

Comparative proteomic analysis of an *Aspergillus fumigatus* mutant deficient in glucosidase I (Afcwh41)

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α -Glucosidase I regulates trimming of the terminal α -1,2-glucose residue in the *N*-glycan processing pathway, which plays an important role in quality control systems in mammalian cells. Previously, we identified the gene encoding α -glucosidase I in the opportunistic human fungal pathogen *Aspergillus fumigatus*, namely *Afcwh41*. Deletion of the *Afcwh41* gene results in a severe reduction of conidia formation, a temperature-sensitive deficiency of cell wall integrity, and abnormalities of polar growth and septation. An upregulation of the genes encoding Rho-type GTPases was also observed, which suggests activation of the cell wall integrity pathway in the mutant. Using 2D gel analysis, we revealed that the proteins involved in protein assembly, ubiquitin-mediated degradation and actin organization are altered in the Δ *Afcwh41* mutant. Evidence was obtained for a defect in the polarized localization of the actin cytoskeleton in the mutant. Our results suggest that blocking of the glucose trimming in *A. fumigatus* might induce accumulation of misfolded proteins in the endoplasmic reticulum; these misfolded proteins are probably required for cell wall synthesis and thus activate the cell wall integrity pathway, which then causes the abnormal polarity associated with the Δ *Afcwh41* mutant.

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INTRODUCTION

Aspergillus fumigatus is the most common opportunistic fungal pathogen of humans, causing fatal invasive aspergillosis (IA) among immunocompromised patients (Latgé, 1999, 2001; Krappmann, 2006). The mortality from IA is above 90%, falling to around 50–70% if treatment is given (Steinbach *et al.*, 2003). The main reason for patient death is the low efficacy of the drug therapies available to treat IA. The *A. fumigatus* cell wall provides both protective and aggressive functions, and thus has been recognized for a long time as an essential and unique specific drug target. The cell wall mainly consists of a covalently connected polysaccharide skeleton interlaced and coated with glycoproteins and GPI (glycosylphosphatidylinositol) proteins, which contain *N*- and *O*-glycans derived primarily from the process of glycosylation (Latgé, 2007). Although these glycoproteins are involved in morphogenesis and cell wall organization (Mouyna *et al.*, 2000, 2005; Bruneau *et al.*,

2001; Chabane *et al.*, 2006; Romano *et al.*, 2006; de Groot *et al.*, 2005; Li *et al.*, 2007), it is poorly understood how glycosylation affects the cell wall organization.

N-Glycan trimming occurring in the endoplasmic reticulum (ER) is one of the most important mechanisms to assure quality control (QC) of glycoproteins in mammalian cells. This QC system, also known as the calnexin–calreticulin cycle, is composed of calnexin, calreticulin, UDP-glucose:glycoprotein glucosyltransferase (GT) and glucosidase II. It has been shown that the calnexin–calreticulin cycle is essential for survival (Helenius & Aebi, 2004; Ruddock & Molinari, 2006). In contrast to mammalian cells, *Saccharomyces cerevisiae* lacks a calnexin and GT, and only has an effective mannosidase I-dependent ERAD (ER-associated degradation) system (Parodi, 2000; Jakob *et al.*, 1998). Filamentous fungi are thought to possess an *N*-glycan-dependent QC system similar to mammalian cells (Banerjee *et al.*, 2007). Indeed calnexin (AAS68033) and GT have been annotated in the latest release of the *A. fumigatus* genomic database (Galagan *et al.*, 2005). Therefore, filamentous fungi are ideal models to investigate the mechanism of glycoprotein folding in eukaryotic cells.

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Abbreviations: AMBP, actin monomer binding protein; CWI, cell wall integrity; ER, endoplasmic reticulum; ERAD, ER-associated degradation; GPI, glycosylphosphatidylinositol; GT, UDP-glucose, glycoprotein glucosyltransferase; QC, quality control.

N-Glycan processing is initiated by the action of α -glucosidase I on the outermost glucose residue. The yeast α -glucosidase I (Cwh1p) is encoded by the *CWH41* gene (Romero *et al.*, 1997). Mutation in the *CWH41* gene only results in selective instability of the glycoprotein Kre6p, which is a Golgi glucan synthase required for β -1,6-glucan synthesis (Ram *et al.*, 1994; Jiang *et al.*, 1996; Abeijon & Chen, 1998). As compared with yeast, deletion of the *Afcwh41* gene in *A. fumigatus* causes more severe phenotypes, including a significant reduction (50% decrease) in conidia formation at the permissive temperature (37 °C) and a severe reduction (99% decrease) in conidia formation at the restrictive temperature (50 °C), as well as defective cell wall integrity at the restrictive temperature. Abnormalities of polar growth and septation were also documented in the mutant (Zhang *et al.*, 2008). These findings suggest that glucose trimming is essential in cell wall synthesis and morphogenesis in *A. fumigatus* and thus more important than that in yeast. To further investigate the molecular basis of the phenotypes associated with the Δ *Afcwh41* mutant, a comparative proteome analysis was carried out in this study.

METHODS

Strains and growth conditions. *Aspergillus fumigatus* strain YJ-407 was maintained on potato glucose (2%) agar slants (Xia *et al.*, 2001). *A. fumigatus* strain Δ *Afcwh41* (*Afcwh41::pyrG⁺*) was constructed as previously described (Zhang *et al.*, 2008) and propagated at 37 °C on complete medium or minimal medium with 0.5 mM sodium glutamate as a nitrogen source (Cove, 1966). Uridine and uracil were added at 5 mM when the Δ *Afcwh41* strain was cultivated. Mycelium was harvested from strains grown in complete liquid medium at 37 °C with shaking at 250 r.p.m.

Protein extraction. Mycelium cultivated at 37 °C for 24 h with shaking (250 r.p.m.) was filtered through Whatman no. 3 MM paper, rinsed four times with superpure H₂O (Ω >18.0) and ground in pre-cooled liquid nitrogen; aliquots were stored at -80 °C until used.

TCA/acetone precipitation was carried out as described by Kniemeyer *et al.* (2006). Briefly, 400 mg homogenate was precipitated overnight with 4 ml acetone/13.3% (w/v) trichloroacetic acid/0.093% (v/v) 2-mercaptoethanol. After centrifugation, the pellet was rinsed in ice-cold acetone containing 0.07% (v/v) 2-mercaptoethanol, air-dried at room temperature and resuspended in 2 ml lysis buffer [8 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% Triton X-100, 0.8% (v/v) ampholytes, 20 mM Tris and 65 mM DTT]. After a brief sonication, the supernatant was collected by centrifugation, quantified with the Quantify kit (GE Healthcare) and stored at -80 °C or -20 °C for further use. The experiment was repeated twice.

