Published in final edited form as:

Fungal Genet Biol. 2015 March ; 76: 57–69. doi:10.1016/j.fgb.2015.02.006.

Kexin-Like Endoprotease KexB is Required for N-Glycan Processing, Morphogenesis and Virulence in *Aspergillus fumigatus*

Jingyang Wang^{1,2}, Hui Zhou¹, Hua Lu¹, Ting Du¹, Yuanming Luo¹, Iain B. H. Wilson³, and Cheng Jin^{1,*}

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

²University of Chinese Academy of Sciences, Vienna A-1190, Austria

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

Abstract

Kexin-like proteins belong to the subtilisin-like family of the proteinases that cleave secretory proproteins to their active forms. Several fungal kexin-like proteins have been investigated. The mutants lacking of kexin-like protein display strong phenotypes such as cell wall defect, abnormal polarity, and, in case of *Candida albicans*, diminished virulence. However, only several proteins have been confirmed as the substrates of kexin-like proteases in these fungal species. It still remains unclear how kexin-like proteins contribute to the morphogenesis in these fungal species. In this study, a *kexB*-null mutant of the human opportunistic fungal pathogen *Aspergillus fumigatus* was constructed and analyzed. The *kexB* mutant showed retarded growth, temperature-sensitive cell wall defect, reduced conidia formation, and abnormal polarity. Biochemical analyses revealed that deletion of the *kexB* gene resulted in impaired N-glycan processing, activation of the MpkA-dependent cell wall integrity signaling pathway, and ER-stress. Results from *in vivo* assays demonstrated that the mutant exhibited an attenuated virulence in immunocompromised mice. Based on our results, the kexin-like endoprotease KexB was involved in the N-glycan processing, which provides a novel insight to understand how kexin-like protein affects the cell-wall modifying enzymes and therefore morphogenesis in fungi.

Keywords

Aspergillus fumigatus; kexin-like protease; morphogenesis; N-glycan processing; virulence

*To whom corresponding should be addressed: Cheng Jin, State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China. Tel: (86)-10-64807425; jinc@im.ac.cn.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

In eukaryotes, site-specific proteolytic cleavage is a common feature in protein maturation and plays a crucial role in activation of many enzymes and in the generation of peptide hormones. In the late secretory pathway of eukaryotic cells this site-specific proteolysis is mainly mediated by kexin-like proteinases, a subfamily of the subtilisin-like serine proteinases such as yeast Kex2-like proteases, mammalian prohormone convertases (PCs), and mammalian furins (Rockwell et al., 2002; Rockwell and Thorner, 2004; Turpeinen et al., 2013). All members of the kexin subfamily share a similar primary structure, including a signal peptide followed by a propeptide, which is autocatalytically removed after its required role in folding, a Ca²⁺-dependent protease catalytic domain, and a P domain, which is essential for catalytic activity and stability (Anderson et al., 2002). The carboxy-terminal region may or may not have a Ser/Thr-rich sequence, a Cys-rich sequence, an amphipathic region, a single transmembrane domain and a cytoplasmic tail (Seidah and Chrétien, 1999). Kexin-like proteinases cleave the secretory proproteins on the carboxyl side of KR or RR in a late Golgi compartment (Bader et al., 2008). The *S. cerevisiae* Kex2 protein contains a Golgi retrieval signal (Wilcox et al., 1992) and localizes in the late trans Golgi network (Redding et al., 1991) and an endocytic, prevacuolar compartment (Blanchette et al., 2004).

Fungi harbor only a single gene coding for a subtilisin-like serine proteinase, such as *kex2* from *Saccharomyces cerevisiae* (Wickner, 1974), *krp1* from *Schizosaccharomyces pombe* (Davey et al., 1994), *KEX1* from *Kluyveromyces lactis* and *Pneumocystis carinii* (Lee et al., 2000; Tanguy-Rougeau et al., 1988), *XPR6* from *Yarrowia lipolytica* (Enderlin and Ogrydziak, 1994), and *KEX2* genes from *Pichia pastoris*, *Candida albicans* and *Candida glabrata* (Bader et al., 2001; Newport and Agabian, 1997). Kexins are also found in various *Aspergillus*, such as *A. nidulans*, *A. niger*, and *A. oryzae* (Bong et al., 2001; Jalving et al., 2000; Punt et al., 2003; Biesebeke et al., 2005; Mizutani et al., 2004). Several kexin substrates have been identified in fungi, such as the *S. cerevisiae* killer toxin or pheromone α -mating factor (Julius et al., 1984), proteins involved in the formation of aerial hyphae (Wosten et al. 1996), zymogens of secreted proteinases (Enderlin and Ogrydziak, 1994; Newport and Agabian, 1997), lipases (Pignède et al., 2000), polysaccharide-degrading enzymes (Goller et al., 1998), peptidase UstA involved in the cyclic peptide ustioxin B synthesis (Umemura et al., 2014), and themselves.

Deletion of the kexin in the yeast *Y. lipolytica* abolished the formation of hyphae (Richard et al., 2001). *kex2*-null mutants of the yeast *S. cerevisiae* were viable but exhibited conditional morphological abnormalities (Komano and Fuller, 1995) and an inhibitory effect on the vacuolar proton-translocating V-ATPase (Oluwatosin and Kane, 1998). The *C. glabrata* *kex2*-deficient mutant was hypersensitive to several antifungal drugs that target the cell membrane (Bader et al., 2001). In *C. albicans*, the *kex2* disruptant showed impaired hyphae production, morphological defects in the cell, and a diminished virulence (Rockwell et al., 2002; Newport et al., 2003; Venancio et al., 2002). Disruption of the *kexB* gene in *A. niger*, *A. oryzae* or *A. nidulans* led to abnormal polarized growth (Punt et al., 2003; Mizutani et al., 2004; Mizutani et al., 2009). The phenotypes of these deletion mutants include morphological changes that are thought to be resulted from the lack of activity from cell-wall modifying enzymes.

In *Aspergillus fumigatus*, one of the most important human opportunistic pathogens and causes life-threatening invasive aspergillosis (IA) in immune-suppressed population (Krappmann, 2006), the *kexB* gene (AFUA_4G12970) was annotated to code for a putative kexin-like endoprotease (Nierman *et al.*, 2005). To evaluate the significance of KexB in *A. fumigatus*, a null mutant was constructed and its phenotypes were analyzed in this study.

Materials and Methods

Strains and growth conditions

Aspergillus fumigatus strain KU80 *pyrG* derived from KU80, a kind gift from Jean-Paul Latgé, Institute Pasteur, France, was propagated at 37°C on YGA (0.5% yeast extract, 2% glucose, 1.5% Bacto-agar), complete medium, 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 5mM when KU80 *pyrG* was grown. Strains were cultured in complete liquid medium at 37°C or 50°C with shaking at 200 r.p.m. Mycelia cultured in different conditions were harvested and washed three times with distilled water, drained and frozen in liquid N₂ and then stored at -70°C for DNA, RNA and protein extraction. Conidia were prepared by growing *A. fumigatus* strains on solid complete medium for 48h at 37°C. The spores were collected, washed three times with saline containing 0.01% Tween-20 and resuspended in saline containing 0.01% Tween-20, and its concentration was confirmed using haemocytometer counting and viable counting. Vectors and plasmids were propagated in *Escherichia coli* DH5a.

Construction of the *kexB* null mutant and complemented strain

In order to delete the *kexB* gene, a deletion construct was designed to replace the entire *kexB* coding region with a *pyrG* cassette by homologous recombination. PCR primers were designed to amplify a 1.5-kb upstream region of the *kexB* before the ATG start codon and a 1.5-kb downstream region of the *kexB* after the terminator codon. The upstream and downstream non-coding regions were separately cloned into pGEM-T Easy (Promega, USA) and confirmed by sequencing. As a fungal selectable marker, the *pyrG* gene cassette (8.3-kb) released by the digestion of pCDA14 (d'Enfert, 1996) with *HpaI* was cloned into the centre of the up- and down-stream flanking regions of the *kexB* to yield the deletion construct. The deletion vector was linearized by *NotI*-digestion and then transformed into *A. fumigatus* strain KU80 *pyrG* protoplasts and plated under uridine and uracil autotrophy selection. Plates were incubated in hypertonic medium at 30°C for 3-5 days, and the transformation was confirmed by PCR and Southern blot analysis (Fig.S1).

