



Phenotypic and Proteomic Analysis of the *Aspergillus fumigatus* Δ PrtT, Δ XprG and Δ XprG/ Δ PrtT Protease-Deficient Mutants

Einav Shemesh^{1†}, Benjamin Hanf^{2,3†}, Shelly Hagag^{1†}, Shani Attias¹, Yana Shadkchan¹, Boris Fichtman⁴, Amnon Harel⁴, Thomas Krüger^{2,3}, Axel A. Brakhage^{2,3}, Olaf Kniemeyer^{2,3} and Nir Osherov^{1*}

OPEN ACCESS

Edited by:

Miguel Cacho Teixeira,
Universidade de Lisboa, Portugal

Reviewed by:

Amariliz Rivera,
New Jersey Medical School,
United States
Izabela Marques Dourado Bastos,
University of Brasília, Brazil
Takahito Toyotome,
Obihiro University of Agriculture
and Veterinary Medicine, Japan

*Correspondence:

Nir Osherov
nosherov@post.tau.ac.il

[†]These authors have contributed
equally to this work.

Specialty section:

This article was submitted to
Infectious Diseases,
a section of the journal
Frontiers in Microbiology

Received: 09 July 2017

Accepted: 30 November 2017

Published: 12 December 2017

Citation:

Shemesh E, Hanf B, Hagag S,
Attias S, Shadkchan Y, Fichtman B,
Harel A, Krüger T, Brakhage AA,
Kniemeyer O and Osherov N (2017)
Phenotypic and Proteomic Analysis
of the *Aspergillus fumigatus*
 Δ PrtT, Δ XprG and Δ XprG/ Δ PrtT
Protease-Deficient Mutants.
Front. Microbiol. 8:2490.
doi: 10.3389/fmicb.2017.02490

¹ *Aspergillus* and Antifungal Research Laboratory, Department of Clinical Microbiology and Immunology, Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel, ² Leibniz Institute for Natural Product Research and Infection Biology, Hans Knöll Institute (HKI), Jena, Germany, ³ Institute of Microbiology, Friedrich Schiller University, Jena, Germany, ⁴ Faculty of Medicine in the Galilee, Bar-Ilan University, Safed, Israel

Aspergillus fumigatus is the most common mold species to cause disease in immunocompromised patients. Infection usually begins when its spores (conidia) are inhaled into the airways, where they germinate, forming hyphae that penetrate and destroy the lungs and disseminate to other organs, leading to high mortality. The ability of hyphae to penetrate the pulmonary epithelium is a key step in the infectious process. *A. fumigatus* produces extracellular proteases that are thought to enhance penetration by degrading host structural barriers. This study explores the role of the *A. fumigatus* transcription factor XprG in controlling secreted proteolytic activity and fungal virulence. We deleted *xprG*, alone and in combination with *prtT*, a transcription factor previously shown to regulate extracellular proteolysis. *xprG* deletion resulted in abnormal conidiogenesis and formation of lighter colored, more fragile conidia and a moderate reduction in the ability of culture filtrates (CFs) to degrade substrate proteins. Deletion of both *xprG* and *prtT* resulted in an additive reduction, generating a mutant strain producing CF with almost no ability to degrade substrate proteins. Detailed proteomic analysis identified numerous secreted proteases regulated by XprG and PrtT, alone and in combination. Interestingly, proteomics also identified reduced levels of secreted cell wall modifying enzymes (glucanases, chitinases) and allergens following deletion of these genes, suggesting they target additional cellular processes. Surprisingly, despite the major alteration in the secretome of the *xprG/prtT* null mutant, including two to fivefold reductions in the level of 24 proteases, 18 glucanases, 6 chitinases, and 19 allergens, it retained wild-type virulence in murine systemic and pulmonary models of infection. This study highlights the extreme adaptability of *A. fumigatus* during infection based on extensive gene redundancy.

Keywords: *Aspergillus fumigatus*, protease secretion, transcription factor, proteomics, virulence

INTRODUCTION

Aspergillus fumigatus is a common saprophytic mold which produces abundant microscopic conidia (2–4 micrometers) that can be inhaled into the pulmonary alveoli to cause a variety of pathological conditions (Kwon-Chung and Sugui, 2013). In the context of the immunocompromised patient, the infection, termed Invasive Pulmonary Aspergillosis (IPA), is life-threatening and severe (Kosmidis and Denning, 2015). The neutropenic status of these patients culminates in their inability to destroy inhaled conidia resulting in fungal growth and penetration through the pulmonary epithelium into the blood stream (Latge, 2001).

The success of *A. fumigatus* as a pathogen is not a result of direct adaptation to the host. Rather it should be viewed as an accidental interaction between a common and hardy environmental mold and a weakened host. *A. fumigatus* survives in the compromised host due to a chance combination of pre-existing capabilities. They include the abundant release of small conidia protected by a non-immunogenic layer of hydrophobins and oxygen-radical quenching pigments. Growing hyphae effectively endure oxidative stress and hypoxia, efficiently collect scarce iron and secrete toxins that further depress host immune function and proteases that degrade host tissue (Abad et al., 2010; Kwon-Chung and Sugui, 2013). In human A549 alveolar epithelial cells, culture filtrates (CFs) of *A. fumigatus* can disrupt the actin cytoskeleton, activate NF κ B signaling and induce the production of proinflammatory cytokines. These cellular events can be prevented by addition of serine protease inhibitors to the secreted CF, implying that they are directly dependent on secreted fungal proteases (Kogan et al., 2004; Sharon et al., 2011). *A. fumigatus*-secreted proteases and gliotoxin also induce platelet activation that may serve as a mechanism for activating the immune defenses and inducing inflammation (Speth et al., 2013). Additionally, the secreted *A. fumigatus* alkaline protease Alp1 cleaves the complement components C3, C4, and C5 that contribute to evasion from the host immune response (Behnsen et al., 2010). Alp1 is also a major allergen (Aspf 13) and promotes airway hyper-responsiveness and bronchoconstriction in asthma (Balenga et al., 2015).

In previous work we have identified the transcription factor PrtT, a positive regulator of secreted proteases in *A. fumigatus*. Deletion of *prtT* results in greatly reduced secreted protease activity and a reduction in the transcription of secreted proteases. Δ *prtT* CF showed reduced killing of A549 lung alveolar cells and erythrocyte lysis (Bergmann et al., 2009; Sharon et al., 2009, 2011). However, the Δ *prtT* strain showed wild-type virulence in infected neutropenic mice suggesting that perhaps residual protease activity was sufficient to enable virulence in this setting (Bergmann et al., 2009; Sharon et al., 2009).

In the related mold *A. nidulans*, that lacks a *prtT* homolog, the transcription factor XprG regulates extracellular protease production in response to nutrient stress (Katz et al., 2013, 2015; Katz and Cooper, 2015). Deletion of *A. nidulans xprG*

resulted in complete loss of halo formation on skimmed milk (SM) agar plates and an inability to grow on medium containing BSA as sole carbon or nitrogen source. XprG is a member of the p53-like transcription factors, also known as the NDT80/PhoG-like family. NDT80 transcription factors are found in animals, fungi, and amoeba. Fungal NDT80 genes were studied in detail in *Saccharomyces cerevisiae* (Pak and Segall, 2002), *Neurospora crassa* (Hutchison and Glass, 2010) *Candida albicans* (Chen et al., 2004; Sellam et al., 2009, 2010) and *A. nidulans* (Katz et al., 2013, 2015; Katz and Cooper, 2015). The consensus is that in response to nutrient stress and deprivation they activate specific target genes such as proteases, phosphatases, secondary metabolites, and genes involved in meiosis (itself a stress response initiated by starvation) and autolysis.

We hypothesized that XprG also co-regulates protease production in *A. fumigatus* and that deletion of both *prtT* and *xprG* would completely abolish secreted protease activity, reducing fungal virulence. To test this hypothesis we prepared *A. fumigatus* strains deleted in *prtT*, *xprG* alone and in combination. The effects of these mutations *in vitro* and during infection *in vivo* are described.

MATERIALS AND METHODS

Strains and Culture Conditions

The strains used in this study are detailed in Table 1. *A. fumigatus* conidia were harvested in 0.2% (vol/vol) Tween 20, resuspended in double-distilled water (DDW) and counted with a hemocytometer. For continuous growth, *A. fumigatus* strains were grown on YAG medium, that consists of 0.5% (wt/vol) yeast extract, 1% (wt/vol) glucose, and 10 mM MgCl₂, supplemented with trace elements, vitamins, and 1.5% (wt/vol) agar when needed (Bainbridge, 1971). SM medium consisted of 1% (wt/vol) glucose, 1% (wt/vol) SM (Difco, Livonia, MI, United States), 0.1% (wt/vol) Casamino Acids (Difco), 7 mM KCl, 2 mM MgSO₄ and 50 mM Na₂HPO₄-NaH₂PO₄ buffer (pH 5.3), supplemented with vitamins, trace elements and 1.5% agar when needed. NaNO₃-depleted *Aspergillus* minimal medium (Weidner et al., 1998) was used for collagen medium with 0.1% (wt/vol) yeast extract, 0.5% (wt/vol) glucose and collagen as sole carbon and nitrogen source. Peptone medium contained 1% (wt/vol) glucose, 0.4% peptone (Difco), 7 mM KCl, 2 mM MgSO₄ and 50 mM Na₂HPO₄-NaH₂PO₄ buffer (pH 5.3), supplemented with vitamins and trace elements. Genetically modified organisms and pathogens used in this study were maintained in accordance with TAU Institutional Policies.

Generation and Verification of *A. fumigatus* Mutant Strains

All strains were prepared in the *Ku80* null background, strain *AkuB^{KU80}* (da Silva Ferreira et al., 2006). Full details of the construction and verification of the strains shown in Table 1, including a list of the primers used (Supplementary Table S1), are provided in the Supplementary Data Section.

TABLE 1 | Strains used in this study.

Strain	Genotype	Source
KU80 (Δ akuB)	CEA17, AFUA_2G02620::pyrG	da Silva Ferreira et al., 2006
Δ PrtT	AFUA_4G10120::hph	This work
Δ XprG	AFUA_8G04050::ptrA	This work
Δ XprG/ Δ PrtT	AFUA_8G04050::ptrA; AFUA_4G10120::hph	This work
PrtT KI	AFUA_4G10120::hph; AFUA_4G10120-phl	This work
XprG KI	AFUA_8G04050::ptrA AFUA_8G04050-hph	This work
Δ XprG/PrtT KI	AFUA_8G04050::ptrA; AFUA_4G10120::hph AFUA_4G10120-phl	This work

Conidial Stability in Detergent Storage

Freshly harvested *A. fumigatus* conidia (10^7 /ml) were suspended in DDW +0.5% (vol/vol) Tween 20 at 37°C. At different time-points, aliquots were diluted, plated on YAG plates and the number of colonies counted.

Conidial Disruption by Glass Beads

Freshly harvested *A. fumigatus* conidia (5×10^7 /ml) were suspended in 0.5 ml DDW +0.1% Tween 20 and mixed with 0.5 ml (packed volume) of acid washed glass beads, 150–212 μ m (Sigma–Aldrich Corp., St. Louis, MO, United States). They were then vortexed on medium strength for up to 10 min. At each time point a sample was taken, diluted and plated on YAG plates. The plates were incubated at 37°C for 24–36 h, colonies were counted and survival rates were calculated as the percentage of viable spores.

