamma-tubulin complex protein 3                            | Green   | -      | SPC98          | Xiong and Oakley, 2009   |
| gcpE   | AN8120 | Gamma-tubulin complex protein 5                            | Green   | +      | N/A            | Xiong and Oakley, 2009   |
| kinA   | AN5343 | Kinesin-family protein; involved in microtubule destabilization | Green   | -      | SMY1           | Requena et al., 2001     |
| migA   | AN2101 | Required for spindle and microtubule positioning           | Green   | -      | KAR9           | Manck et al., 2015       |
| nce102 | AN7683 | Eiosomal protein; regulates sphingolipid biosynthesis      | Purple  | -      | NCE102         | Athanasopoulos et al., 2015 |
| pphA   | AN6391 | Protein phosphatase                                        | Green   | -      | PPH21          | Son and Osmani, 2009     |
| tubB   | AN7570 | Alpha-tubulin, promotes microtubule assembly                | Green   | -      | TUB1           | Kirk and Morris, 1991    |
Multi-omic CWIS Study in *A. nidulans*

### Table VI

| Gene       | Max Avg. Log2FC | Gene       | Max Avg. Log2FC |
|------------|-----------------|------------|-----------------|
| AN0215     | 5.74            | AN3339     | 6.87            |
| ivoB       | 5.30            | AN3996     | 6.15            |
| brnA       | 4.09            | AN4106     | 7.30            |
| AN1261     | 5.90            | AN4111     | 6.76            |
| AN1322     | 4.92            | AN4129     | 5.35            |
| AN1600     | 5.37            | AN4173     | 4.46            |
| agnE       | 5.06            | AN5546     | 4.79            |
| AN1614     | 4.40            | AN5841     | 5.40            |
| AN1677     | 4.73            | AN7557     | 4.51            |
| AN1941     | 5.29            | AN7953     | 5.88            |
| AN2110     | 4.86            | AN7954     | 7.57            |
| AN2116     | 7.36            | furG       | 5.77            |
| AN2118     | 4.28            | AN8084     | 5.71            |
| AN2186     | 5.70            | AN8341     | 4.24            |
| AN2187     | 4.02            | AN8342     | 4.12            |
| AN2197     | 5.49            | AN8593     | 6.16            |
| AN2320     | 5.90            | AN8594     | 4.23            |
| AN2321     | 4.81            | AN8595     | 4.43            |
| AN2469     | 7.07            | AN8597     | 4.08            |
| AN2558     | 7.96            | AN8904     | 4.23            |
| AN2571     | 5.18            | AN8995     | 4.69            |
| AN2598     | 5.53            | AN9004     | 5.61            |
| AN2781     | 4.27            | AN9191     | 4.87            |
| AN2959     | 7.33            | AN9219     | 4.94            |
| AN2960     | 6.63            | AN9490     | 4.09            |

We show many interconnections between various signaling cascades which may account for this. Moreover, many genes that are not directly associated with cell wall repair are expressed at very high level in response to micafungin. Of the 50 most overexpressed genes, 45 of these are uncharacterized (Table VI; supplemental File S5). However, using computational predictions, 14 were predicted to be localized in the membrane (GO cellular component) and associated with transport functions (GO cellular function) (AN2321, AN2598, AN2781, AN2959, AN4106, AN4129, AN4173, AN7557, AN8084, AN8342, AN8595, AN8995, AN9219, and furG). Increased expression of membrane transport genes, in response to micafungin, may facilitate movement of cell wall material through the membrane to facilitate repair of damaged walls.

These hits make up less than 30% of the highly expressed genes. We hypothesize, that these remaining genes are downstream effectors of the CWIS pathway and are involved in cell wall repair. AN2116 and AN3339 were probed by qPCR during micafungin exposure. They were highly expressed in the control strain after 60 min. In the ΔmpkA strain, the expression of these genes changed little. This suggests AN2116 and AN3339 are dependent on MpkA. AN2116 (uncharacterized) is predicted to play a role in coenzyme binding and catalytic activity (49) whereas, AN3339 (uncharacterized), is predicted to have oxidoreductase activity, transferase activity, and zinc-binding activity (49). Because of the overwhelming increase in gene expression of these 50 genes, we hypothesize they are novel downstream effectors of CWIS.

