Published in final edited form as:

Fungal Genet Biol. 2019 July 01; 128: 20–28. doi:10.1016/j.fgb.2019.03.006.

***Aspergillus fumigatus* Mnn9 is responsible for mannan synthesis and required for covalent linkage of mannoprotein to the cell wall**

Ting Du^{1,2}, Haomiao Ouyang¹, Josef Voglmeir^{3,§}, Iain B. H. Wilson³, Cheng Jin^{*,1,4}

¹State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China

²University of Chinese Academy of Sciences, Beijing, China

³Department of Chemistry, University of Natural Resources and Life Sciences, Vienna A-1190, Austria

⁴Guangxi Academy of Sciences, Nanning 530007, Guangxi, China

Summary

Owing to the essential role in protection of the *Aspergillus fumigatus* cell against human defense reactions, its cell wall has long been taken as a promising antifungal target. The cell wall of *A. fumigatus* composed of chitin, glucan and galactomannan and mannoproteins. Although galactomannan has been used as a diagnostic target for a long time, its biosynthesis remains unknown in *A. fumigatus*. In this study, a putative α 1,6-mannosyltransferase gene *mnn9* was identified in *A. fumigatus*. Deletion of the *mnn9* gene resulted in an increased sensitivity to calcofluor white, Congo red, or hygromycin B as well as in reduced cell wall components and abnormal polarity. Although there was no major effect on N-glycan synthesis, covalently-linked cell wall mannoprotein Mp1 was significantly reduced in the mutant. Based on our results, we propose that Mnn9p is a mannosyltransferase responsible for the formation of the α -mannan in cell wall mannoproteins, potentially via elongation of O-linked mannose chains.

Keywords

cell wall; glycosylation; mannan; mannoprotein; polarity

1 Introduction

Invasive fungal infections kill about 1.5 million people every year; most deaths are due to *Cryptococcus*, *Candida*, *Aspergillus*, and *Pneumocystis* spp. (Brown et al., 2012). *Aspergillus fumigatus* is the second clinically important fungal pathogen, accounts for about 65% of all invasive infections in humans (Warris, 2014). Mortality associated with *A.*

*Corresponding author: Cheng Jin, Ph.D., Prof., State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China, jinc@im.ac.cn or jinc@gxas.cn.

§Current address: Glycomics and Glycan Bioengineering Research Center, College of Food Science and Technology, Nanjing Agricultural University, 1 Weigang, Nanjing 210095, China

fumigatus infection exceeds 50% despite treatment with current standard of care antifungal drugs. The fungal cell wall has been thought as an ideal target for drug development since it is essential for growth and morphogenesis and absent in human cells (Lee & Sheppard, 2016). Therefore, understanding the biosynthesis of the *A. fumigatus* cell wall is of utmost importance.

The fungal cell wall possesses a core cell wall structure of chitin and glucan, further decorated by mannoproteins, which play important roles in cell wall biogenesis and cell wall assembly (Orlean & Menon, 2007). The structures of cell wall mannoproteins are best described for the ascomycete yeasts *Saccharomyces cerevisiae* and *Candida* spp., where proteins are decorated with both N- and O-linked glycans. In *S. cerevisiae*, mannoproteins have been shown to carry up to 200 mannose residues on each of their N-linked glycans comprise about 40% of dry cell wall weight (Herscovics & Orlean, 1993). The synthesis of mannoproteins starts by the extension of the α -1,6-linked mannose backbone up to at least 10 residues (Ballou et al., 1980). This reaction is catalyzed by the M-Pol I, a heterodimeric complex formed by mannosyltransferases Mnn9p and Van1p (Hashimoto and Yoda, 1997; Kojima et al., 1999; Hernandez et al., 1989; Jungmann & Munro, 1998). The backbone is further extended with α -1,6-linked mannose residues by the heteropentameric mannosyltransferase complex M-Pol II (Jungmann & Munro, 1998; Kojima et al. 1999; Jungmann et al. 1999). Branches of α -1,2- or α -1,3-linked mannose and phosphomannose is added to this backbone (Rayner & Munro, 1998; Lussier et al., 1999). Finally, mannoproteins are transported to the extracellular space and associated or covalently linked with β -glucan to form the outer layer of the fungal cell wall (Kollár et al., 1997).

The yeast Mnn9p and Van1p have been extensively studied (Hashimoto and Yoda, 1997; Kojima et al., 1999; Hernandez et al., 1989; Jungmann & Munro, 1998). Recently, it has been shown that Mnn9 and its product are necessary for processive M-Pol I mannosyltransferase activity in vitro. Both the presence and the priming activity of Mnn9 are required for the formation of the α -1,6-mannose backbone of mannoproteins (Striebeck et al., 2013). Although mannoproteins are not essential for cell survival of *S. cerevisiae*, strains with defective mannan biosynthesis pathway exhibit higher osmotic sensitivity and slower growth (Ballou et al., 1980; De Nobel et al., 1990). However, intact mannan biosynthesis pathway is important for virulence of *Candida albicans*. In a murine model of systemic infection survival rate of mice infected with a knockout strain, completely lacking mannans (*Caoch1*), was remarkably higher, while tissue burden produced by this *C. albicans* strain was similar to that of the wild-type (Southard et al., 1999; Bates et al., 2006). Disruption of the *Yarrowia lipolytica* *MNN9* leads to phenotypes such as sensitivity to hygromycin B and hypersensitivity to Calcofluor white or Congo red (Jaafar et al., 2003). Disruption of the *Hansenula polymorpha* *MNN9* leads to phenotypic effects suggestive of cell wall defects, including detergent sensitivity and hygromycin B sensitivity (Kim et al., 2001). More recently, a bioinformatics search finds homologues of *S. cerevisiae* Mnn9p present in members of the Zygomycota and Ascomycota phyla, which provides a promising biomarker for detection of fungal infection is the mannoprotein located in fungal cell walls (Burnham-Marusch et al., 2018). Indeed, immunoassays for fungal mannans or galactomannans have been used in diagnosis of several invasive fungal disease, including

aspergillosis (Reiss and Lehmann, 1979; Walsh et al., 2008), candidiasis (Weiner and Yount, 1976), and histoplasmosis (Wheat et al., 1986).

