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Endoplasmic reticulum localized PerA is required for cell wall integrity, azole drug resistance, and virulence in *Aspergillus fumigatus*

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Summary

GPI-anchoring is a universal and critical post-translational protein modification in eukaryotes. In fungi, many cell wall proteins are GPI-anchored, and disruption of GPI-anchored proteins impairs cell wall integrity. After being synthesized and attached to target proteins, GPI anchors undergo modification on lipid moieties. In spite of its importance for GPI-anchored protein functions, our current knowledge of GPI lipid remodeling in pathogenic fungi is limited. In this study, we characterized the role of a putative GPI lipid remodeling protein, designated PerA, in the human pathogenic fungus *Aspergillus fumigatus*. PerA localizes to the endoplasmic reticulum and loss of PerA leads to striking defects in cell wall integrity. A *perA* null mutant has decreased conidia production, increased susceptibility to triazole antifungal drugs, and is avirulent in a murine model of invasive pulmonary aspergillosis. Interestingly, loss of PerA increases exposure of β -glucan and chitin content on the hyphal cell surface, but diminished TNF production by bone marrow derived macrophages relative to wild type. Given the structural specificity of fungal GPI-anchors, which is different from humans, understanding GPI lipid remodeling and PerA function in *A. fumigatus* is a promising research direction to uncover a new fungal specific antifungal drug target.

Introduction

The fungal cell wall is a dynamic structure required for maintaining cell integrity during polarized growth (Smits *et al.*, 1999, Latge, 2010, Latge, 2007). Due to this unique feature of fungi that is absent in humans, the fungal cell wall is an antifungal drug target (Denning, 2002, Pasqualotto & Denning, 2008, Denning & Hope, 2010). Cell wall composition and structure varies depending on fungal species, developmental stages, and environmental conditions. Major constituents of the cell wall are glycoproteins and polysaccharides such as chitin and glucan that are highly cross-linked. Glycoproteins are modified by N- and O-linked oligosaccharides and more than half of the cell wall proteins are modified by

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glycosylphosphatidylinositol (GPI). This posttranslational modification of cell wall proteins is important for integration of glycoproteins into the cell wall (Bowman & Free, 2006, Bernard & Latge, 2001, Free, 2013).

GPI-anchored proteins in yeast are divided into two groups based on their cellular localization. First, GPI-mannoproteins bound to the polysaccharides of the cell wall are involved in adhesion, invasive growth, filamentation, flocculation, and mating (Cormack *et al.*, 1999, Bruneau *et al.*, 2001, Hoyer, 2001, Guo *et al.*, 2000). A second group is composed of proteins with enzymatic activities required for cell wall biosynthesis such as β -glucanotransferase (Hartland *et al.*, 1996, Nakazawa *et al.*, 1998, Popolo *et al.*, 1993, Saporito-Irwin *et al.*, 1995, Mouyna *et al.*, 2000, de Medina-Redondo *et al.*, 2010, Gastebois *et al.*, 2010). Studies on these proteins emphasize the critical and broad role of GPI anchoring in fungal biology. Recently, a novel antifungal drug E1210 (Eisai) was designed to inhibit GPI synthesis in fungi. When orally administered, E1210 inhibits fungal growth and increases survival in mice inoculated with *Candida albicans*, *Aspergillus fumigatus*, and *Fusarium solani* (Hata *et al.*, 2011, Miyazaki *et al.*, 2011, Watanabe *et al.*, 2012). These findings suggest that studies on fungal GPI-anchors are a promising research area for finding new therapeutic targets for human fungal infections.

GPI anchors share a common core structure that includes ethanolamine-phosphate (EtNP), mannose (Man), glucosamine (GlcN), and phosphatidylinositol (PI). In contrast, side chains and lipid moieties at the GPI anchors vary from protein to protein and species to species. After synthesis and attachment to target proteins in the ER, GPI anchors undergo a series of modifications at the lipid moieties. Lipid remodeling of the GPI anchors includes mainly three steps: inositol deacylation, removal of the acyl chain at the *sn*-2 position of the diacylglycerol to form a lyso-GPI, and introduction of a saturated C26 acyl chain at *sn*-2 (Fujita & Kinoshita, 2012, Orlean & Menon, 2007, Fujita & Jigami, 2008, Chen *et al.*, 1998). Genes and organelles responsible for this process have been characterized in mammals and yeast. PGAP1/Bst1p (mammals/yeast) are ER proteins that function as the GPI inositol-deacylase to eliminate the inositol acyl chain. Loss of PGAP1/Bst1p delays maturation of GPI-anchored proteins and fails quality control of misfolded proteins required for ER exit and degradation (Vashist *et al.*, 2001, Tanaka *et al.*, 2004, Fujita *et al.*, 2006b).

Mammalian PERLD1/yeast Per1p ('Protein processing in the ER') functions as a GPI-phospholipase A₂ (GPI-PLA₂)-like protein to form a lyso-GPI. Expression of human PERLD1 in *S. cerevisiae per1* cells complements the deficient yeast mutant phenotypes (Fujita *et al.*, 2006a). PERLD1 has been cloned as PGAP3 in Chinese hamster, a protein involved in lipid remodeling of GPI-anchored proteins (Maeda *et al.*, 2007). Per1p was initially found as Cos16p ('CdcOne Suppressor'), a mutational suppressor of the *cdc1* mutant. Cdc1p is an essential protein required for regulation of cytosolic Mn²⁺ levels, and Cos16p and Cdc1p coordinately mediate Mn²⁺ homeostasis (Paidhungat & Garrett, 1998). *PER1* was also isolated from a genetic screen to identify proteins related to the unfolded protein response in the ER (Ng *et al.*, 2000). Moreover, Per1p was found as a protein that belongs to the same functional cluster with Bst1p and Gup1p from the epistatic miniarray profiles (E-MAP) analysis for the early yeast secretory pathway (Schuldiner *et al.*, 2005). In

yeast, absence of Per1p causes impaired association of GPI-anchored proteins to membranes and alters PI moieties in the GPI anchor (Fujita *et al.*, 2006a).

Unlike in mammals, two distinct types of lipid moieties are found in mature GPI-anchored proteins in yeast. One is a lipid moiety with diacylglycerol with a C26 fatty acid at the *sn*-2 position, and the other is a ceramide-containing moiety composed of phytosphingosine and C26 fatty acid. The latter is not found in mammals. Cwh43p is required for the ceramide type lipid remodeling and loss of Cwh43p causes increased sensitivity to a cell wall disrupting agent, which is consistently observed in other mutants related to the GPI anchor lipid remodeling process (Umemura *et al.*, 2007, Yoko *et al.*, 2013). Overall, these data support that lipid remodeling of the GPI-anchor is critical for proper function of GPI-anchored proteins. However, in spite of the importance in cellular functions, current information on GPI anchor lipid remodeling is still limited in pathogenic fungi and mostly restricted to mammalian and yeast systems.