### CONCLUSION

We have applied a multi-omic methodology for identifying signaling mechanisms in response to cell stimuli. Short time-scale phosphoproteomic sampling, combined with longer-scale transcriptomic sampling, have provided a global picture of the cellular response to fungal cell-wall stress. Our statistical approach in determining dynamic effects resulted in the identification of 431 (794 with Class II) phosphorylation sites and 1810 genes. Cell wall strength and micafungin susceptibility assays provided a relatively rapid means for phenotyping relevant kinase deletion mutants and showed *hk-8–4, stk19, prkA, npkA, nrc2, srnB*, and *kin1* deletion mutants have weaker cell walls than the control. Moreover, ΔprkA, Δyak1 and Δnrc2 are more susceptible to micafungin on solid media, whereas ΔprkA and Δstk19 are susceptible to micafungin in submerged culture. We found many signaling molecules related to Arp2/3 regulation, septation initiation, calmodulin and calcium signaling, and high-osmolarity are changing phosphorylation and most likely activity because of micafungin perturbation. We hypothesize septation formation is being initiated via NimX, SidB, and CaM signaling, whereas PrkA and Kin1 are regulating activity of Arp2/3 and endocytosis. We propose that septation formation is a defense mechanism of fungi under cell wall perturbation to prevent further damage to internal hyphal compartments. Many uncharacterized genes were significantly overexpressed, suggesting novel, downstream CWIS effectors.

### DATA AVAILABILITY

RNA-Seq data has been deposited in the Gene Expression Omnibus (GSE136562) and mass spectrometry phosphoproteomics data in the Proteomics IDENTifications (PRIDE) repository, PXD015038 (113). MS/MS spectra can be viewed using MS-viewer (search key: yhspxctvyc; http://msviewer.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msviewer).

**Funding and additional information**—This work was supported by the National Science Foundation (Awards 1517309, 1517133, and 151690). The UNMC DNA Sequencing Core Facility receives partial support from the Nebraska Research Initiative.

**Author contributions**—C.C., R.S., S.H., and M.R.M. designed research; C.C., W.H., S.R., A.D., K.L., and R.P. performed research; C.C., W.H., and S.R. analyzed data; C.C., S.H., and M.R.M. wrote the paper; W.H., A.D., S.L., B.T., T.G., and R.S. contributed new reagents/analytic tools.
Conflict of interest—Authors declare no competing interests.

Abbreviations—The abbreviations used are: BCA, bicinchoninic acid; FDR, false discovery rate; MAPK, mitogen-activated protein kinase.