2D-PAGE proteomic analysis of the mutant. The protein samples were mixed with rehydration solution. Immobilized dry strips pH 3–10 of 18 cm length were rehydrated passively for 1.5 h in rehydration solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 1% Triton X-100, 20 mM Tris, 0.002% bromophenol blue, 65 mM DTT and 0.8% IPG buffer. Then the IPG strips were rehydrated actively in an IPGphor system for 6 h at 50 V and for 6 h at 100 V. After rehydration, isoelectrofocusing separation was carried out using the following programming steps: 300 V for 4 h, 600 V for 3 h 50 min, 1000 V for 3 h 50 min, gradient 8000 V, 20 000 V h, to achieve a total migration of 28.6 kV h. The IPG strips were then equilibrated in

0.375 M Tris/HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue containing 130 mM DTT for 15 min followed by equilibration buffer containing 2.5% iodoacetamide for 15 min. Equilibrated IPG strips were loaded on the top of 12.5% acrylamide gels and the standard proteins were added at the acidic side. The slab gel was run at 10 mA constant current for 15 min, and then at 20 mA per slab constant current for 6 h at 16.5 °C. The 2D gels were fixed for at least 1 h in fixing solution containing 40% methanol and 7% glacial acetic acid. Then they were stained overnight with gentle shaking in staining solution (10% methanol and 0.1% colloidal Coomassie brilliant blue R350). Finally, the slab gels were destained for about 2 h in destaining solution. The destained gels were stored at -4 °C in fresh film.

Image analysis and spot processing. The 2D-PAGE protein patterns were recorded as digitized images with a Powerlook 1120 scanner (Bio-Rad). Spot detection, quantification and analysis were performed using ImageMaster 2D Platinum version 6.0 (GE Healthcare). To correct for differences in sample loading or staining intensity among gels, the 'total quantity in valid spot' normalization method was used. The duplicate gels of the same sample were grouped together with the 'replicate groups' function that allows the average quantities of their protein spots to be determined.

Protein identification. Protein spots which showed statistically significant differences of fourfold or more in their mean spot volume on all three duplicate gels made from the same culture, or which were present/absent in the mutant, were excised from the gels manually. The gel pieces were washed twice with 0.1 M NH₄HCO₃/50% acetonitrile to remove excess Coomassie brilliant blue and dried completely by speed vacuum. The peptides in the gels were digested with sequencing-grade trypsin (Roche) for 16 h at 37 °C. The peptides were extracted from the gels with 50% acetonitrile and 5% trifluoroacetic acid several times, pooled together and then dried in a speed vacuum.

Dried tryptic peptide mixtures were dissolved in 3 μ l of a saturated solution of α -cyano-4-hydroxycinnamic acid in 50% acetonitrile and were spotted on a MALDI plate. MS and MS² spectra were acquired with a MALDI-TOF-TOF mass spectrometer (Applied Biosystems 4700 Proteomics Analyser) in the positive reflectron mode with a 200 Hz Nd-YAG 355 laser. Conversion of time-of-flight (TOF) to mass (Da) for the monoisotopic ions [M+H]⁺ was based on calibration of the instrument with a peptide standard kit (Applied Biosystems) that included des-Arg¹-bradykinin (*m/z* 904), angiotensin I (*m/z* 1296), Glu¹-fibrinopeptide B (*m/z* 1570), adrenocorticotrophic hormone (ACTH) (1–17, *m/z* 2093), ACTH (18–39, *m/z* 2465) and ACTH (7–38, *m/z* 3657). The resulting MS (PMF) and MS² spectra were submitted to a MASCOT peptide mass fingerprint search using the MASCOT software incorporated in the Applied Biosystems GPS v3.5 Explorer. A local MASCOT server was used for searching a database built from the annotated *A. fumigatus* ORF set in the TIGR database (<http://www.tigr.org/tdb/e2k1/afu1/>).

Proteins secreted by the mutant. The *A. fumigatus* strains were incubated in minimal liquid medium at 37 °C at 250 r.p.m. for 48 h. The secreted proteins in the culture medium were dialysed against double-distilled H₂O at 4 °C overnight and then lyophilized; this process was repeated. The pellet was then resuspended in water, and run on a 12% SDS-PAGE gel. After staining with Coomassie brilliant blue R-250, the protein bands of interest were cut out and analysed by liquid chromatography/tandem MS (LC-MS/MS) (Thermo Finnigan).

The gel pieces were destained and submitted to in-gel digestion as described by Gharahdaghi *et al.* (1999). The digested peptide mixtures for each sample were desalted, dissolved in 20 μ l 0.1% formic acid, and loaded on a reversed-phase column (BioBasic C18, 300 Å, 5 μ m silica, 180 μ m \times 10 cm; ThermoHypersil). The flow rate was

maintained at $100 \mu\text{l min}^{-1}$ before splitting and at $1.0 \mu\text{l min}^{-1}$ after the flow split. The gradient was started at 5% acetonitrile in 0.1% formic acid for 20 min, then ramped to 50% acetonitrile in 80 min, and finally ramped to 95% acetonitrile for an additional 20 min. The resolved peptides were subjected to MS/MS analysis with an LCQ Deca XP Plus ion-trap mass spectrometer (Thermo Finnigan) equipped with a nanospray source.

All MS/MS spectra were searched using Thermo Finnigan Bioworks 3.1 against the protein sequence database of *A. fumigatus* downloaded from NCBI (<http://www.ncbi.nlm.nih.gov/>) containing 21 395 protein entries, with a static modification of +57.0215 Da on cysteine residue and a differential modification of 15.9994 on methionine. The precursor ion mass tolerance was 1.4 Da and the fragment ion mass tolerance was 1.5 Da. We used the following three steps to perform the data processing. First, we used the following SEQUEST criteria to perform an initial filtration: $\Delta\text{Cn} \geq 0.1$; $\text{Rsp} = 1$; $\text{Xcorr} \geq 1.9$ for singly charged fragments; $\text{Xcorr} \geq 2.2$ for doubly charged; $\text{Xcorr} \geq 3.75$ for triply charged. Second, we used AMASS v1.17.0.17 (available via e-mail from gaoyouhe@pumc.cn for non-profit users) to further filter the SEQUEST results based on three parameters (Sun *et al.*, 2004; Li *et al.*, 2004): $\text{MatchPct} \geq 60$, $\text{Cont} \geq 40$, $\text{Rscore} < 2.6$. Finally, proteins with two or more spectra approved by AMASS were accepted as positive identifications. If a protein has multiple isoforms or has multiple entries in the databases, we only specify the major form of the protein unless a specific peptide points to a region of the protein that exists in only one of the isoforms.