Analysis of Fungal Enzymatic Activity

Proteolytic activity on solid medium was assessed by spotting conidia on SM plates containing 0.1% Tween 20. The colonies were grown for 48 h at 37°C, then transferred to room temperature for another 48 h and subsequently photographed. Supernatants were collected from *Aspergillus* cultures grown in liquid SM for 48 h at 37°C. Azocasein (Sigma) was dissolved at a concentration of 5 mg/ml in assay buffer containing 50 mM Tris (pH 7.5), 0.2 M NaCl, 5 mM CaCl₂ and 0.05% Triton X-100 as previously described (Kogan et al., 2004). The azocasein solution (400 μ l) was mixed with 100 μ l portions of supernatants from *Aspergillus* cultures and incubated by shaking for 90 min at 37°C. The reactions were stopped by adding of 150 μ l 12% (vol/vol) trichloroacetic acid, and the reaction mixtures were allowed to stand at room temperature for 30 min. Tubes were then centrifuged for 3 min at 8,000 g, and 100 μ l of each supernatant was added to 100 μ l of 1 M NaOH. The absorbance of released azo dye at 436 nm was determined with a spectrophotometer. Proteolytic activity on bovine serum albumin (BSA) was measured by growing *A. fumigatus* in 24-well plates with 1 ml/well liquid peptone medium containing 0.1% BSA, respectively, for 24–72 h at 37°C. Supernatants were boiled in sample buffer and run on an 8%

SDS-PAGE gel followed by Coomassie staining to visualize the proteins.

Scanning Electron Microscopy (SEM) Analysis

Aspergillus fumigatus wild-type and mutant strains were grown for 72 h at 37°C on YAG agar plates. Fixation and processing of samples for SEM was performed as described earlier (Fichtman et al., 2014; Hover et al., 2016). Briefly, small areas of conidiating mycelium were carefully excised from the zone of interaction and vapor fixed with 8% (vol/vol) paraformaldehyde and 4% (vol/vol) glutaraldehyde dissolved in water for 1 h in a closed chamber. Secondary vapor fixation was then carried out in an aqueous solution of 2% (wt/vol) osmium tetroxide for 1 h. The samples were submerged for 10 min in DDW and then dehydrated (10 min, twice for each step) under a series of ethanol concentrations (7.5, 15, 30, 50, 70, 90, 95, and 100%). Next, samples underwent critical point drying (CPD) using a K850 CPD dryer (Quorum Technologies, United Kingdom). Coating was done with 3 nm iridium using a Q150T coater (Quorum Technologies, United Kingdom). Samples were imaged with a Merlin scanning electron microscope (Zeiss, Germany).

LC-MS/MS Analysis and Identification of Secreted Proteins

Aspergillus fumigatus strains at a concentration of 10^6 conidia/ml were grown in liquid collagen medium for 72 h at 37°C. Total protein was TCA-precipitated from the CFs, digested with trypsin, iTRAQ-labeled and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as detailed in the Supplementary Data.

Murine Models for Invasive Aspergillosis

For the cyclophosphamide/cortisone acetate neutropenic model (Ejzykiewicz et al., 2009), 6-week-old female ICR mice were injected intraperitoneally with cyclophosphamide (150 mg/kg in PBS) at 3 days prior to infection, on the day of infection and at 3 days post-infection. Cortisone acetate (150 mg/kg PBS with 0.1% Tween 20) was injected subcutaneously at 3 days prior to conidial infection.

For intranasal infection, mice were anesthetized by intraperitoneal injection of a solution of 250 μ l xylazine (VMD, Arendonk, Belgium) and ketamine (Imalgene, Fort Dodge, IA, United States) at a concentration of 1.0 and 10 mg/ml, respectively (dissolved in PBS). Following anesthesia, the mice were inoculated intranasally with 2.5×10^5 (intranasal model) or intravenously (IV) through the tail vein (IV model) with freshly harvested conidia of *AkuB*^{KU80}, Δ XprG, or Δ XprG/ Δ PrtT in PBS +0.1% (vol/vol) Tween 20. The inoculum was verified by quantitative culture. The animals were monitored for survival for up to 18 days. For infection of immunocompetent mice, 6-week-old female ICR mice were inoculated intranasally with 1×10^7 conidia and sacrificed 24 h or 48 h later. Excised lungs were ground and plated on YAG agar plates for CFU enumeration. Histological analysis was performed with Gomori methenamine

silver stain (GMS, stains fungal elements black) or haematoxylin and eosin stain (H&E, stains host-cell nuclei purple, cytosol pink). Statistical analysis of mouse survival was performed with GraphPad Prism 4 software (GraphPad Software, San Diego, CA, United States). Animal studies were authorized by the Tel Aviv University Animal Welfare Committee according to the Israel Ministry of Health guidelines and carried out in accordance with Tel Aviv University institutional policies.

RESULTS

Generation of *A. fumigatus prtT*, *xprG*, *xprG/prtT* Null Mutants and Reconstituted Strains

A single *A. fumigatus xprG* ortholog (AFUA_8G04050) was identified by amino-acid similarity to *A. nidulans* XprG (70% identity). It is predicted to be 1752 nucleotides long and to contain one intron. *A. fumigatus* XprG encodes a protein 583 amino acids in length, containing a predicted NDT80/PhoG like DNA-binding domain (amino-acid residues 156–327) and a MAP65/ASE1 domain (amino acid residues 336–505) shared by microtubule-binding proteins. The similarity to NDT80 family genes *vib-1* from *N. crassa* and *S. cerevisiae ndt80* is limited to the PhoG like DNA-binding domain. To analyze the function of XprG and its interaction with PrtT in *A. fumigatus*, we prepared Δ PrtT, Δ XprG, Δ XprG/ Δ PrtT null mutants and corresponding reconstituted strains *PrtT-KI*, *XprG-KI*, and *PrtT-KI/ Δ XprG* by transformation with a circular plasmid containing the *PrtT* gene and the phleomycin resistance cassette for selection (see Supplementary Data for full details about strain construction and verification).

Abnormal Conidiogenesis in the Δ XprG and Δ XprG/ Δ PrtT *A. fumigatus* Mutants

The aforementioned null and reconstituted strains were point inoculated on YAG agar plates and examined for differences in morphology after growth for 72 h at 37°C. Results show that the Δ XprG, Δ XprG/ Δ PrtT null strains produced lighter colored conidia as compared to the *AkuB^{KU80}*, Δ PrtT, and *XprG-KI* strains (Figure 1A). Examination of the conidiophore structure by light microscopy and SEM revealed that the Δ XprG and Δ XprG/ Δ PrtT strains produced significantly smaller, more compact conidiophores ($P < 0.02$) with shorter conidial chains (Figures 1B,E). The conidial hydrophobin outer rodlet layer remained intact and unchanged (Figure 1C) and the surface hydrophobicity of the mutant colonies, based on their ability to exclude water, was unchanged (not shown). Reflecting the shorter conidial chains, the number of conidia produced by the Δ XprG and Δ XprG/ Δ PrtT strains was significantly reduced three–fourfold per plate ($P < 0.05$) compared to *AkuB^{KU80}* and Δ PrtT (Figure 1D). Radial growth rates of the Δ PrtT, Δ XprG, Δ XprG/ Δ PrtT strains were similar to that of *AkuB^{KU80}* at both 37 and 48°C (Data not shown). The lighter conidial color of the *PrtT-KI* complemented strain compared to the deleted strain (Figure 1A) is unexpected and

may be due to multiple integration of the complementing plasmid.

The Conidia Produced by the Δ XprG and Δ XprG/ Δ PrtT *A. fumigatus* Mutants Are More Fragile

We evaluated the ability of the mutant conidia to withstand osmotic or detergent disruption by measuring their survival in water or in water containing Tween 20 detergent. Conidia from the Δ XprG and Δ XprG/ Δ PrtT strains exhibited similar increased susceptibility to the detergent, losing >90% viability after 4 h as compared to loss of only <20% viability in the *AkuB^{KU80}* and Δ PrtT strains (Figure 2A). There were no differences in conidial stability during storage in DDW, suggesting the mutants are not osmotically sensitive (data not shown). The ability of the mutant conidia to withstand physical disruption was assessed by subjecting them to agitation in the presence of glass beads. Conidia from the Δ XprG and Δ XprG/ Δ PrtT strains exhibited increased susceptibility to glass-bead agitation, losing all viability after 4 min agitation, compared to 10 min for the *AkuB^{KU80}* and Δ PrtT strains (Figure 2B).

Taken together these results show that deletion of *xprG* in the *AkuB^{KU80}* and Δ PrtT strains resulted in the production of smaller conidiophores containing less and more fragile spores.

Deletion of *xprG* and *prtT* in *A. fumigatus* Results in an Additive Reduction in the Ability to Degrade Casein and Albumin

To investigate the effect of *xprG* deletion on secreted protease activity, we point inoculated the strains on SM agar plates and visually determined the formation of a proteolytic halo due to casein degradation around the fungal colony. As we have previously shown (Sharon et al., 2009), deletion of *prtT* resulted in the formation of a very weak halo compared to the control strain *AkuB^{KU80}*, whereas deletion of *xprG* (Δ XprG) led to a partially reduced halo. Deletion of both *xprG* and *prtT* (Δ XprG/ Δ PrtT) completely abolished the formation of a proteolytic halo (Figure 3A) and reduced azocasein degradation by over 90% (Figure 3B). Halo formation was restored in the reconstituted strain *XprG-KI* and slightly increased in the *PrtT-KI* and *PrtT-KI/ Δ XprG* strains. This increase, however, was not seen in the azocasein or BSA degradation assays described below. We used a more sensitive SDS-PAGE-based BSA degradation assay to better differentiate between the proteolytic activities of the Δ XprG, Δ PrtT and Δ XprG/ Δ PrtT mutants (Figure 3C). Strains were grown for 24–72 h on peptone medium containing 0.1% BSA. The supernatant was separated on an SDS-PAGE gel followed by Coomassie staining to visualize the degradation of the BSA protein. The control *AkuB^{KU80}* and reconstituted strains substantially degraded the BSA substrate protein after 48 h of incubation, whereas the Δ PrtT, Δ XprG, and Δ XprG/ Δ PrtT strains did not (Figure 3C). After 72 h of incubation, all strains except Δ XprG/ Δ PrtT had completely degraded the BSA. Taken together, our findings indicate that deletion of both *prtT* and *xprG* results in an additive reduction in the