A. fumigatus is a human fungal pathogen that is the major *Aspergillus* species responsible for invasive aspergillosis (IA). IA is a significant life-threatening infection in immunocompromised patients and the mortality rate often exceeds 50% (Brown *et al.*, 2012, Pappas *et al.*, 2010, Dagenais & Keller, 2009). Due to the increasing number of immunosuppressed patients from cancer, AIDS, and organ transplantation, the number of people with a high risk for IA infection continues to increase. To prevent severe disease caused by *Aspergillus spp.*, early diagnosis and suitable antifungal treatments are required. However, timely and accurate diagnosis remains a challenge and clinical use of antifungal drugs is difficult due to host toxicity and emerging antifungal drug resistance (Steinbach, 2013).

In *A. fumigatus*, loss of GPI anchor biosynthesis is not lethal, which is distinct from *S. cerevisiae* (Leidich *et al.*, 1994). PigA is a homolog of yeast Gpi3p (Spt14p), the catalytic subunit of GPI-N-acetylglucosaminyltransferase complex (GPI-GnT). Disruption of PigA leads to completely blocked GPI anchor synthesis, cell wall defects, autophagy, and reduced virulence in *A. fumigatus* (Li *et al.*, 2007, Yan *et al.*, 2013). Interestingly, GPI-anchor lipid moieties of *A. fumigatus* GPI-anchored proteins are found only as the ceramide type, which mammalian GPI anchors lack (Fontaine *et al.*, 2003). Given the distinct characteristic of lipid moieties between *A. fumigatus* and yeast, it is reasonable to hypothesize that the two fungal species utilize different mechanisms for lipid remodeling of GPI anchors. In this study, we investigated a role of a putative ortholog of yeast Per1p, designated PerA in *A. fumigatus*. Taken together, our results strongly suggest that lipid remodeling of the GPI anchor of filamentous pathogenic fungi by PerA is an important virulence attribute and promising antifungal drug target.

Results

Identification of PerA in *A. fumigatus*

BLASTP analysis in the *A. fumigatus* genome database with *S. cerevisiae* Per1p as a query found AFUB_006580 as the best hit (E-value, 2e-40, identity 32.5%, similarity 46.8%). Subsequent BLASTP analysis in the *S. cerevisiae* genome database with *A. fumigatus*

AFUB_006580 as a query identified *PER1* as the best hit (1.6E-32, identity 35%, similarity 50%), which suggests AFUB_006580 and *PER1* are potential orthologs (Figure 1A). Therefore, we designated AFUB_006580, PerA. Additional BLASTP searches using PerA amino acid sequence reveal that this protein is conserved in fission yeast *Schizosaccharomyces pombe*, and several well-established models of filamentous fungi including: *Neurospora crassa*, *Magnaporthe oryzae*, and *Fusarium spp.*, and pathogenic yeasts including *Candida albicans*, *Paracoccidioides brasiliensis*, *Ajellomyces dermatitidis*, *Ajellomyces capsulatus*, and *Coccidioides immitis*.

Per1p and PerA have multiple predicted transmembrane domains based on SMART protein search (<http://smart.embl-heidelberg.de/>) and TMHMM transmembrane search (<http://www.cbs.dtu.dk/services/TMHMM-2.0>). The domains are mainly located between intermediate and C-terminal regions of the protein sequences (Suppl. Figure 1). Recently, a superfamily of putative transmembrane hydrolases called CREST (alkaline ceramidase, PAQR receptor, Per1, SID-1 and TMEM8) was identified by sequence similarity searches. Among these groups, the Per1p family is composed of about 150 eukaryotic proteins in fungi, plants, and metazoans, and *A. fumigatus* PerA was identified as a protein classified in the Per1p family (Pei *et al.*, 2011).

In *S. cerevisiae* cell fraction and light microscopy studies using red fluorescence protein (RFP)-tagging of Per1p, Per1p is observed to localize in the endoplasmic reticulum (ER) and vacuoles (Fujita *et al.*, 2006a, Paidhungat & Garrett, 1998). To better understand the function of PerA in *A. fumigatus*, we studied subcellular localization of PerA by tagging the C-terminus of PerA with green fluorescence protein (GFP). ER structures in *Aspergillus spp.* have been visualized using fluorescent light microscopy, and they localize to a network of strands including domains of nuclear envelopes and peripheral ERs across the cell (Markina-Inarrairaegui *et al.*, 2013, Wang *et al.*, 2010, Willger *et al.*, 2012). PerA:GFP fusion proteins are suggested to be localized in the nuclear envelope/ER (Figure 1B), consistent with localization of Per1p in *S. cerevisiae*.

Loss of PerA causes attenuated growth of *A. fumigatus* on minimal but not osmotically stabilized media

To study the function of PerA in *A. fumigatus*, we generated a *perA* null mutant by homologous gene replacement. A gene replacement construct carrying *Aspergillus parasiticus pyrG* as a selectable marker was generated using the double-joint PCR method (Yu *et al.*, 2004), and replacement of *perA* in the resulting strain with *pyrG* was verified by Southern blot analysis (Figure 2A and B). We also generated a *perA*-reconstituted strain by expressing *perA* ectopically, and a single copy integration of the *perA* reconstitution construct into the null mutant strain was verified using Southern blot analysis (Suppl. Figure 2). Radial growth of *perA* is significantly reduced compared to wild type (CBS144.89) and the reconstituted strain (*perA*-recon) when cultured on glucose minimal media (GMM) at 37 °C (Figure 2C and D). *perA* produces light green colonies compared to dark green colonies of wild type and *perA*-recon. The defective radial growth and abnormal colony color of *perA* was restored when cultured on glucose minimal media containing 1.2 M sorbitol

(SMM) (Figure 2C and D). Nutrient rich Yeast Potato Dextrose (YPD) agar also supported wild type similar growth of *perA* (data not shown).

When cultured in stationary liquid glucose minimal media (LGMM), approximately 11 % of the *perA* germlings show polarity defects including swollen conidia, formation of multiple germ tubes, and abnormal tip branching (Suppl. Figure 3). Moreover, *perA* conidia show reduced viability compared to wild type or the reconstituted strain when stored in 0.01 % tween 80 at 4 °C for 7 days. Ninety seven percent of wild type conidia remain viable after 7 days whereas 80 % of *perA* conidia are viable at the same time point. In contrast, conidia viability does not differ in all strains tested when conidia are stored in sterile H₂O at 4 °C for 7 days (Suppl. Figure 4). To rule out the effect of conidia viability on subsequent experiments, freshly collected *perA* conidia with either sterile H₂O or 0.01 % tween 80 were always utilized.

