

Afcwh41* is required for cell wall synthesis, conidiation, and polarity in *Aspergillus fumigatus

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Introduction

In a mammalian cell, N-glycan trimming in the endoplasmic reticulum (ER) is one of the most important mechanisms to assure quality control (QC) of glycoproteins, which is composed of calnexin, calreticulin, UDP-glucose: glycoprotein glucosyltransferase, and glucosidase II, and is essential for survival (Mesaeli *et al.*, 1999; Helenius & Aebi, 2004; Ruddock & Molinari, 2006). Unlike the mammalian cells, *Saccharomyces cerevisiae* lacks a calnexin cycle and glucosyltransferase, and only has an effective mannosidase I-dependent ERAD (ER-associated degradation) system (Jakob *et al.*, 1998; Parodi, 2000). In contrast to *S. cerevisiae*, some filamentous fungi are proposed to possess N-glycan-dependent QC of glycoprotein folding (Banerjee *et al.*, 2007). However, the consequence of the N-glycan trimming in these species is still unclear.

The trimming of N-glycan is initiated by the action of glucosidase I. Blocking of the outermost glucose removal causes accumulation and degradation of some glycoproteins in the ER (Datema *et al.*, 1987; Elbein, 1991). An inherited glucosidase I deficiency has been reported to be involved in a neonate born with severe generalized hypotonia and dys-

Abstract

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 the quality control system in mammalian cells. However, the consequence of glucose trimming of the N-glycan in filamentous fungi is unclear. We identified the gene encoding α -glucosidase I in the human opportunistic fungal pathogen *Aspergillus fumigatus*, namely *Afcwh41*. Deletion of the *Afcwh41* gene resulted in a defective N-glycan processing of the proteins secreted by *A. fumigatus*. Although the *Afcwh41* was not essential for hyphal growth and virulence, a severe reduction in conidia formation and a temperature-sensitive deficiency of cell wall integrity (CWI) were observed. Also, abnormalities of polar growth and septation were observed during conidial germination and hyphal elongation of the mutant. Our results suggest that *Afcwh41* was involved in CWI, polarity, septation, and conidiation in *A. fumigatus*, probably by affecting the proper function of the proteins that are required for cell wall synthesis.

morphic features (de Praeter *et al.*, 2000). In yeast, glucosidase I (Cwh1p) is encoded by the *CWH41* gene (Romero *et al.*, 1997). Mutational defects in the *CWH41* gene cause severe and selective instability of a glycoprotein Kre6p, a putative Golgi glucan synthase (GS) required for β -1,6-glucan synthesis (Ram *et al.*, 1994; Jiang *et al.*, 1996; Abeijon & Chen, 1998).

Aspergillus fumigatus is known to cause fatal invasive aspergillosis (IA) among immunocompromised patients (Latgé, 1999; Steinbach *et al.*, 2003). It has been shown that the structures of the N-glycans on proteins secreted by *A. fumigatus* are mostly Man₈GlcNAc and Man₉GlcNAc, and the addition of castanospermine, a specific inhibitor for glucosidase I, leads to the presence of Glc₃Man₇GlcNAc and Glc₃Man₈GlcNAc on mature glycoproteins (Elbein *et al.*, 1984). Indeed, this is the only report of N-glycan trimming in *A. fumigatus* so far. Because many glycoproteins are directly or indirectly involved in the synthesis and organization of the fungal cell wall, which is essential for the fungal viability, it therefore represents a unique specific target for antifungal drug development; it is of importance to assess the roles of glucose trimming in cell wall synthesis in *A. fumigatus*. Here, we report the

identification, deletion, and phenotypic analyses of the *Afcwh41* gene in *A. fumigatus*.

Materials and methods

Strains and growth conditions

Aspergillus fumigatus strain YJ-407 (China General Microbiological Culture Collection Center, CGMCC0386) was maintained on potato glucose (2%) agar slant (Xia *et al.*, 2001). *Aspergillus fumigatus* strain CEA17 (Weidner *et al.*, 1998), a kind gift from C. d'Enfert, Institute of Pasteur, France, was propagated at 37 °C on YGA (0.5% yeast extract, 2% glucose, and 1.5% Bacto agar), complete medium (Cove, 1966), or minimal medium with 0.5 mM sodium glutamate as a nitrogen source (Cove, 1966). Uridine and uracil were added to the medium at a concentration of 5 mM when CEA-17 or complemented strain was grown. Mycelium was harvested from strains grown in complete liquid medium at 37 °C with shaking at 250 r.p.m. At the specified culture time point, mycelium was harvested and washed with distilled water, and then frozen in liquid N₂ and ground by hand. The powder was stored at -70 °C for DNA, RNA, and protein extraction. Conidia were prepared by growing *A. fumigatus* strains on solid complete medium with uridine and uracil (CMU) for 48 h at 37 °C. The spores were collected, washed twice with 0.01% Tween-20 in physiological saline and resuspended in 0.01% Tween-20 in saline, and its concentration was confirmed using haemocytometer counting and viable counting. Vectors and plasmids were propagated in *Escherichia coli* DH5 α (BRL).

Molecular cloning of *A. fumigatus Afcwh41*

The *Afcwh41* genomic sequence was identified in a search of the *A. fumigatus* genome database (<http://www.tigr.org/tldb/e2k1/afu1/>), using a TBLASTN program for sequences corresponding to the conserved amino acid sequences of α -glucosidase I that were homologous between human, mice, yeast, and *Neurospora crassa*. A 2.8-kb genomic DNA fragment was found to contain the entire ORF for the glucosidase I homologue and designated as *Afcwh41*, which encodes for a putative 93-kDa protein that has an overall protein sequence homology of 56% and an identity of 36% to the yeast Cwh41p. Based on the nucleotide sequence, the forward primer (5'-ATGCATTCCCCGAACTTACGTT-3') and the reverse primer (5'-TTACAACATCATGGCCATATC-3') were designed for cloning of the cDNA of the *Afcwh41* (AY461807) using PCR. The PCR products were subcloned into pGEM-T easy Vector (Promega) and sequenced. The position of the intron was determined by comparing the cDNA with the genomic sequence.