REFERENCES

1. Ubersax, J. A., and Ferrell, J. E. J. (2007) Mechanisms of specificity in protein phosphorylation. Nat. Rev. Mol. Cell Biol. 8, 530–541
2. Manning, G., Plowman, G. D., Hunter, T., and Sudarsanam, S. (2002) Evolution of protein kinase signaling from yeast to man. Trends Biochem. Sci. 27, 514–520
3. Levin, D. E. (2005) Cell wall integrity signaling in Saccharomyces cerevisiae. Microbiol Mol. Biol. Rev. 69, 262
4. Gow, N. A. R., Latge, J. P., and Munro, C. A. (2017) The fungal cell wall: structure, biosynthesis, and function. Microbiol. Spectrum 5
5. Latge, J. P. (2007) The cell wall: a carbohydrate armour for the fungal cell. Mol. Microbiol. 66, 279–290
6. Bowman, S. M., and Frey, S. J. (2006) The structure and synthesis of the fungal cell wall. Bioessays 28, 799–808
7. Yoshimi, A., Fujioka, T., Mizutani, O., Marui, J., Hagiwara, D., and Abe, K. (2015) Mitogen-activated protein kinases MpkA and MpkB independently affect mictalin sensitivity in Aspergillus nidulans. Biosci. Biotechnol. Biochem. 79, 836–844
8. Valiante, V., Heinekamp, T., Jain, R., Hartl, A., and Brakhage, A. A. (2008) The mitogen-activated protein kinase MpkA of Aspergillus fumigatus regulates cell wall signaling and oxidative stress response. Fungal Genet. Biol. 45, 618–627
9. Chauvin, D., Hutz, M., Schutte, M., Chesnay, A., Parent, C., Moreira, G., Arroyo, J., Sanz, A. B., Pugniere, M., Martinez, P., Chardenier, J., Heuze-Vourch, N., and Desoubeaux, G. (2019) Targeting Aspergillus fumigatus Crf transglycosylases with neutralizing antibody is relevant but not sufficient to erase fungal burden in a neutropenic rat model. Front. Microbiol. 10, 600
10. Desoubeaux, G., Chauvin, D., Piqueras, M. D., Bronson, E., Bhattacharya, S. K., Sirpenski, G., Bailly, E., and Cray, C. (2018) Translational proteomic study to address host protein changes during aspergillosis. PloS One 13, e0200843
11. Fujitaka, T., Mizutani, O., Furukawa, K., Sato, N., Yoshimi, A., Yamagata, Y., Nakajima, T., and Abe, K. (2007) MpkA-dependent and -independent cell wall integrity signaling in Aspergillus nidulans. Eukaryotic Cell 6, 1497–1510
12. Valiante, V., Jain, R., Heinekamp, T., and Brakhage, A. A. (2009) The MpkA MAP kinase module regulates cell wall integrity signaling and pyomelanin formation in Aspergillus fumigatus. Fungal Genet. Biol. 46, 909–918
13. Mizutani, O., Nojima, A., Yamamoto, M., Furukawa, K., Fujitaka, T., Yamagata, Y., Abe, K., and Nakajima, T. (2004) Disordered cell integrity signaling caused by disruption of the kexB gene in Aspergillus oryzae. Eukaryotic Cell 3, 1038–1048
14. Madrid, M., Soto, T., Khong, H. K., Franco, A., Vicente, J., Perez, P., Gacto, M., and Cansado, J. (2006) Stress-induced response, localization, and regulation of the Pmk1 cell integrity pathway in Schizosaccharomyces pombe. J. Biol. Chem. 281, 2033–2043
15. Navarragarcia, F., Sanchez, M., Pla, J., and Nombeila, C. (1995) Functional characterization of the Mck1 gene of Candida albicans, which encodes a mitogen-activated protein-kinase homolog related to cell integrity. Mol. Cell Biol. 15, 2197–2206
16. Futagawa, T., and Goto, M. (2012) Putative cell wall integrity sensor proteins in Aspergillus nidulans. Commun. Integrative Biol. 5, 206–208
17. Malavazi, I., Goldmann, G. H., and Brown, N. A. (2014) The importance of connections between the cell wall integrity pathway and the unfolded protein response in filamentous fungi. Briefings Funct. Genomics 13, 456–470
18. Guest, G. M., and Momany, M. (2000) Analysis of cell wall sugars in the pathogen Aspergillus fumigatus and the saprophyte Aspergillus nidulans. Mycologia 92, 1047–1050
19. Zhang, X., Jia, X. D., Tian, S. G., Zhang, C. J., Lu, Z. Y., Chen, Y., Chen, F. Y., Li, Z. W., Su, X. T., Han, X. L., Sun, Y. S., and Han, L. (2018) Role of the small GTPase Rho1 in cell wall integrity, stress response, and pathogenesis of Aspergillus fumigatus. Fungal Genetics Biol. 120, 30–41
20. Bussink, H. J., and Osmani, S. A. (1999) A mitogen-activated protein kinase (MPKA) is involved in polarized growth in the filamentous fungus, Aspergillus nidulans. Fems Microbiol. Lett. 173, 117–125
21. Teepe, A. G., Loprete, D. M., He, Z. M., Hoggard, T. A., and Hill, T. W. (2007) The protein kinase C orthologue PkcA plays a role in cell wall integrity and polarized growth in Aspergillus nidulans. Fungal Genetics 44, 554–562
22. Katayama, T., Ohta, A., and Horikuchi, H. (2015) Protein kinase C regulates the expression of cell wall-related genes in RlmA-dependent and independent manners in Aspergillus nidulans. Biosci. Biotechnol. Biochem. 79, 321–330
23. Park, B. C., Park, Y. H., Yi, S., Choi, Y. K., Kang, E. H., and Park, H. M. (2014) Transcriptional regulation of RlsA, a beta-1,3-glucan synthase gene, by the APSES protein StuA during Aspergillus nidulans development. J. Microbiol. 52, 940–947
24. Choi, Y. K., Kang, E. H., and Park, H. M. (2014) Role of LAMMER kinase in cell wall biogenesis during vegetative growth of Aspergillus nidulans. Mycobiology 42, 422–426
25. Cramer, R. A., Perfect, B. Z., Pinchall, N., Park, S., Perlin, D. S., Asfaw, Y. G., Heitman, J., Perfect, J. R., and Steinbach, W. J. (2008) Calcineurin target CrzA regulates conidial germination, hypothalamic and test-pathogenesis of Aspergillus fumigatus. Eukaryotic Cell 7, 1085–1097
26. Guerriero, G., Silvestrini, L., Obersiebng, M., Hausmann, J. F., Strauss, J., and Ezcurra, I. (2018) A WDR gene is a conserved member of a chitin synthase gene cluster and influences the cell wall in Aspergillus nidulans. Int. J. Mol. Sci. 17, 1031
27. Futagami, T., Seto, K., Kajiwara, Y., Takahashi, H., Omori, T., Takegawa, K., and Goto, M. (2014) The putative stress sensor protein MIA is required for conidia formation, cell wall stress tolerance, and cell wall integrity in Aspergillus nidulans. Biosci. Biotechnol. Biochem. 78, 325–335
28. Juvvadi, P. R., Ma, Y., Richards, A. D., Soderblom, E. J., Moseley, M. A., Lamoth, F., and Steinbach, W. J. (2015) Identification and mutational analyses of phosphorylation sites of the calcineurin-binding protein CbpA and the identification of domains required for calcineurin binding in Aspergillus fumigatus. Fungal Genetics Biol. 125, 1–12
29. Ribeiro, L. F. C., and Chelli, C. L., Harris, S. D., and Marten, M. R. (2017) Insights regarding fungal phosphoproteomic analysis. Fungal Genetics Biol. 104, 38–44
30. Fuchs, B. B., and Mylonakis, E. (2009) Our paths might cross: the role of the fungal cell wall integrity pathway in stress response and cross talk with other stress response pathways. Eukaryotic Cell 8, 1616–1625
31. Ribeiro, L. F. C., Chelli, C. L., Harris, S. D., and Marten, M. R. (2017) Multi-omic CWIS Study in A. nidulans.
49. Cerqueira, G. C., Arnaud, M. B., Inglis, D. O., Skrzypek, M. S., Binkley, G., Luciani, A., Potter, S., Finn, R. D., Urban, M., Hammond-Kosack, K. E., Carvalho-Silva, D., Christensen, M., Davis, P., Grabmueller, C., Kumar, S., Remm, M., and Rozen, S. G. (2012) Primer3-new capabilities and improvements. Nucleic Acids Res. 40, D191–D198