***perA* has impaired cell wall integrity**

Sorbitol is an osmotic stabilizer and several mutants deficient in cell wall integrity have restored growth rates on media containing sorbitol (Borgia & Dodge, 1992, de Medina-Redondo *et al.*, 2010, Lee *et al.*, 2002, Puttikamonkul *et al.*, 2010). Based on the restored growth of *perA* on GMM containing sorbitol, we examined cell wall integrity in *perA* using three cell wall perturbing agents including Calcofluor white (CFW), Congo red (CR), and caspofungin. CFW and CR inhibit enzymes involved in assembly of chitin to β -1,3-glucan and β -1,6-glucan, and CFW preferentially binds to chitin (Ram & Klis, 2006). Caspofungin belongs to the echinocandins, a class of clinically licensed antifungal drugs, and inhibits synthesis of β -1,3-glucan, a major component of the fungal cell wall (Cappelletty & Eiselstein-McKittrick, 2007, Kahn *et al.*, 2006). Compared to the wild type and reconstituted strain, *perA* is strikingly more susceptible to all cell wall perturbing agents tested, which suggests that PerA has a major role in cell wall integrity in *A. fumigatus* (Figure 3).

Transcript levels of *perA* increase as fungal development proceeds

Because the cell wall is a continuously changing structure over time (Latge, 2010, Latge, 2007) and PerA is important for cell wall integrity, *perA* transcript levels in different developmental stages in *A. fumigatus* wild type were quantified. Three developmental stages, dormant conidia, germlings, and hyphae/mycelia were examined for *perA* transcript levels. Conidia were harvested from a colony grown on solid GMM and were applied to liquid media to harvest germlings and hyphae/mycelia. The transcriptional levels of *perA* differ with respect to developmental stage and increase as fungal polarized growth proceeds. *PerA* transcript is most abundant in hyphae or mycelia compared to dormant conidia or germlings by 20- and 3- fold, respectively (Figure 4A). As hyphae are the predominant morphological form found at sites of infection in patients with IA, *perA* is a potential drug target.

PerA is important for conidia production and germination, but dispensable for biomass production

We questioned if the light green colony of *perA* is caused by deficient conidia production. To address this, we investigated the number of conidia produced in *perA*. Because *perA* displays reduced radial growth compared to wild type on glucose minimal medium, the overlay method was utilized to rule out the possibility that conidia production is affected by radial growth rate. *perA* produces a significantly decreased number of conidia that accounts for only 8% of wild type conidia production (Figure 4B). Germination of spores is the first step for fungal colonization (d'Enfert & Fontaine, 1997). Initiation of germination occurs 1 hour earlier in *perA* compared to other strains. All strains tested reach the stationary germination stage after incubation for 8 hours (Figure 4C). Intriguingly, biomass production is not significantly altered by loss of PerA (Figure 4D). Finally, unlike the *S. cerevisiae* *PER1* null mutant, *A. fumigatus perA* does not display enhanced susceptibility to increased Mn^{2+} levels (data not shown).

PerA mediates resistance to the triazole antifungal drug voriconazole

We tested susceptibility of *perA* to the antifungal triazole, voriconazole (VCZ) using E-test strips. Minimum inhibitory concentrations (MICs) based on cleared areas that represent fungal growth inhibition by VCZ were observed. *perA* shows a significantly lower MIC (MIC = 0.064 $\mu g\ ml^{-1}$) compared to wild type (MIC = 0.272 $\mu g\ ml^{-1}$) or the reconstituted strain (MIC = 0.293 $\mu g\ ml^{-1}$) that suggests loss of PerA causes increased susceptibility to VCZ (Figure 5A). Despite the increased susceptibility to VCZ, total ergosterol production is not altered in *perA* compared to wild type (Figure 5B). We interpret this latter finding as evidence that *perA* does not contribute directly to ergosterol production *per se*.

The triazole antifungal drugs including VCZ inhibit 14- α sterol demethylase required for ergosterol biosynthesis pathway by binding to an iron atom in the active site of the enzyme (Kelly *et al.*, 1995). In *A. fumigatus*, 14- α sterol demethylase is encoded by two genes *cyp51A* and *cyp51B* (also known as *erg11A* and *erg11B*) (Mellado *et al.*, 2001). Previously it has been reported that *cyp51A* transcript levels increase in response to VCZ (Blosser & Cramer, 2012, Song *et al.*, 2004, da Silva Ferreira *et al.*, 2006). Correspondingly, *cyp51A* transcript levels in *perA* in response to VCZ are 2-fold higher relative to wild type (Figure 5C). These data are consistent with a working hypothesis that suggests the altered cell wall integrity of *perA* impacts intracellular levels of VCZ leading to increased susceptibility and subsequent transcriptional responses in the sterol biosynthesis pathway. Altered localization/function of a drug efflux pump is also a viable alternative hypothesis.

PerA is required for virulence

A. fumigatus mutants that have deficiencies in cell wall integrity or GPI-anchor biosynthesis are attenuated in virulence (Mouyna *et al.*, 2005, Li *et al.*, 2007, Beauvais *et al.*, 2013). Since *perA* has cell wall defects, we examined its virulence using a steroid murine model of invasive pulmonary aspergillosis (IPA) (Grahl *et al.*, 2011). Twelve outbred CD-1 mice were immunocompromised with triamcinolone (40 mg kg⁻¹) on day -1, followed by intranasal fungal inoculation. Fungal strains were recovered from the sacrificed mouse lungs and tested by PCR to confirm the inoculated strain (Suppl. Figure 5A). Mice inoculated with

wild type or the *perA*-reconstituted strain began to die at day 2 post-inoculation and the number of survivors continued to decrease over the time course of the experiment as expected. The mortality levels between wild type and the reconstituted strain were not statistically significant ($P = 0.15$ from log-rank analysis). In contrast, most of the mice inoculated with *perA* survived to the experimental end point, which was statistically similar to mock controls ($P = 0.56$ from log-rank analysis) (Figure 6A). Although *perA* shows drastically attenuated virulence, it is able to colonize mouse lungs in an *ex-vivo* lung growth assay, which suggests *perA* does not have defects in nutrition uptake and utilization from murine lung tissue (Suppl. Figure 5B).

Using lung tissue collected from mice inoculated with each strain, we conducted histopathological analyses. Mice were sacrificed at day 3 post-inoculation and the collected lungs were stained using hematoxylin and eosin (H&E) and Gomori methenamine silver (GMS). The lungs from mice inoculated with wild type or the reconstituted strain display aggressive fungal growth spreading into the lung parenchyma and an inflammatory cellular infiltrate in areas where fungal growth is present. In contrast, no apparent fungal growth is detected and inflammatory responses are reduced in the lungs inoculated with *perA* (Figure 6B and C), which implies *perA* was efficiently eliminated by host immune system. Overall, these data suggest PerA is required for *A. fumigatus* virulence.