In *A. fumigatus*, the cell wall mannan is a linear polysaccharide with a tetra mannoside repeating oligosaccharide composed of α -1,6- and α -1,2-linked mannose units. It represents the linear backbone of the galactomannan covalently bound to the cell wall β -1,3-glucans (Fontaine et al., 2000; Latgé et al., 1994). Mnn9 is confirmed as an α -1,6-mannosyltransferase, which transfers the mannose from GDP-Man onto the acceptor mannose. However, no growth phenotype is observed when the *mnn9* is deleted in *A. fumigatus* parent strain CEA17 - Δ kuB^{ku80pyrG} (Henry et al., 2016). In this study, the *A. fumigatus* homolog of the *S. cerevisiae* Mnn9p was investigated. By deletion of the *mnn9* gene in CEA17, another parent *A. fumigatus*, we found that Mnn9 was essential for cell wall integrity, morphogenesis, and proper localization of cell wall mannoprotein.

2 Materials and methods

2.1 Strains and growth conditions

Aspergillus fumigatus strain YJ-407 (China General Microbiological Culture Collection Center, CGMCC0386) was maintained on potato glucose (2%) agar slant (Weidner et al., 1998). *A. fumigatus* strain CEA17 which is a *pyrG* mutant strain (Cove, 1966), a kind gift from C. d'Enfert, Institut Pasteur, France. The bacterial strain used for transformation and amplification of recombinant DNA was *E. coli* DH5. *A. fumigatus* strain YJ-407 was grown at 37°C on complete medium (CM), or minimal medium (MM) with 0.5 mM sodium glutamate as a nitrogen source (Cove, 1966), solidified with 1.5% (w/v) agar when required. Uridine and uracil were added at a concentration of 5mM for strain CEA17. Mycelia were harvested from strains grown in complete liquid medium at 37°C with shaking at 200 rpm. Conidia for spore inoculation were prepared by growing *A. fumigatus* strains on solid complete medium with uridine and uracil (CMU) for 48 hours at 37°C, harvested with 0.1% Tween 20 and washed twice with distilled water. Its concentration was confirmed by haemocytometer and flat dilution counting.

2.2 Construction of the mutant and revertant

A 1.5-Kb of the upstream flanking region of the *mnn9* gene was amplified from *A. fumigatus* strain YJ-407 genomic DNA using primer 7 and 8. A 1.2-Kb of the downstream flanking region of the *mnn9* gene was amplified using primer 9 and 10 (Table S1). By insertion of the flanking regions into the corresponding sites of pGEM-T Easy Vector (Stratagene), T-vector-mnn9 carrying the flanking regions of the *mnn9* gene was obtained. The *pyrG*-blaster cassette (8.6 kb) released by digestion of pCDA14 (kindly provided by C. d'Enfert, Institut Pasteur, France) (d'Enfert, 1996) with *Hpa* I was cloned into the site between the up- and down-stream non-coding regions of the *mnn9* to yield pCDmnn9. The linearized pCDmnn9 at a unique *Not* I site was transformed into strain CEA17 by protoplast transformation (Yelton et al., 1984).

0.55 mg/ml of 5-fluorotic acid (5-FOA) was used to obtain *mnn9pyrG* from *mnn9*. The revertant strain was constructed by the replacement of the *pyrG* in the *mnn9* mutant with

the up- stream non-coding region, *mnn9*, *pyrG* and down-stream non-coding region of the *mnn9*. The upstream flanking region of the *mnn9* gene was amplified from *A. fumigatus* genomic DNA using primer 11 and 12. The downstream flanking region of the *mnn9* gene was amplified using primer 13 and 14 (Table S1). The *mnn9* with its up-stream and down-stream non-coding regions was cloned into the corresponding sites of pGEM-T Easy Vector. Then, the *pyrG*-blaster cassette was inserted into the site between *mnn9* and down-stream non-coding region of the *mnn9* to yield pCDmnn9-RE. The linearized pCDmnn9-RE at a unique *Not I* site was transformed into strain CEA17 by protoplast transformation (Yelton et al., 1984).

The transformants were primarily screened by PCR using primer 1 and 2 for amplification of the *mnn9* gene. Further confirmation was carried out by PCR using primer *pyrG*-up and *pyrG*-down for amplification of the *pyrG* selective marker and primer *neo*-dd5 and *mnn*-dd3 for amplification of the exchange cassette and *mnn9* down-stream fragment. The candidate transformants were then confirmed by Southern blot using the down-stream non-coding region as a probe, which was labeled following the protocol of DIG labeled hybridization kit (Roche Applied science Cat.NO.1093657).

2.3 Phenotype analysis of the mutant

2×10^8 spores were inoculated into 200 ml liquid CM and incubated at 37 °C at 200 rpm, then stained with 10 µg/ml Calcofluor white (Sigma) and 1 mg/ml 4'-6-diamidino-2-phenylindole (DAPI) (Sigma) as previously described (Wang et al., 2015), and detected by fluorescence microscope (Zeiss Imager A2, Japan). For chemical analysis of the cell wall, cell wall components were isolated and determined as previously described (Yan et al., 2013).

For transmission electron microscopy (TEM), the mycelia cultivated in liquid CM at 37°C were collected and fixed as previously described (Yan et al., 2013). The section was examined with a Tecnai Spirit (120kV) transmission electron microscope (FEI, USA).