Construction of the $\Delta Afcwh41$ mutant and complemented strain

To delete *Afcwh41*, a deletion construct was designed to replace the entire coding region of *Afcwh41* with a *pyrG* cassette by homologous recombination (d'Enfert *et al.*, 1996). PCR primers were designed to amplify a 2.0-kb upstream noncoding region of the *Afcwh1* before the ATG start codon (5' primer pair 5'-GGTGGTGC GGCCGCTTCTGACTGCCCCGATAT-3' and 5'-GGTGGTTCTAGAGACCCGATCTTGGCGCTCTTT-3') and a 2.0-kb downstream noncoding region of the *Afcwh1* after the stop codon (3' primer pair 5'-GGTGGTTCTAGATTTGTGAAGAATCGGCCACTACT-3' and 5'-GGTGGTGGTACCACAGTGGCGGCCGCGCGGATTT-3'). The upstream and downstream noncoding regions were digested with NotI/XbaI and XbaI/KpnI, respectively, and then cloned into the relevant sites of pBlueScript II SK (Stratagene). The *pyrG* blaster cassette (8.6 kb) released by the digestion of pCDA14 (d'Enfert *et al.*, 1996) with HpaI was cloned into the site between the up- and downstream noncoding regions of the *Afcwh41*, to yield the deletion construct pAFGI-pyrG. At a unique NotI site, the linearized pAFGI-pyrG was transformed into strain CEA17 by protoplast transformation (Yelton *et al.*, 1984) and screened for mutants with uridine and uracil autotrophy. The deletions in the mutants were confirmed using PCR and Southern blotting (Fig. 1).

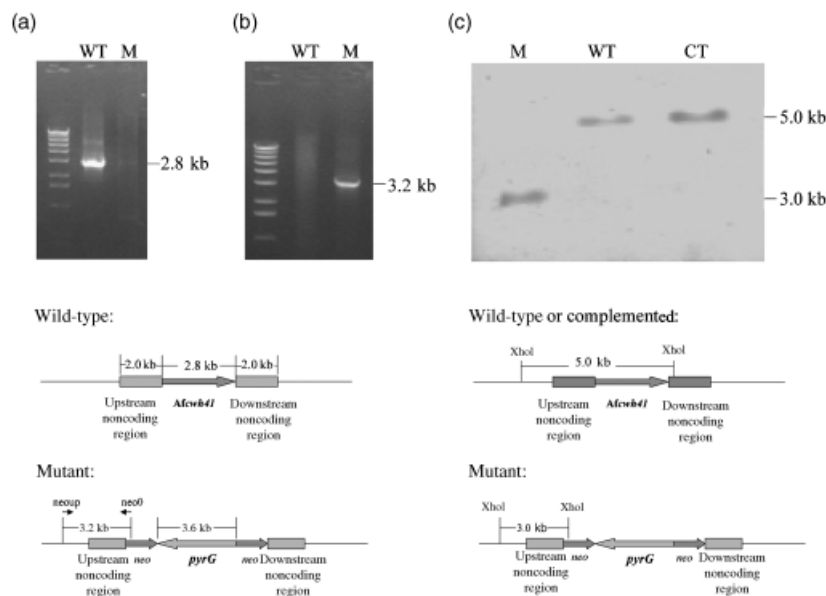
The complemented strain was constructed by the replacement of *pyrG* with a wild-type copy of *Afcwh41* in the $\Delta Afcwh41$ mutant. *Afcwh41*, with its 2.0 kb upstream and 2.0 kb downstream noncoding regions, was amplified using PCR (primer pair 5'-GGTGGTGC GGCCGCTTCTGACTGCCCCGATAT-3' and 5'-GGTGGTGGTACCACAGTGGCCGCGCCGCGGAGTTT-3'). The product was cloned into pGEM-T Easy Vector and sequenced. The transformants were chosen using PCR, and then the transformation was confirmed using Southern blot analysis, using the upstream noncoding region as a probe (Fig. 1c). The probe was labelled by following the protocol of the DIG-labelled hybridization kit (catalogue no. 1093657; Roche Applied Science).

Phenotypic analyses of the mutant

Growth kinetics of *A. fumigatus* strains were assayed as follows: 1×10^8 spores were inoculated into 100 mL liquid CMU medium. After incubation at 37 °C with shaking (250 r.p.m.), three 1 mL aliquots of liquid were taken for each strain at set time intervals, and dried and weighed. The mean weight was used to plot the growth kinetics. The experiment was repeated three times.

To test the sensitivities of the mutant to antifungal reagents, conidiospores were collected from the wild type, the mutant, and the complemented strain, and, for each strain, similar numbers of conidiospores were spotted on

Fig. 1. Confirmation of the $\Delta Afcwh41$ mutant and complemented using PCR (a and b) and Southern blot (c). (a) A pair of primers were used to generate the 2.8-kb *Afcwh41* gene by PCR as described in Materials and methods. (b) A pair of primers (neop: 5'-AGGAAGGCTTTGTGTTT GATC-3' and neo0: 5'-GGTTCAGCTGCTGCCT GAGGCTGGACG-3') were used to generate the mutant 3.2-kb fragment that includes the 2.0-kb upstream noncoding regions of the *Afcwh41* and a fragment of *neo*. (c) Genomic DNA digested with *Xho*I was probed with an upstream noncoding region of the *Afcwh41*. The electrophoretic positions and sizes of DNA are indicated both in (a) and (b). M, mutant; WT, wild type; CT, complemented.