***perA* triggers a decreased inflammatory response from bone marrow derived macrophage (BMDM)**

Fungal cell wall components serve as pathogen associated molecular patterns (PAMPs) recognized by hosts to initiate immune responses (Janeway & Medzhitov, 2002, Lamarinis *et al.*, 2008, Hohl *et al.*, 2008). The deficient cell wall integrity and drastically attenuated virulence in *perA* led us to investigate immune responses triggered by *perA*. Tumor necrosis factor alpha (TNF) is a critical inflammatory mediator secreted by macrophages when exposed to fungal pathogens. TNF plays an important role as a proximal signal in induction of the innate immune response to *A. fumigatus* (Mehrad *et al.*, 1999, Taramelli *et al.*, 1996). We examined TNF levels released from BMDMs after co-incubation with *A. fumigatus* hyphae. BMDMs co-cultured with *perA* show decreased TNF production to approximately 70% of BMDMs cultured with wild type or the reconstituted strain (Figure 7A).

We also assessed the ability of macrophages (J774.1 murine macrophage (MΦ)-like cell line) to damage *perA* hyphae by using a colorimetric 2,3-bis-(2-methoxy-4-nitro-5-sulphenyl)-(2H)-tetrazolium-5-carboxanilide (XTT) assay. XTT substitutes as the final electron acceptor (oxygen) and is reduced to a water-soluble formazan in metabolically active cells (Meshulam *et al.*, 1995, Meletiadis *et al.*, 2001). Intriguingly, the level of damage to *perA* by MΦs is similar to that of other strains ($P = 0.1739$ by one-way ANOVA) (Figure 7B). As the reactive oxygen species (ROS) burst is a primary mechanism that host phagocytes use to damage fungal pathogens (Philippe *et al.*, 2003, Forman & Torres, 2002) we examined sensitivity of *perA* to oxidative stress using menadione. Loss of PerA does not affect sensitivity to oxidative stress *in vitro* consistent with the *in vitro* macrophage XTT assay (Suppl. Figure 6). These results suggest that PerA is important for

inducing inflammatory responses but dispensable for resistance to the antifungal activities of a murine MΦ like cell line.

Loss of PerA alters content and exposure of PAMPs

It has been reported that secretion of TNF by MΦs correlates with β -glucan exposure on the cell surface of *A. fumigatus* (Hohl *et al.*, 2008, Shepardson *et al.*, 2013, Steele *et al.*, 2005). As we have observed decreased TNF production by MΦs in *perA*, we hypothesized that exposure of β -glucan on the cell surface would be diminished in *perA* compared to wild type. Unexpectedly, soluble dectin-1 staining on the fungal cell surface revealed that *perA* has more abundant β -glucan relative to wild type or the reconstituted strain. The intensity of dectin-1 stain per the fungal area is higher in *perA* by 2.7-fold compared to wild type (Figure 8A). In *C. albicans*, chitin does not directly induce cytokine production but blocks fungal recognition by human peripheral blood mononuclear cells (PBMCs) and murine MΦs, which leads to drastic attenuation of cytokine production including TNF (Mora-Montes *et al.*, 2011). In addition, treatment of *C. albicans* with caspofungin increases chitin content on the cell surface and decreases cytokine secretion by PBMCs (Mora-Montes *et al.*, 2011). We questioned if decreased secretion of TNF observed in *perA* in spite of increased exposure of β -glucan on the cell surface is due to the altered chitin level in *perA* compared to wild type. To address this question, we examined chitin levels on the cell surface using CFW staining. The intensity of CFW stain per the fungal area in *perA* is higher by 3.1-fold than wild type (Figure 8B), which suggests that *perA* cell surface has more abundant chitin compared to wild type.

Furthermore, we examined the overall cell surface proteins in *perA* compared to wild type by extraction of cell surface proteins using 0.5 M NaCl (Beauvais *et al.*, 2013). Proteins isolated from conidial surface were separated and visualized in SDS-PAGE. Densitometric analysis showed that more abundant total proteins were isolated from the cell surface of conidia in *perA* compared to wild type and the *perA*-reconstituted strain by 2-fold (Figure 8C). This suggests that loss of PerA alters cell surface nature and compromises surface protein integrity in response to NaCl.

Heterologous expression of *S. cerevisiae* Per1p in *perA* partially restores cell wall integrity and drug resistance

To confirm further the orthology of PerA to Per1p, *S. cerevisiae* *PER1* was expressed using the *PER1* promoter (*ScPER1*(p):*PER1*) or the *perA* promoter (*AfperA*(p):*PER1*) in the *perA* null mutant (Figure 9A). Transcription of *PER1* in the *ScPER1*(p):*PER1* and *AfperA*(p):*PER1* strains was verified by quantitative real-time PCR (qRT-PCR) (Figure 9B). Expression of *PER1* using its native promoter did not restore defective radial growth and conidiation in *perA*. However, resistance to the cell wall perturbing agent, CFW and a triazole antifungal drug, VCZ was partially restored in *perA* by expression of *PER1* (Figure 9C and Suppl. Figure 7). Interestingly, expression of *PER1* using the *A. fumigatus* *perA* promoter did not complement deficiency of *perA* in cell wall integrity and azole resistance as well as in radial growth and conidiation (data not shown).

Discussion

GPI-anchoring of proteins is a universal and critical post-translational process in eukaryotes. In fungi, many cell wall-related proteins are GPI-anchored. Because it is a unique structure that humans lack, the fungal cell wall is an antifungal drug target. However, to date the echinocandin class of antifungal drugs is the only clinically in use drug that targets the fungal cell wall. Therefore, research on the GPI anchoring of fungal proteins has potential to uncover critical information that may improve or augment antifungal therapies.

Lipid remodeling of GPI anchors is critical for sorting, transport, and degradation of GPI-anchored proteins (Vashist *et al.*, 2001, Tanaka *et al.*, 2004, Fujita *et al.*, 2006a, Fujita *et al.*, 2006b). To date, our knowledge of GPI anchor lipid remodeling is mostly restricted to human and yeast in spite of the important role of this mechanism for function of GPI-anchored proteins. Notably, *S. cerevisiae* and *A. fumigatus* have distinct types of lipid moieties on the GPI-anchored proteins. Yeast shows both diacylglycerol- and ceramide-types of lipid moieties in the GPI-anchors. In contrast, *A. fumigatus* GPI-anchored proteins carry only the ceramide type, which is absent in human. Therefore, current knowledge of lipid remodeling of GPI anchors that is mostly based on yeast research might not directly apply to *A. fumigatus*. In this study, we elucidated a role of the previously unstudied PerA in *A. fumigatus*, a putative ortholog of Per1p in *S. cerevisiae*. Per1p is one of the four major proteins (Bst1p, Per1p, Gup1p, Cwh43p) that have been characterized in yeast to regulate lipid remodeling on the GPI anchor. We demonstrated that expression of *ScPER1* using its native promoter partially complemented defective resistance to the cell wall perturbing agent, Calcofluor white (CFW) and the antifungal drug voriconazole (VCZ) in *perA*. Given the distant evolutionary relationship and ecological differences between *S. cerevisiae* and *A. fumigatus*, it is perhaps not surprising that the degree of complementation is moderate. These results further illustrate the point that it is critical to study gene and protein function in human fungal pathogens and not fully rely on previous functional characterizations in model yeast.