2.4 Membrane and cell wall protein preparation

A total of 10^8 conidia of *A. fumigatus* wild-type, mutant, or revertant strain were inoculated into CM and cultured at 37°C for 56 h with shaking at 250 rpm. Mycelia were collected by paper filtration under vacuum, extensively washed with distilled water, and then saved at -80°C for further use. Extracellular proteins were precipitated from the culture supernatant, and intracellular proteins were precipitated from the cell lysate with 4 volumes of ethanol. The membrane fraction was prepared using the method described by Fontaine et al. (Fontaine et al., 2003). The cell wall was isolated as described by Damveld et al. (Damveld et al., 2005). Ground mycelia were lyophilized, weighed, and resuspended in 25 µl of Tris buffer (0.05 M Tris-HCl, pH 7.8) per mg (dry weight). The cytosolic fraction was separated from the cell wall and membrane by centrifugation (13,000 rpm) at 4°C for 10 min. To remove residual cytosolic contaminants, membrane proteins, and disulfide-linked cell wall proteins, the pellets were boiled three times in 25 µl of sodium dodecyl sulfate (SDS) extraction buffer (50 mM Tris-HCl, pH 7.8, 2% [wt/vol] SDS, 20 mM sodium EDTA, and 40 mM β-mercaptoethanol) per mg (dry weight). The supernatants were stored as SDS fractions

(SDS1 to SDS3) (Montijn et al., 1994). The cell wall pellet was recovered by centrifugation (12,000 rpm) at 4°C for 10 min, washed three times with deionized water, and then freeze-dried.

Freeze-dried SDS/ β -mercaptoethanol-extracted cell walls were treated in two ways. (i) The cell-wall sample was subjected to Quantazyme digestion (recombinant endo-1,3- β -glucanase; Quantum Biogene). First, 10 mg of lyophilized cell wall was resuspended in 300 μ l of buffer (10 mM Na₃PO₄, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 50 U of Quantazyme), followed by incubation at 37°C for overnight (Terashima et al., 2003). Enzymatic hydrolysis was stopped by boiling the samples for 10 min in the extraction buffer. Solubilized proteins were recovered in the supernatant after centrifugation at 4,000 rpm for 10 min. (ii) The cell wall sample was incubated with 10 μ l of hydrofluoride (HF)-pyridine per mg (dry weight) for 3 h at 0°C (De Groot et al., 2004). After centrifugation, the supernatant containing the HF-extracted proteins was collected (in 100- μ l aliquots), and proteins were precipitated by the addition of 9 volumes of 100% methanol buffer (100% [vol/vol] methanol, 50 mM Tris-HCl, pH 7.8) and subsequently incubated at 0°C for 2 h. Precipitated proteins were collected by centrifugation (13,000 rpm, 10 min, at 4°C). The pellet was washed three times with 90% methanol buffer (90% [vol/vol] methanol, 50 mM Tris-HCl, pH 7.8) and lyophilized. The Quantazyme-/HF-extracted proteins were detected by SDS-PAGE and Western blotting.

2.5 Western blotting

Proteins were loaded to 12% polyacrylamide gel. After separation, the proteins were electrotransferred to nitrocellular membrane. After blocking in 5% defatted dried milk in Tris-buffered saline (TBS; 10 mM Tris-HCl, 150 mM NaCl, pH 8.0), the membrane was probed with antibodies at 1:1,000 dilution in TBS containing 1% dried milk. The membrane was then washed for 15 min each in TBS plus 0.05% Triton X-100 (TBST), TBST plus 0.5 M NaCl, and TBST before incubating with alkaline phosphatase-conjugated anti-rabbit IgG 1:5,000 in TBS with 1% dried milk. After washing as described above, immunolabeling was visualized by using NCIP/NBT solution (Amersco). Customer-designed antibody against the *A/Gel1* or *A/Mp1*, which were developed in specific-pathogen-free rabbit antiserum using synthesized peptide (*A/Gel1*, CPAKDAPNWDVDNDALPA; *A/Mp1*, DKFVAANAGGTVYEDLK), were obtained from B&M.

2.6 Analysis of glycans

The cell lysate was extracted from 1-2 g of wet mycelia in liquid nitrogen. The pH value of the cell lysate was adjusted to pH 1.0-2.0 with 5% (v/v) formic acid; 1.5 mg of pepsin was added to the cell lysate and the mixture was incubated at 37 °C overnight. Then the mixture was centrifuged at 12000 rpm for 5 min and the supernatant was applied to a Dowex AG50W column; the glycopeptides eluted with 0.5 M ammonium acetate (detection by orcinol staining), pooled, lyophilized, de-salted with Sephadex G25, and lyophilized again (Paschinger et al, 2012).

The glycopeptides were suspended in 120 μ l of ddH₂O and incubated at 95 °C for 10 min. 75 μ l 0.1 M of ammonium bicarbonate (pH 8.0) were added to the suspended glycopeptides

and 10 U PNGase F (Roche) were added. The mixture was incubated 37 °C overnight and the N-glycans were separated from remaining glycopeptides by a second round of Dowex chromatography. The N-glycans were then lyophilized and dissolved in 80 µl of 2-aminopyridine solution (100 mg in 76 µl HCl and 152 µl ddH₂O) prior to boiling for 15 min. Thereafter, 4 µl of sodium cyanoborohydride/2-aminopyridine solution (a mixture of the aforementioned 9 µl 2-aminopyridine solution, 13 µl ddH₂O, and 4.4 mg NaBH₃CN) were added and the solution was incubated at 90 °C overnight. The labeled products were purified with Sephadex G15 (pooled on the basis of fluorescence at 320/400 nm) and analysed by MALDI-TOF MS using a Bruker Autoflex Speed in positive ion mode (Paschinger et al, 2012). O-glycans were purified and analyzed as described by Lu et al. (Lu et al., 2015).