CMU plates in the presence of 250 μg Calcofluor white mL^{-1} or 250 μg Congo red mL^{-1} . After incubation at 37 or 50 °C for 24–48 h, the plates were taken out and photographed.

For examination of conidial germination, 10 mL complete liquid medium was inoculated with 10^7 freshly harvested conidia, poured into a Petri dish containing a glass coverslip, and incubated at 37 °C for the time indicated in each experiment. At the specified times, the coverslips with adhering germlings were removed, and spore germination was observed and counted under a differential interference contrast microscope.

For examination of nuclei, septa, and cell-wall staining at the germination stage, the coverslips with adherent germlings were removed and fixed in a fixative solution (4% formaldehyde, 50 mM phosphate buffer, pH 7.0, and 0.2% Triton X-100) for 30 min. The coverslips were then washed with phosphate-buffered saline (PBS), incubated for 15 min with 1 mg 4',6-diamidino-2-phenylindole (DAPI) mL^{-1} (Sigma), washed with PBS, then incubated for 5 min with a 10 mg mL^{-1} solution of fluorescent brightener 28 (Sigma), washed again, and the germlings were photographed using a microscope.

For examination of nuclei, septa, and cell wall staining at the conidiation stage, 100 mL complete liquid medium was inoculated with 1×10^6 conidia and incubated with shaking (200 r.p.m.) at 37 °C for 17 h. The mycelia were taken and placed on a glass coverslip. The glass coverslip was then placed in a Petri dish containing two layers of filter paper saturated with complete liquid medium. After incubation at 37 °C for 2–8 h, the coverslip was removed and stained with DAPI and a fluorescent brightener.

A 100 mL volume of complete liquid medium was inoculated with 1×10^8 freshly harvested conidia and incubated at

37 or 50 °C with shaking (250 r.p.m.) for 12 h. Mycelia were harvested by filtering the culture through two layers of Miracloth (Calbiochem), washed twice with distilled water, and streaked on minimal medium agar. After incubation at 37 or 50 °C for 2, 4, 6, and 8 h, mycelia were suspended in 5 mL distilled water, and conidial production was expressed as the mean number of conidia per volume (millilitre).

Chemical analysis of cell wall

Conidia were inoculated into 100 mL complete medium at a concentration of 1×10^6 conidia mL^{-1} and incubated with shaking (250 r.p.m.) at 37 °C for 28 h or at 50 °C for 60 h. The mycelium was harvested by filtering the culture through two layers of Miracloth (Calbiochem), washed twice with distilled water, and lyophilized. Three aliquots of 10 mg dry mycelium were used as independent samples for cell wall analysis, and the experiment was repeated twice. To remove unbound cell wall proteins and water-soluble sugar, each sample was boiled for 5 min in 2 mL 2% sodium dodecyl sulfate (SDS) in 50 mM Tris/HCl buffer supplemented with 100 mM Na-EDTA, 40 mM β -mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride (Elorza *et al.*, 1985; Hearn & Sietsma, 1994; Schoffemeer *et al.*, 1999). Mannoprotein was extracted with 3% NaOH at 75 °C for 1 h, and determined quantitatively using the Lowry protein assay (Lowry *et al.*, 1951). Glucan and chitin were digested in 96% formic acid at 100 °C for 4 h. Formic acid was evaporated by lyophilization, and the residues were dissolved in 1 mL distilled water. Glucan and chitin were estimated by measuring the glucose and *N*-acetylglucosamine released after digestion. Glucose was measured by the phenol–sulfuric acid method (Dubois

et al., 1956). *N*-Acetylglucosamine was measured by the method described by Lee *et al.* (2005).

Protein extracts and Western analysis

Chitinase AfChiB1, secreted by *A. fumigatus*, was induced and purified as described previously (Xia *et al.*, 2001). Anti-AfChiB1 antibody developed in mouse was used in Western blotting. Proteins in the cell lysate or the culture supernatant were run on a 12% SDS-polyacrylamide gel electrophoresis (PAGE) gel and transferred to polyvinylidene difluoride (Bio-Rad) at 300 mA for 1.5 h. The anti-AfChiB1 mouse serum was diluted at 1 : 5000. Protein was detected with the enhanced chemoluminescence substrate (Pierce) and autoradiography on a film.

N-glycan analysis

For N-glycan analysis, the AfChiB1 was induced and purified as described previously (Xia *et al.*, 2001). Fifty micrograms of the AfChiB1 from different strains was incubated with PNGase F (New England BioLabs) at 37 °C overnight to release N-glycans. The oligosaccharide released from the proteins was dried in a centrifugal vacuum device. Oligosaccharides were labelled with 0.15 M ANTS (8-amino-phthalene-1,3,6-trisulfonate, Sigma) and 1 M NaBCNH₃ (Glyko) and run on a 30% polyacrylamide gel at 4 °C. The gel was imaged with a fluorescent scanner (Glyko).

Quantitative real-time reverse transcription-PCR analysis

Total RNAs from the spores cultured at specific times were extracted with 1 mL of Trizol reagent (Invitrogen). The cDNA synthesis was performed with 5 µg RNA using the SuperScript-First-Strand Synthesis System (Promega). Specific primers (see Supporting Information, Table S1) were designed using PRIMER EXPRESS 2 software (Applied Biosystems), with the product sizes of all being about 80 bp. To exclude contamination of cDNA preparations with genomic DNA, primers were designed to amplify regions containing one intron in the gene (Bustin, 2000, 2002). The PCR reaction components were 1 µL cDNA, 12.5 µL SYBR Green-ER qPCR SuperMix (Invitrogen), and 0.6 µM of each pair of primers. Thermal cycling conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Samples isolated from different strains and different times were tested in triplicate.