To complement the *kexB* mutant, the *kexB pyrG* strain was generated by growing the *kexB* mutant conidia on minimal medium agar plates supplemented with 1 mg/mL 5-fluoro-orotic acid (5-FOA, sigma), 5 mM uracil and 5 mM uridine. The complemented strain was constructed by replacing the *pyrG* gene cassette in the mutant with the *kexB* gene and the *pyrG* gene. PCR primers were designed to amplify a 4.8-kb sequence containing an up-stream noncoding region and coding region of the *kexB* gene. The PCR product was cloned into pGEM-T Easy Vector and sequenced. The *pyrG* gene was released from pCDA14 by *XbaI*, and then inserted between the terminator codon and downstream non-

coding regions of the *kexB* gene. The resulting vector harboring *kexB* and *pyrG* gene was linearized by *NorI*-digestion and transformed into the *kexB pyrG* strain. Transformants were screened by PCR and confirmed by Southern blotting (Fig.S1).

For Southern blotting, genomic DNA was extracted and digested with *PstI*, separated by electrophoresis and transferred to a Zeta-probe blotting membrane (Bio-Rad). The 1-kb fragment of the *kexB* down-stream non-coding region was used as a probe, and labeled by following the protocol of the DIG-labeled hybridization kit (Roche Applied Science, Germany).

Chemical analysis of the cell wall

Cell wall components were isolated and determined as described by Yan et al. (2013).

Quantitative real-time PCR

1×10^7 conidia were inoculated into 100 ml complete liquid medium and incubated in a shaker (200 r.p.m) at 37°C for 24 h. Total RNA was isolated using TRIZOL (Invitrogen). The cDNA synthesis was performed with 5 µg RNA using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). To exclude contamination of cDNA preparations with genomic DNA, primers were designed to amplify regions containing one intron in the gene. The quantitative PCR reaction was done with SYBR® *Premix Ex Taq*™ (Takara) in a reaction mixture containing 11.5 µl cDNA, 10 µl SYBR® *Premix Ex Taq*™ (2×) and 0.2 µM of each pair of primers. Thermal cycling conditions were 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 58°C for 30 s. The 18s rRNA gene was used to standardize the mRNA levels of the target genes. Quantification of mRNA levels of different genes was performed using the 2^{-Ct} method. A triplicate of samples was tested in each assay and each experiment was repeated 3 times. The primers used in this test are listed in Supplementary Table S2.

Phosphorylation of the MpkA

To detect the activated form of MpkA, 1×10^7 conidia were inoculated into 100 ml liquid complete medium and cultured at 37°C for 22 h or 50°C for 46 h. Cell wall damage was induced by the addition of 100 µg ml⁻¹ Congo red for 2 h. Mycelia were harvested, ground in liquid nitrogen to a fine powder using a mortar and pestle, and immediately suspended in lysis buffer (200 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1 mM phenyl- methanesulphonyl fluoride) and incubated on ice for 20 min. Proteins in the supernatant were collected by centrifugation (16 000 *g* at 4°C for 10 min). Protein concentration was determined by the Lowry method. Thirty micrograms of cellular proteins was separated by 12% SDS-PAGE and transferred to PVDF membrane (Millipore, USA). After blocking in 5% skimmed milk in Tris-buffered saline (TBS; 10 mM Tris-HCl, 150 mM NaCl, pH 8.0), the membrane was probed with anti-phospho-p44/42 MAPK antibody (Cell Signaling Technology, USA) in TBS containing 1% skimmed 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. Anti-Mn-SOD antibody was used as control. The primary antibody was detected using Anti-Rabbit IgG Horseradish peroxidase (HRP) Conjugate (Promega) and Enlight™ reagents (Engreen Biosystem, China).

Protein extraction and Western blotting

1×10^8 conidia were inoculated into 200 ml complete medium and incubated at 37°C for 48 h with shaking at 200 r. p. m. Mycelia were harvested and extensively washed with distilled water for three times, then frozen in liquid N₂ and stored at -70°C for protein extraction. To isolate the extracellular protein, freshly prepared 2% (w/v) sodium deoxycholate was added to the complete medium (1/100 in volume), mixed and placed at 4°C for 30 min. Protein in the culture supernatant was precipitated with 100% trichloroacetic acid (1/10 in volume) at 4°C for 40 min, collected by centrifugation (15,000g at 4°C for 15 min), washed three times with acetone and dried.

Cytosolic and membrane proteins were extracted from ground mycelia with buffer I (200 mM Tris-HCl, 50 mM EDTA, protease inhibitor cocktail) at 4°C for 2 h, then centrifuged 4,000 g for 10 min. The supernatant containing cytosolic and membrane proteins was ultra-centrifuged 50,000 g at 4°C for 1 h. The cytosolic proteins in the supernatant and the membrane proteins in the precipitate were collected separately.

To extract the cell wall proteins, the precipitate was isolated, dried, weighted, and resuspended in 25 µl of Tris buffer (0.05 M Tris-HCl, pH 7.8) mg⁻¹. Then the pellets were boiled three times in 25 µl of sodium dodecyl sulfate extraction buffer (50 mM Tris-HCl, 2% SDS, 20 mM Na-EDTA, and 40 mM β-mercaptoethanol) mg⁻¹. After centrifugation (12 000 r.p.m) at 4°C for 10 min, the precipitate was washed three times with distilled water, dried, and treated with 10 µl of hydrofluoride (HF)-pyridine mg⁻¹ on ice for 3 h. The supernatant was collected and added 9 volumes of 100% methanol buffer (100% methanol, 50 mM Tris-HCl, pH 7.8) at 0°C for 2 h. The cell wall proteins were collected by centrifugation (12 000 r.p.m) at 4°C for 10 min and washed three times with 90% methanol buffer (90% methanol, 50 mM Tris-HCl, pH 7.8).

Protein concentration was determined by Bradford protein assay. Equal amounts of extracellular, cell wall, membrane and cytosolic proteins from the wild type and mutant were separated by SDS-PAGE and Western blotting. The antibodies against Gel1p, Gel4p and Ecm33p were developed with synthesized peptides from B&M Company (Gelp, CPAKDAPNWD VDNDALPA; Gel4p, AKWEASNKLP PSPNSELG; Ecm33p, TITISSQSDA DGYSSC).

Analysis of N-glycan

N-glycans were released by peptide N-glycosidase F (PNGase F, NEB, P0704) from cell wall proteins of the wild-type and the *kexB* mutant. The enzyme reaction was carried out after denaturation of cell wall proteins at 95°C for 5 minutes and then 10% Nonidet P40 (NP40) was added before the addition of PNGase F. The sample was centrifuged and the supernatant was applied to the C8 column to separate N-glycans from cell wall proteins. The C8 column was washed with 100% acetonitrile (ACN) and equilibrated with 0.1% trifluoroacetic acid (TFA). N-glycans were collected and applied to a carbon column. The carbon column was washed with 0.1% TFA to remove salts and then eluted with elution buffer (60% ACN, 0.1% TFA) to collect N-glycans in a clean eppendorf tube. The structure of N-glycans was analyzed by MALDI-TOF-MS.

TFMS digestion of cell wall proteins

To remove both N- and O-glycans from proteins, the cell wall proteins were treated with TFMS (trifluoromethanesulfonate) (Sigma Chemical Co.) as previously described (Edge, 2003; Ouyang et al., 2013).

Analysis of virulence

Virulence of the wild-type, the *kexB* mutant or the complemented strain was detected with immunosuppressed mice as described by Li et al. (2007). Four groups of each containing 20 mice were inoculated and monitored twice each day for 30 days after inoculation and mortality was recorded. Mice surviving the course of the experiment were humanely terminated on day 30. The right lung from each mouse was dissected at day 3 post infection, fixed in 10% (v/v) formaldehyde in physiological saline. Sections were stained with haematoxylin and eosin by standard techniques.