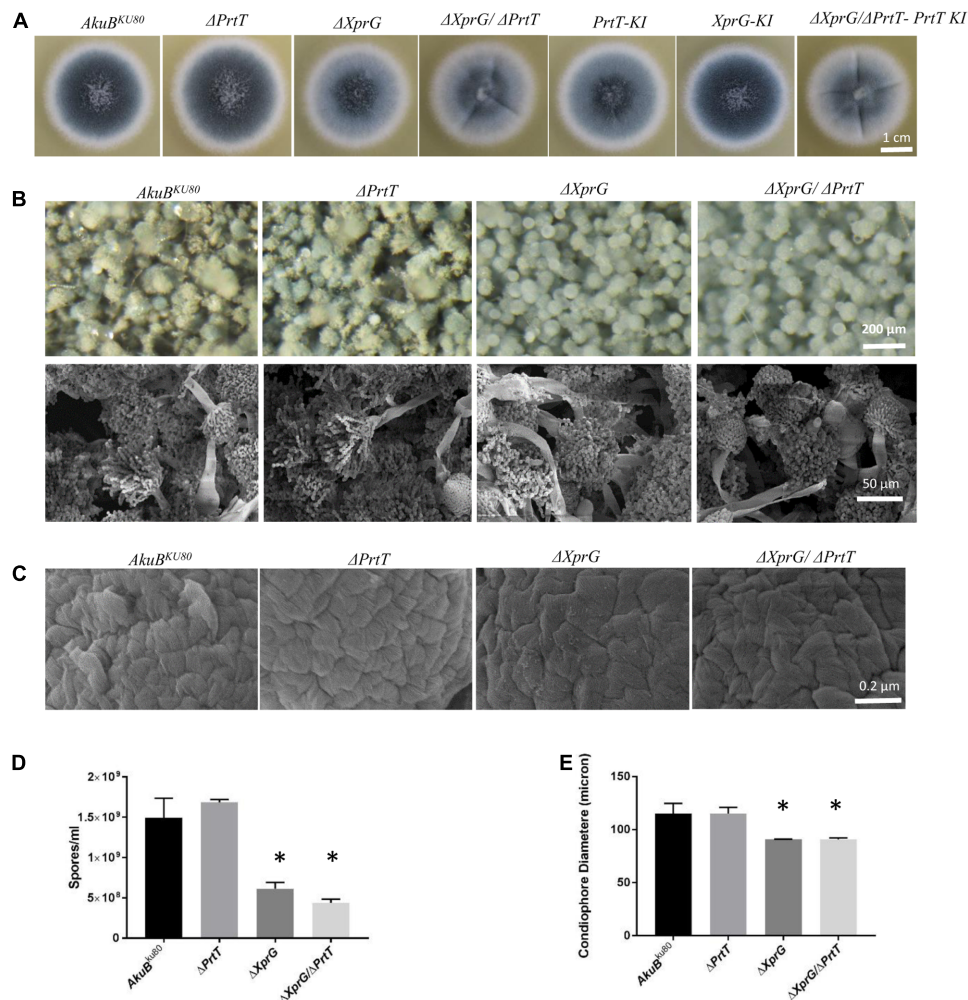


FIGURE 1 | Deletion of *xprG* and *xprG/prtT* leads to abnormal conidiogenesis. Phenotypic analysis of the $\Delta XprG$ and $\Delta XprG/\Delta PrtT$ strains compared to the wild-type *AkuB^{KU80}* and $\Delta PrtT$ strains shows that they produce (A) lighter colored conidia when growing on YAG agar plates (B) conidiophores with reduced diameter and shorter spore chains (Light microscopy and SEM) (C) conidia with a normal rodlet outer layer (SEM, high magnification), (D) a significantly reduced number of conidia (* $P < 0.005$). (E) Quantification of reduction in conidiophore diameter (based on the diameter of 50 conidiophores per strain) (* $P < 0.02$).

ability to degrade casein or albumin. Previous analyses using protease inhibitors and deletion mutants have demonstrated that secreted *A. fumigatus* serine proteases, and in particular Alp1, are primarily responsible for the degradation of these substrates (Jaton-Ogay et al., 1994; Tomee et al., 1997; Kogan et al., 2004).

Proteomic Analysis of the $\Delta PrtT$, $\Delta XprG$, and $\Delta XprG/\Delta PrtT$ Secretomes

To identify the repertoire of proteins secreted by the $\Delta PrtT$, $\Delta XprG$, and $\Delta XprG/\Delta PrtT$ mutants compared to the control *AkuB^{KU80}* strain, conidia were cultured in liquid MM containing collagen for 72 h at 37°C with shaking. Supernatants were TCA-precipitated, treated after Yeung and Stanley (2009), followed by a Wessel-Flügge precipitation, labeled with iTRAQ 4-plex isobaric labeling for quantitative proteomics approach and analyzed by LC-MS/MS. A total of 938 proteins were identified in the fungal

supernatants, 274 of which were predicted to be secreted by *in silico* analysis (secretion signal found by signalP for 236 proteins and targetP for 274 proteins) (Supplementary Table S2).

Cell lysis cannot be completely excluded during cultivation and sample preparation. It is therefore possible that a proportion of the secretome consisted of proteins without a secretion signal, which had been released by cell lysis. However, the comparison of the abundance (based on normalized PSM values) of typical intracellular, non-secreted proteins like actin (*Afu6g04740*) or tubulin (*Afu1g02550*) with the abundance of a secreted protein like lap2 (*Afu3g00650*) revealed a depletion by a factor between 12 and 106. In addition, fungi are able to release typical intracellular proteins via extracellular vesicles to the extracellular space (Joffe et al., 2016). It can be therefore assumed that the contamination of the secretome by cell lysis was relatively low.

Deletion of *prtT* resulted in strongly reduced secretion of 22 proteases, including Lap1, Lap2, Cp1, Cp3 Alp1, Alp2, and Mep

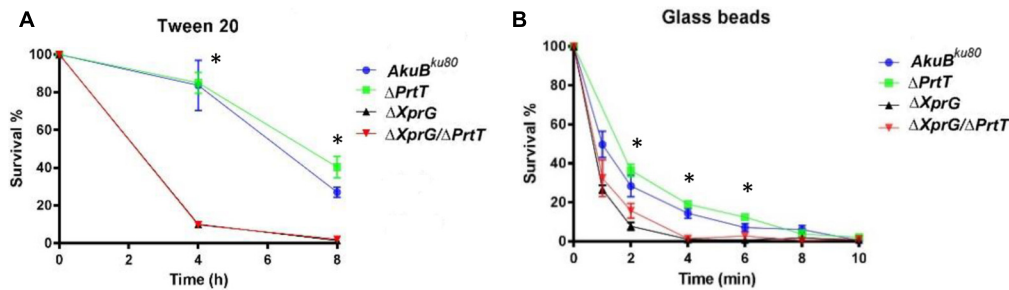


FIGURE 2 | The Δ *XprG* and Δ *XprG*/ Δ *PrtT* mutants produce conidia that are more sensitive to chemical and mechanical stress. **(A)** 10^3 conidia/ml were suspended in 0.5% Tween 20 for the time indicated, plated and the number of colonies counted in comparison to controls suspended in DDW alone. $*P < 0.001$ *AkuB^{ku80}* vs. Δ *XprG* and Δ *XprG*/ Δ *PrtT* mutants. **(B)** 5×10^7 conidia/ml were agitated in the presence of acid-washed glass beads for the time indicated, plated and the number of colonies counted in comparison to untreated controls. $*P < 0.05$ *AkuB^{ku80}* vs. Δ *XprG* and Δ *XprG*/ Δ *PrtT* mutants.

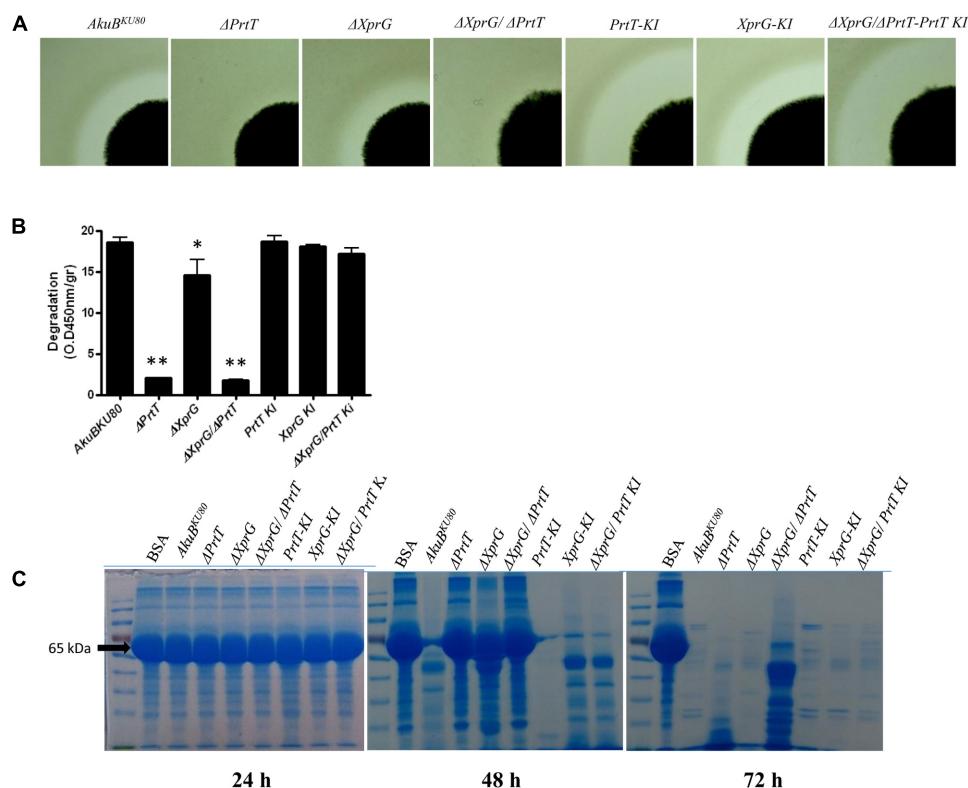


FIGURE 3 | Proteolytic activity following deletion of *prtT* and *xprG*. Proteolytic activity of the fungal strains was assessed by **(A)** Halo formation on SM agar plates containing 0.1% Tween 20 following 48 h of growth at 37°C followed by another 48 h at room temperature. **(B)** Degradation of azocasein by *A. fumigatus* culture filtrates (CFs). The absorbance of the released azo dye was measured spectrophotometrically at 450 nm. ($*P < 0.05$, $**P < 0.0001$). **(C)** BSA proteolysis by *A. fumigatus* CFs followed by SDS-PAGE analysis of the cleaved substrate. BSA (65 kDa, arrow) and other proteins were stained by Coomassie.