Analysis of virulence of the mutant

The wild-type and mutant strains were used for experimental infections in white male BALB/c mice (18–20 g). Conidia were suspended in 0.01% Tween-20 in saline to yield a challenge inoculum of 3×10^5 CFU (g body wt)⁻¹ in a 30 mL

volume. Mice were immunosuppressed by an intraperitoneal injection of cyclophosphamide [150 mg (kg body wt)⁻¹] on days 23 and 21, and one subcutaneous injection of hydrocortisone acetate [40 mg (kg body wt)⁻¹] on day -1. On day 0, mice were anaesthetized by inhalation of diethyl ether and infected intranasally with a 30 mL spore suspension containing 6×10^6 conidia. A concurrent control group consisted of mice that had been immunosuppressed and then inoculated with 30 mL 0.01% Tween 20 in saline. Immunosuppression was prolonged by cyclophosphamide injections [150 mg (kg body wt)⁻¹] on days 3, 6, and 9. Mice were kept in sterile cages with filter tops, and they received sterile food and bedding. Tetracycline (1 mg mL⁻¹) was added to the drinking water, which was changed twice daily. Four groups, each containing 20 mice, were inoculated, monitored twice daily for 30 days after inoculation, and the mortality was recorded. Mice surviving the course of the experiment were killed humanely on day 30. The survival rate was analysed statistically using the methods of Kaplan–Meier, with SPSS13 software. *P* values of < 0.05 were considered significant in this analysis.

Results

Defect of glucose trimming in the Δ Afchw41 mutant

As shown in Fig. 2a, although the proteins secreted by the mutant were similar to those of the wild-type or the complemented strain, they appeared larger than their counterparts from the wild-type or the complemented strain. The AfChiB1, an indicator molecule used in this study (Xia *et al.*, 2001; Hu *et al.*, 2004; Wang *et al.*, 2004), was secreted to a lesser extent and was larger in the mutant as compared with the one from the wild type (Fig. 2b and c). The N-glycan attached to the AfChiB1 from the mutant was determined as Glc₃Man₉GlcNAc₂, while the wild type one was Man₈GlcNAc₂, Man₇GlcNAc₂, and Man₆GlcNAc₂ (Fig. 2d). In addition, two AfChiB1 bands were detected in the cell lysate of the mutant (Fig. 2e). The smaller one, which moved to the position corresponding to the wild type one, was not secreted because no such molecule was detected in the culture supernatant. One plausible explanation is that the smaller one, probably misfolded AfChiB1, was degraded by ERAD. These results suggested that the glucose trimming and subsequent QC were not essential for secretion of extracellular glycoproteins in *A. fumigatus*, at least for the AfChiB1.

Growth phenotypes and virulence of the Δ Afchw41 mutant

When the growth kinetics were determined, as described in Materials and methods, the mutant mycelia did not show any significant difference in the growth rate as compared

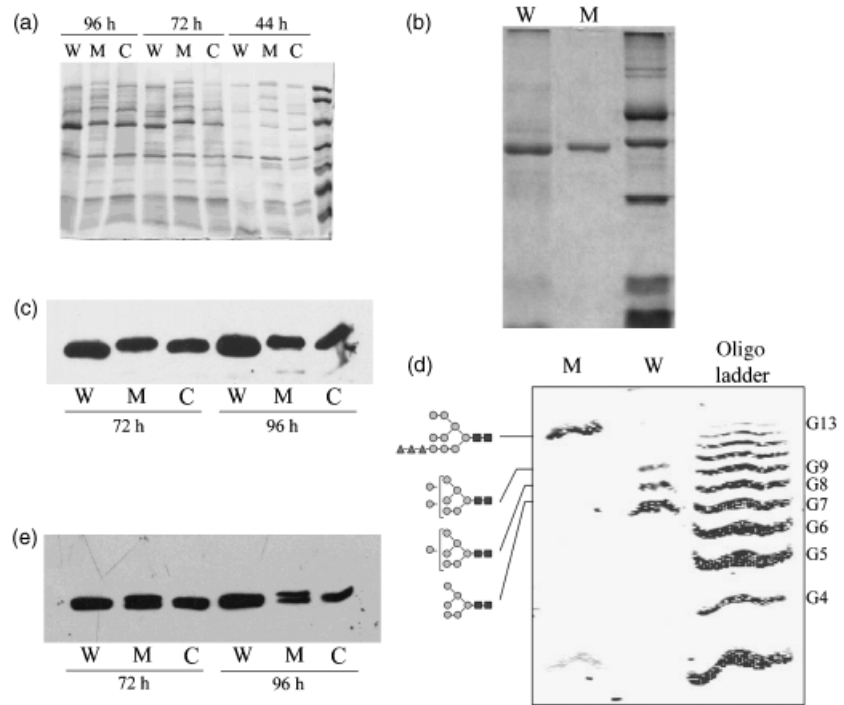


Fig. 2. Glucose trimming of glycoproteins in the mutant. (a) SDS-PAGE analysis of glycoproteins secreted by the $\Delta Afcwh41$; (b) SDS-PAGE analysis of purified chitinase AfChiB1 from the $\Delta Afcwh41$; (c) Western blot of AfChiB1 secreted by the $\Delta Afcwh41$; (d) N-glycan determination. ■, N-Acetylglucosamine; ●, mannose; ▲, glucose. (e) Western blot of intracellular AfChiB1 in the $\Delta Afcwh41$. W, wild type; M, mutant; C, complemented.