50. Quintanilla, D., Chelius, C., Iambamrung, S., Nelson, S., Thomas, D., and Mering, C. (2019) STRING v11: protein-protein association network inference for non-vertebrate species. Nucleic Acids Res. 46, D802–D808

51. Gnadt, F., Gunawardena, J., and Mann, M. (2011) PHOSIDA 2011: the web-based infrastructure for the interpretation of phosphoproteomics data. Nucleic Acids Res. 39, D253–D260

52. Schwartz, D., and Gygi, S. P. (2005) An iterative statistical approach to the confident identification of protein biomarkers. Mol. Cell. Proteomics 4, 111–125

53. Kim, D., Landmead, B., and Salzberg, S. L. (2015) HISAT: a fast spliced alignment algorithm. Bioinformatics 31, 142–143

54. Si, H. Y., Rittenour, W. R., Xu, K. M., Nicksrallian, M., Calvo, A. M., and Harris, S. D. (2012) Morphogenetic and developmental functions of the Aspergillus nidulans homologues of the yeast bud site selection proteins Bud4 and Axl2. Mol. Microbiol. 85, 252–270

55. Gnad, F., Gunawardena, J., and Mann, M. (2011) PHOSIDA 2011: the posttranslational modification database. Nucleic Acids Res. 39, D253–D260

56. Love, M. I., Huber, W., and Anders, S. (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Bioinformatics 30, 155–162

57. Olsen, J. V., Blagoev, B., Nisimuk, R., Macek, B., Kumar, C., Mortensen, P., and Mann, M. (2006) Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. Cell 127, 635–648