(Figure 4 and Supplementary Table S3). Deletion of *xprG* resulted in reductions in the secretion of 19 proteases, including most strongly Lap1, Sxa2, DppV, DppIV, Mep, and SedC. Notably, several proteases that were strongly reduced in the Δ *PrtT* mutant were only slightly reduced (Pep2, DapB, Cps1, Cp1, Cp3) or not reduced (CtsD, Ape3, Lap2, Alp1, Alp2) in the Δ *XprG* strain (Figure 4 and Supplementary Table S3). This provides a good explanation for the higher secreted protease activity

of the Δ *XprG* vs. the Δ *PrtT* mutant, especially the lack of reduction in the major neutral serine protease Alp1 (Behnsen et al., 2010). Deletion of both *prtT* and *xprG* resulted in the strongest reductions in the secretion of 24 proteases comprising the entire joint dataset of the Δ *PrtT* and Δ *XprG* mutants, including 14 serine proteases, 7 metalloproteases and 3 aspartic proteases (Figure 4 and Supplementary Table S3). Interestingly, in all three mutant strains, the secretion of numerous glucanases,

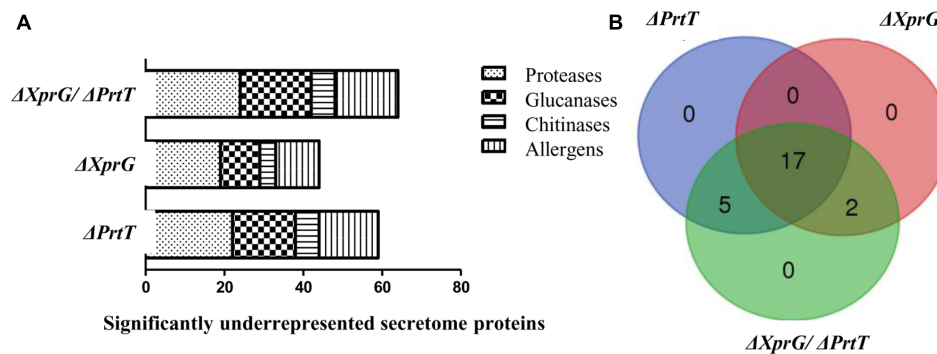


FIGURE 4 | Effect of the deletion of *prtT*, *xprG* and *xprG/prtT* on the secretome of *A. fumigatus*. Deletion of *prtT*, *xprG* and *xprG/prtT* (A) reduces the secretion not only of proteases but also of glucanases and chitinases participating in cell wall biosynthesis and of numerous allergenic proteins. (B) Venn diagram illustrating the degree of overlap in the secretome datasets of the mutant strains.

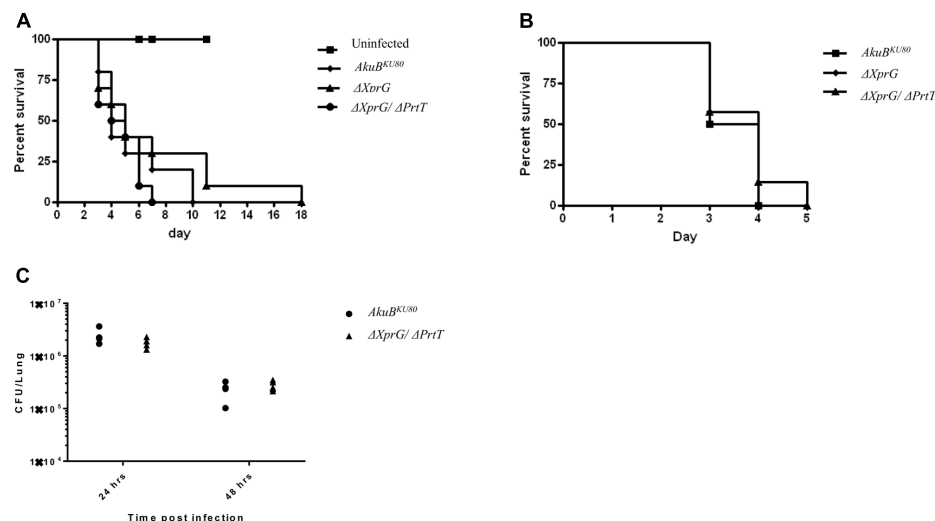


FIGURE 5 | Deletion of *xprG* and *xprG/prtT*, does not affect virulence in a neutropenic murine model of (A) pulmonary aspergillosis, (B) disseminated aspergillosis and (C) pulmonary fungal load in immunocompetent mice. Cortisone acetate plus cyclophosphamide (neutropenia model) were used to immunocompromise the infected mice (A,B). Survival curves are shown for mice infected (A) intranasally with an inoculum of 2.5×10^5 conidia per mouse ($n = 10$ mice/group) and (B) intravenously via the lateral tail vein with an inoculum of 2.5×10^5 conidia per mouse ($n = 7$ mice/group). These experiments were repeated twice with similar results. (C) Immunocompetent mice were infected intranasally with an inoculum of 1×10^7 conidia per mouse ($n = 5$ mice/group) and total lung fungal load (CFU/lung) assessed 24 and 48 h post-infection. (Additional histological analysis is presented in the Supplements- Supplementary Figure S6).

chitinases, and allergens was also reduced, indicating that these transcription factors not only regulate the expression of secreted proteases but also of cell wall enzymes and antigenic proteins (Supplementary Table S3). The significance of these results will be explained in the discussion. No proteins showed increased secretion in the mutants vs. the control $AkuB^{KU80}$ strain.

The $\Delta XprG$ and $\Delta XprG/\Delta PrtT$ Strains Exhibit Normal Virulence in Infected Mice

Previous studies have shown that deletion of *prtT* in *A. fumigatus* does not affect virulence in lung-infected neutropenic mice (Bergmann et al., 2009; Sharon et al., 2009). We hypothesized that the almost complete lack of secreted protease activity in the $\Delta XprG/\Delta PrtT$ strain would result in a noticeable

reduction in virulence. To test this, we compared the virulence of the control $AkuB^{KU80}$, $\Delta XprG$ and $\Delta XprG/\Delta PrtT$ strains in two mouse models of invasive aspergillosis. Both models reproduce profound neutropenia (as found, for example following chemotherapy in leukemic patients), by immunosuppressing the mice with a combination of cortisone acetate and cyclophosphamide. In the first model, the mice were infected intranasally with freshly harvested conidia. In the second model reproducing disseminated infection, mice were infected IV. The number of live mice in each group was recorded daily throughout the experiment. The $\Delta PrtT$ strain was not included as we have previously shown it to be fully virulent in these models (Sharon et al., 2009). Figure 5 shows survival curves obtained during the course

of the experiment for the lung (**Figure 5A**) and disseminated (**Figure 5B**) models. No significant differences in virulence were found for the $\Delta XprG$ and $\Delta XprG/\Delta PrtT$ mutants compared to the control $AkuB^{KU80}$ strain (P -value > 0.3). Next, we analyzed the response of immunocompetent mice to intranasal pulmonary infection (10^7 conidia/mouse) with $\Delta XprG/\Delta PrtT$ and control $AkuB^{KU80}$ strains. Outputs included lung CFU (**Figure 5C**) and histology (Supplementary Figure S6), 24 and 48 h following infection. There was no significant difference in lung fungal load (P -value > 0.2) between the $\Delta XprG/\Delta PrtT$ and control $AkuB^{KU80}$ strains (**Figure 5C**). Histology revealed abundant conidia in the bronchi of both mutant and control strains 24 h after infection (GMS stain, right panel, arrows) accompanied by a considerable influx of red blood cells and lymphocytes (H&E stain, left panel) (Supplementary Figure S6). As expected in immunocompetent mice, the number of conidia strongly decreased 48 h after infection, as did the number of red blood cells and lymphocytes (Supplementary Figure S6). Taken together, these results suggest that immunocompetent mice mount a similar and effective immune response to both $\Delta XprG/\Delta PrtT$ and control $AkuB^{KU80}$ infection.

DISCUSSION

This study describes the disruption of the putative transcriptional activator XprG in *A. fumigatus*, alone and in combination with the previously described transcription factor PrtT. We chose to study XprG in *A. fumigatus* because (i) it regulates protease expression in *A. nidulans* and we reasoned it may also do so, in combination with PrtT, in *A. fumigatus*, (ii) regulation of protease expression by combinations of transcription factors in pathogenic filamentous fungi is poorly understood and (iii) we hypothesized that deletion of two transcriptional regulators controlling the expression of multiple proteases could shed more light on the involvement of secreted proteases in the pathogenesis of *A. fumigatus*.

We gained several important insights from this study. First, we found considerable divergence in the role of XprG in *A. nidulans* and *A. fumigatus*. In *A. nidulans* that lacks a PrtT homolog XprG is the major activator of secreted protease activity under starvation. In *A. fumigatus*, this function is carried out by PrtT, with XprG performing a secondary role. Increasing functional divergence is found in the *N. crassa* XprG homolog vib-1 – in addition to controlling conidial pigmentation and increasing extracellular protease activity it also controls protoperithecial sexual development (Hutchison and Glass, 2010). Further functional separation has occurred in *S. cerevisiae* ndt80 (Pak and Segall, 2002) that controls meiosis and *C. albicans* CaNdt80 (Chen et al., 2004; Sellam et al., 2009, 2010), that regulates azole resistance, hyphal growth and virulence. The basis for this diversity likely stems from the extreme sequence divergence outside of the conserved NDT80/PhoG like DNA-binding domain.

We also found evidence for overlap in ndt80/vib-1/XprG function. As previously shown in *A. nidulans* xprG and *N. crassa* vib-1 null mutants, deletion of *A. fumigatus* xprG resulted in the formation of pale conidia, suggesting a role in melanin biosynthesis. We further showed that in *A. fumigatus*, conidiogenesis is impaired and the resulting conidia are weaker and more prone to chemical and physical disruption. Most interestingly, we show here that *A. fumigatus* XprG and PrtT activate expression of glucanases, chitinases and numerous allergens. *C. albicans* CaNdt80 also activates genes encoding cell wall components including expression of chitinase Cht3p and cell wall glucosidase Sun41p that are essential for the completion of cell separation (Sellam et al., 2010). However, despite reduced levels of numerous secreted glucanases and chitinases in *A. fumigatus* $\Delta XprG$, we found no obvious evidence for cell wall alterations by either SEM, transmission electron microscopy (TEM) or cell wall inhibitor analysis (data not shown). CaNdt80 also activates CDR1 efflux pump and ergosterol biosynthesis and its deletion results in azole sensitivity. Nevertheless, deletion of *A. fumigatus* xprG did not increase azole sensitivity (data not shown) suggesting that it does not affect these targets.

Our analysis of XprG and PrtT showed that both genes are not essential for maintaining virulence. Considering that the double-mutant has almost no detectable secreted protease activity *in vitro* and exhibits strong (two–fivefold) reductions in 24 secreted proteases [including all 5 proteases that are activated during *in vivo* infection (McDonagh et al., 2008)] as well as 18 glucanases and 6 chitinases, the unaffected virulence in the mouse model is unexpected. The *A. fumigatus* genome encodes approximately 50 putative secreted proteases, and some may be alternatively activated *in vivo*, compensating for the reductions seen in $\Delta PrtT/\Delta XprG$. Another possible explanation is that under *in vivo* stress, $\Delta XprG/\Delta PrtT$ activates alternative virulence determinants that are not activated in the wild type. For example, we have previously shown that deletion of *prtT* resulted in the upregulation of four secondary metabolite clusters, including genes for the biosynthesis of toxic pseurotin A (Hagag et al., 2012). These factors could increase its virulence to wild type levels, despite its inability to produce secreted proteases. Functional gene redundancy could also account for our findings. Such redundancy has been repeatedly shown in *A. fumigatus*, following multiple deletions in large gene families encoding chitin synthases (Muszkieta et al., 2014), α -1,3-glucan synthases (Henry et al., 2012), and oligopeptide transporters (Hartmann et al., 2011).