To define a role of PerA in *A. fumigatus*, a *perA* genetic null mutant was generated. *perA* displays reduced radial growth on glucose minimal media (GMM) and conidia production. Reduced growth of *perA* on GMM plates is restored when grown on GMM containing the osmotic stabilizer sorbitol or on nutrient rich media. This phenotype has been reported in other *Aspergillus* mutants that have defects in cell wall integrity (Borgia & Dodge, 1992, Lee *et al.*, 2002, Dichtl *et al.*, 2012, Puttikamonkul *et al.*, 2010). Indeed, *perA* displays impaired resistance to cell wall perturbing agents, CFW, Congo red, and caspofungin. Given the role of GPI-anchor lipid remodeling in sorting and transporting GPI-anchored proteins, it is plausible to think that this deficiency on certain GPI-anchored proteins leads to the cell wall phenotype in *perA*. A *per1* null mutant in yeast also has increased susceptibility to CFW and PER1 is responsible for localization of β -(1,3)-glucanosyltransferase Gas1p to the cell surface (Fujita *et al.*, 2006a).

Gas1p is one of the most abundant GPI-anchored proteins in yeast and 5 'Gas' proteins are present in *S. cerevisiae*. β -(1,3)-glucanosyltransferases are involved in cell wall synthesis during vegetative growth by elongation of β -(1,3)-glucan chains in the cell wall (Ragni *et*

al., 2007, Popolo *et al.*, 1993, Nuoffer *et al.*, 1991, Vai *et al.*, 1990). Homologs of ‘Gas’ proteins have been characterized in *S. pombe*, *C. albicans*, *Candida maltosa*, *N. crassa*, and *A. fumigatus* (de Medina-Redondo *et al.*, 2008, de Medina-Redondo *et al.*, 2010, Saporito-Irwin *et al.*, 1995, Muhlschlegel & Fonzi, 1997, Nakazawa *et al.*, 1998, Nakazawa *et al.*, 2000, Kamei *et al.*, 2013, Hartland *et al.*, 1996, Mouyna *et al.*, 2000, Mouyna *et al.*, 2005, Zhao *et al.*, 2013). ‘Gas’ proteins are designated ‘Gel’ proteins in *A. fumigatus* and 7 ‘Gel’ proteins have been identified. To date, Gel1, Gel2, and Gel4 have been studied and they show glucanosyltransferase activities. A *gel2* null mutant exhibits altered cell wall composition and reduced virulence in a murine model of IA, whereas *gel1* does not have appreciable phenotype. Gel4 is essential in *A. fumigatus* (Mouyna *et al.*, 2000, Mouyna *et al.*, 2005, Gastebois *et al.*, 2010). *perA* shows increased susceptibility to caspofungin which specifically inhibits β -(1,3)-glucan synthesis in the cell wall. Whether any of the ‘Gel’ proteins are dysregulated in *perA* is under investigation. Fungal cell wall biosynthesis is a dynamic process that continues to change as polarized growth proceeds (Smits *et al.*, 1999, Latge, 2007, Latge, 2010). For example, *gel1* and *gel4* are highly expressed in mycelia compared to resting conidia or germlings in *A. fumigatus* (Gastebois *et al.*, 2010). Similarly, transcript abundance of *perA* increases as development proceeds. Thus, *perA* expression pattern is potentially related to its role in cell wall integrity.

perA displays a significant increase in susceptibility to the triazole drug, VCZ. Probably, the simplest explanation for this phenotype is that VCZ penetrates more efficiently into *perA* due to altered cell wall integrity compared to wild type. To address other possibilities, we studied transcript levels of *erg11A* (*cyp51A*) in *perA*. Altered *erg11* transcripts affect resistance to the triazole drugs in *C. albicans* (Sanglard *et al.*, 2003, Song *et al.*, 2004, Harry *et al.*, 2002). *A. fumigatus* has two genes encoding 14- α -sterol demethylases (Mellado *et al.*, 2001) and we tested *cyp51A* expression in *perA* in response to VCZ. *Cyp51A* transcript increases in response to VCZ in wild type as previously reported (Blosser & Cramer, 2012, Song *et al.*, 2004), and also does in *perA*. The degree of increased *erg11A* transcripts in response to VCZ is significantly higher in *perA* by 2.3-fold relative to wild type. Possibly, the non-*cyp51A*-mediated drug susceptibility observed in *perA* might be associated with altered localization and/or functions of transporters in the plasma membrane. In *A. fumigatus*, overproduction of ATP-binding cassette transporter AbcA leads to increased azole resistance, and clinical azole-resistant isolates display over-induction of efflux transporters such as Cdr1B (Paul *et al.*, 2013, Fraczek *et al.*, 2013).

Our data suggest that *perA* has impaired cell wall integrity, which is associated with fungal virulence. In the corticosteroid murine model of IPA, *perA* is essentially avirulent compared to the wild type and reconstituted strains. Histological analyses revealed that lungs inoculated with *perA* were essentially clear of fungus and inflammation 3 days after inoculation. *In vitro* studies suggest that *perA* can grow well on lung explants and thus the lack of fungal growth observed *in vivo* may be due to increased susceptibility to immune effector cell killing. *In vivo*, resident alveolar M Φ s encounter conidia and produce cytokines and chemokines to initiate a protective immune response (Taramelli *et al.*, 1996). Consistent with the histology result showing limited inflammation in mice inoculated with *perA*, secretion of TNF, one of the major inflammatory mediators in response to fungal hyphae,

was reduced in *perA* compared to wild type. We hypothesized that β -glucan exposure was likely decreased in *perA* as β -glucan is a potent stimulator of the TNF response in fungi (Faro-Trindade *et al.*, 2012, Huang *et al.*, 2009, Hohl *et al.*, 2005, Steele *et al.*, 2005). Surprisingly, soluble dectin-1 staining shows that exposed β -glucan, a known stimulator of TNF secretion by M Φ s, is more abundant in the *perA* cell surface than wild type.