3 Results

3.1 Deletion of the *mnn9* gene in *A. fumigatus*

To evaluate *mnn9* gene (AFUA_2G01450) in vivo function, we deleted it in *A. fumigatus* by replacing the *mnn9* gene with *pyrG* as described under Materials and Methods. To construct the revertant strain, the *mnn9* mutant was incubated with 5-FOA to generate the *mnn9pyrG* strain, which was then used as host cell to re-introduce the *mnn9* and *pyrG* to yield the revertant strain (d'Enfert, 1996). The deletion mutant and revertant strain were first confirmed by PCR. A 1.1-kb fragment of the *mnn9* was amplified in the wild-type (WT) strain, while a 1.5-Kb fragment of the *pyrG* and a 2.4-Kb fragment of containing partial *neo* gene and the down-stream non-coding region of the *mnn9* were amplified in the mutant strain. Both 1.1-Kb and 1.5-Kb fragments were amplified in the revertant strain (Fig.1). Furthermore, Southern blotting was carried out by detection of the *Apa* I-digested genomic DNA with a 1.2-Kb probe derived from the down-stream non-coding region of the *mnn9* gene. The 4.3-Kb, 7.8-Kb, and 6.3-Kb fragments were detected in genomic DNA from the WT, mutant, and revertant strain, respectively. These results confirmed that the *mnn9* gene was deleted in the *mnn9* mutant and restored in the revertant strain.

3.2 Morphogenesis of the mutant

Morphogenesis of *A. fumigatus* initiates with conidia germination (Momany and Taylor, 2000). Typically, after the first mitosis conidium begins its polarized growth. The first germling tube emerges after the second mitosis and the second germling tube occurs after the third mitosis. The septum is formed at the base of the first germling tube after the fourth mitosis. As shown in Fig.2, the first mitosis of the WT and revertant conidia occurred at 5 h and the first germling tube was formed after the second mitosis at 6 h. After four rounds of mitosis (8 h) the second germling tube formed at an angle of 180° with the first germling tube and the septa was formed at the base of the first germling tube. As compared with the WT and revertant, in the mutant the first and the second mitosis occurred at 4 h and 5 h, respectively. 68% of the mutant conidia formed the first germling tube. After 6-7 h, 53% of the mutant conidia formed the second germling tube and septum form at 8 h. Additionally, 28% of conidia displayed a random budding (Fig.2 and Fig.3). These observations suggested an abnormal polarity associated with the mutant.

3.3 Cell wall of the mutant

Under TEM, the thickness of the mutant conidial cell wall reduced by 28% at 37°C and 35% at 50°C, respectively. Similarly, the thickness of the mutant mycelial cell wall reduced by 26% at 37°C and 30% at 50°C, respectively (Fig.4 and Table 1). Which suggested that deletion of the *mnn9* in *A. fumigatus* led to a defect of cell wall. We further analyzed the cell wall components of the mutant. As summarized in Table 2, the cell wall components of the mutant were reduced at either 37°C or 50°C. Especially, the contents of Man and Gal on cell wall mannoproteins were significantly decreased by 32% at either 37°C and 50°C ($p < 0.05$). As compared with the WT and revertant, the mutant displayed slightly increased sensitivity to Congo red and hygromycin at either 37 and 50 °C, and a slight increase of sensitivity to calcofluor white at 50 °C (Fig.5). Taken together, these data show that deletion of the *mnn9* led to a defect in cell wall integrity in *A. fumigatus*.

3.4 Analysis of the cell wall glycoproteins in the mutant

In *S. cerevisiae*, Mnn9p involves in elongation of the N-glycans in Golgi. To test if Mnn9 is involved in elongation of the N-glycans in *A. fumigatus*, we also analyzed the N-glycome of the mutant by MALDI-TOF MS. As shown in Fig.6, the N-glycomes of all three examined strains were very similar, especially those of the mutant and revertant, suggesting that Mnn9 has no significant role in the biosynthesis of N-glycans in *A. fumigatus*.

As the cell wall integrity was affected in the mutant, we further analyzed the glycoproteins involved in the synthesis and organization of the cell wall in the mutant using cell wall glucan synthesis enzyme Gel1 and cell wall serine-threonine-rich galactomannoprotein Mp1 (Ouyang et al., 2013) as reporters. As shown in Fig.7, the amounts of Gel1 in membrane and cell wall were slightly decreased in the mutant, whereas extracellular Gel1 was significantly increased and degraded. These results suggested that Gel1 was more sensitive to proteolytic degradation in the mutant. On the other hand, the content of Mp1 released from the mutant cell wall by SDS was increased, suggesting an increase of non-covalently linked Mp1 in the cell wall. When the cell wall proteins were released by Quantazyme, Mp1 protein was mainly detected as 55-70 KD in the WT and significantly decreased in the mutant. These results suggested that the covalent linkage of Mp1 to cell wall β -glucan were reduced in the mutant. In addition, 45 KD of Mp1 released from the mutant cell wall by HF-pyridine, which cleaves the bond between peptide and GPI anchor, was increased as compared with the WT and revertant, while extracellular Mp1 (35 KD), which is a proteolytical form of Mp1, was significantly increased. These observations indicated that Mnn9 was involved in the covalent link of the Mp1 to cell wall β -glucan. It is likely that Mnn9 plays a priming role in the formation of the α -mannan, through which the cell wall mannoproteins are further linked to cell wall β -glucan.

4 Discussion

Mannan is a common cell wall structural component in Zygomycetes and ascomycetes, which account for the majority of fungal causes of human, animal, and plant disease. Therefore, the cell wall mannans or galactomannans have been treated as a diagnostic target applicable to multiple pathogenic fungi, including aspergillosis, candidiasis, and

histoplasmosis (Weiner and Yount, 1976; Reiss and Lehmann, 1979; Wheat et al., 1986; Walsh et al., 2008; Burnham-Marusich et al., 2018). As the homologues of *S. cerevisiae* Mnn9p, the α -mannosyltransferase necessary for α 1,6-mannan production, are found to present in members of the Zygomycota and Ascomycota phyla, Mnn9p is also a promising target for development of anti-fungal drug.