Results

Growth phenotypes of the *kexB* mutant

In *A. fumigatus* genome database, the open reading frame AFUA_4G12970 was annotated to encode a putative pheromone processing endoprotease KexB, which is 841 amino acids in length and contains a predicted self-cleavage site on the carboxyl side of ₁₂₂LVKR₁₂₅. In the putative cytosolic tail, the peptide sequence ₇₆₈YDFEMI₇₇₃ is identified, in which the underlined amino acid residues are identical to the late Golgi retention signal (consensus, YXFXXI) in the cytosolic tail of the *S. cerevisiae* Kex2 (Wilcox et al., 1992; Redding et al., 1991). To evaluate its significance, the *kexB* gene (AFUA_4G12970) was deleted in *A. fumigatus*. As compared with the wild-type strain, the *kexB* null mutant displayed a severely retarded growth on complete solid medium, especially at 50°C (Fig. 1A). Under scanning electron microscopy, the *kexB* mutant showed a severe reduction and abnormality in conidia formation (Fig. 1B). The amount of phialides formed by the *kexB* mutant was greatly reduced at 37°C. When the temperature was elevated to 50°C, the mutant lost its ability to form vesicles. Conidia counting confirmed that the *kexB* mutant exhibited a remarkably reduced conidiation at 37°C (Table 2) and was unable to produce conidiospore at 50°C.

In *A. fumigatus*, spore germination undergoes a brief period of isotropic growth, polarity establishment, and emergence of the germ tube that elongates by tip growth. It has been shown that the switch from isotropic to polar growth precedes the first mitotic division of the nuclear during early stage of germination. The earliest emergence of second germ tube from the conidia occurs after the third mitosis, and the first septation usually occurs in germ tube that has undergone four rounds of mitosis (Momany and Taylor, 2000; Zhang et al., 2008). As shown in Fig. 2, when conidia were cultivated in complete liquid medium at 37°C, the wild-type germinated in a typical bipolar pattern at an angle of 180°, and formed the second germ tube and the septum which formed at the basal end of the first germ tube. While the earliest emergence of the second germ tube occurred in the mutant only after the second mitotic division (5 h), and the hyper-branching was found after 7 h. As summarized in Table 3, 27% of the mutant conidia formed the first germ tube at 5 h, while 20% were

found with the hyper-branching at 8 h. Furthermore, about 30% of septum formed at the wrong places, suggesting that septation was less controlled in the mutant. These results demonstrated that deletion of the *kexB* gene led to abnormal polarized growth in *A. fumigatus*.

Cell wall defect of the *kexB* mutant

As shown in Fig.3, the mutant was hyper-sensitive to calcofluor white and Congo red at 50°C. The hyper-sensitivity of the *kexB* mutant to Congo red can be complemented by the addition of 1.5 M sorbitol to the medium, suggesting a temperature-sensitive defect of cell wall integrity in the mutant. The mutant also showed an increased sensitivity to hygromycin B, an aminoglycoside antibiotic inhibits protein synthesis (Brodersen et al., 2000). To examine whether alkaline conditions influenced the mutant or not, conidia were grown on solid medium containing 0.1 M Tris (pH 9.0). The growth of the mutant was greatly inhibited at pH 9.0, indicating that the *kexB* mutant was susceptible to alkaline pH. The mutant also showed an increased sensitivity to 2 mM H₂O₂ as compared with the wild-type.

Transmission electron microscope (TEM) revealed morphological changes in ultra-structure of mycelial cell wall of the mutant. As shown in Fig.4, after cultivation in complete liquid medium at 37°C for 24 h, the mutant had a slightly thickened hyphal cell wall. When the temperature was elevated to 50°C, the thickness of the mycelial cell wall was increased by 80% in the mutant as compared with the wild-type. Additionally, the cell wall of the mutant conidia formed at 37°C was also significantly thickened and the melanin layer was shedding.

We further analyzed the cell wall components of the *kexB* mutant. As summarized in Table 4, the glycoprotein and α -glucan in the *kexB* mutant were decreased by 28% and 39%, respectively, whereas β -glucan and chitin were increased by 35% and 28% as compared with the wild-type at 37°C. When the *kexB* mutant was cultured at 50°C, the amount of α -glucan, β -glucan and chitin were decreased by 47%, 28% and 9%, respectively, whereas the content of glycoprotein was increased by 11%. These results suggested that the *kexB* mutant might compensate for its reduction of glycoprotein and α -glucan by the up-regulation of β -glucan and chitin synthesis to strengthen its cell wall at 37°C. However, when the temperature was elevated to 50°C, the mutant was not able to up-regulate the synthesis of β -glucan and chitin, and displayed a temperature-sensitive defect of the cell wall integrity, which is consistent with the hyper-sensitivity of the *kexB* mutant to calcofluor white and Congo red at 50°C.

Activation of the CWI pathway in the *kexB* mutant

In *A. fumigatus*, the compensatory mechanism for cell wall defect requires an activation of the cell wall integrity (CWI) signaling pathway, which mainly consists of a family of cell-surface sensors, a small G protein Rho1, the mitogen-activated protein kinase (MAPK) cascade Pkc1-Bck1-Mkk2-MpkA/Slk2 (Dichtl et al., 2012; Dirr et al., 2010; Malavazi et al., 2014). Quantitative real-time PCR analysis revealed that transcription levels of the genes in this pathway were up-regulated in the mutant except *pkc1* (Fig.5A). We further detected the phosphorylation of MpkA in the mutant using the anti-phospho-p44/42 MAPK (Erk1/2)

antibody (Jain et al., 2011). Our results showed that Congo red-induced cell wall defect triggered an activation of MpkA in the wild-type, while MpkA was constitutively activated in the mutant at 37°C or 50°C (Fig.5B). These results demonstrated that cell wall defect in the *kexB* mutant induced an activation of the MpkA-dependent CWI signaling pathway.

As the CWI signaling was activated in the mutant, we further examined the expression of the genes responsible for cell wall biogenesis, such as β -1,3-glucan synthase FksA, β -1,3-glucan glucanotransferases Gel1-7, α -1,3-glucan synthases Ags1-3, and chitin synthases ChsA-F. As a result, when the mutant was grown at 37°C, the expression of the *gel* genes was induced in the mutant only with an exception of the *gel2*. Among these genes, the *gel5* and *gel6* were significantly induced (Fig.5C). Although *chsD* and *chsE* were suppressed in the mutant, the expression the *chsA* and *chsC* were induced 1.4- and 3.0-fold as compared with the wild-type. On the other hand, the expression of the *ags1* was only 30% of the wild-type, while the expression of the *ags3* was not changed and the *ags2* was slightly induced (1.5-fold). These results indicated that activation of the CWI signaling induced up-regulation of the β -1,3-glucan and chitin synthesis and led to an increase of the β -1,3-glucan and chitin in the cell wall of the mutant.