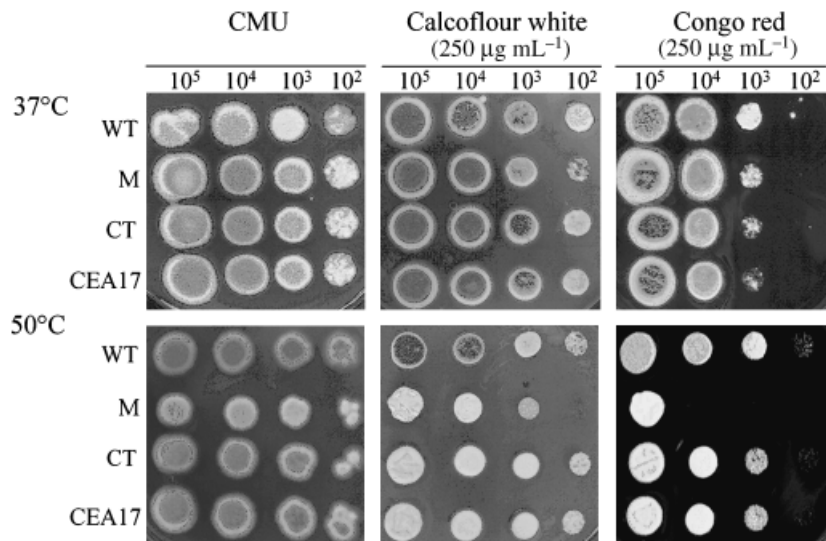


Fig. 3. Hyphal growth and sensitivity to antifungal reagents of the mutant at 37 or 50 °C. A series of 10-fold dilutions (10^5 – 10^2 cells) of the wild type, $\Delta Afcwh41$, and complemented strain were spotted on CMU plate with or without antifungal reagent and cultivated at 37 or 50 °C for 24–48 h. WT, wild type; M, mutant; RT, complemented.

with that of the wild-type or the complemented strain (data not shown). Because *A. fumigatus* can grow comfortably at temperatures from 30 to 50 °C, we also examined the growth rate of the mutant at both 37 and 50 °C on solid CMU. As shown in Fig. 3, the hyphal growth of the $\Delta Afcwh41$ mutant was similar to that of the wild type or the complemented strain. The only difference was that the mutant produced lighter green colour in comparison with the dark green color of the wild type at 50 °C. Conidia counting revealed that the amount of conidia (1.4×10^9) produced by the mutant was one half of that (2.9×10^9) produced by the wild type at

37 °C, while at 50 °C the conidia (1.6×10^6) produced by the mutant were only 1% of those produced by the wild type (1.7×10^8), suggesting a severe reduction of conidia formation, especially at a higher temperature.

When the mutant was grown in the presence of Calcofluor white or Congo red, hyphal growth was not affected at 37 °C (Fig. 3). However, the mutant showed a slightly increased sensitivity to Calcofluor white and a significantly increased sensitivity to Congo red at 50 °C, suggesting that the cell wall integrity (CWI) of the mutant was affected at a higher temperature. To further investigate the effect of the

Table 1. Cell wall components of the mutant

Temperature (°C)	Strain	Alkali soluble		Alkali insoluble	
		α -Glucan (μ g)	Mannoprotein (μ g)	β -Glucan (μ g)	Chitin (μ g)
37	Wild-type	5465 \pm 111	286 \pm 7	8610 \pm 284	471 \pm 13
	Δ Afcwh41	4617 \pm 247	205 \pm 7	8054 \pm 283	419 \pm 7
50	Wild-type	6267 \pm 260	238 \pm 5	12 663 \pm 394	626 \pm 40
	Δ Afcwh41	5653 \pm 300	183 \pm 5	7642 \pm 663	524 \pm 14

Three aliquots of 10 mg dry mycelium were used as independent samples for the analyses of unbound cell wall proteins and water-soluble sugar as described under Materials and methods. The same experiment was repeated three times. The numbers are shown as micrograms cell wall component per 10 mg dry mycelium.

Afcwh41 on the cell wall synthesis, we analysed the cell wall contents. When the mutant was grown at 37 °C, the content of mannoprotein, α -glucan, β -glucan, and chitin was decreased by 28%, 16%, 6%, and 11%, respectively, while at 50 °C, the content of cell wall components was reduced by 23%, 10%, 40%, and 16%, respectively (Table 1). It is interesting to note that the elevated temperature could induce an increased content of β -glucan, α -glucan, and chitin in the wild type by 47%, 15%, and 33%, respectively, whereas the content of mannoprotein was reduced by 36% upon heat induction, suggesting that *A. fumigatus* might compensate the reduction of mannoprotein by upregulation of β -glucan, α -glucan, and chitin to strengthen its cell wall and survive at higher temperatures. In contrast to the wild type, although a 22% increase of α -glucan and a 25% increase of chitin were observed upon heat-induction, the mutant showed a heat induced reduction of β -glucan (by 5%) as compared with the one cultivated at 37 °C. These results were consistent with the remarkably increased sensitivity of the mutant to Congo red at 50 °C. Thus, we concluded that the temperature-sensitive-deficient CWI was mainly due to a failure in the upregulation of the β -glucan content at a higher temperature.

Moreover, the difference in virulence between the wild-type and the Δ Afcwh41 mutant was not statistically significant ($P > 0.05$) in the immunocompromised mouse model (see Fig. S1).

Taken together, we concluded that the *Afcwh41* gene was not essential for the growth and virulence of *A. fumigatus*, and deletion of this gene led to a severe defect in conidia formation and a temperature-sensitive deficiency of CWI.