In *S. cerevisiae*, upon entry into the cis-Golgi, core N-linked glycans receive an α 1,6-linked mannose by the transferase Och1p (Nakayama et al., 1992; Nakanishi-Shindo et al., 1993). This residue is extended with an α 1,2- and α 1,3-linked mannose in proteins targeted for retention in cellular organelles (Hernández et al., 1989). The M-Pol I complex, a heterodimeric complex formed by mannosyltransferases Mnn9p and Van1p, is responsible for synthesis of the α 1,6-linked mannose backbone attached to α 1,3-linked mannose (Hashimoto and Yoda, 1997; Kojima et al., 1999; Hernandez et al., 1989; Jungmann & Munro, 1998); specifically, *S. cerevisiae* Mnn9p may act as both a primer and an allosteric activator of Van1P in mannoprotein modification (Striebeck et al., 2013). Disruption of the *mnn9* gene in *S. cerevisiae*, *C. albicans*, *Y. lipolytica*, and *H. polymorpha* leads to a defect in cell wall integrity such as higher sensitivity to osmotic, hygromycin B, calcofluor white or Congo red (Ballou et al., 1980; De Nobel et al., 1990; Southard et al., 1999; Jaafar et al., 2003; Kim et al., 2001). Furthermore, *mnn9* is required for virulence in *C. albicans* (Bates et al., 2006).

In contrast to yeasts, the biosynthesis of mannan and mannoprotein remained unknown in filamentous fungi such *A. fumigatus*. More recently, in vitro analysis confirms that Mnn9 is an α -1,6-mannosyltransferase, however, deletion of the *mnn9* gene does not lead to any visible growth phenotype in *A. fumigatus* (Henry et al., 2016). In this study, the *A. fumigatus* homolog of the *S. cerevisiae* Mnn9p was investigated. In contrast to the results reported by Henry et al., we found that the mutant displayed an abnormal polarity and a slight increase of sensitivity to calcofluor white at 50 °C. The difference between the previous report and this study is the parent strains used in these two studies. In previous study (Henry et al., 2016), CEA17- *akuB*^{ku80pyrg}- strain is used as parent strain to delete the *mnn9* gene. Therefore, the mutant is CEA17- *akuB*^{ku80mnn9}-. As *ku80*-knock-out is also a mutant, it is likely that the phenotype associated with the *mnn9* deletion is buried. In our study, we used CEA17 as parent strain to delete the *mnn9* gene. When the *mnn9* gene was replaced by *pyrG* gene, the mutant strain only lacked the *mnn9* gene as compared with wild-type *A. fumigatus*.

Moreover, several lines of indirect evidence suggest that Mnn9 is involved in the synthesis of α -mannan: (i) previously we have shown that mature glycoproteins present in *A. fumigatus* bear N-glycan of Man₆GlcNAc₂ instead of a hypermannosylated type (Zhang et al., 2008) and deletion of the *mnn9* did not cause any significant change in N-glycome (Fig. 6), indicating that Mnn9 is not involved in elongation of the N-glycan in Golgi; (ii) the *mnn9* mutant showed a significant reduction of covalently-linked Mp1, a cell wall serine-threonine-rich galactomannoprotein, which suggests that Mnn9 is involved in covalent linkage of Mp1 to the cell wall; (iii) as the product of yeast Mnn9 plays a priming role in the formation of the α -mannan in cell wall mannoproteins (Striebeck et al., 2013) and the *A. fumigatus* Mp1 does not contain any potential N-glycosylation site, it is also possible for the

A. fumigatus Mnn9 to play an essential role in elongation of O-glycan, through which Mp1 is attached to cell wall β -glucan; and (iv) the *mnn9* displayed phenotypes such as defect in cell wall and aberrant polarized growth, which are similar to that in yeasts. Interestingly, deletion of the *mnn9* led to an increase of 45 kDa of Mp1 in the mutant, which was released by HF-pyridine, a reagent cleaves the phosphoester bond between peptide and GPI anchor. This observation indicates that Mp1 present in the mutant is mainly proteins with its remaining mannose residues, which are released from the cell membrane by the removal of phosphoinositol.

In conclusion, our genetic analysis revealed that Mnn9 was essential for cell wall integrity, morphogenesis, and proper localization of cell wall mannoprotein. Thereby, it is interesting to note that deletion of eleven *A. fumigatus* mannosyltransferase genes (including *mnn9*) results in lower survival of conidia both in storage and in mice as well as in a reduction in both conidial alkali-soluble mannan and cell wall thickness (Henry et al, 2016); we have examined an overlapping set of parameters on a single mutant and come to broadly similar, but not identical, results. Certainly, both the studies by us (solely on Mnn9) and others (via single and multiple knock-outs) indicate that Mnn9 is required for mannan and mannoprotein biosynthesis, but the exact molecular basis remains to be investigated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This project was supported by the National Natural Science Foundation of China (31320103901) and the Austrian Science Fund (I391).