Interestingly, the *A. fumigatus* strains generated here showed reductions in the expression of 21 of the 23 allergens identified in *A. fumigatus*, including those encoding the proteases Alp1/Aspf13, Alp2/Aspf18, Pep1/Aspf10, and Mep/Aspf5. Allergens encoded by fungal proteases activate an allergic Th-2 response *in vivo* by cleaving airway fibrinogen and generating fragments that act as TLR4 ligands on alveolar macrophages and airway epithelium (Millien et al., 2013). While secreted *A. fumigatus* proteases are not critical for infection of the immunocompromised host, our findings propose that they

could be highly important in determining the response in fungal allergies. This possibility can be further explored with the *A. fumigatus* strains generated in this study.

AUTHOR CONTRIBUTIONS

ES, BH, SH, SA, YS, BE, and TK performed the experiments. AH, AB, OK, and NO conceived and planned the project and wrote the manuscript.

FUNDING

This study was provided by the Israel Ministry of Health Infect-ERA (Grant 11080) to NO the Israel Science Foundation (985/15)

REFERENCES

- Abad, A., Fernandez-Molina, J. V., Bikandi, J., Ramirez, A., Margareto, J., Sendino, J., et al. (2010). What makes *Aspergillus fumigatus* a successful pathogen? Genes and molecules involved in invasive aspergillosis. *Rev. Iberoam. Micol.* 27, 155–182. doi: 10.1016/j.riam.2010.10.003
- Bainbridge, B. W. (1971). Macromolecular composition and nuclear division during spore germination in *Aspergillus nidulans*. *J. Gen. Microbiol.* 66, 319–325. doi: 10.1099/00221287-66-3-319
- Balenga, N. A., Klichinsky, M., Xie, Z., Chan, E. C., Zhao, M., Jude, J., et al. (2015). A fungal protease allergen provokes airway hyper-responsiveness in asthma. *Nat. Commun.* 6:6763. doi: 10.1038/ncomms7763
- Behnsen, J., Lessing, F., Schindler, S., Wartenberg, D., Jacobsen, I. D., Thoen, M., et al. (2010). Secreted *Aspergillus fumigatus* protease Alp1 degrades human complement proteins C3, C4, and C5. *Infect. Immun.* 78, 3585–3594. doi: 10.1128/IAI.01353-09
- Bergmann, A., Hartmann, T., Cairns, T., Bignell, E. M., and Krappmann, S. (2009). A regulator of *Aspergillus fumigatus* extracellular proteolytic activity is dispensable for virulence. *Infect. Immun.* 77, 4041–4050. doi: 10.1128/IAI.00425-09
- Chen, C. G., Yang, Y. L., Shih, H. I., Su, C. L., and Lo, H. J. (2004). CaNdt80 is involved in drug resistance in *Candida albicans* by regulating *CDR1*. *Antimicrob. Agents Chemother.* 48, 4505–4512. doi: 10.1128/AAC.48.12.4505-4512.2004
- da Silva Ferreira, M. E., Kress, M. R., Savoldi, M., Goldman, M. H., Hartl, A., Heinekamp, T., et al. (2006). The akuB(KU80) mutant deficient for nonhomologous end joining is a powerful tool for analyzing pathogenicity in *Aspergillus fumigatus*. *Eukaryot. Cell* 5, 207–211. doi: 10.1128/EC.5.1.207-211.2006
- Ejzykowicz, D. E., Cunha, M. M., Rozental, S., Solis, N. V., Gravelat, F. N., Sheppard, D. C., et al. (2009). The *Aspergillus fumigatus* transcription factor Ace2 governs pigment production, conidiation and virulence. *Mol. Microbiol.* 72, 155–169. doi: 10.1111/j.1365-2958.2009.06631.x
- Fichtman, B., Shaulov, L., and Harel, A. (2014). Imaging metazoan nuclear pore complexes by field emission scanning electron microscopy. *Methods Cell Biol.* 122, 41–58. doi: 10.1016/B978-0-12-417160-2.00002-3
- Hagag, S., Kubitschek-Barreira, P., Neves, G. W., Amar, D., Nierman, W., Shalit, I., et al. (2012). Transcriptional and proteomic analysis of the *Aspergillus fumigatus* DeltaprtT protease-deficient mutant. *PLOS ONE* 7:e33604. doi: 10.1371/journal.pone.0033604
- Hartmann, T., Cairns, T. C., Olbermann, P., Morschhauser, J., Bignell, E. M., and Krappmann, S. (2011). Oligopeptide transport and regulation of extracellular proteolysis are required for growth of *Aspergillus fumigatus* on complex substrates but not for virulence. *Mol. Microbiol.* 82, 917–935. doi: 10.1111/j.1365-2958.2011.07868.x
- Henry, C., Latge, J. P., and Beauvais, A. (2012). alpha1,3 glucans are dispensable in *Aspergillus fumigatus*. *Eukaryot. Cell* 11, 26–29. doi: 10.1128/EC.05270-11
- to AH and by the Deutsche Forschungsgemeinschaft within the Collaborative Research Center TR124 FungiNet (project Z2) to TK and OK.
- Hover, T., Maya, T., Ron, S., Sandovsky, H., Shadkchan, Y., Kijner, N., et al. (2016). Mechanisms of bacterial (*Serratia marcescens*) attachment to, migration along, and killing of fungal hyphae. *Appl. Environ. Microbiol.* 82, 2585–2594. doi: 10.1128/AEM.04070-15
- Hutchison, E. A., and Glass, N. L. (2010). Meiotic regulators Ndt80 and ime2 have different roles in *Saccharomyces* and *Neurospora*. *Genetics* 185, 1271–1282. doi: 10.1534/genetics.110.117184
- Jaton-Ogay, K., Paris, S., Huerre, M., Quadroni, M., Falchetto, R., Togni, G., et al. (1994). Cloning and disruption of the gene encoding an extracellular metalloprotease of *Aspergillus fumigatus*. *Mol. Microbiol.* 14, 917–928. doi: 10.1111/j.1365-2958.1994.tb01327.x
- Joffe, L. S., Nimrichter, L., Rodrigues, M. L., and Del Poeta, M. (2016). Potential roles of fungal extracellular vesicles during infection. *mSphere* 1:e00099-16. doi: 10.1128/mSphere.00099-16
- Katz, M. E., Braunberger, K., Yi, G., Cooper, S., Nonhebel, H. M., and Gondro, C. (2013). A p53-like transcription factor similar to Ndt80 controls the response to nutrient stress in the filamentous fungus, *Aspergillus nidulans*. *F1000Res.* 2:72. doi: 10.12688/f1000research.2-72.v1
- Katz, M. E., Buckland, R., Hunter, C. C., and Todd, R. B. (2015). Distinct roles for the p53-like transcription factor XprG and autophagy genes in the response to starvation. *Fungal Genet. Biol.* 83, 10–18. doi: 10.1016/j.fgb.2015.08.006
- Katz, M. E., and Cooper, S. (2015). Extreme diversity in the regulation of Ndt80-Like transcription factors in fungi. *G3* 5, 2783–2792. doi: 10.1534/g3.115.021378
- Kogan, T. V., Jadoun, J., Mittelman, L., Hirschberg, K., and Oshero, N. (2004). Involvement of secreted *Aspergillus fumigatus* proteases in disruption of the actin fiber cytoskeleton and loss of focal adhesion sites in infected A549 lung pneumocytes. *J. Infect. Dis.* 189, 1965–1973. doi: 10.1086/420850
- Kosmidis, C., and Denning, D. W. (2015). The clinical spectrum of pulmonary aspergillosis. *Thorax* 70, 270–277. doi: 10.1136/thoraxjnl-2014-206291
- Kwon-Chung, K. J., and Sugui, J. A. (2013). *Aspergillus fumigatus*—what makes the species a ubiquitous human fungal pathogen? *PLOS Pathog.* 9:e1003743. doi: 10.1371/journal.ppat.1003743
- Latge, J. P. (2001). The pathobiology of *Aspergillus fumigatus*. *Trends Microbiol.* 9, 382–389. doi: 10.1016/S0966-842X(01)02104-7
- McDonagh, A., Fedorova, N. D., Crabtree, J., Yu, Y., Kim, S., Chen, D., et al. (2008). Sub-telomere directed gene expression during initiation of invasive aspergillosis. *PLOS Pathog.* 4:e1000154. doi: 10.1371/journal.ppat.1000154
- Millien, V. O., Lu, W., Shaw, J., Yuan, X., Mak, G., Roberts, L., et al. (2013). Cleavage of fibrinogen by proteinases elicits allergic responses through Toll-like receptor 4. *Science* 341, 792–796. doi: 10.1126/science.1240342
- Muszyńska, L., Aimaniananda, V., Mellado, E., Gribaldo, S., Alcazar-Fuoli, L., Szcwyczyk, E., et al. (2014). Deciphering the role of the chitin synthase families 1 and 2 in the in vivo and in vitro growth of *Aspergillus fumigatus* by multiple gene targeting deletion. *Cell. Microbiol.* 16, 1784–1805. doi: 10.1111/cmi.12326
- Pak, J., and Segall, J. E. (2002). Role of Ndt80, Sum1, and Swe1 as targets of the meiotic recombination checkpoint that control exit from pachytene and spore

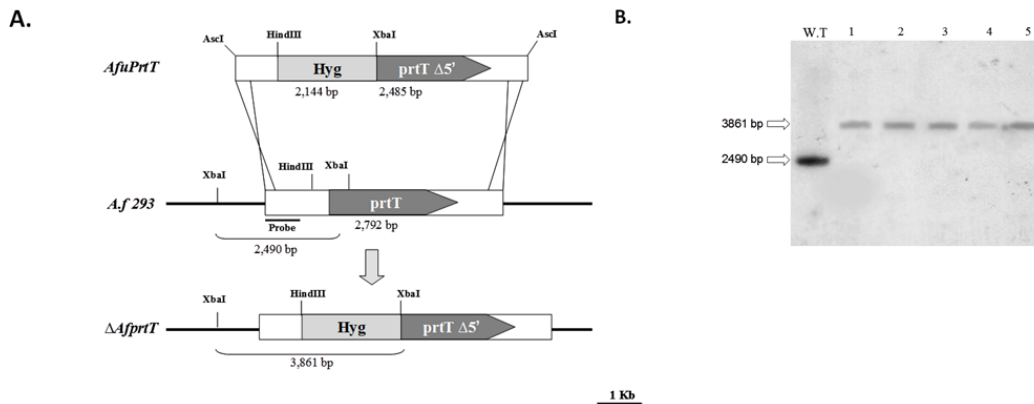
- formation in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 22, 6430–6440. doi: 10.1128/MCB.22.18.6430-6440.2002
- Sellam, A., Askew, C., Epp, E., Tebbji, F., Mullick, A., Whiteway, M., et al. (2010). Role of transcription factor CaNdt80p in cell separation, hyphal growth, and virulence in *Candida albicans*. *Eukaryot. Cell* 9, 634–644. doi: 10.1128/EC.00325-09
- Sellam, A., Tebbji, F., and Nantel, A. (2009). Role of Ndt80p in sterol metabolism regulation and azole resistance in *Candida albicans*. *Eukaryot. Cell* 8, 1174–1183. doi: 10.1128/EC.00074-09
- Sharon, H., Amar, D., Leviansky, E., Mircus, G., Shadkchan, Y., Shamir, R., et al. (2011). PrtT-regulated proteins secreted by *Aspergillus fumigatus* activate MAPK signaling in exposed A549 lung cells leading to necrotic cell death. *PLOS ONE* 6:e17509. doi: 10.1371/journal.pone.0017509
- Sharon, H., Hagag, S., and Osherov, N. (2009). Transcription factor PrtT controls expression of multiple secreted proteases in the human pathogenic mold *Aspergillus fumigatus*. *Infect. Immun.* 77, 4051–4060. doi: 10.1128/IAI.00426-09
- Speth, C., Hagleitner, M., Ott, H. W., Wurzner, R., Lass-Flörl, C., and Rambach, G. (2013). *Aspergillus fumigatus* activates thrombocytes by secretion of soluble compounds. *J. Infect. Dis.* 207, 823–833. doi: 10.1093/infdis/jis743
- Tomee, J. F., Wierenga, A. T., Hiemstra, P. S., and Kauffman, H. K. (1997). Proteases from *Aspergillus fumigatus* induce release of proinflammatory cytokines and cell detachment in airway epithelial cell lines. *J. Infect. Dis.* 176, 300–303. doi: 10.1086/517272
- Weidner, G., d'Enfert, C., Koch, A., Mol, P. C., and Brakhage, A. A. (1998). 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.* 33, 378–385. doi: 10.1007/s002940050350
- Yeung, Y. G., and Stanley, E. R. (2009). A solution for stripping antibodies from polyvinylidene fluoride immunoblots for multiple reprobing. *Anal. Biochem.* 389, 89–91. doi: 10.1016/j.ab.2009.03.017