To seek the explanation for this result, we studied the content of chitin that has been reported to suppress TNF production by M Φ s in *C. albicans* (Mora-Montes *et al.*, 2011). Intriguingly, the cell surface of *perA* contains higher levels of chitin compared to wild type as qualitatively assessed using CFW staining. Therefore, it is possible that the increased chitin content on the cell surface interferes with stimulation of TNF secretion by M Φ s in response to the *perA* cells. However, other explanations on the *perA* phenotype are still possible as many components of the fungal cell wall and its surface can affect the immune response (Latge, 2010, Bozza *et al.*, 2009, Chai *et al.*, 2010, Levitz, 2010, Aimagani *et al.*, 2009). For example, the recently characterized cell wall polysaccharide, galactosaminogalactan (GAG) plays a role in mediating host immune responses during *A. fumigatus* infection. Loss of GAG leads to increased β -glucan exposure on the cell surface, which results in hyperinflammatory responses by bone marrow derived dendritic cells (Fontaine *et al.*, 2011, Gravelat *et al.*, 2013).

Moreover, we observed that surface proteins extracted by NaCl are more abundant in *perA* compared to wild type and the reconstituted strain. This suggests that the overall cell surface was changed by loss of PerA, which could include alterations in glycolipid containing proteins that could impact host defense responses. Quantitative analyses of cell wall components in *perA* remain for future investigation. Taken together it is not entirely clear why the *perA* strain is avirulent in our murine model. *In vitro*, the mutant does not show enhanced susceptibility to ROS stress that is used by immune effector cells to damage fungal conidia and hyphae. Consistent with this, a macrophage cell line is able to damage *perA* to a similar extent as wild type. What is unclear is whether *perA* has enhanced susceptibility to immune effector cells inhibited by steroid treatment through non-oxidative anti-fungal mechanisms including antimicrobial peptides or hydrolases from neutrophils (Brown, 2011). Further *in vivo* studies of *perA*'s interaction with the immune system are ongoing.

In conclusion, analysis of a genetic null mutant of *perA* in *A. fumigatus* strongly suggests that understanding lipid remodeling of GPI anchors in *A. fumigatus* is a promising research direction for increasing our knowledge of fungal pathogenesis mechanisms. Moreover, given the specificity of lipid remodeling of GPI anchor moieties between yeast, molds and humans, research developing antifungal therapies with high drug specificity that target these underlying mechanisms is warranted.

Experimental Procedures

Fungal strains, media, and growth conditions

All fungal strains used in this study are listed in Supplemental Table 1. Media and supplements were prepared as previously described unless otherwise stated (Shimizu &

Keller, 2001). Fungal strains were grown at glucose minimal media (GMM) at 37 °C, harvested using sterile H₂O or 0.01 % tween 80 and stored in 50 % glycerol at –80 °C for long-term storage. For a radial growth assay on GMM compared to GMM containing 1.2M sorbitol, 10⁴ spores were inoculated at the center of the plate and cultured at 37 °C for 96 hours. A diameter of colonies was recorded daily, and this tested was conducted in triplicate. For a growth test in hypoxia, 1 % O₂ and 5 % CO₂ was provided. For DNA or RNA extraction, 10⁶ spores/ml conidia were cultured in liquid GMM at 37 °C at 250 rpm for 18 hours. Mycelia were harvested, lyophilized and utilized for isolation of DNA or RNA. For the *ex-vivo* growth assay, lungs collected from C57BL/6 mice were utilized. Two thousand conidia were inoculated on YPD (control) or the mice lung tissue on 1% agarose media, and cultured at 37 °C for 3 days.

Construct design and fungal transformation

Plasmids used in this study are listed in Supplemental Table 2. In order to generate constructs for a *perA* (AFUB_006580/Afu1g06200) null mutant (*perA*) and a *perA*-reconstituted strain (*perA*-recon), the double-joint PCR method was used as previously described (Yu *et al.*, 2004). Primers used to design constructs are listed in Supplemental Table 3. For creating a *perA* construct, a 0.8 kb (5' flank) and a 0.6 kb (3' flank) DNA fragments were amplified using the primers RAC1472 and 1473 and RAC1474 and 1475, respectively from genomic DNA (gDNA) of *A. fumigatus* wild type. As a selectable marker, a 3.2 kb DNA fragment of *A. parasiticus pyrG* was amplified from the plasmid pJW24 using the primers RAC1468 and 1469. The three PCR products were combined and used as a template to generate a 4.4 kb DNA fragment using the primers RAC1476 and 1477. The final PCR product was transformed into a wild type strain, CEA17 that resulted in *perA*. Fungal transformation was performed as described previously (Bok & Keller, 2004). Transformants were recovered by their uracil prototrophy on GMM containing 1.2 M sorbitol (SMM) in the absence of uracil and uridine.

To design a *perA*-recon construct, a 2.5 kb DNA fragment including a 0.8 kb promoter region, a 1.2 kb coding sequence, and a 0.5 kb 3' flank was amplified using the primers RAC1472 and 1526 from *A. fumigatus* gDNA. As a selectable marker, a 3.0 kb *hygB* (*Neurospora crassa cpc-1(p):hygB:A. nidulans trpC(t)*) fragment was amplified using the primers RAC1523 and 1524 from the plasmid pBC-hygro. The two PCR products were used as a template to generate a 5.2 kb DNA fragment using the primers RAC1476 and 1525. The final PCR product was transformed into the *perA* strain that resulted in the *perA*-recon strain. To recover *perA*-recon transformants that carry *hygB* in the genome, hygromycin B (Calbiochem #400052) was added into media at 150 µg ml⁻¹.

To generate a strain expressing *perA* tagged with green fluorescence protein (GFP) at the C-terminus, a 1.9 kb DNA fragment including a 0.7 kb 5' flank and a 1.2 kb *perA* coding sequence was amplified from *A. fumigatus* gDNA using the primers RAC1472 and 1707. A 2.7 kb DNA fragment containing a Gly-Ala linker, eGFP, and *A. fumigatus pyrG* was amplified from the plasmid pFNO3 using the primers RAC1318 and 1319. A 0.6 kb of *perA* 3' flank sequence was amplified from *A. fumigatus* gDNA using the primers RAC1708 and 1475. The final 5.0 kb PCR product was generated from the three PCR products as a

template using the primers RAC1476 and 1477, and transformed into a wild type strain, CEA17. To confirm functionality of PerA-GFP fusion proteins, the final PerA:GFP construct was co-transformed with pyrithiamine resistance gene (*ptrA*) fragment (Kubodera *et al.*, 2000) to *perA*. To select pyrithiamine-resistant transformants, pyrithiamine hydrobromide (Sigma, # P0256) was added into media at 0.1 mg l⁻¹. Then, the selected transformants were further screened by PCR. Gene replacement for *perA* and single copy insertion for *perA*-recon were verified by PCR and Southern blot analyses (Figure 2 and Suppl. Figure 2).