References

- Ballou L, Cohen RE, Ballou CE. *Saccharomyces cerevisiae* mutants that make mannoproteins with a truncated carbohydrate outer chain. J Biol Chem. 1980; 255:5986–5991. [PubMed: 6991499]
- Bates S, Hughes HB, Munro CA, Thomas WPH, MacCallum DM, et al. Outer chain N-glycans are required for cell wall integrity and virulence of *Candida albicans*. J Biol Chem. 2006; 281:90–98. [PubMed: 16263704]
- Brown GD, Denning DW, Gow NA, Levitz SM, Netea MG, et al. Hidden killers: human fungal infections. Sci Transl Med. 2012; 4:165rv13.
- Burnham-Marusich AR, Hubbard B, Kvam AJ, Gates-Hollingsworth M, Green HR, et al. Conservation of mannan synthesis in fungi of the Zygomycota and Ascomycota reveals a broad diagnostic target. mSphere. 2018; 3:e00094–18. [PubMed: 29720523]
- Cove DJ. The induction and repression of nitrate reductase in the fungus *Aspergillus nidulans*. Biochim Biophys Acta. 1966; 113:51–56. [PubMed: 5940632]
- Damveld RA, Arentshorst M, VanKuyk PA, Klis FM, van den Hondel CAMJJ, et al. Characterisation of CwpA, a putative glycosylphosphatidylinositol-anchored cell wall mannoprotein in the filamentous fungus *Aspergillus niger*. Fungal Genet Biol. 2005; 42:873–885. [PubMed: 16154783]
- d'Enfert C. Selection of multiple disruption events in *Aspergillus fumigatus* using the orotidine-5'-decarboxylase gene, *pyrG*, as a unique transformation marker. Curr Genet. 1996; 30:76–82. [PubMed: 8662213]

- De Groot PW, de Boer AD, Cunningham J, Dekker HL, de Jong L, et al. Proteomic analysis of *Candida albicans* cell walls reveals covalently bound carbohydrate-active enzymes and adhesins. *Eukaryot Cell*. 2004; 3:955–965. [PubMed: 15302828]
- De Nobel JG, Klis FM, Priem J, Munnik T, Van Den Ende H. The glucanase-soluble mannoproteins limit cell wall porosity in *Saccharomyces cerevisiae*. *Yeast*. 1990; 6:491–499. [PubMed: 2080666]
- Fontaine T, Simenel C, Dubreucq G, Adam O, Delepierre M, et al. Molecular organization of the alkali-insoluble fraction of *Aspergillus fumigatus* cell wall. *J Biol Chem*. 2000; 275:41528.
- Fontaine T, Magnin T, Melhert A, Lamont D, Latgé J-P, et al. Structures of the glycosylphosphatidylinositol membrane anchors from *Aspergillus fumigatus* membrane proteins. *Glycobiology*. 2003; 13:169–177. [PubMed: 12626404]
- Hashimoto H, Yoda K. Novel membrane protein complexes for protein glycosylation in the yeast Golgi apparatus. *Biochem Biophys Res Commun*. 1997; 241:682–686. [PubMed: 9434768]
- Henry C, Fontaine T, Heddergott C, Robinet P, Aïmanianda V, et al. Biosynthesis of cell wall mannan in the conidium and the mycelium of *Aspergillus fumigatus*. *Cellular Microbiology*. 2016; 18:1881–1891. [PubMed: 27603677]
- Hernandez LM, Ballou L, Alvarado E, Tsai PK, Ballou CE. Structure of the phosphorylated N-linked oligosaccharides from the *mnn9* and *mnn10* mutants of *Saccharomyces cerevisiae*. *J Biol Chem*. 1989; 264:13648–13659. [PubMed: 2668269]
- Hernández LM, Ballou L, Alvarado E, Gillece-Castro BL, Burlingame AL, et al. A new *Saccharomyces cerevisiae* *mnn* mutant N-linked oligosaccharide structure. *J Biol Chem*. 1989; 264:11849–11856.
- Herscovics A, Orlean P. Glycoprotein biosynthesis in yeast. *FASEB J*. 1993; 6:540–550.
- Jaafar L, León M, Zueco J. Isolation of the *MNN9* gene of *Yarrowia lipolytica* (*YIMNN9*) and phenotype analysis of a mutant *ylmnn9* strain. *Yeast*. 2003; 20:633–644. [PubMed: 12734801]
- Jungmann J, Munro S. Multi-protein complexes in the cis Golgi of *Saccharomyces cerevisiae* with α -1,6-mannosyltransferase activity. *EMBO J*. 1998; 2:423–434.
- Jungmann J, Rayner JC, Munro S. The *Saccharomyces cerevisiae* protein Mnn10p/Bed1p is a subunit of a Golgi mannosyltransferase complex. *J Biol Chem*. 1999; 274:6579–6585.
- Kim S-Y, Sohn J-H, Kang HA, Yoo S-K, Pyun Y-R, et al. Cloning and characterization of the *Hansenula polymorpha* homologue of the *Saccharomyces cerevisiae* *MNN9* gene. *Yeast*. 2001; 18:455–461. [PubMed: 11255253]
- Kojima H, Hashimoto H, Yoda K. Interaction among the subunits of golgi membrane mannosyltransferase complexes of the yeast *Saccharomyces cerevisiae*. *Biosci Biotechnol Biochem*. 1999; 63:1970–1976. [PubMed: 10635561]
- Kollár R, Reinhold BB, Petráková E, Yeh HJC, Ashwell G, et al. Architecture of the yeast cell wall: α -(1-6)-glucan interconnects mannoprotein, β -(1-3)-glucan, and chitin. *J Biol Chem*. 1997; 272:17762–17775.
- Latgé JP, Kobayashi H, Debeaupuis JP, Diaquin M, Sarfati J, et al. Chemical and immunological characterization of the extracellular galactomannan of *Aspergillus fumigatus*. *Infection and Immunity*. 1994; 62:5424–5433. [PubMed: 7960122]
- Lee MJ, Shepard DC. Recent advances in the understanding of the *Aspergillus fumigatus* cell wall. *J Microbiol*. 2016; 54:232–242. [PubMed: 26920883]
- Lu H, Lü Y, Ren J, Wang Z, Wang Q, et al. Identification of the S-layer glycoproteins and their covalently linked glycans in the halophilic archaeon *Haloarcula hispanica*. *Glycobiology*. 2015; 25(11):1150–1162. [PubMed: 26170448]
- Lussier M, Sdicu AM, Bussey H. The KTR and MNN1 mannosyltransferase families of *Saccharomyces cerevisiae*. *Biochim Biophys Acta*. 1999; 2:323–334.
- Montijn RC, van Rinsum J, van Schagen FA, Klis FM. Glucomannoproteins in the cell wall of *Saccharomyces cerevisiae* contain a novel type of carbohydrate side chain. *J Biol Chem*. 1994; 269:19338–19342. [PubMed: 8034698]
- Momany M, Taylor I. Landmarks in the early duplication cycles of *Aspergillus fumigatus* and *Aspergillus nidulans*: polarity, germ tube emergence and septation. *Microbiology*. 2000; 146(Part 12):3279–3284. [PubMed: 11101686]