58. Rose, C. M., Venkateshwaran, M., Volkening, J. D., Grimsrud, P. A., Maeda, J., Bailey, D. J., Park, K., Howes-Poddol, M., den Os, D., Yeun, L. H., Westphal, M. S., Sussman, M. R., Ane, J.-M., and Coon, J. J. (2012) Rapid phosphoproteomic and transcriptomic changes in the rhizobia-legume symbiosis. Mol. Cell. Proteomics 11, 724–744

59. Humphrey, S. J., Yang, G., Yang, P. Y., Fazakerley, D. J., Stockill, J., Yang, J. Y., and James, D. E. (2013) Dynamic adipocyte phosphoproteome reveals that Akt directly regulates mTORC2. Cell Metabolism 17, 1009–1020

60. Tan, H. Y., Yang, K., Li, Y. X., Shaw, T. I., Wang, Y. Y., Blasco, D. B., Wang, X. S., Cho, J. H., Wang, H., Rankin, S., Guy, C., Peng, J. M., and Chen, H. B. (2017) Integrative proteomics and phosphoproteomics profiling reveals dynamic signaling networks and bioenergetics pathways underlying t cell activation. Immunity 46, 488–503

61. Licona-Cassani, C., Lim, S., Marcellin, E., and Nielsen, L. K. (2014) Temporal dynamics of the Saccharopolyspora erythraea phosphoproteome. Mol. Cell. Proteomics 13, 1219–1230

62. Hagiwara, D., Asano, Y., Marui, J., Furukawa, K., Kanamaru, K., Kato, M., Abe, K., Kobayashi, T., Yamashimo, T., and Mizuno, T. (2007) The SaKa and SmnA response regulators are implicated in oxidative stress responses of hyphae and assexual spores in the phosporelay signaling network of Aspergillus nidulans. Biosci. Biotechnol. Biochem. 71, 1003–1014

63. Hagiwara, D., Mizuno, T., and Abe, K. (2011) Characterization of the conserved phosphorylation site in the Aspergillus nidulans response regulator SmnA. Current Genetics 57, 103–114

64. Ramamoorthy, V., Santappan, S., Dhingra, S., and Calvo, A. M. (2012) vea-dependent RNA-poly II transcription elongation factor-like protein, RftA, is associated with secondary metabolism and morphological development in Aspergillus nidulans. Mol. Microbiol. 85, 795–814

65. Myers, R. R., Smith, T. D., Elsaawa, S. F., Puel, O., Tadrits, S., and Calvo, A. M. (2017) rftA controls development, secondary metabolism, and virulence in Aspergillus fumigatus. Plos One 12, e0176702

66. Colabardini, A. C., de Castro, P. A., de Gouveia, P. F., Savoldi, M., Malavazi, I., Goldman, M. H. S., and Goldman, G. H. (2010) Involvement of the Aspergillus nidulans protein kinase Cc with farnesol tolerance is related to the unfolded protein response. Mol. Microbiol. 78, 1259–1279

67. Steidi, S., Papagiannopoulos, P., Litzka, O., Andrianopoulos, A., Davis, M. A., Brakhage, A. A., and Hynes, M. J. (1999) AnCo, the CCAAT binding complex of Aspergillus nidulans, contains products of the hapB, hapC, and hapE genes and is required for activation by the pathway-specific regulatory gene amdR. Mol. Cell. Biol. 19, 99–106

68. Kwon, N. J., Park, J. H., Jung, S., Kim, S. C., and Yu, J. H. (2012) The putative guanine nucleotide exchange factor RicA mediates upstream signaling for growth and development in Aspergillus. Eukaryotic Cell 11, 1399–1412

69. Yang, Y., El-Ganiny, A. M., Bray, G. E., Sanders, D. A. R., and Kaminskyj, S. G. W. (2008) Aspergillus nidulans hypB encodes a Sec7-domain protein.
protein important for hyphal morphogenesis. *Fungal Genetics Biol.* 45, 749–759

75. Wolkow, T. D., Harris, S. D., and Hamer, J. E. (1996) Cytokinesis in *Aspergillus nidulans* is controlled by cell size, nuclear positioning and mitosis. *J. Cell Sci.* 109, 2179–2188

76. Harris, S. D. (2001) Septum formation in *Aspergillus nidulans*. *Curr. Opinion Microbiol.* 4, 736–739

77. Kraus, P. R., and Harris, S. D. (2001) The *Aspergillus nidulans* sept genes are required for the regulation of septum formation and cell cycle checkpoints. *Genetics* 159, 557–569