Cytoskeletal staining. A 10 ml volume of complete liquid medium was inoculated with 10^7 freshly harvested conidia, poured into a Petri dish containing glass coverslips and incubated at 37°C for the time

indicated in each experiment. At the specified times, coverslips with adhering germlings were removed and fixed. Briefly, coverslips were transferred to 3.7% formaldehyde in PBS and incubated for 30 min at room temperature, then washed twice. For cell wall digestion, coverslips were overlaid for 40 min at room temperature with a solution of 10 mg Novozym 234 per ml PBS containing 2% BSA (Esnault *et al.*, 1999). After three washes, coverslips were immersed in absolute ethanol at -20°C for 10 min. After several washes in PBS, the coverslips were incubated for 1 h at room temperature with mouse anti-actin C4 monoclonal antibody (MP Biomedicals) at a 1:400 dilution in PBS-BSA containing 0.5% Nonidet P-40. After four 10 min washes with PBS, coverslips were then stained for 1 h in the dark with Alexa Fluor 488 goat anti-mouse IgG antibody (Invitrogen) at a 1:400 dilution in PBS-BSA. Finally, the coverslips were washed for 10 min with PBS, mounted on glass slides in 25% glycerol and examined under a Leica TCS SP2 microscope.

RESULTS

Proteomic analysis of the $\Delta Afcwh41$ mutant

A comparative proteomic analysis of the $\Delta Afcwh41$ mutant and wild-type was carried out as described in Methods. We detected approximately 700 protein spots with molecular masses of 14–100 kDa and a pI range of 3.0–10.0 on 2D gels. A total of 78 proteins were found to change their expression levels up to fourfold. Seventeen proteins (Fig. 1A, spots 20–36) showed reduced expression and 19

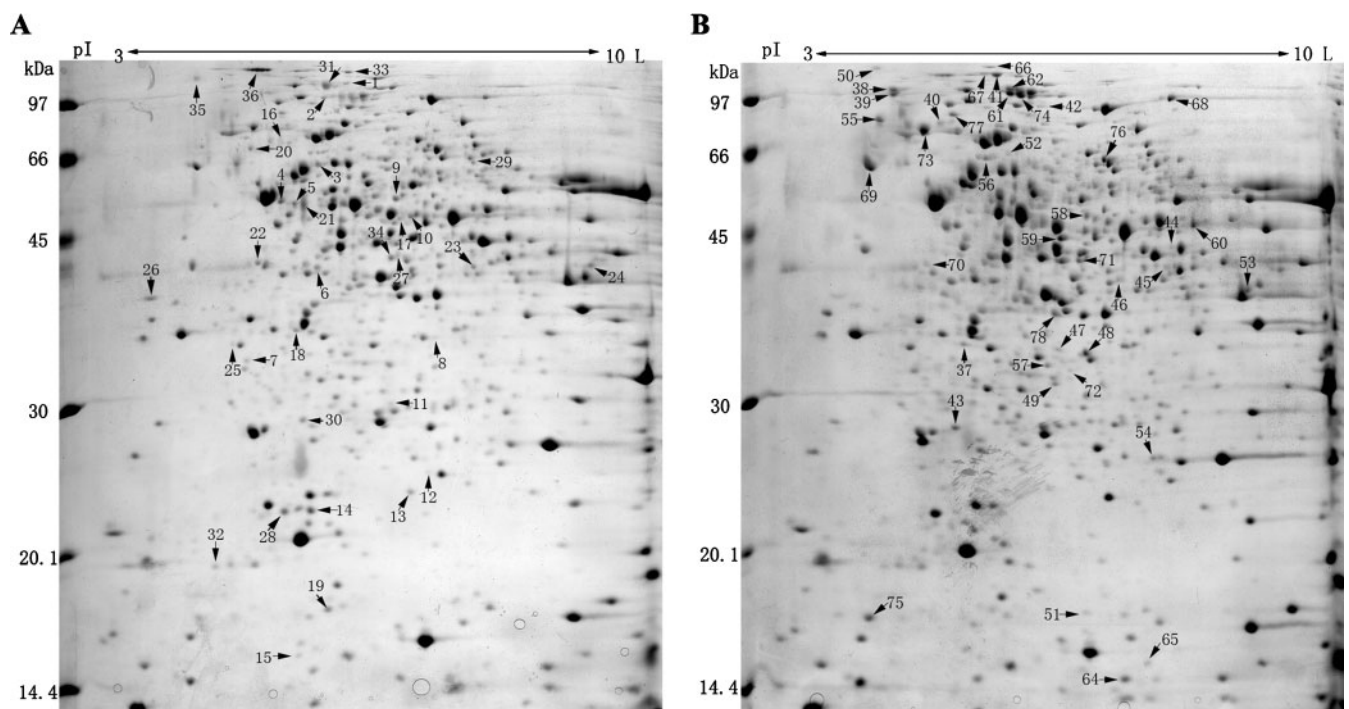


Fig. 1. 2D-PAGE map of proteins in the wild-type (A) and mutant (B) strain. After incubation at 37°C for 24h, the mycelium of the wild-type or mutant strain was harvested and subjected to protein extraction. 2D gel electrophoresis of extracted proteins was performed as described in Methods. The gels were scanned with a Powerlook 1120 scanner (Bio-Rad) at 300 d.p.i. and gel images were analysed by ImageMaster 2D Platinum version 6.0 (GE Healthcare). Differentially expressed proteins were excised from the gels manually and identified with MALDI TOF/TOF and MASCOT.

proteins (Fig. 1A, spots 1–19) were not detected in the mutant. Forty-two proteins (Fig. 1B, spots 37–78) increased their expression levels in the mutant, and 18 new proteins (Fig. 1B, spots 37–54) were induced. Interestingly, some proteins showed the same pI value but different molecular mass, and the molecular mass of some proteins in the mutant was higher than that of their counterparts in the wild-type. Unfortunately, we identified only two of these proteins (spots 35 and 50, spots 70 and 22) by MS, which might be due to the technical difficulty of glycoprotein identification (Oda *et al.*, 2006).