- Nakanishi-Shindo Y, Nakayama K, Tanaka A, Toda Y, Jigami Y. Structure of the N-linked oligosaccharides that show the complete loss of alpha-1,6-polymannose outer chain from *och1*, *och1mn1*, and *och1mn1alg3* mutants of *Saccharomyces cerevisiae*. *J Biol Chem*. 1993; 35:26338–26345.
- Nakayama K, Nagasu T, Shimma Y, Kuromitsu J, Jigami Y. OCH1 encodes a novel membrane bound mannosyltransferase: outer chain elongation of asparagine-linked oligosaccharides. *EMBO J*. 1992; 7:2511–2519.
- Ouyang H, Chen X, Lü Y, Wilson IBH, Tang G, et al. One single basic amino acid at the ω -1 or ω -2 site is a signal that retains glycosylphosphatidylinositol-anchored protein in the plasma membrane of *Aspergillus fumigatus*. *Eukaryotic Cell*. 2013; 12:889–899. [PubMed: 23584992]
- Paschinger K, Hykollari A, Razzazi-Fazeli E, Greenwell P, Leitsch D, et al. The N-glycans of *Trichomonas vaginalis* contain variable core and antennal modifications. *Glycobiology*. 2012; 22:300–313. [PubMed: 21983210]
- Rayner JC, Munro S. Identification of the MNN2 and MNN5 mannosyltransferases required for forming and extending the mannose branches of the outer chain mannans of *Saccharomyces cerevisiae*. *J Biol Chem*. 1998; 41:26836–26843.
- Reiss E, Lehmann PF. Galactomannan antigenemia in invasive aspergillosis. *Infect Immun*. 1979; 25:357–365. [PubMed: 383620]
- Southard SB, Specht CA, Mishra C, Chen-Weiner J, Robbins PW. Molecular analysis of the *Candida albicans* homolog of *Saccharomyces cerevisiae* MNN9, required for glycosylation of cell wall mannoproteins. *J Bacteriol*. 1999; 181(24):7439–7448. [PubMed: 10601199]
- Striebeck A, Robinson DA, Schüttelkopf AW, van Aalten DMF. Yeast Mnn9 is both a priming glycosyltransferase and an allosteric activator of mannan biosynthesis. *Open Biol*. 2013; 3
- Terashima H, Hamada K, Kitada K. The localization change of Ybr078w/Ecm33, a yeast GPI-associated protein, from the plasma membrane to the cell wall, affecting the cellular function. *FEMS Microbiol Lett*. 2003; 218:175–180. [PubMed: 12583915]
- Walsh TJ, Anaissie EJ, Denning DW, Herbrecht R, Kontoyiannis DP, et al. Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. *Clin Infect Dis*. 2008; 46:327–360. [PubMed: 18177225]
- Wang J, Zhou H, Lua H, Du T, Luo Y, et al. Kexin-like endoprotease KexB is required for N-glycan processing, morphogenesis and virulence in *Aspergillus fumigatus*. *Fungal Genet Biol*. 2015; 76:57–69. [PubMed: 25687931]
- Warris A. The biology of pulmonary aspergillus infections. *J Infect*. 2014; 69:S36–S41. [PubMed: 25135079]
- Weiner MH, Yount WJ. Mannan antigenemia in the diagnosis of invasive *Candida* infections. *J Clin Invest*. 1976; 58:1045–1053. [PubMed: 993329]
- Weidner G, d'Enfert C, Koch A, Mol PC, Brakhage AA. Development of a homologous transformation system for the human pathogenic fungus *Aspergillus fumigatus* based on the *pyrG* gene encoding orotidine 5'-monophosphate decarboxylase. *Curr Genet*. 1998; 33:378–385. [PubMed: 9618589]
- Wheat LJ, Kohler RB, Tewari RP. Diagnosis of disseminated histoplasmosis by detection of *Histoplasma capsulatum* antigen in serum and urine specimens. *N Engl J Med*. 1986; 314:83–88. [PubMed: 3941695]
- Yan J, Du T, Zhao W, Hartmann T, Lu H, et al. Transcriptome and biochemical analysis reveals that suppression of GPI-anchor synthesis leads to autophagy and possible necroptosis in *Aspergillus fumigatus*. *PLoS ONE*. 2013; 8(3):e59013.doi: 10.1371/journal.pone.0059013 [PubMed: 23527074]
- Yelton M, Hamer J, Timberlake W. Transformation of *Aspergillus nidulans* by using a *trpC* plasmid. *Proc Natl Acad Sci USA*. 1984; 81:1470–1474. [PubMed: 6324193]
- Zhang L, Zhou H, Ouyang H, Li Y, Jin C. *Afcwh41* is required for cell wall synthesis, conidiation, and polarity in *Aspergillus fumigatus*. *FEMS Microbiol Lett*. 2008; 289(2):155–166. [PubMed: 19090038]
- Orlean P, Menon AK. Thematic review series: lipid posttranslational modifications. GPI anchoring of protein in yeast and mammalian cells, or: how we learned to stop worrying and love glycosphospholipids. *J Lipid Res*. 2007; 48:993–1011. [PubMed: 17361015]

Fig.1. Confirmation of the mutant and revertant strain.