78. Kim, J. M., Lu, L., Shao, R. Z., Chin, J. L., and Liu, B. (2006) Isolation of mutations that bypass the requirement of the septation initiation network for septum formation and conidiation in *Aspergillus nidulans*. *Genetics* 173, 685–696

79. De Souza, C. P., Hashmi, S. B., Osmani, A. H., Andrews, P., Ringelberg, C. S., Dunlap, J. C., and Osmani, S. A. (2013) Functional analysis of the *Aspergillus nidulans* kinome. *Plos One* 8, e58008

80. Roberts-Galbraith, R. H., Oh, M. D., Ballif, B. A., Chen, J. S., McLeod, I., McDonald, W. H., Gygi, S. P., Yates, J. R., and Gould, K. L. (2010) Diphosphorylation of F-BAR Protein Cdc15 modulates its conformation and stimulates its scaffolding activity at the cell division site. *Molecular Cell* 39, 86–99

81. Taheri-Talesh, N., Horio, T., Araujo-Bazan, L., Dou, X. W., Espeso, E. A., Matzno, S., Tsujimoto, S., Kita, A., and Sugiiura, R. (2016) Skb5, an SH3 adaptor protein, regulates Pmk1 MAPK signaling by controlling the intracellular localization of the MAPKKK Mkh1. *J. Cell Sci.* 129, 3189–3202

82. Xu, G. X., Ye, J., Zheng, L. K., Jiang, P., and Lu, L. (2019) A new identified suppressor of Cdc7p/Sept2 kinase, PomA, regulates fungal asexual reproduction via affecting phosphorylation of MAPK-HogA. *Plos Genetics* 15, e1008206

83. Raitt, D. C., Posas, F., and Saito, H. (2000) Yeast Cdc42 GTPase and Ste20 PAK-like kinase regulate Sho1-dependent activation of the Hog1 MAPK pathway. *EMBO J.* 19, 4623–4631

84. Biwa, A., Jia, Y. H., Poh, S. L., Karuriti, R. K. M., den Elzen, B., Peng, X., Zheng, L. L., O’Connell, M., Liu, E. T., Balasubramanian, M. K., and Liu, J. H. (2005) Systematic deletion analysis of fission yeast protein kinases. *Eukaryotic Cell* 4, 799–813

85. Yoshikawa, K., Tanaka, T., Ida, Y., Furusawa, C., Hirasawa, T., and Shimizu, H. (2011) Comprehensive phenotypic analysis of single-gene deletion and overexpression strains of Saccharomyces cerevisiae. *Yeast* 28, 349–361

86. Wilson, L. S., Reyes, C. M., Stolzmann, P., Speckman, J., Allen, K., and Beney, J. (2002) The direct cost and incidence of systemic fungal infections. *Value in Health* 5, 26–34

87. Roelants, F. M., Torrance, P. D., Bezman, N., and Thorell, J. (2002) Pkh1 and Pkh2 differentially phosphorylate and activate Ypk1 and Ykr2 and define protein kinase modules required for maintenance of cell wall integrity. *Mol. Biol. Cell* 13, 3005–3028

88. Enke, C., Zekert, N., Veith, D., Schaaf, C., Konzack, S., and Fischer, R. (2007) *Aspergillus nidulans* Ntl1/XMAP215 protein AlpA localizes to spindle pole bodies and microtubule plus ends and contributes to growth directionality. *Eukaryotic Cell* 6, 555–562

89. Harris, S. D., Turner, G., Meyer, V., Espeso, E. A., Specht, T., Takeshita, N., and Helmstedt, K. (2009) Morphology and development in *Aspergillus nidulans*: A complex puzzle. *Fungal Genetics Biol.* 46, S82–S92

90. Lu, K. P., and Means, A. R. (1993) Regulation of the cell-cycle by calcium and calmodulin. *Endocrin. Rev.* 14, 40–58

91. Chen, S. C., Song, Y. J., Cao, J. L., Wang, G., Wei, H., Xu, X. S., and Lu, L. (2010) Localization and function of calmodulin in live-cells of *Aspergillus nidulans*. *Fungal Genetics Biol.* 47, 268–278

92. Joseph, J. D., and Means, A. R. (2000) Identification and characterization of two Ca2+–CAM-dependent protein kinases required for normal nuclear division in *Aspergillus nidulans*. *J. Biol. Chem.* 275, 35230–35238