Identification of potential KexB substrates

In *S. cerevisiae*, proproteins such as the killer toxin or the pheromone α -mating factor are processed in the trans-Golgi network (TGN) by the Kex2p, which cleaves after KR motifs or, to a lesser degree, RR paired basic residue sites (Julius et al., 1984). Other kexin substrates identified in fungi include proteins involved in the formation of aerial hyphae, zymogens, peptidase, lipases, polysaccharide-degrading enzymes, and themselves (Wosten et al. 1996; Enderlin and Ogrydziak, 1994; Newport and Agabian, 1997; Umemura et al., 2014; Pignède et al., 2000; Goller et al., 1998). Based on these studies, Newport et al. (2003) proposed that potential substrates for kexin should be featured with: i) a total length longer than 100 amino acids, ii) a leader peptide, but not necessarily a signal peptidase cleavage site, iii) a propeptide less than 150 amino acids that P₁ must be R, P₂ can be R or K, P₃ can be any amino acid and P₄ cannot be CDFGPS or W, iv) cell membrane, cell wall or secreted proteins. Using the criteria proposed by Newport et al. (2003), we identified 213 candidate KexB substrates from the 9631 predicted proteins in the *A. fumigatus* proteome database (Nierman et al., 2005). Among 213 candidate substrates, 49 proteins are hypothetical proteins with unknown function, 49 proteins contain low-frequency RR cleavage sites, and, as summarized in Table S1, 115 proteins contain high-frequency KR cleavage sites (Bader et al., 2008). As the abnormal polarized growth associated with the fungal *kexB* disruptants are thought to be resulted from the lack of activity from cell-wall modifying enzymes (Punt et al., 2003; Mizutani et al., 2004; Mizutani et al., 2009), we paid a special attention to the cell-wall modifying enzymes in these predicted substrates. It turns out that β -1,3-glucanotransferases Gel4, Gel5, and Gel7 were predicted as candidate substrates of KexB in *A. fumigatus*. The potential cleavage site for Gel4, Gel5, and Gel7 lies at the carboxyl side of ₁₅₀LYKR₁₅₃, ₇₁LCKR₇₄, and ₇₁ACKR₇₄, respectively.

Protein detection in the *kexB* mutant

Using Anti-Gel4 antibody (Ouyang et al., 2013; Yan et al., 2013), we were able to detect if Gel4 protein was cleaved by KexB. Ecm33, a membrane glycoprotein without KexB-cleavage site, were used as a negative control. As shown in Fig.6A (upper panel), the molecular mass of Gel4 in the mutant was slightly higher than that in the wild-type, however, this minor difference was not expected to be contributed by the KexB-cleavage. Meanwhile, Ecm33 in the mutant was also slightly bigger than that in the wild-type. When the proteins were treated with trifluoromethanesulfonate (TFMS) to remove both N- and O-glycan or with PNase F to remove N-glycan (Fig.6A, lower panel), the molecular mass of Gel4 or Ecm33 from the mutant became the same as that from the wild-type, indicating that differences in molecular mass were due to different N-glycosylation. This finding was further confirmed by MALDI-TOF analysis of the N-glycans on cell wall proteins. In *A. fumigatus*, the N-glycans attached to mature secreted proteins are determined as Man₆GlcNAc₂, Man₇GlcNAc₂, and Man₈GlcNAc₂, in which Man₆GlcNAc₂ is the major glycoform (Zhang et al., 2008). As shown in Fig.6B, strong signals corresponding to Man₅₋₈GlcNAc₂ were detected in the wild-type, while strong signals corresponding to Man₅₋₉GlcNAc₂ were detected in the mutant. Although the abundances were quite lower, GlcMan₉GlcNAc₂ and Glc₂Man₉GlcNAc₂ were detected in the wild-type. In contrast, GlcMan₉GlcNAc₂ and Glc₂Man₉GlcNAc₂ were detected with a relative higher abundance in the mutant. In addition, the precursor N-glycan Glc₃Man₉GlcNAc₂ was only detected in the mutant. These results demonstrated that N-glycans extracted from the mutant were bigger than those from the wild-type, suggesting an impaired N-glycan processing occurred in the *kexB* mutant. In *A. fumigatus*, Ecm33, Gel1 and Gel4 have been confirmed as GPI-anchored membrane glycoproteins and can be detected in the cytosol, membrane, cell wall and culture supernatant (Ouyang et al., 2013; Yan et al., 2013). As an impaired N-glycan processing was observed to be associated with the mutant, we further examined the sub-cellular localization of these glycoproteins. As revealed in Fig.7, the amount of Gel1 or Gel4 protein was increased in the cytosol, but decreased in the cell wall as compared with the wild-type, indicating that the increased expression of Gel1 or Gel4 did not completely restore the cell wall bound Gel1 or Gel4 to a level similar to the wild-type and led to an accumulation of Gel1 and Gel4 inside the mutant cells. The similar result was also found in Ecm33. These results suggested that impaired N-glycan processing led to an accumulation of intracellular glycoproteins in the mutant.

Endoplasmic reticulum stress in the *kexB* mutant

Previously, we have shown that deletion of the *cwh41* gene, which encodes α -glucosidase I that regulates trimming of the terminal α -1,2-glucose residue in the N-glycan-processing pathway, leads to a severe reduction in conidia formation, a temperature-sensitive deficiency of cell wall integrity and abnormalities of polar growth and septation in *A. fumigatus* (Zhang et al., 2008). Further analyses reveal that misfolded proteins, especially Gel1 and Gel2, are accumulated in the endoplasmic reticulum (ER) in the *cwh41* mutant, which leads to ER-stress featured with over-expression of the histone ubiquitination protein Bre1, calnexin, and Hsp70 (Zhang et al., 2009; Zhao et al., 2013). Since the phenotypes, as well as un-trimmed N-glycan on secreted proteins, associated with the *kexB* mutant were similar to those

observed in the *cwh41* mutant, it is reasonable to expect an occurrence of ER-stress in the *kexB* mutant.

Several chemical compounds are known to induce ER stress, such as DTT, tunicamycin (TM) and brefeldin A (BFA) (Back et al., 2005). DTT unfolds proteins directly by reducing disulfide bonds, TM impairs protein folding by inhibiting N-glycosylation, and BFA impairs anterograde protein transport from the ER to the Golgi (Richie et al., 2011). When the *kexB* mutant was cultivated in the presence of DTT, TM or BFA at 37°C or 50°C, an increased sensitivity was observed (Fig.8A). Furthermore, Real-time PCR analysis revealed that the genes involved in proteasome and ubiquitin-mediated proteolysis, such as *pre6*, *ump1* and *cwf8* (Yan et al., 2013), were induced in the mutant (Fig.8B). The expression levels of the histone ubiquitination protein Bre1, Hsp70 and calnexin were also increased in the mutant. These results confirmed an occurrence of ER stress in the *kexB* mutant.

As ER-stress can activate the unfolded protein response (UPR) in *A. fumigatus* (Richie et al., 2011; Malavazi et al., 2014), we further measured the expression levels of four known UPR-related genes, including *hacA*, *bipA*, *pdiA* and *tigA*. The expression levels of these genes were 17.3-, 2.5-, 25.1- and 18.5-fold of that in the wild-type respectively, suggesting an activation of the UPR triggered by ER stress in the mutant.

Virulence of the *kexB* mutant

To evaluate the contribution of KexB to the virulence of *A. fumigatus*, freshly harvested conidia from the wild-type, the *kexB* mutant and the complemented were inoculated into immune-compromised mice. The survival rate of the mice inoculated with conidia from the *kexB* mutant was significantly higher than those inoculated with the wild-type or complemented conidia (Fig.9A). Micrograph showed that the lesion of experimental invasive pulmonary aspergillosis (IPA) was developed in mice inoculated with conidia intranasally and not found in mice inoculated with saline (Fig.9B). In the lung tissues from mice inoculated with conidia of the wild-type and the complemented, mycelial invasion of the bronchial epithelium and alveolae could be readily observed and *A. fumigatus* hyphae could be easily identified. Histologically these fungus-induced lesions were characterized by extensive necrosis and an influx of neutrophils and macrophages. Although hyphae could also be found in the lung tissues from mice infected with the *kexB* conidia, the neutrophilic infiltration and necrosis were much gentle, suggesting an attenuated virulence of the mutant.

Discussion

Some kexin substrates, such as pheromone α -mating factor, proteinases, lipases, and polysaccharide-degrading enzymes, have been identified in yeast and several other fungi (Julius et al., 1984; Enderlin and Ogrydziak, 1994; Newport and Agabian, 1997; Pignède et al., 2000; Goller et al., 1998). By comparing those experimentally confirmed kexin substrates, Newport et al. proposed several criteria to predict potential kexin substrates and identified 130 potential substrates for Kex2p in *C. albicans* (Newport et al., 2003). However, only few of them have been experimentally confirmed as substrates of Kex2p. It still remains unclear why deletion of the *kex2* gene in *C. albicans* causes severe phenotypes

such as impaired hyphae production, morphological defects in the cell, and a diminished virulence.