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Shemesh, Hanf, Hagag, Attias, Shadkchan, Fichtman, Harel, Krüger, Brakhage, Kniemeyer and Osherov. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

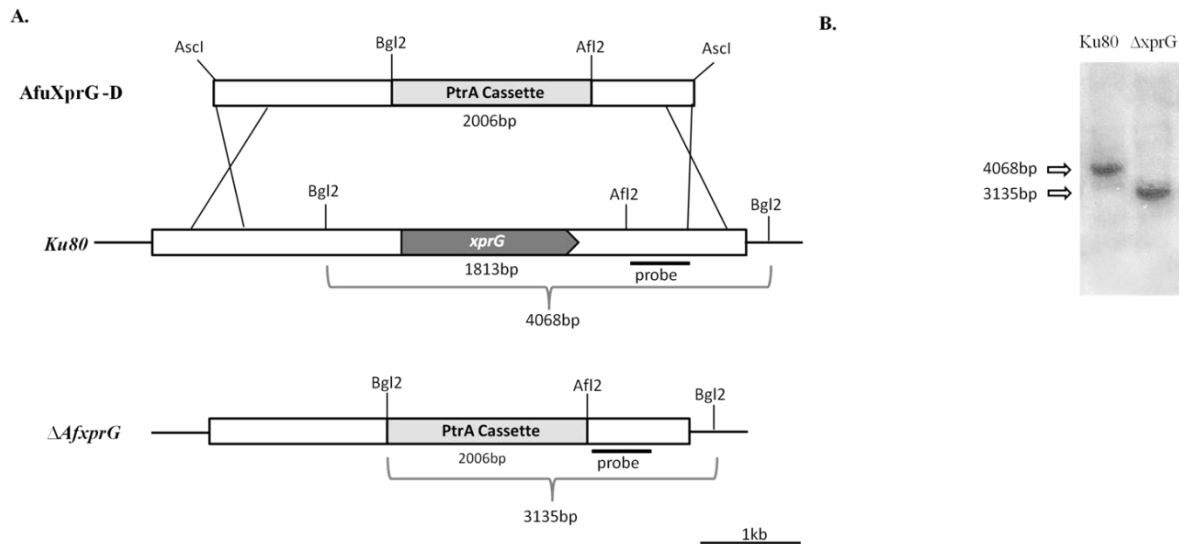
Supplementary Data.

Construction and verification of the *A. fumigatus* *PrtT* disruption mutant. A- 5120-bp DNA fragment flanking the *A. fumigatus* *PrtT* gene (AFUA_4G10120) was generated by PCR, using the Expand high-fidelity PCR system (Roche Diagnostics), genomic DNA template from *AkuB*^{KU80} and primers *PrtT* outer -5' and *PrtT* outer -3' designed to contain an *AscI* restriction site at their 5' end (Supplementary Table S1). The product of this PCR was cloned into the TA vector pGEM-T-Easy (Promega). A 773-bp fragment, which included 307 bp of the N-terminal *PrtT* open reading frame, was then removed by digestion with *XbaI* and *HindIII* and replaced with a hygromycin-selectable marker to produce the p*PrtT*-D plasmid. The hygromycin cassette, containing 5' and 3' *HindIII* and *XbaI* restriction sites, respectively, was generated by PCR amplification using primers *Hyg* 5' and *Hyg* 3' (Table 3). For transformation, 10 µg of spin-purified *AscI*-digested p*PrtT*-D plasmid was used. Transformation was performed as previously described by Romano et al (17). For Southern blot analysis, genomic DNA was extracted from the *A. fumigatus* *Ku80* strain and five independent transformants (Δ *PrtT*-D1-5). Southern hybridization analysis was performed as previously described (18). Briefly, 10 µg fungal genomic DNA samples were digested with *XbaI* and run on a 1% (w/v) agarose gel. The restricted DNA was transferred to a Nytran N nylon membrane (Schleicher & Schuell Bioscience, Keen, NH, USA) and hybridized with an [α -³²P]dCTP- radiolabeled *PrtT* 5' probe at 65°C. The probe (785 bp) was generated by PCR with primers *PrtT* outer 5' and *PrtT* up 3' (Supplementary Table S1). Disruption of *PrtT* was verified by Southern blotting (Supplementary Fig. S1).



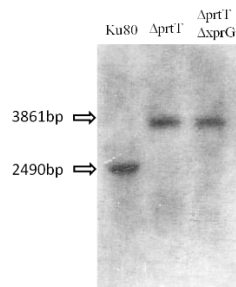
Supplementary Figure S1. Disruption of the *prtT* gene in *A. fumigatus*. (A) Schematic representation of the *prtT* WT locus and the *AscI*-cut insert of plasmid pPrtT used for disruption. (B) Southern blot verification of the Δ *prtT*-D1-5 isolates and control *AkuB*^{KU80} strain. For the Southern blot analysis, genomic DNA (10 µg per well) was digested with *XbaI*, blotted, and hybridized with a ³²P-labeled *PrtT* 5'-flanking DNA probe, resulting in fragments of ~3.8 kb for the *PrtT* disrupted strains and ~2.5 kb for the *AkuB*^{KU80} control strain.

Construction and verification of the *A. fumigatus* *XprG* disruption mutant. A- 5750-bp DNA fragment flanking the *A. fumigatus* *xprG* homologe gene (AFUA_8G04050) was generated by PCR, using the Expand high-fidelity PCR system (Roche Diagnostics), genomic DNA template from *AkuB*^{KU80} and primers *XprG* up f and *XprG* D r designed to contain an *AscI* restriction site at their 5' end (Supplementary Table S1). The product of this PCR was cloned into the TA vector pGEM-T-Easy (Promega). A 2945-bp fragment, which included the entire *xprG* open reading frame, was then removed by digestion with *Bgl*III and *Afl*III and replaced with a pyrithiamine -selectable marker to produce the p*XprG* -D plasmid. The pyrithiamine resistance cassette, containing 5' and 3' *Bgl*II and *Afl*II restriction sites, respectively, was generated by PCR amplification using primers PtrA Cassette f and PtrA Afl2 r and psk275 as template (19) (Supplementary Table S1). *AscI*-digested p*XprG* -D plasmid was transformed into the *A. fumigatus* *AkuB*^{KU80} strain. Transformation was performed as previously described. For Southern blot analysis, genomic DNA was extracted from *AkuB*^{KU80} and two independent transformants (Δ *xprG*-D1-2). Southern hybridization analysis was performed as previously described (18). Briefly, 10 µg fungal genomic DNA samples were digested with *Bgl*III and run on a 1% (w/v) agarose gel. The restricted DNA was transferred to a Nytran N nylon membrane (Schleicher & Schuell Bioscience, Keen, NH, USA) and hybridized with an [α -³²P]dCTP- radio labeled *XprG* flanking region probe at 65°C. The probe (617 bp) was generated by PCR with primers Prob *Bgl*II and Prob *Bgl*II-R2 (Supplementary Table S1). Disruption of *XprG* was verified by Southern blotting (Supplementary Fig. S2)



Supplementary Fig. S2. Deletion of the *xprG* gene in *A. fumigatus*. (A) Schematic representation of the *XprG* WT locus and the *Ascl* cut insert of plasmid pXprG-D used for disruption. (B) Southern blot verification of the $\Delta XprG$ -D1 and control *AkuB*^{KU80} strain. For the Southern blot analysis, genomic DNA (10 μ g per well) was digested with *Bgl*II, blotted, and hybridized with a ³²P-labeled DNA probe, resulting in fragments of for the 4068 bp for the *AkuB*^{KU80} strain and 3135 bp for the *XprG* disrupted strain.

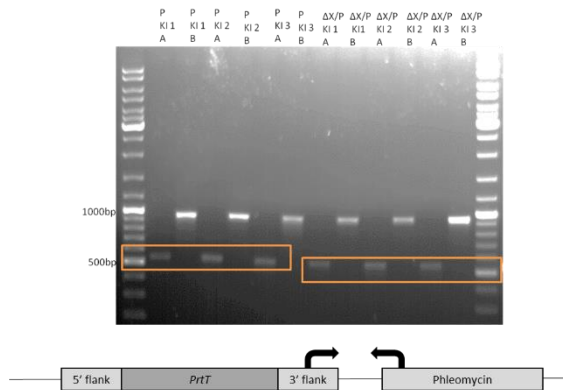
Construction and verification of the *A. fumigatus* *PrtT/XprG* disrupted mutant. We created this mutant by transforming the plasmid used for the deletion of *PrtT* into the $\Delta XprG$ -D1 mutant. After transformation, hygromycin-resistant transformants were purified and screened by PCR for putative insertion mutants. Mutation was further confirmed and verified for single integration of the transforming construct by Southern blotting (Supplementary Fig. S3).