Up to 28 differentially expressed protein spots were identified. As summarized in Table 1, proteins concerned with protein assembly, transport (spots 60, 66 and 77) and ubiquitin-mediated degradation (spots 35 and 51) were overexpressed in the mutant. Spot 25, identified as chitin synthase ChsE, was underexpressed in the mutant, which is consistent with the reduced chitin content in the mutant (Zhang *et al.*, 2008).

We also analysed the proteins secreted by the mutant. To avoid contamination with the proteins contained in the complete medium, we cultivated the mutant in liquid minimal medium at 37 °C for 48 h to obtain adequate secreted proteins for analysis. As shown in Fig. 2, as compared with the wild-type, a significant increase of secretory proteins was observed in culture supernatant of the mutant. However, the proteins that were not detected or were underexpressed inside the mutant were not secreted into the culture supernatant. Interestingly, some GPI proteins involved in cell wall synthesis were secreted by the mutant, such as Gell1, ECM33 and β -1,3-glucanoyltransferase (Mouyna *et al.*, 2000, 2005; Chabane *et al.*, 2006; Romano *et al.*, 2006). As we have previously shown that cell wall mannoprotein, α -glucan, β -glucan and chitin were decreased by 28 %, 16 %, 6 % and 11 %, respectively, in the mutant grown at 37 °C (Zhang *et al.*, 2008), it is reasonable to conclude that the defect in cell wall integrity associated with the mutant is the cause of the release of these GPI proteins, probably due to the decrease in cross-linking of the cell wall structure.

Proteins involved in ER stress

Among the differentially expressed proteins in the mutant, Lhslp and calnexin were overexpressed. Lhslp, a member of the Hsp70 chaperone family, which participates in both polypeptide translocation and subsequent protein folding, is a stress-response protein and is regulated by the unfolded protein response pathway (Craven *et al.*, 1996). Calnexin acts as a chaperone to bind glycoproteins with their *N*-linked oligosaccharide side chains to ensure proper folding and assembly of newly synthesized glycoproteins, and serves a key role in QC in the ER (Hammond *et al.*, 1994). The finding that Lhslp and calnexin were overexpressed in the mutant might suggest that deletion of α -glucosidase I led to an increased accumulation of misfolded proteins in the ER lumen.

Several proteins involved in ubiquitin-mediated degradation were also overexpressed in the mutant, including protease regulatory particle subunit, E3 ubiquitin–protein ligase Bre1 and histone ubiquitination protein. The regulatory particle is a subunit of the proteasome that is responsible for degrading proteins conjugated to ubiquitin and governs the entry of improperly folded proteins into an interior chamber within the particle that contains the proteolytic active sites (Glickman *et al.*, 1998). Thus, it is likely that the overexpression of proteins involved in ubiquitin-mediated degradation was induced by the accumulation of misfolded proteins in the mutant.

Several chemical compounds are known to induce ER stress; these include DTT, tunicamycin and brefeldin A (Back *et al.*, 2005). To gain evidence to support our hypothesis that the Δ Afcwh41 mutation causes ER stress, the *A. fumigatus* wild-type strain was cultivated in the presence of DTT, which unfolds proteins directly by reducing disulfide bonds. As shown in Fig. 3, 18 proteins (Fig. 3A, spots 1–18) showed decreased expression and 24 proteins (Fig. 3B, spots 19–42) showed increased expression in wild-type *A. fumigatus* following DTT treatment. Among these differentially expressed protein spots, 18 proteins were identified. As summarized in Table 2, DTT-induced ER stress led to overexpression of molecular chaperone Hsp70 (spot 20) and proteasome regulatory particle subunit RpnL (spot 33). Although DTT disrupts ER homeostasis by reducing disulfide bonds, a different mechanism from inhibition of *N*-linked glycosylation, our results show that overexpression of proteins belonging to the Hsp70 chaperone family and the protease regulatory particle complex is a feature of ER stress; this indirectly supports our hypothesis that deletion of α -glucosidase I leads to an ER stress in *A. fumigatus*.

Proteins involved in polarity

Protein spots 6 and 34 were identified as actin monomer binding protein (AMBP) and actin-bundling protein Sac6, respectively. The former was not detected and the latter was underexpressed in the mutant. AMBP is essential in cytoskeletal reorganization and in regulating the assembly and disassembly rate of highly dynamic actin. Protein Sac6 cooperates with Scp1p (calponin/transgelin) in the organization and maintenance of the actin cytoskeleton. Since the actin cytoskeleton is both necessary and sufficient for cell polarization and polarized hyphal growth in filamentous fungi (Heath *et al.*, 2000), the aberrant polar growth and septation associated with the mutant might mainly arise from diminished AMBP and Sac6.

To gain evidence to support our hypothesis, the actin skeleton of the mutant was stained. As shown in Fig. 4, during early stages of germination (7 h), the actin patches accumulated in the isotropically swollen conidium and the expanding tip of the germ tube (Fig. 4A, B) in a polarized manner in the wild-type; also the actin ring formed at the neck site of the first germ tube, where the first septation

Table 1. Identification by MALDI-TOF/TOF and MASCOT of proteins differentially expressed in the $\Delta Afcwh41$ mutant of *A. fumigatus*