Morphogenesis of the Δ Afcwh41 mutant

In filamentous fungi, the life cycle initiates from spore germination. Spore germination undergoes a brief period of isotropic growth, polarity establishment, and emergence of the germ tube that elongates by tip growth. The nucleus, meanwhile, undergoes several mitotic divisions, and new nuclei move out into the germ tube. Septation takes place after the third nuclear division by placement of a cross-wall

at the basal end of the germ tube (Harris *et al.*, 1997, 1999; Momany & Taylor, 2000).

Although the growth rate of the mutant was apparently normal at 37 °C, we observed abnormalities of polar growth and septation at the early stage of germination. As shown in Fig. 4, when *A. fumigatus* conidia were cultivated at 37 °C, the wild-type conidium germinated in a typical bipolar pattern at an angle of 180°, and the second germ tube and the first septation occurred after four rounds of mitosis (7–8 h). The septum formed at the basal end of the first germ tube. In comparison with the wild type, the earliest emergence of the second germ tube occurred in the mutant at a 120° angle only after the second mitotic division (5 h), and the third germ tube or branching of germling was found after the third or the fourth nuclear division (6–7 h). Indeed, after three rounds of mitotic division (6 h), 41% of the mutant conidia formed the second germ tube, while 31% were found with the third germ tube or first branching after four rounds of mitosis (7 h) (Table 2). Most mutant germlings did not form septation even after four rounds of mitosis. Less than 10% of the mutant conidia had only one germ tube, in which the first septation formed after four rounds of mitosis. In addition, about 50% of the mutant conidia germinated in an apparently normal bipolar pattern; however, the first septum was formed randomly, and also lateral branching was observed (Fig. 4 and Table 2).

Once the polarity was established, the hyphal tip of the wild type formed septum after three rounds of nuclear division and usually contained five nuclei, while the hyphal tip of the mutant formed a septum after the fourth mitotic division and contained 11 nuclei, suggesting a slightly lagged septation during hyphal elongation. Furthermore, a random branching was also observed in the hyphal tip of the mutant. These observations demonstrated that deletion of the *Afcwh41* gene led to abnormalities of polarity and septation of *A. fumigatus*.

Gene expression in the Δ Afcwh41 mutant

In yeast, the defect of the cell wall requires the cells to induce the CWI pathway to survive and a compensatory

Table 2. Statistics of germination of the $\Delta Afcwh41$ mutant

Time (h)	Wild-type				$\Delta Afcwh41$			
	Number of germ tube				Number of germ tube			
	0	1	2	3 or more	0	1	2	3 or more
5	33 ± 5	67 ± 7	0	0	27 ± 6	57 ± 7	16 ± 4	0
6	10 ± 3	90 ± 6	0	0	3 ± 2	55 ± 8	41 ± 5	1 ± 0
7	2 ± 2	86 ± 7	7 ± 2	3 ± 1	2 ± 1	24 ± 5	43 ± 6	31 ± 4
8	0	76 ± 9	21 ± 5	1 ± 0	0	9 ± 3	50 ± 5	41 ± 6

Freshly harvested conidia (10^7) were poured into a Petri dish containing a glass coverslip and incubated in 10 mL of complete liquid medium at 37 °C. The coverslips with adhering germlings were removed and counted under microscope. For each independent experiment 100 conidia were counted and three independent experiments were carried out.