Supplementary Figure S3. Southern blot verification of the $\Delta PrtT/\Delta XprG$ double mutant and control *AkuB*^{KU80} strain. For the Southern blot analysis, genomic DNA (10 μ g per well) was digested with *Xba*I,

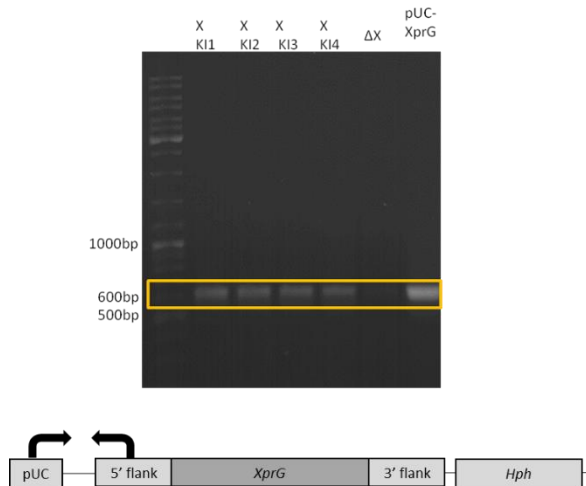
blotted, and hybridized with a ^{32}P -labeled *PrtT* 5'-flanking DNA probe, resulting in fragments of ~3.8 kb for the *PrtT*-disrupted strains and ~2.5 kb for the Ku80 control strain.

Construction and verification of the *A. fumigatus* complemented strains. The *PrtT*-KI (KI denotes knock-in or complemented) and $\Delta XprG$ /*PrtT* KI control strains were prepared by complementing the $\Delta PrtT$ and $\Delta XprG$ /*PrtT* strains with a circular plasmid containing the *pgpdA-phleomycin* cassette and the 5120-bp DNA fragment flanking (2 kb upstream and 0.4 kb downstream of the *PrtT* ORF) the above-described *A. fumigatus* *PrtT* gene. This plasmid (p*PrtT*-KI) was generated by cloning a *pgpdA-phleomycin* cassette into the p*PrtT*-D plasmid via an *NdeI* restriction site. The primers used to amplify the *pgpdA-phleomycin* cassette from pAN-8.1(20) were pAN-8.1 *NdeI*-5' and pAN-8.1 *NdeI*-3' (Supplementary Table S1). Verification of integration of p*PrtT*-D into the $\Delta PrtT$ and $\Delta XprG$ /*PrtT* strains was verified by colony PCR of transformants (Supplementary Fig. S4).



Supplementary Figure S4. PCR verification of integration of p*PrtT*-D into the $\Delta PrtT$ and $\Delta XprG$ / $\Delta PrtT$ strains to generate the *PrtT*-KI (PKI1-3) and $\Delta XprG$ /*PrtT* KI ($\Delta X/P$ KI1-3) rescued strains. Lanes A- PCR amplification with primers 5'*PrtT* and 3'*PrtT* validation to generate a 500 bp fragment (Supplementary Table S1). Lanes B-Control PCR performed using primers for Afu6g12750 (*RbdA*).

The *XprG*-KI control strain was prepared by complementing the $\Delta XprG$ strain with a circular plasmid containing the *puc-hph* cassette and a 3862 bp DNA fragment flanking the *A. fumigatus* *XprG* gene. The 3862 bp *XprG*-containing DNA fragment was cloned by PCR from *AkuB*^{KU80} genomic DNA using primers 5'*HindXprG* and 3'*XbaXprG* (Supplementary Table S1). Verification of integration of p*XprG*-D into the $\Delta XprG$ strain was verified by colony PCR of transformants (Supplementary Fig. S5).



Supplementary Figure S5. PCR verification of integration of pXprG-D into the $\Delta XprG$ strain to generate the *XprG* KI (X KI1-4) rescued strains. PCR amplification with primers 5'*XprG* and 3'*XprG* validation to generate a 600 bp fragment (Supplementary Table S1). Controls- ΔX = $\Delta XprG$ strain, pUC *XprG* plasmid as template.

Proteomic analysis.

Cultures were harvested and filtered through Miracloth (Calbiochem). 10 % (w/v) trichloroacetic acid was added to the supernatant for 12 h at 4 °C in an ice-water bath with gentle shaking (15 rpm) to precipitate the secreted proteins. After centrifugation for 1h at 30000 $\times$ g, the pellets were emulsified in 0.5 ml supernatant and centrifuged at 14000 $\times$ g for 30 min. To remove detergents and other contaminants, the samples were treated with chloroform-methanol purification after Wessel-Flügge (1) and water-saturated ethyl acetate clean-up after Yeung and Stanley (2). The samples were vacuum-dried for approximately 1-5 min to almost dryness. Proteins were solubilised in 200 μ l ammonium bicarbonate (50 mM) / 2,2,2-trifluoroethanol 1:1 (w/v) and sonicated in a water-bath sonicator for 10 min. Samples were centrifuged for 20 min at 20,000 $\times$ g, 20 °C. Supernatant was transferred in a new 1.5 ml microcentrifuge tube. Protein concentration was detected using Millipore DirectDetect and 70 mg of samples were denatured at 90 °C, 500 rpm for 10 min. Disulphide bonds were reduced with 5 μ l of 100 mM tris(2-carboxyethyl)phosphine and reduced cysteine residues were alkylated with 5 μ l 500 mM iodoacetamide for 30 min at room temperature, 500 rpm. Samples were evaporated until a final volume of about 5 μ l was left and diluted in 100 μ l 50 mM ammonium bicarbonate. Proteins were digested over night with trypsin in a ratio of 1:25 trypsin to protein at 37 °C, 400 rpm. Samples were labelled with iTRAQ isobaric labelling for quantitative proteomics

approach to analyse the protein ratios between the knock-out mutants and the wild type. The iTRAQ labelling was conducted according to the manufacturer's instructions (Applied Biosystems). The labeling reactions were incubated for 1 h at room temperature, subsequently quenched with water, combined and further fractionated on a strong cation exchanger (Thermo Fischer HyperSep SCX SPE 50 mg/ml) in five different fractions. The initial equilibrium buffer for the SCX fractioning was 25 % ACN v/v, 0.05 % HCOOH v/v and 0 mM ammonium bicarbonate, the four following fractions were eluted from the SCX column with 25mM, 50 mM, 100 mM and 400 mM ammonium bicarbonate solutions. The last fraction (400 mM ammonium bicarbonate) and the flow-through (0 mM ammonium bicarbonate, 25 % ACN v/v, 0.05 % HCOOH v/v) were combined. The volume of the fractions were reduced in a vacuum centrifuge, peptides were diluted in 20 µl of 0.05 % TFA v/v in 2/98 v/v ACN/H₂O (0.05 % TFA in 98/2 H₂O/ACN) and sonicated in a water bath sonicator for 15 min. An Ultimate 3000 nano RSLC system coupled to a QExactive Plus mass spectrometer (both Thermo Scientific Fischer) was used as a mass analyser. 4 µl sample was injected for each analysis and per biological replicate, three analytical replicates were applied. For enrichment and desalting, an online pre-concentration set-up was used with a nano trap column (Acclaim PepMap 100, 2 cm x 75 µm, 3 µm) at a flow rate of 5 µl / min for 4 min. The pre-concentrated sample was eluted on the analytical column (Acclaim PepMap RSLC, 50 cm x 75 µm, 2 µm). The binary mobile phase consisted of ((A) 0.1% v/v formic acid in H₂O and (B) 0.1% v/v formic acid in 90/10 ACN/H₂O) to separate the peptides with a non-linear 135 min gradient elution at 40°C: 0-5 min at 4% B, 10 min at 6.5% B, 15 min at 7.5% B, 20 min at 8% B, 25 min at 8.6% B, 30 min at 9.2% B, 35 min at 9.9% B, 40 min at 10.6% B, 45 min at 11.3% B, 50 min at 12.2% B, 55 min at 13.4% B, 60 min at 14.9% B, 65 min at 17% B, 70 min at 19.1% B, 94 min at 40% B, 199 min at 52% B, 103 min at 68% B, 106-114 min at 96% B, and 115-135 min at 4% B. . Positive charged ions were generated at the Nanospray Flex Ion Source (Thermo Fisher Scientific) with a stainless steel emitter at 2.2 kV spray voltage. The capillary temperature was set to 220 °C, the S-lens RV level to 50. The calibration was carried out with the positive CalMix solution (external calibration) and with the lock mass of m/z 445.12003 amu for [C₂H₆SiO]₆ (polydimethylsiloxane) (internal calibration). In turns with the samples, Cytochrome C and blind injections were used to monitor the performance of the LC during the whole analysis. Further on, the UV absorption at 214 nm wavelength was recorded. The mass range of the precursor ions was set to m/z 300 – 1600 at a

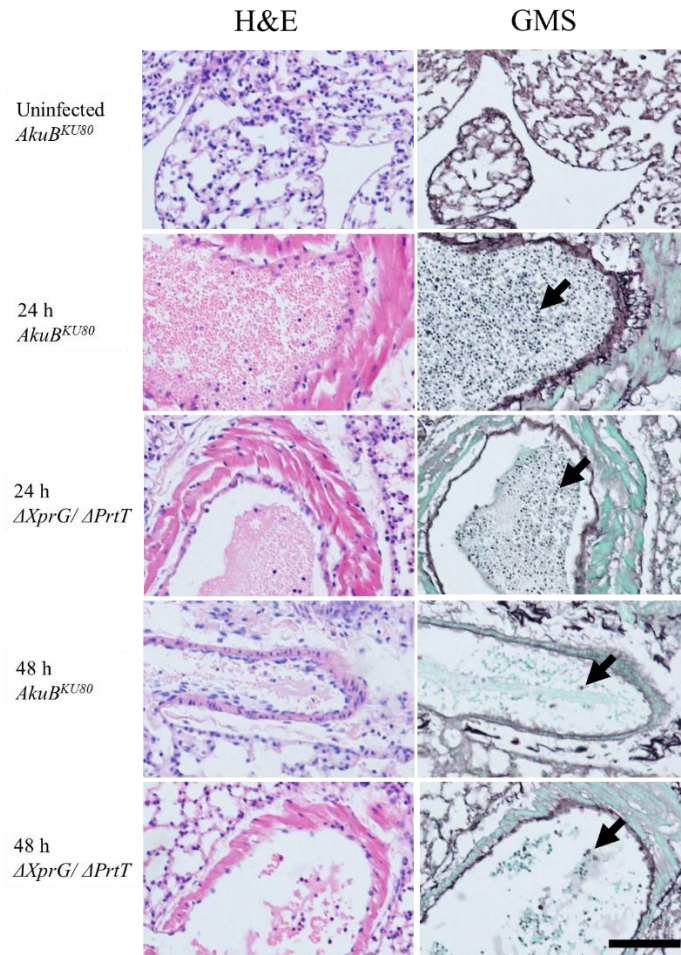
resolution of 70k full width at half maximum (FWHM) with a maximum injection time of 120 ms and an automatic gain control target of 1×10^6 . (The quadrupole / orbitrap mass analyser worked in Full MS/ddMS² (TopN) mode.) For data-dependent acquisition, up to eight most abundant precursor ions with an assigned charge of $z = 2-6$ for further fragmentation, selected by the quadrupole in each scan cycle with an isolation width of m/z 2.0. In the collision cell, the fragments were generated at a normalised collision energy of 36 V with nitrogen gas. Precursor ions were excluded dynamically for 35 s. With a maximum injection time of 120 ms and an automated gain control target of 2×10^5 , the fragment ion resolution was set to 17.5k FWHM. The software Thermo/Dionex Chromeleon Xpress v6.80 SR13 build 3818 and the Thermo QExactive Plus Tune/Xcalibur v3.0.63 2.3 build 1765 graphical interface software was used on the LC-MS/MS instrument. The raw files generated by the LC-MS/MS were further processed by the software Proteome Discoverer v1.4.0.288 (Thermo). Tandem mass spectra were searched against the AspGD *Aspergillus fumigatus* Af293