93. Hernandez-Ortiz, P., and Espeso, E. A. (2013) Phospho-regulation and nucleocytoplasmic trafficking of CrzA in response to calcium and alkaline pH stress in *Aspergillus nidulans*. *Mol. Microbiol.* 89, 532–551

94. Spielvogel, A., Findon, H., Arst, H. N., Araujio-Bazan, L., Hernandez-Ortiz, P., Stahl, U., Meyer, V., and Espeso, E. A. (2008) Two zinc finger transcription factors, CrzA and SitA, are involved in calcium homeostasis and detoxification in *Aspergillus nidulans*. *Biochem. J.* 414, 419–429

95. Steinbach, W. J., Cramer, R. A., Perfect, B. Z., Henn, C., Nielsen, K., Heitman, J., and Perfect, J. R. (2007) Calcineurin inhibition of mutation enhances cell wall inhibitors against *Aspergillus fumigatus*. *Antimicrobial Agents and Chemotherapy* 51, 2979–2981

96. Ries, L. N. A., Rocha, M. C., de Castro, P. A., Silva-Rocha, R., Silva, R. N., Freitas, F. de, Assis, L. J., Bertolini, M. C., Malavazi, I., and Goldman, G. H. (2017) The *Aspergillus fumigatus* CrzA transcription factor activates chitin synthase gene expression during the caspofungin paradoxical effect. *Mbio* 8, e00705–17

97. Winkelströter, L. K., Bom, V. L. P., de Castro, P. A., Ramalho, L. N. M., Goldman, M. H. S., Brown, N. A., Rajendran, R., Ramage, G., Bowier, E., dos Reis, T. F., Savoldi, M., Hagiwara, D., and Goldman, G. H. (2015) High osmolarity glycerol response PtcB phosphatase is important for *Aspergillus fumigatus* virulence. *Mol. Microbiol.* 96, 42–54

98. Sacristán-Reviriego, A., Martín, H., and Molina, M. (2015) Identification of putative negative regulators of yeast signaling through a screening for protein phosphatases acting on cell wall integrity and mating MAPK pathways. *Fungal Genetics Biol.* 77, 1–11

99. Du, Y. R., Walker, L., Novick, P., and Ferro-Novick, S. (2006) Ptc1p regulates cortical ER inheritance via Sit2p. *EMBO J.* 25, 4413–4422

100. Stanger, K., Gorelik, M., and Davidson, A. R. (2012) Yeast adaptin protein, Nbp2p, is conserved regulator of fungal Ptc1p phosphatases and is involved in multiple signaling pathways. *J. Biol. Chem.* 287, 22213–22214

101. Kassis, S., Melhuish, T., Annan, R. S., Chen, S. L., Lee, J. C., Livi, G. P., Kedersha, N., Kundu, D. J., Inuganti, A., Griss, J., Mayer, G., Eisenacher, M., Niedermann, T., Bader, C., and Garbe, M. (2011) Comprehensive analysis of the *Saccharomyces cerevisiae* cell wall stress response. *EMBO J.* 30, 1285–1299

102. Zhou, X. G., Ye, J., Zheng, L. K., Jiang, P., and Lu, L. (2019) A new identified suppressor of Cdc7p/Sept2 kinase, PomA, regulates fungal asexual reproduction via affecting phosphorylation of MAPK-HogA. *Plos Genetics* 15, e1008206

103. Raitt, D. C., Posas, F., and Saito, H. (2000) Yeast Cdc42 GTPase and Ste20 PAK-like kinase regulate Sho1-dependent activation of the Hog1 MAPK pathway. *EMBO J.* 19, 4623–4631

104. Biwa, A., Jia, Y. H., Poh, S. L., Caruruti, R. K. M., den Elzen, B., Peng, X., Zheng, L. L., O’Connell, M., Liu, E. T., Balasubramanian, M. K., and Liu, J. H. (2005) Systematic deletion analysis of fission yeast protein kinases. *Eukaryotic Cell* 4, 799–813

105. Zhou, X. G., Ye, J., Zheng, L. K., Jiang, P., and Lu, L. (2019) A new identified suppressor of Cdc7p/Sept2 kinase, PomA, regulates fungal asexual reproduction via affecting phosphorylation of MAPK-HogA. *Plos Genetics* 15, e1008206