To express *S. cerevisiae* *PER1* in *perA*, two constructs were generated. First, a 2.1 kb DNA fragment including a 500 bp 5' flank, a 1.1 kb coding region, and a 500 bp 3' flank of *PER1* sequence were PCR-amplified using the primers RAC2225 and RAC2226 from *S. cerevisiae* wild type gDNA (gDNA was utilized for cloning as *PER1* does not include introns). A 2.9 kb *hygB* fragment was PCR-amplified from the plasmid pBC-hygro using the primers RAC1523 and RAC1524. The two PCR products were used as a template for the final PCR to generate a 5.0 kb DNA fragment using the primers RAC2227 and RAC1525. Second, a 650 bp 5' flank and a 520 bp 3' flank of *A. fumigatus perA* were PCR-amplified from *A. fumigatus* gDNA using the primers RAC1472/1473 and RAC2223/2224, respectively. A 1.1kb coding genomic sequence was PCR-amplified from *S. cerevisiae* wild type gDNA using the primers RAC2221 and RAC2222. A 2.9 kb *hygB* fragment was PCR-amplified as described earlier. The four PCR products (5' and 3' flank of *perA*, *PER1* coding region, and *hygB*) were used as a template for the final PCR to generate a 5.1 kb DNA fragment using the primers RAC1476 and RAC1525. Each construct for *PER1* expression was transformed into *perA*, and transformants were recovered on SMM containing hygromycin B at 150 µg ml⁻¹.

Conidia production

Five milliliters of melted 0.7% agar in GMM containing 10⁶ conidia of each strain were overlaid onto GMM agar plates followed by incubation at 37 °C for 3 days. Conidia were collected using 10 ml of 0.01 % tween 80, vortexed for 30 seconds, and filtered using miracloth. The collected conidia suspensions were centrifuged at 5000 rpm for 8 minutes, and pellets were resuspended using 10 ml of H₂O. The number of conidia was counted using a hemacytometer. This experiment was performed in triplicate.

Germination assay

Conidia (1 × 10⁷ conidia ml⁻¹) were inoculated in 10 ml liquid GMM and cultured at 300 rpm at 37 °C. Culture samples were taken at each time point and fungal clumps were separated by bead beating. One hundred fungal cells in each strain were observed under microscope and counted for germination. This experiment was performed in triplicate with independent biological cultures.

Viability test

Conidia of *perA*, *perA*-reconstituted, and wild type strains were collected using sterile water and counted. Ten million conidia were added to 5 ml of either sterile water or 0.01% tween 80 and stored at 4 °C. Spore suspensions of identical volume by serial dilution were

plated on GMM at day 0 and 7. After 36 hour incubation at 37 °C, the number of recovered colonies was counted. Viability was calculated by the ratio of the number of recovered colonies at day 7 to day 0. This experiment was performed in triplicates.

Cell wall integrity test

To test the cell wall integrity of *perA*, three cell wall perturbing agents, Calcofluor white (CFW, Fluorescent brightener 28, Sigma F3543), Congo red (CR, Sigma C6277), and caspofungin (Cancidas, MERCK & CO., INC.) were added to 25 mL of GMM plates in appropriate concentrations. CFW stock solution was made in 10 mg mL⁻¹ in 25 mM NaOH solution, and added to GMM at 5 µg mL⁻¹ for the final concentration. CR stock solution was made at 10 mg mL⁻¹ in sterile H₂O and added to GMM at 0.25 mg mL⁻¹ for the final concentration. Caspofungin stock solution (5 mg mL⁻¹ in PBS) was added to GMM to make 0.5 µg mL⁻¹ for the final concentration. Conidia in 5 µl of sterile H₂O were inoculated onto GMM by serial dilutions and cultured at 37 °C for 2 days.

Antifungal drug test using voriconazole

Five milliliters of melted 0.7% agar in GMM containing 10⁶ conidia were overlaid onto GMM agar plates. E-test strips (BIOMÉRIUX #532840) were placed on the center of the plate and strains were culture at 37 °C for 2 days. The minimum inhibitory concentrations (MICs) based on cleared areas by VCZ were recorded. This experiment was performed in triplicate.

Light microscopy

To visualize localization of PerA:GFP, conidia were incubated in 200 µl of liquid GMM on coverslips at 30 °C for 12 hours. Microscopic observations were performed with a Zeiss HAL 100 microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA) equipped with a Zeiss Axiocam MRm camera. A filter for GFP visualization was used with excitation wavelength of 470 nm, and emission wavelength of 509 nm. A filter for DAPI (4',6-diamidino-2-phenylindole) visualization was used with excitation wavelength of 359 nm and emission wavelength of 461 nm. Image acquisition and processing were performed using Zeiss AxioVision Rel 4.8 software. Bright-field images of histological samples were taken using a Zeiss Axioplan2 microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA) equipped with a Qimaging Fast 1394 camera (Qimaging, Surrey BC, Canada). Imaging acquisition and processing was performed by Zeiss Phylum software. Photoshop CS (Adobe, Mountain View, CA) was used for preparing images for publication.

Total ergosterol extraction

For total ergosterol extraction from *A. fumigatus* strains, mycelia were harvested from a culture of 2 × 10⁸ conidia in 500 ml liquid GMM after 24 hour growth at 37 °C, 250 rpm. The harvested mycelia were collected via vacuum filtration, lyophilized, and weighed. Two hundred milligrams of dry fungal tissue was extracted as previously described (Alcazar-Fuoli *et al.*, 2008). Briefly, tissues were treated with 3 ml of 25 % alcoholic potassium hydroxide (3:2, methanol to ethanol) and then vortexed vigorously for 1 min. Samples were incubated at 85 °C for 1 h. Sterols were extracted with 1 ml of distilled water and 3 ml of

pentane and vortexed for 3 min. The upper aqueous layer was allowed to separate from the cellular debris, and 1 ml of this extract was transferred to a clean glass tube and evaporated in a fume hood at room temperature. Upon preparation for injection, samples were dissolved in 1 ml of methanol and syringe filtered through a 0.2 µm-pore-size filter (Acrodisc; Waters). Total ergosterol was measured using a Shimadzu CLASS-VP high-performance liquid chromatograph (HPLC) and detected via an SPD-M10AVP photo diode array at 280 nm on a µBondapak C18 column (300 mm by 3.9 mm, 10 µm). Running conditions and solvents utilized for HPLC were followed as previously described with minor modification using 100% methanol throughout the entire performance (Mejanelle *et al.*, 2001). The quantity of ergosterol per strain was calculated from a standard curve of ergosterol (Acros Organics, catalog no. 117810250). This experiment was performed in triplicate.

Ethics statement

We carried out our animal studies in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council (Council, 1996). The animal experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Dartmouth College (protocol number cram.ra1).