In this study, we identified 213 candidate KexB substrates in the *A. fumigatus*. Among them, 115 proteins contain high-frequency KR cleavage sites (Table S1). Although none of them has been experimentally confirmed as substrate of KexB in *A. fumigatus*, it is interesting to note that these predicted substrates include β -1,3-glucanotransferases Gel4, Gel5, and Gel7. It should be pointed out that the predicted self-cleavage site of KexB is ¹²²LVKR-VPP₁₂₈ (cleavage-site is shown as dash), while the predicted cleavage site of Gel4, Gel5, and Gel7 are ¹⁵⁰LYKR-YTS₁₅₆, ⁷¹LCKR-DVP₇₇, and ⁷¹ACKR-DVP₇₇, respectively. Theoretically, Gel5 possesses a cleavage site similar to that in KexB and should be a good substrate for KexB. Indeed, we observed a 56-fold increase in expression of the *gel5* in the mutant (Fig.5C), which might be a compensatory response for the dysfunction of Gel5 in the mutant.

As Gel4 was predicted as one of the potential KexB substrates in *A. fumigatus*, we first tried to determine whether Gel4 was substrate of KexB by using antibody available in our lab. To our surprise, we only observed a slight increase of molecular mass of the Gel4 from the mutant strain, which was not contributed by a typical Kexin-cleavage. As a negative control, a slight up-shift of molecular mass was also observed with Ecm33 from the mutant. Therefore, we assumed that this slight difference in molecular mass might be resulted from altered glycosylation. After treating with TFMS or PNase F, as expected, Gel4 and Ecm33 in the mutant moved to the same positions corresponding to those in the wild-type (Fig.6A). Further analysis confirmed that increase of molecular weight was contributed by un-trimmed N-glycan (Fig.6B). Moreover, Western blot results showed that Gel1, Gel4 and Ecm33 were accumulated inside the mutant cells. These results demonstrated an occurrence of the impaired N-glycan processing and defective protein folding in the *kexB* mutant.

In a mammalian cell, N-glycan processing in the 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 and Aebi, 2004; Ruddock and Molinari, 2006). This QC system 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-associated degradation (ERAD) system takes care of the misfolded proteins (Ruddock and Molinari, 2006; Tamura et al., 2010). The processing of N-glycan is initiated by the action of α -glucosidase I to remove the terminal glucose. *A. fumigatus* possesses a similar QC system, which is composed of calnexin, UDP-glucose:glycoprotein glucosyltransferase, and glucosidase II. We have previously shown that deletion of the *cwh41*, a gene encoding *A. fumigatus* α -glucosidase I, results in a conversion of N-glycan on mature glycoprotein from the wild-type Man₆ GlcNAc₂ to the mutant Glc₃Man₉GlcNAc₂ and the phenotypes such as temperature-sensitive cell wall defect, reduced conidiation, abnormal polarity, and activation of both UPR and CWI signaling pathway (Zhang et al., 2008; Zhang et al., 2009). Recently, we confirmed that β -1,3-glucanotransferases Gel1 and Gel2 required N-glycosylation for their proper folding and function in *A. fumigatus*.

Deletion of the *cwh41* led to mis-folding and degradation of Gel1 and Gel2, which is the main factor causing the phenotypes associated with the *cwh41* mutant (Zhao et al., 2013).

In this study we showed that deletion of the *kexB* gene resulted in a temperature-sensitive defect of cell wall integrity, severe reduction of conidiation, and abnormalities of polarized growth in *A. fumigatus*, which are quite similar to the phenotypes associated with the *cwh41* mutant, but more severe (Zhang et al., 2008). As blocking of the N-glycan processing leads to activation of the CWI signaling and ER-stress in the *cwh41* mutant, which is featured with over-expression of Hsp70, calnexin and histone ubiquitination protein Bre1 (Zhang et al., 2009), we also examined the CWI pathway and ER-stress in the *kexB* mutant. It turns out that in the *kexB* mutant the CWI signaling was activated (Fig.5A&B) and ER-stress was induced (Fig.8). In addition, the *hacA*, *bipA*, *pdiA* and *tigA* were also induced (Fig.8B). Although the UPR has been proposed to be a regulatory hub in *A. fumigatus*, either with or without connection with the CWI (Malavazi et al., 2014), in our case the UPR should be connected to the CWI and the ER-stress in the *kexB* mutant.

As the Kexin-like proteins localize in the late trans Golgi network, endocytic compartment, and prevacuolar compartment (Redding et al., 1991; Blanchette et al., 2004), their target spectrum should be limited to proteins attached to the cell surface, those proteins which are secreted into the environment or to the luminal domains of integral membrane proteins passing through these compartments. Interestingly, ER α -glucosidase II (AFUA_5G03500), one of members of the protein folding QC system in *A. fumigatus*, was predicted as one of the candidate substrates of KexB with a predicted cleavage site at ₁₁₆KR-MKG (Table S1). Although it is not understood whether and how α -glucosidase II is processed by KexB, together with the evidences we obtained, it is reasonable to postulate that deletion of the *kexB* might lead to the loss-of-function of α -glucosidase II and the impaired N-glycan processing in the ER, which then leads to a failure of glycoprotein folding, especially the enzymes required for cell wall synthesis. Our hypothesis are supported by several lines of evidences: (i) the *kexB* mutant exhibited phenotypes similar to those associated with the *cwh41* mutant; (ii) the N-glycans on membrane proteins from the mutant were slightly larger than those from the wild-type, which is consistent with that observed in the *cwh41* mutant; (iii) the occurrence of ER-stress and UPR in the mutant suggested an accumulation of misfolded proteins in the ER; and (iv) expression of the gene encoding α -glucosidase II in the mutant was 6.7-fold of that in the wild-type.

Despite the *kexB* mutant and the *cwh41* mutant share similar phenotypes, in contrast to the *cwh41* (Zhang et al., 2008), the *kexB* is essential for virulence of *A. fumigatus*. It's likely that N-glycan processing is not the only one pathway affected in the *kexB* mutant and some virulence factors (Rementeria et al., 2005) that are predicted as the substrates of KexB are also affected (Table S1). Furthermore, we observed that melanin layer was unable to effectively attach to the cell wall of the mutant (Fig.4). As the melanin is an important virulence factor and sequesters the reactive oxygen species (ROS) of the phagocytes (Rementeria et al., 2005; Malavazi et al., 2014), it is likely that shedding of melanin from the surface of the mutant conidia is one of reasons that causes attenuated virulence of the mutant.

In summary, our results showed that disruption of the *kexB* gene led to a retarded growth, reduced conidia formation, temperature-sensitive defect in cell wall integrity, abnormal polarity and attenuated virulence. For the first time, the kexin-like endoprotease was found to be involved in the N-glycan processing and thus the N-glycan-dependent protein folding. It is likely that phenotypes associated with the *kexB* mutant were mainly due to impaired N-glycan processing, which probably results from the loss-of-function of α -glucosidase II, a predicted candidate substrate of KexB. On the other hand, some virulence factors that require KexB-cleavage for their activation might contribute to the attenuated virulence of the *kexB* mutant. To gain evidence to support our hypothesis, the native substrates of KexB need to be identified in *A. fumigatus*.

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 (313101010) to C. Jin and partially by the Austrian Science Fund (FWF) Grant I 391 to I. Wilson. The authors would thank Dr. Lei Sun, Institute of Biophysics, Chinese Academy of Sciences, for TEM analysis.