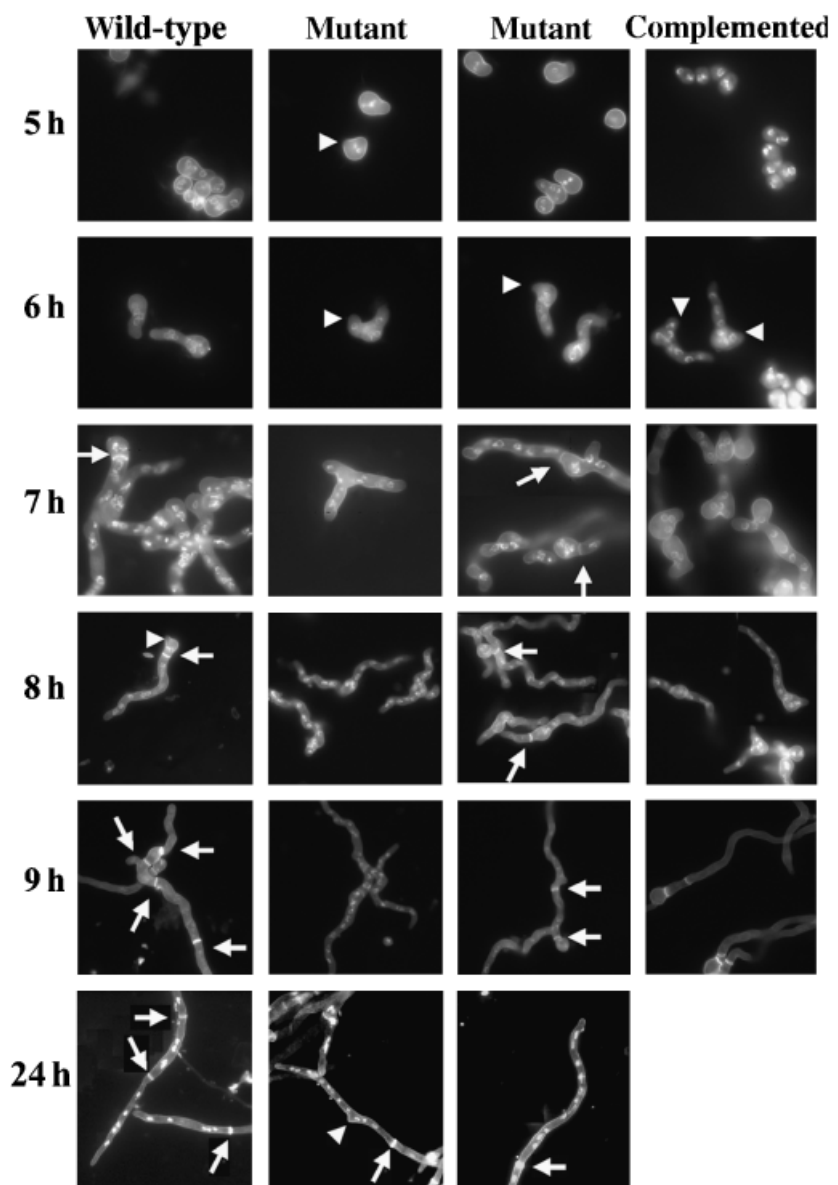


Fig. 4. Germination and septation of the $\Delta Afcwh41$ mutant. Freshly harvested conidia (10^7) were poured into a Petri dish containing a glass coverslip and incubated in 10 mL of complete liquid medium at 37 °C. The coverslips with adhering germlings were fixed in fixative solution and stained with Calcofluor white and DAPI as described in Materials and methods. The stained germlings were photographed using microscope. The sites of germ tube emergence and septum formation are indicated by triangle and arrow, respectively.

mechanism featuring increased chitin content is triggered (Carotti *et al.*, 2002). The CWI pathway used by *S. cerevisiae* has been studied in great detail (Levin, 2005). CWI signalling is induced in response to several environmental stimuli such as elevated temperature, hypo-osmotic shock, mating pheromone, and agents that cause cell wall stress. Briefly, upon environmental stimuli or cell wall stress, the small G-protein Rho1p is activated, which then activates downstream effectors such as Pkc1-MAP kinase cascade, β -1,3-GS, and Bni1/SepA forming protein. Thus, Rho1p 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 addition to Rho1p, *S. cerevisiae* also possesses five other Rho-type GTPases, named Rho2p to Rho5p and Cdc42p. Rho2p is nonessential and partially redundant with Rho1p. Rho3p is nearly essential for cell growth – conditional mutants display cell polarity and lysis defects (Matsui & Toh-e, 1992). It has been suggested that Rho3p shares a role in actin polarization and bud formation with the nonessential Rho4p. Cdc42p function is important for bud site assembly and is essential for the establishment of polarized growth (Johnson & Pringle, 1990; Johnson, 1999). Analysis of Rho protein modules in *Ashbya gossypii* reveals the critical roles of Cdc42 in polarity establishment, Rho1 and Rho3 in polar growth, and Rho1 in CWI (Wendland & Philipson, 2001), suggesting a similar role of these proteins in filamentous fungi. Thus, we also analysed the expression of *Afcdc42/CDC42*, *Afrho1/RHO1*, and *Afrho3/RHO3* in the mutant. When the mutant conidia were incubated at 37 °C for 4 h, the transcripts of the *Afcdc42*, *Afrho1*, and *Afrho3* genes were 1.8-, 2.4-, and 1.1-fold those of the wild type, respectively. After incubation at 37 °C for 6 h, the time point at which the second germ tube occurred in the mutant, much higher levels of *Afcdc42*, *Afrho1*, and *Afrho3* were detected, which were 174-, 29-, and 47-fold those in the wild type, respectively. At the time point of 9 h, the transcripts of these three genes were slightly higher than those of the wild type (1.2–1.5-fold) (Table 3). These results indicate that deletion of the $\Delta Afcwh41$ gene induced higher levels of *Afcdc42*, *Afrho1*, or *Afrho3* at the early stage of germination and a slight increase after germination, which are consistent with severe abnormalities of polarity during the early stage of germination and slight abnormalities of polarity during the stages of hyphal elongation.

Discussion

Some fungi are proposed to be active in N-glycan-dependent QC of glycoprotein folding (Banerjee *et al.*, 2007). However, their consequence remains unclear. Thus, it is of importance to assess the roles of N-glycan in protein

Table 3. Quantitative real-time PCR analysis of gene expression in the mutant

Time (h)	Ratio of expression level (mutant/wild-type)		
	<i>CDC42</i>	<i>RHO1</i>	<i>RHO3</i>
4	1.8	2.4	1.1
6	174	29.0	47.4
9	1.2	1.5	1.3

The spores cultured at 37 °C for 4, 6, and 9 h were used for total RNAs extraction and cDNA synthesis as described in Materials and methods. PCR reaction was carried out in a final volume of 25 μ L using 0.6 μ M of each pair of primers. Samples isolated from different strains and different times were tested in triplicate. TATA box-binding protein was used as parameter. At specific time, the gene expression level of the wild type was taken as onefold.

trafficking, localization, and function in fungi. To this end, we investigated the N-glycan trimming in *A. fumigatus*. Our investigation was initiated before the completion of the genome sequencing of *A. fumigatus*. At that time, we could only identify glucosidase I (AAR23808) and the calnexin (AAS68033) homologue. In the last release of the TIGR database (Galagan *et al.*, 2005), a putative glucosyltransferase has been annotated, suggesting the existence of N-glycan-dependent QC in *A. fumigatus*.

Although the $\Delta Afcwh41$ mutant did not show retarded growth, it displayed a severe reduction of conidia formation, especially at a higher temperature. When we compared the cell wall components of the mutant with those of the wild type at 37 °C, we found that the contents of cell wall mannoprotein, glucans, and chitin were reduced. However, the CWI of the mutant was not affected. When the culture temperature was increased to 50 °C, the wild-type strain displayed a significant increase in α -glucan (15%), β -glucan (47%), and chitin (33%) as compared with the one cultivated at 37 °C. This heat-induced increase of α -glucan, β -glucan, and chitin contents suggests that a compensatory mechanism is triggered, that allows *A. fumigatus* to survive at higher temperatures. However, the elevated temperature did not induce an increase of β -glucan in the mutant. On the contrary, the content of β -glucan in the mutant decreased to 95% of that at 37 °C. This failure in the heat-induced increase of β -glucan was consistent with the hypersensitivity of the mutant to Congo red at 50 °C. Previously, it has been shown that mutational defects in the yeast *CWH41* gene causes severe and selective instability of a glycoprotein Kre6p, a putative GS, and leads to a defect in cell wall β -1,6-glucan synthesis (Ram *et al.*, 1994; Jiang *et al.*, 1996). Thus, it is possible that temperature-sensitive CWI associated with the $\Delta Afcwh41$ mutant also resulted from the instability of the protein involved in β -glucan synthesis.