Spot no.	Protein identification	Theoretical mol. mass (kDa)/pI	Experimental mol. mass (kDa)/pI	Regulation in the mutant*	Function
77	Calnexin	62/4.97	84/5.19	Up	Interacts with newly synthesized glycoproteins in the ER to play a major role in QC by the retention of incorrectly folded proteins
66	Hsp70 family chaperone Lhs1/Orp150	109/5.29	125/5.52	Up	Chaperone activity, response to unfolded protein
35	E3 ubiquitin-protein ligase Bre1	82/6.39	118/4.48	Up	Transcriptional activator through direct activator interactions; chromosome segregation ATPases (cell division and chromosome partitioning)
51	Histone ubiquitination protein Bre1	84/5.95	17/6.42	+	E3 ubiquitin ligase shown to act as a transcriptional activator through direct activator interactions
59	Protease regulatory particle subunit Rpt6	43/8.24	49/6.32	Up	ATPase activity, endopeptidase activity
6	Actin monomer binding protein (AMBP)	34/5.25	42/5.6	-	Actin depolymerization factor that interacts with actin monomers and actin filaments to enhance the turnover rate of actin
34	Actin-bundling protein Sac6	72/5.78	82/6.32	Down	Similar to fimbrin; cooperates with Scp1p (calponin/transgelin) in the organization and maintenance of the actin cytoskeleton
60	Dynactin	153/5.51	51/7.49	Up	ER to Golgi vesicle-mediated transport, protein complex assembly
64	CipC protein	15/5.84	14/5.43	Up	Polarized growth
17	UPF0160 domain protein MYG1	40/5.85	50/6.37	-	This family of proteins contains a large number of metal-binding residues; the patterns are suggestive of a phosphoesterase function
25	Chitin synthase ChsE	205/7.43	35/6.21	Down	Chitin synthase activity
22	GTP-binding protein YchF	43/7.62	43/5.05	Down	YchF is a member of the Obg family, which includes four other subfamilies of GTPases. <i>OBG</i> is an essential gene involved in DNA replication
48	Translation elongation factor EF-2 subunit	93/6.51	34/6.42	+	Translation elongation factor activity
47	Nuclear export protein Noc3	78/5.8	34/6.16	+	Involved in the nuclear export of pre-ribosomes
71	Negative regulation of gluconeogenesis	47/8.69	42/6.36	Up	Negative regulation of gluconeogenesis
68	Cobalamin-independent methionine synthase	87/6.33	100/7.21	Up	5-Methyltetrahydropteroyltrimethylglutamate-homocysteine S-methyltransferase activity
61	Extracellular phytase	73/5.63	106/5.72	Up	Alkaline phosphatase homologue; catalyses the hydrolysis reaction via a phosphoserine intermediate to produce P _i and the corresponding alcohol
56	Aryl-alcohol dehydrogenase	65/5.3	69/5.6	Up	Aryl-alcohol dehydrogenase
58	FAD-dependent oxidoreductase	51/5.7	54/6.52	Up	Fumarate reductase (NADH) activity
24	Mitochondrial enoyl reductase	46/8.24	42/8.13	Up	Enoyl-(acyl carrier protein) reductase
15	Calmodulin-binding protein Sha1	114/9.91	15/5.43	-	A class V myosin Sha1/Myo2p, an essential protein that moves a series of cargos, including secretory vesicles, the vacuole, late compartments of the Golgi, and peroxisomes along the actin track
30	NADH-quinone oxidoreductase, 23 kDa subunit	26/6.63	29/5.55	Down	Similar to NADH dehydrogenase (ubiquinone) (NADH-coenzyme Q reductase)
33	Carbamoyl-phosphate synthase, large subunit	129/5.87	123/5.89	Down	Catalyses the ATP-dependent synthesis of carbamyl phosphate from glutamine or ammonia and bicarbonate
18	Homocysteine S-methyltransferase	34/5.11	36/5.4	Down	Homocysteine S-methyltransferase
43	Conserved hypothetical protein	29/7.74	29/5.47	+	

* '+' and '-' indicate that the protein was only present or absent in the mutant.

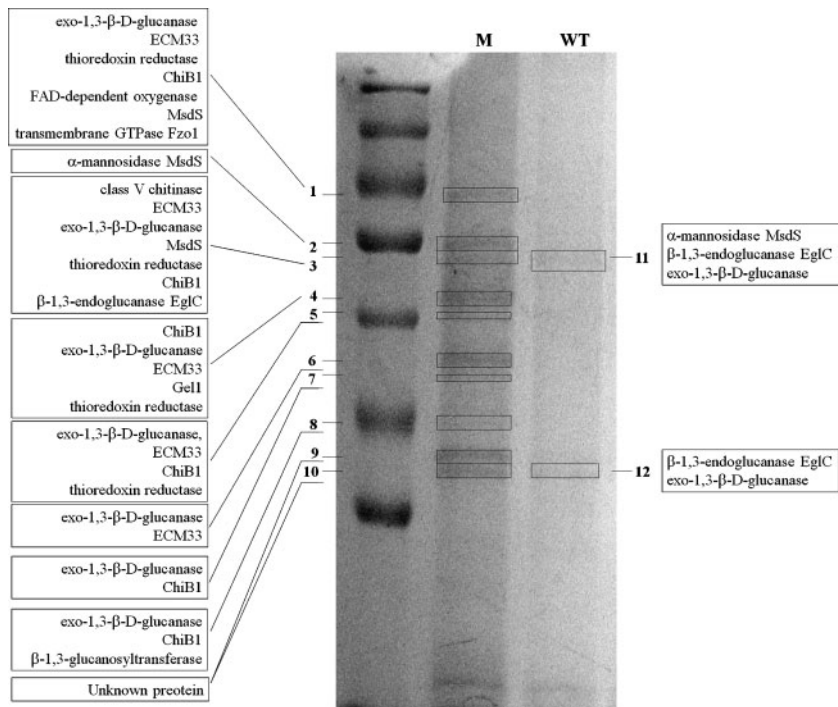


Fig. 2. The proteins secreted by the mutant. The mutant (M) and wild-type (WT) *A. fumigatus* strains were incubated in minimal liquid medium at 37 °C at 250 r.p.m. for 48 h, and the secreted proteins in the culture medium were separated by 12% SDS-PAGE. Twelve bands of protein were cut out and divided based on similar staining intensity, and subjected to MS/MS analysis as described in Methods. Proteins containing at least two peptides detected with MS/MS spectra are shown.