References

1. Anderson ED, Molloy SS, Jean F, Fei H, Shimamura S, et al. The ordered and compartment-specific autoproteolytic removal of the furin intramolecular chaperone is required for enzyme activation. *J Biol Chem.* 2002; 277(15):12879–90. [PubMed: 11799113]
2. Back SH, Schröder M, Lee K, Zhang K, Kaufman RJ. ER stress signaling by regulated splicing: IRE1/HAC1/XBP1. *Methods.* 2005; 35:395–416. [PubMed: 15804613]
3. Bader O, Krauke Y, Hube B. Processing of predicted substrates of fungal Kex2 proteinases from *Candida albicans*, *C. glabrata*, *Saccharomyces cerevisiae* and *Pichia pastoris*. *BMC microbiology.* 2008; 8:116. [PubMed: 18625069]
4. Bader O, Schaller M, Klein S, Kukula J, Haack K, et al. The *KEX2* gene of *Candida glabrata* is required for cell surface integrity. *Molecular Microbiology.* 2001; 41:1431–1444. [PubMed: 11580846]
5. Biesebeke R, Record E, Biezen N, Heerikhuisen M, Franken A, et al. Branching mutants of *Aspergillus oryzae* with improved amylase and protease production on solid substrates. *Appl Microbiol Biotechnol.* 2005; 69:44–50. [PubMed: 15909137]
6. Blanchette JM, Abazeed ME, Fuller RS. Cell-free reconstitution of transport from the trans-golgi network to the late endosome/prevacuolar compartment. *J Biol Chem.* 2004; 279:48767–48773. [PubMed: 15364946]
7. Bong KK, Kap HH, Kyu YH, Sung MJ, Sang GH, et al. Molecular Cloning of *kpcA* Gene Encoding a Kex2p-like Endoprotease from *Aspergillus nidulans*. *Mol Cells.* 2001; 12:142–147. [PubMed: 11561725]
8. Brodersen DE, Clemons WM Jr, Carter AP, Morgan-Warren RJ, Wimberly BT, et al. The structural basis for the action of the antibiotics tetracycline, pactamycin, and hygromycin B on the 30S ribosomal subunit. *Cell.* 2000; 103:1143–1154. [PubMed: 11163189]
9. Cove D. The induction and repression of nitrate reductase in the fungus *Aspergillus nidulans*. *Biochimica et Biophysica Acta.* 1966; 113:51.
10. Davey J, Davis K, Imai Y, Yamamoto M, Matthews G. Isolation and characterization of *krp*, a dibasic endopeptidase required for cell viability in the fission yeast *Schizosaccharomyces pombe*. *The EMBO journal.* 1994; 13:5910–5921. [PubMed: 7813430]

11. 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]
12. Dichtl K, Helmschrott C, Dirr F, Wagener J. Deciphering cell wall integrity signalling in *Aspergillus fumigatus*: identification and functional characterization of cell wall stress sensors and relevant Rho GTPases. *Molecular Microbiology*. 2012; 83:506–519. [PubMed: 22220813]
13. Dirr F, Echtenacher B, Heesemann J, Hoffmann P, Ebel F, et al. *AfMkk2* is required for cell wall integrity signaling, adhesion, and full virulence of the human pathogen *Aspergillus fumigatus*. *Int J Med Microbiol*. 2010; 300:496–502. [PubMed: 20452278]
14. Edge ASB. Deglycosylation of glycoproteins with trifluoromethanesulphonic acid: elucidation of molecular structure and function. *Biochem J*. 2003; 376:339–350. [PubMed: 12974674]
15. Enderlin CS, Ogrydziak DM. Cloning, nucleotide-sequence and functions of *Xpr6*, which codes for a dibasic processing endoprotease from the yeast *Yarrowia lipolytica*. *Yeast*. 1994; 10:67–79. [PubMed: 8203153]
16. Goller SP, Schoisswohl D, Baron M, Parriche M, Kubicek CP. Role of endoproteolytic dibasic proprotein processing in maturation of secretory proteins in *Trichoderma reesei*. *Applied and Environmental Microbiology*. 1998; 64:3202–3208. [PubMed: 9726860]
17. Helenius A, Aebi M. Roles of N-linked glycans in the endoplasmic reticulum. *Ann Rev Biochem*. 2004; 73:1019–1049. [PubMed: 15189166]
18. Jain R, Valiante V, Remme N, Docimo T, Heinekamp T, et al. The MAP kinase MpkA controls cell wall integrity, oxidative stress response, gliotoxin production and iron adaptation in *Aspergillus fumigatus*. *Mol Microbiol*. 2011; 82:39–53. [PubMed: 21883519]
19. Jalving R, van de Vondervoort PJJ, Visser J, Schaap PJ. Characterization of the kexin-like maturase of *Aspergillus niger*. *Applied and Environmental Microbiology*. 2000; 66:363–368. [PubMed: 10618249]
20. Julius D, Brake A, Blair L, Kunisawa R, Thorner J. Isolation of the putative structural gene for the lysine-arginine-cleaving endopeptidase required for processing of yeast prepro- α -factor. *Cell*. 1984; 37:1075–1089. [PubMed: 6430565]
21. Komano H, Fuller RS. Shared functions in vivo of a glycosyl-phosphatidylinositol-linked aspartyl protease, Mkc7, and the proprotein processing protease Kex2 in yeast. *Proc Natl Acad Sci USA*. 1995; 92:10752–10756. [PubMed: 7479877]
22. Krappmann S. Tools to study molecular mechanisms of *Aspergillus* pathogenicity. *Trends in Microbiology*. 2006; 14:356–364. [PubMed: 16806936]
23. Lee LH, Gigliotti F, Wright TW, Simpson-Haidaris PJ, Weinberg GA, et al. Molecular characterization of KEX1, a kexin-like protease in mouse *Pneumocystis carinii*. *Gene*. 2000; 242:141–150. [PubMed: 10721706]
24. Li H, Zhou H, Luo Y, Ouyang H, Hu H, Jin C. Glycosylphosphatidylinositol (GPI)-anchor is required in *Aspergillus fumigatus* for morphogenesis and virulence. *Mol. Microbiol*. 2007; 64(4): 1014–1027. [PubMed: 17501924]
25. Malavazi I, Goldman GH, Brown NA. The importance of connections between the cell wall integrity pathway and the unfolded protein response in filamentous fungi. *Brief Funct Genomics*. 2014; 13(6):456–470. [PubMed: 25060881]
26. Mesaeli N, Nakamura K, Zvaritch E, Dickie P, Dziak E, et al. Calreticulin is essential for cardiac development. *J Cell Biol*. 1999; 144:857–868. [PubMed: 10085286]
27. Mizutani O, Furukawa K, Ichianagi S, Matsuda Y, Tokuoka M, et al. Alternative processing of proproteins in *Aspergilli kexB* gene disruptants under hyperosmotic conditions. *Bioscience, biotechnology, and biochemistry*. 2009; 73:40–46.
28. Mizutani O, Nojima A, Yamamoto M, Furukawa K, Fujioka T, et al. Disordered cell integrity signaling caused by disruption of the *kexB* Gene in *Aspergillus oryzae*. *Eukaryot Cell*. 2004; 3:1036–1048. [PubMed: 15302836]
29. Momany M, Taylor I. Landmarks in the early duplication cycles of *Aspergillus fumigatus* and *Aspergillus nidulans*: polarity, germ tube emergence and septation. *Microbiology*. 2000; 146:3279–3284. [PubMed: 11101686]