(www.aspergillusgenome.org/download/sequence/A_fumigatus_Af293/current/A_fumigatus_Af293_current_orf_trans_all.fasta.gz; status 2015/07/05) protein database with the algorithm of MASCOT v2.4.1 (Matrix Science, UK), SEQUEST HT, and MS Amanda for database analysis with a maximum of two missed cleavages. The mass tolerances were set to 10 ppm. for the precursor ion and to 0.02 Da as a fragment mass tolerance. As modifications, oxidation of Met (dynamic), 4-plex peptide iTRAQ labeling of the N-termini of peptides (dynamic), such as of the side chains of lysine (dynamic) and carbamidomethylation of Cys (static, due to usage of idoacetamide) were considered. iTRAQ reporter ion ratios were calculated by comparison of all biological replicates of the single (Δ prtT / WT and Δ xprG / WT) or double (Δ prtT Δ xprG / WT) mutant strains against the wild type (115/114, 116/114, 117/114, 116/115, 117/115, 114/115, 117/116, 114/116, 115/116). For validation (q value) of the peptide spectra matches, a reverse decoy database and a percolator node were used with a maximum Δ Cn of 0.05 and a target false discovery rate of 0.01 (strict) and 0.05 (relaxed). A minimum of 2 peptides per protein hit were required and only unique peptides were used for the quantification. A regulation ≥ 1.5 -fold was considered as significant threshold for up- or down-regulations. Calculated ratios that are based on 2-4 reporter ions (average) and an average variability 30 - 50% were considered as medium confidence, ≥ 4 reporter ions (average) and $\leq 30\%$ were considered as high confident data. The deletion of the transcription factors XprG

and PrtT led to a strong reduction in the amount of secreted proteins. Due to this reduced levels of secreted proteins in the deletion mutant strains, up to three replicates were required and were pooled to obtain the minimal total protein amount of 70 µg for LC-MS/MS analysis. In order to take into consideration the significant reduction of protein secretion, the protein ratios were afterwards normalized to the protein concentration of secreted proteins determined in the medium. To determine these normalization factors, the protein concentrations measured in the culture supernatant were divided by the protein concentration in the culture supernatant of the wild type strain.

Histological analysis of lung sections following intranasal murine infection with *A. fumigatus* conidia.

Six-week-old immunocompetent female ICR mice were inoculated intranasally with 1×10^7 conidia and sacrificed after 24 h or 48 h. Histological analysis was performed with gomori methenamine silver stain (GMS, stains fungal elements black) or haematoxylin and eosin stain (H&E, stains host-cell nuclei purple, cytosol pink).



Supplementary Figure S6. Immunocompetent mice mount a similar immune response to $\Delta XprG/\Delta PrtT$ and control *AkuB^{KU80}* pulmonary infection. 6-week old female ICR mice were infected intranasally with 10^7 $\Delta XprG/\Delta PrtT$ or control *AkuB^{KU80}* conidia. The mice ($n = 5$ each group) were sacrificed 24 h or 48 h following infection. Their lungs were surgically excised and histological sections were stained with GMS (stains fungi black, right panel, black arrow) or H&E (stains host cell nuclei purple, cytosol in pink, left panel). Size-bar = 100 μm .

References

1. Wessel D, Flügge UI. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal Biochem*. 1984 138:141-3.
2. Yeung YG, Stanley ER. Rapid detergent removal from peptide samples with ethyl acetate for mass spectrometry analysis. *Curr Protoc Protein Sci*. 2010. Chapter 16: Unit 16.12.

Supplementary Table S1: Primers used in this study

Oligonucleotide	Sequence 5'→3'
XprG up f	ATGGCGCGCCCATGCATACGTTAGGTGGTT
XprG D r	ATGGCGCGCCACTCTGTTGCCACGTACCA
PtrA Cassette f	TAAGATCTAGGCCAATTGATTACGGGAT
PtrA Afl2 r	ATCTTAAGCCTAGATGGCCTCTTGCAT
Prob Bgl2	AGTAATGACTCGTGGCAGCT
Prob Bgl2-R2	TGAACCCTCGAGCTTACCGA
PrtT outer 5'	ATGGCGCGCCGCACTGATCTCGTCCTATCGGTT
PrtT outer 3'	ATGGCGCGCCCTAAAGTCTCCTACCTACCTCAG
Hyg 5'	ATAAGCTTGTCGACAGAAGATGATAT
Hyg 3'	ATAAGCTTGCTCTCCCTTATGCGACTCCTGCA
PrtT up 3'	AGTGACAGAACAGGCGACAG
pAN-8.1 NdeI 5'	ATCATATGGAATTCCTTGTATCTCTACACAC
pAN-8.1 NdeI 3'	TGCACCATATGCGGTGTGAAATA
gpdA 5'	TCTCCAACGTTCTTGCACC
gpdA 3'	CCACTCGTTGTCTGACCAGG
prtT inner 5'	GGTGATAGCGAAACAGAAGAGACAAG
prtT inner 3'	ATAGACTCCACTGGTAGTTAGGTGTTCTG
5'HindXprG	TAAAGCTTCAGCCTACAGACTGTCTTTCTC
3'XbaXprG	TATCTAGAGCAGAGGGACTGGGATATTTG
5'PrtT validation	GTAACGACTGAGGTAGGTAGGA
3'PrtT validation	TTTAAGGCTGCCAACAGCT
5'XprG validation	CAATGGCTATCGGCTCTCTTT
3'XprG validation	TGGTTAGCGTCTGACTTTG

Supplementary Table S3

Category	Sub-Category	Gene number/Name	$\Delta PrtT$	$\Delta XprG$	$\Delta XprG/PrtT$
Proteases (n=24)	Serine Proteases	<i>DppV/Afu2g09030</i>	NS*	0.51†	0.29
		<i>DppIV/Afu4g09320</i>	0.63	0.42	0.3
		<i>Cp6/Afu5g01200</i>	0.47	0.45	0.18
		<i>Sxa2/Afu2g03510</i>	0.44	0.37	0.24
		<i>DapB/Afu3g07850</i>	0.42	0.64	0.28
		<i>Cp3/Afu6g13540</i>	0.32	0.51	0.29
		<i>Cp1/Afu5g07330</i>	0.32	0.53	0.27
		<i>Afu2g01250</i>	0.26	0.48	0.32
		<i>AorB/Afu6g10250</i>	0.52	0.57	0.36
		<i>KexB/Afu4g12970</i>	0.45	0.56	0.31
		<i>SedC/Afu3g08930</i>	0.41	0.32	0.36
		<i>Sed/Afu4g03490</i>	0.32	0.42	0.25
		<i>Alp1/Aspf13/Afu4g11800</i>	0.31	NS	0.34
		<i>Alp2/Aspf18/Afu5g09210</i>	0.31	NS	0.29
	Metalloproteases	<i>Afu1g05960</i>	0.46	0.59	0.29
		<i>Ape3/Afu2g00220</i>	0.4	NS	0.35
		<i>Cps1/Afu3g07040</i>	0.36	0.6	0.32
		<i>Afu6g06800</i>	0.34	0.47	0.35
		<i>Lap1/Afu4g04210</i>	0.32	0.32	0.25
		<i>Lap2/Afu3g00650</i>	0.27	NS	0.24
		<i>Mep/Aspf5/Afu8g07080</i>	0.39	0.3	0.36
		<i>CtsD/Afu4g07040</i>	0.46	NS	0.4
	Aspartic Proteases	<i>Pep1/Aspf10/Afu5g13300</i>	NS	0.46	0.29
		<i>Pep2/Afu3g11400</i> 1	0.35	0.64	0.31
Glucanases					
(n=18)		<i>Eng6/Afu6g14540</i>	NS	0.42	0.32
		<i>BtgE/Afu8g05610</i>	NS	NS	0.55
		<i>Exg6/Afu6g13270</i>	0.54	0.62	0.37
		<i>Afu2g03980</i>	0.53	NS	0.31
		<i>Utr2/Afu2g03120</i>	0.51	NS	0.37
		<i>Neg1/Afu8g07120</i>	0.48	0.59	0.29
		<i>Eng7/Afu3g03080</i>	0.48	NS	0.32
		<i>Afu7g06150</i>	0.4	0.53	0.29
		<i>Egl2/Afu6g11600</i>	0.4	0.65	0.35
		<i>Engl1/Afu1g04260</i>	0.35	NS	0.31
		<i>Afu3g03870</i>	0.34	0.35	0.34
		<i>Afu8g06830</i>	0.33	NS	0.35
		<i>ExgO/Afu1g14450</i>	0.32	NS	0.29
		<i>Afu6g03230</i>	0.31	0.52	0.38

		<i>Bgt3/Afu5g08780</i>	0.31	0.51	0.3
		<i>Aspf9/Afu1g16190</i>	0.26	0.52	0.26
		<i>Bgt2/Afu3g00270</i>	0.25	NS	0.31
		<i>Afu1g12560</i>	0.23	0.33	0.32
Chitinases					
(n=6)		<i>Afu3g07160</i>	0.46	0.42	0.42
		<i>ChiB1/Afu8g01410</i>	0.41	NS	0.36
		<i>Chi3/Afu7g05140</i>	0.37	NS	0.33
		<i>Afu3g11280</i>	0.36	NS	0.33
		<i>Afu6g09307</i>	0.33	0.64	0.32
		<i>ChiA1/Afu5g03760</i>	0.32	0.6	0.3
Allergens					
(n=17)		<i>Aspf4/Afu2g03830</i>	NS	NS	0.65
		<i>Aspf28/Afu6g10300</i>	NS	0.51	0.51
		<i>Aspf12/Afu5g04170</i>	0.6	0.48	NS
		<i>Aspf11/Afu2g03720</i>	0.59	0.56	0.45
		<i>Aspf3/Afu6g02280</i>	0.55	NS	0.48
		<i>Aspf27/Afu3g07430</i>	0.54	NS	0.42
		<i>Aspf29/Afu5g11320</i>	0.51	0.46	0.5
		<i>Aspf7/Afu4g06670</i>	0.48	NS	0.35
		<i>Aspf6/Afu1g14550</i>	0.45	0.35	0.63
		<i>Aspf23/Afu2g11850</i>	0.41	0.53	0.24
		<i>Aspf1/Afu5g02330</i>	0.4	0.48	0.35
		<i>Aspf2/Afu4g09580</i>	0.39	0.41	0.29
		<i>Aspf22/Afu6g06770</i>	0.38	0.49	0.52
		<i>Aspf13/Afu2g12630</i>	0.34	0.38	0.39
		<i>Aspf8/Afu2g10100</i>	0.3	0.31	0.34
		<i>Aspf34/Afu3g03060</i>	0.28	NS	0.3
		<i>Aspf17/Afu4g08960</i>	0.28	NS	0.26

NS-not significant

† Fold change vs. *AkuB*^{KU80} wild-type strain