In addition to the defective conidiation and temperature-sensitive CWI, the mutant also showed abnormalities of

polar growth and septation during germination and hyphal elongation. Our observations suggest that *A. fumigatus* is more dependent on the glucose trimming than yeast. In the budding yeast *S. cerevisiae*, the cell wall is required to maintain the cell shape, which is essential for the formation of a bud and hence cell division. The yeast cell remodels its rigid structure to accommodate cell expansion during vegetative proliferation, mating pheromone-induced morphogenesis, and nutrient-driven filamentation through CWI signalling pathway. The CWI signalling pathway is comprised of a family of cell surface sensors coupled to a small G-protein called Rho1p, which activates the CWI mitogen-activated protein kinase cascade via protein kinase C (Pkc1p) and allows the specific activation of genes encoding cell wall proteins required to stabilize the cell wall in response to low osmolarity, thermal stress, or mating pheromone and polarized growth. Meanwhile, activated Rho1p also activates a set of effectors, which regulate a diverse set of processes including β -glucan synthesis at the site of wall remodelling, gene expression related to cell wall biogenesis, organization of the actin cytoskeleton, and secretory vesicle targeting to the growth site (Levin, 2005). A family of cell surface sensors has been implicated in detecting and transmitting cell wall status to Rho1p (Levin, 2005). These include Wsc1 (Hcs77/Slg1) (Gray *et al.*, 1997; Verna *et al.*, 1997; Jacoby *et al.*, 1998), Wsc2 and Wsc3 (Verna *et al.*, 1997), and Mid2 and Mtl1 (Ketela *et al.*, 1999; Rajavel *et al.*, 1999). Among these cell wall stress sensors, Wsc1 and Mid2 appear to be the most important and serve a partially overlapping role in CWI signalling. The extensive O-mannosylation of Mid2 and Wsc1 is important to their function (Philip & Levin, 2001; Lommel *et al.*, 2004). More recently, the N-glycan was also shown to be directly involved in Mid2 sensing (Hutzler *et al.*, 2008). These observations demonstrate that N- and O-glycosylation are important for CWI sensing and are thus important for polarized growth in yeast. In contrast to yeast, little is known about the cell wall stress sensors and the CWI signalling pathway in *A. fumigatus*. Indeed, in the last release of the *A. fumigatus* genomic database (<http://www.tigr.org/tldb/e2k1/afu1/>), only one protein (AFUA_5G09020) is annotated as a homologue of the Wsc4, which does not appear to contribute to CWI signalling in yeast. Therefore, the *A. fumigatus* cell wall stress sensor molecule remains to be investigated. Somehow, it is likely that the N-glycan is also important for the function of this unidentified molecule.

Although the analysis regarding the CWI signaling pathway in *A. fumigatus* has yet to be initiated, the presence of *A. fumigatus* genes coding for proteins homologues to the yeast Rho1p, Rho3p, and Cdc42p suggests a similar mechanism for the CWI pathway. In this study, we found that *Afcdc42/CDC42*, *Afrho1/RHO1*, and *Afrho3/RHO3* were highly expressed in the mutant during the stage of polarity

establishment (Table 2). After four rounds of mitotic division, the expression levels of these genes were slightly higher than those of the wild type. At this stage, we still do not know how these Rho-type GTPases are activated in the $\Delta Afcwh41$ mutant. However, it is likely that, as in yeast, a defect in the cell wall may also trigger the CWI signalling pathway in *A. fumigatus*, which activates downstream effectors that regulate cell wall biogenesis and polarized growth. A future challenge is to identify proteins that are located upstream and downstream of the Rho-type GTPases, especially upstream mechanosensors.

To ensure that all phenotypes observed in the $\Delta Afcwh41$ strain were due to specific deletion of the *Afcwh41*, the complemented strain was constructed by replacement of the *pyrG* in the mutant strain with the *Afcwh41* gene to yield a genotype of *Afcwh41*⁺*pyrG*⁻. When the complemented strain was used as one of the controls, however, it also displayed abnormalities of polarity (Fig. 4). Previously, it has been shown that uridine-uracil deprivation in *A. fumigatus* CEA17 results in a low rate of conidium swelling and the inability of the mutant conidia to produce germ tubes (d'Enfert *et al.*, 1996). The *pyrG* gene encodes for orotidine-5'-phosphate decarboxylase that catalyses the last step of *de novo* UMP biosynthesis, which is a precursor for the synthesis of glycoproteins. Therefore, the similar phenotypes displayed by the complemented or CEA17 strain could be explained by the depletion of glycoprotein synthesis.

In summary, we have shown that the *Afcwh41* gene is involved in CWI, conidiation, and polarity in *A. fumigatus*. We propose that proteins involved in cell wall synthesis or cell wall stress sensing are substrates of the AfCwh41 and require glucose trimming for their proper localization, stability, and function. Obviously, an understanding of the role of the *Afcwh41* will depend on identification of key substrates of the AfCwh41 and their roles in these processes.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Virulence of the $\Delta Afcwh41$ mutant.

Table S1. Primers used in quantitative real-time PCR analysis

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