30. Newport G, Agabian N. *KEX2* Influences *Candida albicans* proteinase secretion and hyphal formation. *J Biol Chem*. 1997; 272:28954–28961. [PubMed: 9360967]
31. Newport G, Kuo A, Flattery A, Gill C, Blake JJ, et al. Inactivation of Kex2p diminishes the virulence of *Candida albicans*. *J Biol Chem*. 2003; 278:1713–1720. [PubMed: 12419804]
32. Nierman WC, Pain A, Anderson MJ, Wortman JR, Kim HS, et al. Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. *Nature*. 2005; 438:1151–1156. [PubMed: 16372009]
33. Oluwatosin YE, Kane PM. Mutations in the yeast *KEX2* gene cause a Vma(–)-like phenotype: a possible role for the Kex2 endoprotease in vacuolar acidification. *Molecular and cellular biology*. 1998; 18:1534–1543. [PubMed: 9488470]
34. Ouyang H, Chen X, Lu 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*. *Eukaryot Cell*. 2013; 12:889–899. [PubMed: 23584992]
35. Pignède G, Wang H, Fudalej F, Gaillardin C, Seman M, et al. Characterization of an extracellular lipase encoded by *LIP2* in *Yarrowia lipolytica*. *Journal of Bacteriology*. 2000; 182:2802–2810. [PubMed: 10781549]
36. Punt PJ, Drint-Kuijvenhoven A, Lokman BC, Spencer JA, Jeenes D, et al. The role of the *Aspergillus niger* furin-type protease gene in processing of fungal proproteins and fusion proteins: Evidence for alternative processing of recombinant (fusion-) proteins. *Journal of Biotechnology*. 2003; 106:23–32. [PubMed: 14636707]
37. Redding K, Holcomb C, Fuller RS. Immunolocalization of Kex2 protease identifies a putative late Golgi compartment in the yeast *Saccharomyces cerevisiae*. *J Cell Biol*. 1991; 113:527–538. [PubMed: 2016334]
38. Rementeria A, Lopez-Molina N, Ludwig A, Vivanco AB, Bikandi J, et al. Genes and molecules involved in *Aspergillus fumigatus* virulence. *Revista iberoamericana de micologia*. 2005; 22:1–23. [PubMed: 15813678]
39. Richard M, Quijano RR, Bezzate S, Bordon-Pallier F, Gaillardin C. Tagging morphogenetic genes by insertional mutagenesis in the yeast *Yarrowia lipolytica*. *Journal of Bacteriology*. 2001; 183:3098–3107. [PubMed: 11325938]
40. Richie DL, Feng XZ, Hartl L, Aimaganianda V, Krishnan K, et al. The virulence of the opportunistic fungal pathogen *Aspergillus fumigatus* requires cooperation between the endoplasmic reticulum-associated degradation pathway (ERAD) and the unfolded protein response (UPR). *Virulence*. 2011; 2:12–21. [PubMed: 21217201]
41. Rockwell NC, Krysan DJ, Komiyama T, Fuller RS. Precursor processing by Kex2/Furin proteases. *Chemical Reviews*. 2002; 102:4525–4548. [PubMed: 12475200]
42. Rockwell NC, Thorner JW. The kindest cuts of all: crystal structures of Kex2 and furin reveal secrets of precursor processing. *Trends in Biochemical Sciences*. 2004; 29:80–87. [PubMed: 15102434]
43. Ruddock LW, Molinari M. N-glycan processing in ER quality control. *Journal of Cell Science*. 2006; 119:4373–4380. [PubMed: 17074831]
44. Seidah NG, Chrétien M. Proprotein and prohormone convertases: a family of subtilases generating diverse bioactive polypeptides. *Brain Research*. 1999; 848:45–62. [PubMed: 10701998]
45. Tamura T, Sunryd JC, Hebert DN. Sorting things out through endoplasmic reticulum quality control. *Molecular Membrane Biology*. 2010; 27:412–427. [PubMed: 20553226]
46. Tanguy-Rougeau C, Wésolowski-Louvel M, Fukuhara H. The *Kluyveromyces lactis KEX1* gene encodes a subtilisin-type serine proteinase. *FEBS Letters*. 1988; 234:464–470. [PubMed: 3292294]
47. Turpeinen H, Ortutay Z, Pesu M. Genetics of the first seven proprotein convertase enzymes in health and disease. *Current Genomics*. 2013; 14:453–467. [PubMed: 24396277]
48. Umemura M, Nagano N, Koike H, Kawano J, Ishii T, et al. Characterization of the biosynthetic gene cluster for the ribosomally synthesized cyclic peptide ustiloxin B in *Aspergillus flavus*. *Fungal Genetics and Biology*. 2014; 68:23–30. [PubMed: 24841822]

49. Venancio EJ, Daher BS, Andrade RV, Soares CMA, Pereira IS, et al. The *kex2* gene from the dimorphic and human pathogenic fungus *Paracoccidioides brasiliensis*. *Yeast*. 2002; 19:1221–1231. [PubMed: 12271458]
50. Wickner RB. Chromosomal and nonchromosomal mutations affecting the “killer character” of *Saccharomyces cerevisiae*. *Genetics*. 1974; 76:423–432. [PubMed: 4364866]
51. Wilcox CA, Rdedding R, Wright R, Fuller RS. Mutation of a tyrosine localization signal in the cytosolic tail of yeast Kex2 protease disrupts Golgi retention and results in default transportation to the vacuole. *Mol Biol Cell*. 1992; 3:1353–1371. [PubMed: 1493334]
52. Wosten HA, Bohlmann R, Eckerskorn C, Lottspeich F, Bolker M, et al. A novel class of small amphipathic peptides affect aerial hyphal growth and surface hydrophobicity in *Ustilago maydis*. *The EMBO journal*. 1996; 15:4274–4281. [PubMed: 8861956]
53. 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]
54. 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; 289:155–165. [PubMed: 19090038]
55. Zhang L, Feng D, Fang W, Ouyang H, Luo Y, et al. Comparative proteomic analysis of an *Aspergillus fumigatus* mutant deficient in glucosidase I (AfcWh41). *Microbiol-SGM*. 2009; 155:2157–2167.
56. Zhao W, Lü Y, Ouyang H, Zhou H, Yan J, et al. N-glycosylation of Gel1 or Gel2 is vital for cell wall β -glucan synthesis in *Aspergillus fumigatus*. *Glycobiology*. 2013; 23(8):955–968. [PubMed: 23650256]

Highlights

- The KexB is required for morphogenesis such as hyphal growth, conidiation, and polarity in *A. fumigatus*.
- Deletion of the *kexB* gene results in a temperature-sensitive cell wall defect.
- MpkA-dependent cell wall integrity signaling pathway is activated and ER-stress is induced in the *kexB* mutant.
- An impaired N-glycan processing is associated with the *kexB* mutant.
- The *kexB* mutant exhibits an attenuated virulence in immunocompromised mice.

Fig.1. Growth (A) and morphology (B) of the *kexB* mutant at 37°C or 50°C

In A, a 10 μ l drop containing 1×10^3 freshly harvested conidia were dotted on solid complete medium and incubated at 37°C or 50°C. The colony diameter was monitored intermittently and the mean diameter was used to plot the radial growth curves. Each point represents one growth diameter measurement (three experiments) and the tendency curves are indicated. In B, strains were cultivated on solid medium at 37°C or 50°C. Colonies were fixed in phosphate buffered glutaraldehyde followed by OsO_4 , impregnated with uranyl acetate during ethanol dehydration, critical point dried, and sputter coated with gold-palladium and examined with a Quanta200 scanning electron microscope (SEM).

Fig.2. Germination of the *kexB* mutant

10 ml of complete liquid medium was inoculated with 10^6 freshly harvested conidia in a Petri dish containing five glass coverslips at 37°C. At the specified times, the coverslips with adhering germinated conidia were taken out, fixed in fixative solution (4% formaldehyde, 50mM phosphate buffer, pH 7.0, and 0.3% Triton X-100). After 30 min, coverslips were washed with phosphate-buffered saline (PBS), incubated for 20 min with 1 μg 4',6-diamidino-2-phenylindole (DAPI) ml^{-1} (Sigma), washed with PBS, then dyed for 5 min with a 5 μg ml^{-1} solution of fluorescent brightener 28 (Sigma) and washed for 5 times, observed and photographed using a fluorescence microscope (Axiovert 200M, Carl Zeiss). Typical photographs are shown. WT, wild-type; M, mutant; C, complemented strain. Bar, 10 μm .

Fig.3. Sensitivity to cell wall perturbing compounds and conditions of the *kexB* mutant
 A series of 10-fold dilutions (10^5 - 10^2 cells) of spores were spotted onto the solid complement medium containing $100 \mu\text{g ml}^{-1}$ Congo red, $100 \mu\text{g ml}^{-1}$ Calcofluor white (Sigma), 1.5 M sorbitol (Sigma), $20 \mu\text{g ml}^{-1}$ hygromycin B (Sigma), 0.1 M Tris (Amresco) or 2 mM H_2O_2 , respectively. After incubation at 37°C for 24 h or 50°C for 48 h, the plates were taken out and photographed. WT, wild-type; M, mutant; C, complemented strain.