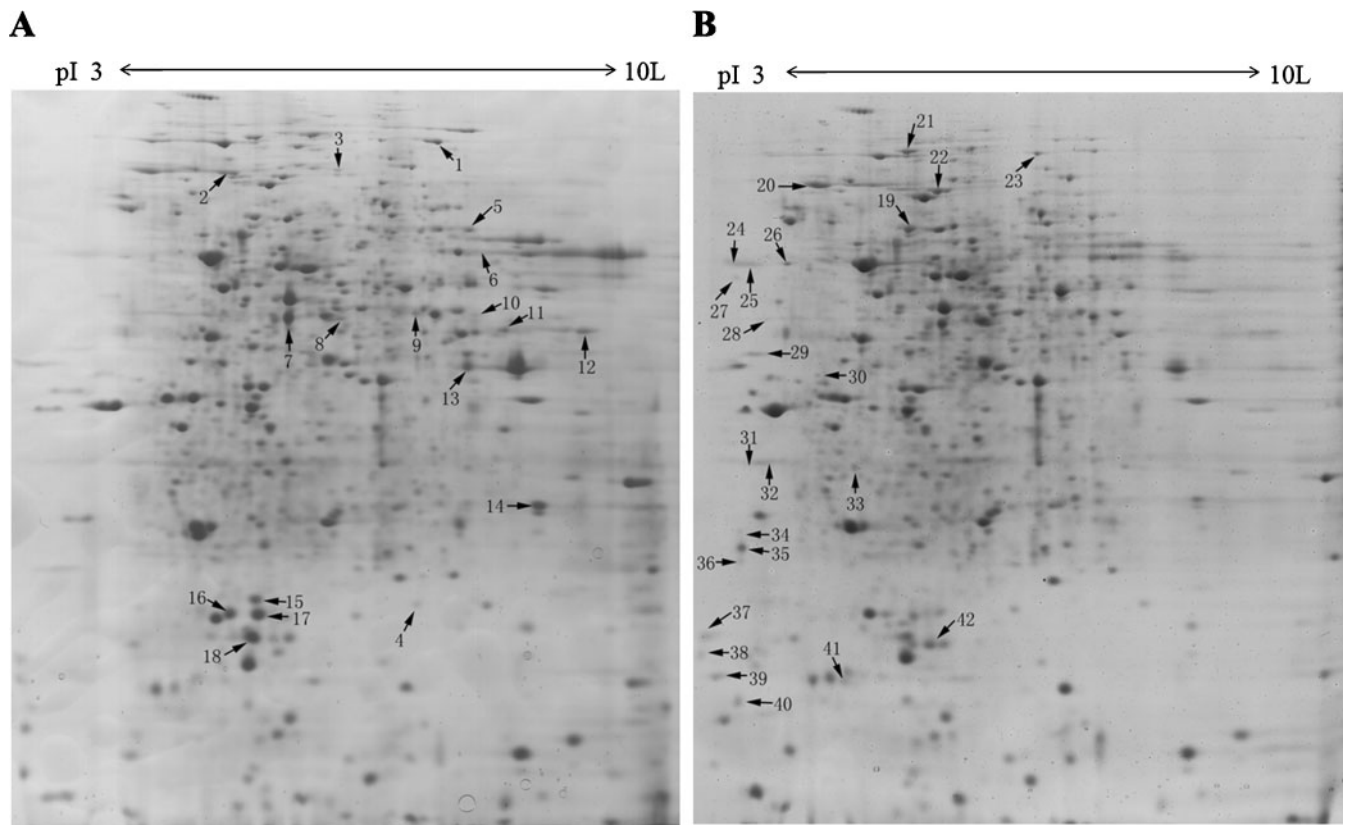


Fig. 3. 2D-PAGE map of proteins in the wild-type cultivated in complete medium without DTT (A) or with 1 mM DTT (B). After incubation at 37 °C for 24 h, the mycelium of the wild-type was harvested and subjected to protein extraction. 2D gel electrophoresis of extracted proteins was performed as described in Methods. Other details as for Fig. 1.

Table 2. Identification by MALDI-TOF/TOF and MASCOT of proteins showing differential expression under DTT-induced ER stress in *A. fumigatus*

A. fumigatus YJ-407 (wild-type) was incubated for 24 h in complete medium with or without 1 mM DTT at 37 °C. The protein extracted from mycelium was separated by 2D-PAGE as described in Methods. Differentially expressed proteins were excised from the gels manually and identified with MALDI TOF/TOF and MASCOT.

Spot no.	Protein identification	Theoretical mol. mass (kDa)/pI	Regulation of protein	Function
7	Glutamine synthetase	40/5.48	Down	Involved in glutamate metabolism, peptidoglycan biosynthesis, nitrogen metabolism
8	Flavo haemoprotein	45/5.72	Down	Nitric oxide dioxygenase
9	NAD-dependent formate dehydrogenase AciA/Fdh	46/8.42	Down	Lactate dehydrogenase and related dehydrogenases
10	Acetyl-CoA-acetyltransferase, putative	45/9.04	Down	Catalyses the reversible thiolytic cleavage of 3-ketoacyl-CoA into acyl-CoA and acetyl-CoA, a two-step reaction involving a covalent intermediate formed with a catalytic cysteine
11	Aspartate transaminase, putative	51/8.60	Down	Aspartate aminotransferase family; this family belongs to the pyridoxal phosphate-dependent aspartate aminotransferase superfamily (fold I).
12	Aspartate aminotransferase, putative	48/8.94	Down	Aspartate aminotransferase family
13	Glyceraldehyde 3-phosphate dehydrogenase GpdA	36/6.96	Down	Essential in glycolysis and gluconeogenesis
14	Hex1	49/6.56	Down	The major component of the Woronin body in filamentous fungi; the Woronin body plays a vital role in cell integrity in filamentous fungi
	Woronin body protein HexA, putative	61/8.83	Down	Woronin body protein HexA
16	Conserved hypothetical protein	21/6.2	Down	Cupin domain protein
	Cupin domain protein	14/4.87	Down	Cupin domain protein
17	Conserved hypothetical protein	21/6.2	Down	Cupin domain protein
	Cupin domain protein	14/4.87	Down	Cupin domain protein
19	DUF967 domain protein	20/6.43	Up	Unknown function
20	Molecular chaperone Hsp70	70/5.09	Up	ATP-dependent stabilization of hydrophobic regions in extended polypeptide segments
21	Hsp70 family chaperone Hsp88	80/5.08	Up	Conformational maturation and unfolding for degradation
22	Mitochondrial Hsp70 chaperone (Ssc70), putative		Up	Molecular chaperone DnaK, a ubiquitous protein involved in the formation of filaments that are a major component of the cytoskeleton; interaction with myosin provides the basis of muscular contraction and many aspects of cell motility
23	Mitochondrial aconitate hydratase, putative	86/6.26	Up	Aconitase (also known as aconitate hydratase and citrate hydrolyase) catalyses the reversible isomerization of citrate and isocitrate as part of the TCA cycle.
26	NAP family protein	41/4.4	Up	Nucleosome assembly protein (NAP)
33	Proteasome regulatory particle subunit (RpnL), putative	31/4.90	Up	Similar to 26S proteasome regulatory subunit RPN12 of <i>S. cerevisiae</i>
42	ADP-ribosylation factor, putative	21/5.5	Up	Arf1-Arf5-like subfamily; this subfamily is crucial for assembling coat proteins during vesicle formation