The *mnn9* mutant was constructed by replacing of the *mnn9* gene with *pyrG* and the revertant strain was constructed by introducing of the *mnn9* gene into the mutant as described under Materials and Methods. Both *mnn9* mutant (M) and revertant (RT) strains were confirmed by PCR (A) and Southern blot (B). A fragment amplified from the downstream non-coding sequence of the *mnn9* were used as probes. Genomic DNA digested with *Apa I* was probed with a DIG-labelled probe.

Fig.2. Morphogenesis of the *mnn9* mutant during the germination of conidia.

2×10^8 spores of the wild-type (WT), *mnn9* mutant (M), or revertant (RT) strain were inoculated into 200 ml liquid CM and incubated at 37 °C for indicated times; after staining with calcofluor white and DAPI, the conidia were viewed by fluorescence microscopy (1000×). Bar = 10 µm.

Fig.3. Statistics of conidial germination of the *mnn9* mutant.

Freshly harvested conidia (10^7) were poured into a Petri dish containing a glass coverslip and incubated in complete liquid medium at 37 °C; the number of germ tubes were observed and counted per hour after cultured for 4 h (A, 5 h; B, 6 h; C, 7 h; D, 8 h). For each independent experiment, 100 conidia were counted, and three independent experiments were carried out. The values shown are means $\pm$ SD.

Fig.4. Morphology of the mutant conidia (A) and hyphae (B).

The conidia or hyphae of the wild-type, mutant, or revertant strain were fixed and examined by electron microscopy as described under Materials and Methods. The arrows indicate the thickness of the cell wall. Bar = 200 nM.

Fig.5. Sensitivity of the mutant to chemical compounds.

A serial dilution of conidia of 1×10^5 - 1×10^2 from the wild-type (WT), mutant (M), and revertant (RT) strain was grown on CM plate supplied with 150 $\mu\text{g/ml}$ Congo red, 60 $\mu\text{g/ml}$ calcofluor white, or 20 $\mu\text{g/ml}$ hygromycin at 37 or 50 °C.

Fig.6. N-glycome of the wild-type, mutant and revertant strains.

PNGase F-released N-glycans prepared from mycelia were labelled with 2-aminopyridine and analysed by MALDI-TOF MS. N-glycans were detected as $[M+Na]^+$ and are annotated as HxN2 ($Hex_{5-12}HexNAc_2$), whereby the hexose residues are primarily mannose, but also one galactofuranose may be present.

Fig.7. Distribution of the cell membrane and cell wall proteins.

The same amount of intracellular, membrane, cell wall, or extracellular proteins from *A. fumigatus* strains was analyzed by Western blotting and probed with anti-Gel1p or anti-Mp1 antibody. Cell wall proteins were released by SDS, HF-pyridine (HF), or Quantazyme (endo-1,3- β -glucanase).

Table 1
Thickness of conidia and hyphae cell wall (nM)

		Wild-type	<i>mnn9</i>	Revertant
37°C	conidia	155.9±12.9	100.4±9.6	120.6±7.1
	hyphae	212.1±4.6	155.7± 7.7	183.92±3.6
50°C	conidia	177.1±10.2	113.5±6.3	149.1±11. 7
	hyphae	321.3±3.8	224.2±15.8	295.3±14.7

Experiment was carried as described for Figure 4. Values are presented as mean ± standard error. The width was measured for n = 15.

Table 2
Chemical analysis of the *mnn9* mutant cell wall.

Strain		Alkali-soluble				Alkali-insoluble		
		Mannoprotein				α -glucan (μ g)	Chitin (μ g)	β -glucan (μ g)
		Protein (μ g)	GlcNAc (μ g)	Gal (μ g)	Man (μ g)			
37°C	WT	181 $\pm$ 4	0.36 $\pm$ 0.01	2.5 $\pm$ 0.2	1.7 $\pm$ 0.1	379 $\pm$ 18	344 $\pm$ 1	1242 $\pm$ 73
	<i>mnn9</i>	147 $\pm$ 1	0.31 $\pm$ 0.02	1.7 $\pm$ 0.1	1.2 $\pm$ 0.1	299 $\pm$ 12	282 $\pm$ 1	1046 $\pm$ 81
	Revertant	191 $\pm$ 2	0.35 $\pm$ 0.02	2.2 $\pm$ 0.21	1.5 $\pm$ 0.1	347 $\pm$ 13	332 $\pm$ 1	1283 $\pm$ 99
50°C	WT	282 $\pm$ 13	0.59 $\pm$ 0.03	6.5 $\pm$ 0.3	3.6 $\pm$ 0.3	461 $\pm$ 8	404 $\pm$ 7	1931 $\pm$ 52
	<i>mnn9</i>	242 $\pm$ 6	0.35 $\pm$ 0.02	4.2 $\pm$ 0.3	2.5 $\pm$ 0.2	417 $\pm$ 17	366 $\pm$ 1	1755 $\pm$ 72
	Revertant	275 $\pm$ 18	0.41 $\pm$ 0.03	5.6 $\pm$ 0.3	3.1 $\pm$ 0.2	459 $\pm$ 23	405 $\pm$ 1	1935 $\pm$ 19

Conidia were inoculated into 100 ml liquid CM or CMU at a concentration of 10^6 conidia ml⁻¹, and incubated at 37 or 50°C with shaking (200 rpm) for 48 h. The mycelium was then harvested and lyophilized, and three aliquots of 10 mg dry mycelium were used as independent samples for the analyses of unbound cell wall proteins and water-soluble sugars, as described in Materials and Methods. The experiment was repeated three times. Student's t-test was used for statistical analyses of data. The values shown are mg cell wall component per 10 mg dry mycelium ($\pm$ SD).