Fig.4. Transmission electron microscopy (TEM) of the mycelia and conidial cell wall in the *kexB* mutant

The mycelia cultivated in liquid complement medium at 37°C for 24 h and 50°C for 48 h were collected and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 4 h or overnight at 4°C, washed 3 times in 0.1 M phosphate, post-fixed in 1% osmium tetroxide, and incubated for 2-4 h in 0.1 M phosphate. After 15-20 min in methanol 30%, 50%, 70%, 85%, 95%, and 100% sequentially, the mycelia were post-fixed in 2% of 30% uranyl acetate-methanol, rinsed, dehydrated and embedded in Epon 812 for the floating sheet method. The thin sections of the cell samples were examined with a transmission electron microscope (Tecnai Spirit model, FEI) operating at an accelerating speed of 120 kV. WT, wild-type; M, mutant; C, complemented strain.

Fig.5. Activation of the genes involved in cell wall biogenesis in the *kexB* mutant

In A, conidia (10^7) cultured at 37°C for 24 h were used for total RNA extraction and cDNA synthesis as described under Materials and Methods. Results are presented as mean $\pm$ SD (B). In B, conidia (10^7) were cultured at 37°C for 22 h or 50°C for 46 h. Cell wall damage was induced by the addition of Congo red for 2 h. Cell extracts were prepared and detected using anti-phospho-p44/42 MAPK antibody. As a control, an anti-Mn-SOD antibody was used to detect an unrelated protein. In C, conidia (10^7) cultured at 37°C for 24 h were used

for total RNA extraction and cDNA synthesis as described under Materials and Methods. Results are presented as mean $\pm$ SD.

Fig.6. Detection of protein glycosylation (A) and N-glycans (B) in the *kexB* mutant
Cell wall proteins were extracted from different strains. In A, cell wall proteins was treated with or without PNGase F or TFMS, separated by SDS-PAGE and detected with Western blotting using anti-Gel1, anti-Gel4, or anti-Ecm33 antibody. In B, N-glycans on the cell wall proteins were released by PNGase F and subject to Mass spectrometry analysis.

Fig.7. Distribution of β -1,3-glucan glucanosyltransferase Gel1 and Gel4

Extracellular, cell wall, membrane and cytosolic proteins were extracted as described under Material and Methods. Equal amounts of extracellular, cell wall, membrane and cytosolic proteins from the wild-type and mutant were separated by SDS-PAGE and detected by Western blotting using anti-Gel1, anti-Gel4, or anti-Ecm33 antibody.

Fig.8. Occurrence of ER-stress and UPR in the *kexB* mutant

In A, 5,000 conidia were inoculated onto the center of the complement medium containing Brefeldin A (Sigma), dithiothreitol (Sigma) or tunicamycin (Sigma) and incubated at 37°C for 24 h or 50°C for 48 h. The experiment was repeated three times. In B, conidia (10^7) cultured at 37°C for 24 h were used for total RNA extraction and cDNA synthesis as described under Materials and Methods. Results are presented as mean $\pm$ SD. WT, wild-type; M, mutant; C, complemented strain.

Fig.9. Virulence of the *kexB* mutant

In A, survival of immune-suppressed mice infected intranasally with 6×10^6 spores of the wild-type, the *kexB* mutant and the complemented strain. This figure only shows survival until day 14 post infection, as there was no further change from day 8 post infection until the end of the experiment. In B, lung tissue of immune-suppressed mice infected with the mutant at day 3 post infection. Saline containing 0.2% Tween 20 served as the negative control. The right lung from each mouse was dissected, fixed in 10% (v/v) formaldehyde in physiological saline and was stained with haematoxylin and eosin using standard techniques. Paraffin sections were prepared, cut and slices were observed under a light microscope. WT, wild-type; M, mutant; C, complemented strain.

Table 1
Primers used in this study.

Oligonucleotide primer	Sequence (5'-3')
Del-kexB-up-5'	TGAGGTTGACCTCCCACTTTACG
Del-kexB-up-3'	CTTGGATGTGTAGGACGCCTTT
Del-kexB-down-5'	CCTGCTTCTGTACTTAGTAGGCG
Del-kexB-down-3'	GGCTTTGACGGAGACAATGTATG
Rev-kexB-up-5'	TTGTCATCGTGCAAATAGTCC
Rev-kexB-up-3'	CTATCGGGTGCTGGTGGGTG
Rev-kexB-down-5'	GATATATTCCATATTCTCGT
Rev-kexB-down-3'	TTATCCGTTCAAAGTCGGCTA
Probe-5'	CTTTGATAGCGTCTCATGGAG
Probe-3'	TGATCCGTGTGGTCTTTGAGC
PyrG-5'	CCAGTGGAAGCCTCTGAAGGAG
PyrG-3'	ACTATGCGTGCTGCTAGGGTCG
KexB-5'	GGAGTATAGCTCTAGTCTTGAGC
KexB-3'	TCAGTGGTCACCGTGTCTCAG

Table 2
Conidia counting of the *kexB* mutant at 37°C.

Time (h)	Number of conidia ($\times 10^5$)	
	Wild-type	Mutant
24	217 $\pm$ 7	5.3 $\pm$ 0.1
36	2833 $\pm$ 81	44.4 $\pm$ 4.2
48	9937 $\pm$ 679	180.5 $\pm$ 13.8
60	26583 $\pm$ 2358	215 $\pm$ 12.3
72	34500 $\pm$ 2119	248.7 $\pm$ 8.8
84	38083 $\pm$ 1305	261.2 $\pm$ 2.3
96	78125 $\pm$ 4065	271.9 $\pm$ 2.6

The conidia (10^3) were spotted on solid complete medium and incubated at 37°C. At the specified times, conidia were harvested, resuspended in saline containing 0.01% Tween-20, and counted under microscopy using haemocytometer. A triplet of each strain was counted. The same experiment was repeated twice.

Table 3

Statistics of germination of the *kexB* mutant

Time (h)	Wild-type				<i>kexB</i>				Complemented strain			
	Number of germ tube				Number of germ tube				Number of germ tube			
	0	1	2	hyper-branching	0	1	2	hyper-branching	0	1	2	hyper-branching
5	85±3	15±1	0	0	72±2	27±1	1±0	0	88±5	12±1	0	0
6	45±1	54±2	1±0	0	41±2	57±4	2±0	0	45±3	54±5	1±0	0
7	7±1	78±4	15±1	0	14±1	72±3	13±1	1±0	6±1	80±6	14±2	0
8	4±0	73±5	22±3	1±0	6±0	60±2	14±1	20±2	1±0	67±3	30±2	2±0

Freshly harvested conidia (10^6) were poured into a Petri dish containing five glass coverslips and incubated in 10 ml of complete liquid medium at 37°C. The coverslips with adhering germings were removed and counted under microscope. For each independent experiment 100 conidia were counted and three independent experiments were carried out.

Table 4
Cell wall components of the *kexB* mutant at 37°C and 50°C.

Temperature	Strain	Alkali soluble		Alkali insoluble	
		Glycoprotein (μg)	α-glycan (μg)	β-glycan (μg)	Chitin (μg)
37°C	WT	148±6	71±3	258±13	204±11
	M	106±3	43±3	349±18	262±4
	C	153±12	58±1	294±4	221±9
50°C	WT	137±9	171±5	977±30	482±7
	M	152±6	90±7	700±23	439±8
	C	128±10	123±4	771±14	452±11

Three aliquots of 10 mg lyophilized mycelia were used as independent samples for cell wall analysis, and the experiment was repeated three times from different biological samples. The values shown are microgram of cell wall component per 10 mg dry mycelia (± SD). WT, wild-type; M, mutant; C, complemented strain.