occurred (Fig. 4B). By contrast, in the mutant the actin patches were mainly visualized in the isotropically swollen conidium and no obvious accumulation was found in the first and second germ tubes, especially at their growing tips (Fig. 4E, F). At the hyphal elongation stage (24 h), the accumulation of actin patches was found at the expanding tip (Fig. 4C, D) and septation site (Fig. 4D) in the wild-type, suggesting a polarized localization of the actin

skeleton. In contrast to the wild-type, in the mutant the actin patches were randomly or evenly distributed in the hyphae during hyphal elongation (Fig. 4G, H). No obvious actin patch accumulated at the hyphal tip of the mutant. These observations clearly demonstrated that abnormalities of polarity associated with the mutant were due to a failure of polarized localization of the actin cytoskeleton.

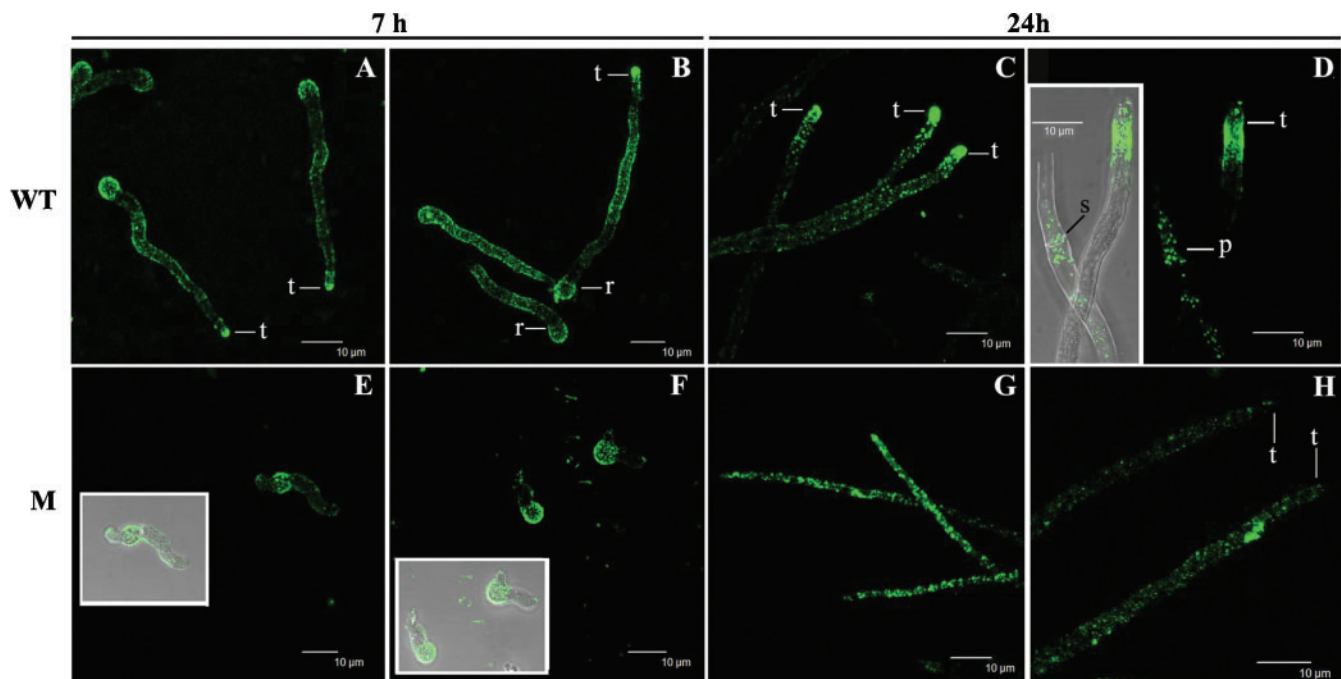


Fig. 4. Actin staining of the mutant during development from a spore to a mature mycelium. A 10 ml volume of complete liquid medium was inoculated with 10^7 freshly harvested conidia, poured into a Petri dish containing glass coverslips and incubated at 37 °C for 7–24 h. At the specified times, coverslips with adhering germlings were removed and fixed as described in Methods. The coverslips were incubated for 1 h at room temperature with mouse anti-actin C4 monoclonal antibody (MP Biomedicals) at a 1 : 400 dilution in PBS-BSA containing 0.5% Nonidet P-40 and then stained for 1 h in the dark with Alexa Fluor 488 goat anti-mouse IgG antibody (Invitrogen) at a 1 : 400 dilution in PBS-BSA. Finally, the coverslips were examined under a Leica TCS SP2 microscope. WT, wild-type; M, mutant; t, hyphal tip; s, septation site; r, actin ring; p, actin patch.

DISCUSSION

In mammalian cells, *N*-linked glycan plays an important role in the QC of the folding of secretory proteins, which ensures that only correctly folded proteins are delivered to the secretory pathway from the ER lumen to the cell surface. When correct folding is not achieved, an ER-specific *N*-glycan-dependent pathway of degradation takes care of the misfolded proteins (Helenius & Aebi, 2004; Ruddock & Molinari, 2006). Once *N*-glycosylation is inhibited, the most commonly observed effect is the generation of misfolded, aggregated proteins that fail to reach a functional state. It has been shown that the QC system of glycoprotein folding is essential for survival of mammalian cells (Mesaali *et al.*, 1999), whereas a yeast mutant devoid of glucose trimming showed only a defect in cell wall β -1,6-glucan synthesis (Ram *et al.*, 1994; Jiang *et al.*, 1996). It appears that the consequence of the added *N*-glycans varies between proteins and species (Weerapana & Imperiali, 2006; Banerjee *et al.*, 2007).

Unlike the case in yeast, the finding of a calnexin (AAS68033) and GT gene in the *A. fumigatus* genome suggests that *A. fumigatus* has evolved a QC system of glycoprotein folding. Previously, we showed that deletion of the *Afcwh41* gene led to an accumulation of two forms

of intracellular AfChiB1 protein in the mutant cell: one is bigger than the wild-type AfChiB1 and the other is the same size as the wild-type protein. The bigger one was *N*-glycosylated by an untrimmed *N*-glycan ($\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$) and some of this form could be secreted by the mutant cells, while the smaller one that is similar to the wild-type AfChiB1 was only found inside the cell and decreased when the mutant was cultivated for a prolonged time (up to 96 h). Thus, we postulated that the smaller protein was probably misfolded AfChiB1 and degraded by ERAD (Zhang *et al.*, 2008). As revealed by 2D-PAGE analysis, Lhslp and calnexin were both overexpressed. Rpt6, E3 ubiquitin-protein ligase Bre1 and histone ubiquitination protein, which are involved in ubiquitin-mediated degradation, were also overexpressed in the mutant. Based on these results, we propose that deletion of the *Afcwh41* gene leads to an ER stress in *A. fumigatus*. Although the mechanisms are different, the overexpression of the Hsp70 chaperone and protease regulatory particle subunit induced by DTT indirectly confirms that blocking of the glucose trimming induces an accumulation of misfolded proteins in the mutant and thus leads to an ER stress. Furthermore, the reduced content of mannoprotein, chitin and glucan in the cell wall of the $\Delta\text{Afcwh41}$ mutant also suggests that the proteins involved

in cell wall biosynthesis in *A. fumigatus* are more dependent on the *N*-glycan-dependent folding system than those in yeast.

It has been shown that in yeast the cell wall integrity (CWI) pathway triggered by cell wall stress is also important for cell growth, bud site assembly and establishment of polarized growth. The yeast CWI signalling pathway comprises a family of cell-surface sensors coupled to a small G-protein Rho1, Rho3 and Cdc42 (Johnson & Pringle, 1990; Matsui & Toh-e, 1992; Johnson, 1999; Levin, 2005). Two cell-surface sensors, Wsc1 (Hcs77/Slg1) and Mid2, have been extensively studied in yeast (Gray *et al.*, 1997; Verna *et al.*, 1997; Jacoby *et al.*, 1998; Ketela *et al.*, 1999; Rajavel *et al.*, 1999). Wsc1 and Mid2 serve a partially overlapping role in detecting and transmitting cell wall status to Rho1. *N*- or *O*-glycans of these two molecules are important for their function (Lommel *et al.*, 2004; Philip & Levin, 2001; Hutzler *et al.*, 2008). Upon environmental stimuli or cell wall stress, Rho1 is activated, which then activates downstream effectors such as the Pkc1-MAP kinase cascade, β -1,3-glucan synthase and Bni1/SepA formin protein (Walther & Wendland, 2003; Longtine & Bi, 2003; Guest *et al.*, 2004). Thus, Rho1 is considered the master regulator of CWI signalling not only because it receives the major inputs from the cell surface but also because it regulates a variety of outputs involved in cell wall biogenesis, actin organization and polarized secretion.

In contrast to yeast, little is known about the cell wall stress sensors and CWI signalling pathway in *A. fumigatus*. Indeed, in the last release of *A. fumigatus* genomic database (<http://www.tigr.org/tdb/e2k1/afu1/>), only one protein (AFUA_5G09020) is annotated as a homologue of Wsc4, which does not appear to contribute to CWI signalling in yeast. Although the *A. fumigatus* cell wall stress sensor molecule remains to be investigated, it is likely that glycosylation of some unknown cell surface sensors is important for their functions. Unfortunately, we failed to identify those differentially expressed glycoproteins in this study. On the other hand, the presence of genes coding for proteins homologous to the yeast Rho1p, Rho3p and Cdc42p suggests a similar mechanism for the CWI pathway in *A. fumigatus*. In our previous study, we showed that *Afcdc42*, *Afrho1* and *Afrho3* are overexpressed in the $\Delta Afcwh41$ mutant (Zhang *et al.*, 2008). It is likely that the abnormal polarity associated with the $\Delta Afcwh41$ mutant could also be induced by the activation of the CWI pathway.

In the $\Delta Afcwh41$ mutant, AMBP was absent and Sac6 was reduced. In addition, a class V myosin Sha1/Myo2p, an essential protein that moves a series of cargos, including secretory vesicles, the vacuole, late compartments of the Golgi and peroxisomes, along the actin track (Fagarasanu *et al.*, 2006), was not detected. These observations are consistent with the lagged septation and random branching associated with the $\Delta Afcwh41$ mutant (Zhang *et al.*, 2008),

suggesting a failure of actin reorganization. We obtained clear evidence for a defect in polarized localization of the actin cytoskeleton in the mutant (Fig. 4). However, the mechanism by which activation of the CWI pathway triggers the aberrant expression of proteins involved in actin reorganization is not clear.

In addition to AMBP and Sac6, several other proteins involved in polarized growth were over- or underexpressed in the mutant. Dynactin, an integral part of the cytoplasmic molecular motor protein dynein which presides over transport of cellular and neuronal vesicles, organelles and other particles along microtubules (Quintyne *et al.*, 1999; Mallik & Gross, 2004), was overexpressed in the mutant. Possibly, the overexpression of the dynactin is triggered by an unknown mechanism to compensate for subcellular organelle transportation. We also observed an elevated expression of CipC protein in the mutant. In *Aspergillus nidulans*, increased branching and reduced growth following treatment with concanamycin, an inhibitor of vacuolar ATPases from *Streptomyces* species, have been related to upregulation of the CipC (Melin *et al.*, 2002). Therefore, the upregulation of CipC protein in the $\Delta Afcwh41$ mutant could be one of factors that contributed to the hyperbranching. Moreover, it is interesting to note that the UPF0160 domain protein MYG1 was diminished in the mutant. Although the function of MYG1 is unclear, MYG1 is a newly identified novel melanocyte-related gene and shows elevated expression in vitiligo (Kingo *et al.*, 2006).

In summary, our results indicate that deletion of the *Afcwh41* gene induces overexpression of proteins involved in protein assembly and degradation, underexpression of proteins involved in actin organization, and overexpression of proteins involved in subcellular organelle transportation and hyphal branching. Based on our observations, we propose that the proteins that are required for cell wall synthesis or cell wall stress sensing are substrates of *Afcwh41* and require glucose trimming for their proper localization and function. Misfolding of these proteins would cause cell wall defects, which would then lead to activation of the Rho-type GTPases-mediated CWI pathway and thus cause abnormalities of polarized growth in *A. fumigatus*. Obviously, an understanding of the role of the *Afcwh41* will depend on identification of key substrates of *Afcwh41* and their roles in these processes.

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