Murine model of aspergillosis

Six to eight week old CD1 female mice were obtained from Charles River Laboratories (Raleigh, NC). Mice were housed four per cage in an environment of HEPA-filtered air, with access to autoclaved food and sterile acidified water ad libitum. Triamcinolone acetonide (Kenalog-10, Bristol-Myers Squibb Company, Princeton, NJ) was injected subcutaneously at 40 mg kg⁻¹ dose for immunosuppression of mice 1 day prior to inoculation. For survival tests, 10 to 20 mice per strain were inoculated with 2×10^6 *A. fumigatus* conidia in 40 µl of PBS intranasally. Inoculated mice were monitored 3 times a day for 14 days. Animal experiments were performed in duplicate.

Enzyme-linked immunosorbent assay (ELISA)

Monitoring TNF levels in macrophages by co-incubation with *A. fumigatus* was performed as described (Shepardson *et al.*, 2013). Briefly, bone marrow (BM) cells were eluted from C57BL/6 mice and cultured for macrophages. After 6 days, BM-derived macrophages (BMDMs) were harvested. Cells were plated in tissue culture media in 96 well plates at a density of 5×10^5 cells ml⁻¹. Each fungal strain was cultured for 18 hours up to a hyphal stage at a density of 2×10^4 per well, UV-irradiated, and used to stimulate macrophages. After co-incubation for 18 hours, supernatants were applied to ELISA (eBioscience, San Diego, California, United States).

XTT assay

This study was performed as described recently (Shepardson *et al.*, 2013). J774.1 macrophage (MΦ)-like cell lines were co-cultured with *A. fumigatus* hyphae in a 9:1 (effector:target) ratio. After 3 hour incubation, cells were lysed and treated with 0.4 mg/ml XTT (2,3-bis(2-methoxy-4-nitro-5-[(sulphenylamino)carbonyl])-2H-tetrazolium-hydroxide)

solution with 0.5 mg/ml of the electron coupling agent coenzyme Q. The remaining alive fungal cells were quantitated by measuring the OD at 450 nm. The percent fungal damage was calculated by the equation:

$$(1 - [A_{450} \text{ of fungi with M}\Phi - A_{450} \text{ of M}\Phi \text{ alone}] / [A_{450} \text{ of fungi alone}]) \times 100.$$

Analysis of conidia surface proteins

Surface proteins of fungal strains were extracted as described in Beauvais *et al.* (Beauvais *et al.*, 2013). Briefly, 2×10^9 conidia were incubated with 200 μ l of 0.5 M NaCl solution for 2 hours at room temperature. The supernatant was collected by centrifugation at 12,000 rpm for 5 min, and 25 μ l of the supernatant was directly loaded to a 10% SDS-PAGE gel. Proteins were visualized by GelCode™ Blue protein stain (Thermo Scientific, #1860957) and imaged using FluorChem® FC2 (Alpha Innotech).

Staining for soluble dectin-1 and chitin

Culture conditions and staining procedures for soluble dectin-1 were followed as described recently (Shepardson *et al.*, 2013). *A. fumigatus* conidia were cultured to hyphae at 30 °C, UV-irradiated, blocked using blocking solution (RPMI media containing 10% fetal bovine serum with low IgG and 0.025 % Tween-20) for 1 hour at room temperature, and stained with conditioned medium containing s-dectin-hFc followed by DyLight 594-conjugated, goat anti-human IgG1 (Graham *et al.*, 2006). For chitin staining, UV-irradiated hyphae were treated with Calcofluor white (Sigma, final conc. 2 μ g ml⁻¹) for 1 hr. After being washed, stained cells were visualized under identical imaging conditions for parallel comparison using a Zeiss HAL 100 microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA) equipped with a Zeiss Axiocam MRm camera. Staining was quantified as the averaged amount of staining to the total fungal area using ImageJ software.

Quantitative real-time PCR (qRT-PCR)

To examine expression of *erg11A* (*cyp51A*) in response to voriconazole (VCZ) treatment, 5×10^7 conidia were incubated in 50 mL of liquid GMM at 37 °C, 250 rpm for 18 hours. Then VCZ was added to each culture at 0.0625 μ g ml⁻¹ (wild type and the reconstituted strain) or 0.03125 μ g ml⁻¹ (*perA*), and further cultured for 6 hours. Mycelia were collected, lyophilized for isolation of RNA. qRT-PCR was conducted as previously described (Grahl *et al.*, 2011). Data were analyzed from 2 biological and 3 technical replicates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Sequence analysis of PerA

(A) Amino acid sequences of *S. cerevisiae* Per1p (Sc) and *A. fumigatus* PerA (Af) were aligned using GeneDoc. Black and grey areas represent identical and similar amino acid residues, respectively. (B) PerA tagged using GFP (PerA:GFP) was ectopically expressed in wild type. DAPI was used to visualize nuclei. DIC (differential interference contrast). PerA:GFP is localized to the nuclear envelope/ER in *A. fumigatus*. Scale bar = 10 μ m.

Figure 2. Generation of a *perA* null mutant and radial growth

(A and B) A *perA* replacement construct was generated using the double-joint PCR method. Gene replacement with a selectable marker, *A. parasiticus pyrG* was confirmed by Southern blot analysis. (C and D) Growth of *perA* on glucose minimal media (GMM) or GMM containing 1.2 M sorbitol (SMM) was compared to wild type and the *perA*-reconstituted (*perA*-recon) strain. On GMM, *perA* radial growth is significantly reduced compared to wild type and *perA*-recon (by two-way ANOVA followed by the Bonferroni post-test at 48, 72 and 96 hour. $P < 0.0001$). However, the reduced growth of *perA* is restored when cultured on SMM. This experiment was performed in triplicate, and the results are displayed as mean values with standard errors.

Figure 3. *perA* has increased susceptibility to cell wall perturbing agents

Five microliters of H₂O containing 10⁵, 10⁴, and 10³ conidia of each strain was inoculated on GMM with Calcofluor white (5 $\mu\text{g ml}^{-1}$), Congo red (0.25 mg ml^{-1}), or caspofungin (0.5 $\mu\text{g ml}^{-1}$) and incubated at 37 °C for 2 days. Growth was compared to that on GMM with no drugs. In all tested conditions, *perA* is more susceptible to the drugs compared to wild type and the *perA*-reconstituted (*perA*-recon) strain. Experiments were performed in biological triplicate with similar results.

Figure 4. Phenotypic Characterization of *A. fumigatus* PerA

(A) Transcription of *perA* was studied in developmental stages including resting conidia, germings, and hyphae or mycelia using quantitative real-time PCR. Expression levels were normalized to *tefA* encoding translation elongation factor EF-1 alpha subunit. Transcript levels of *perA* increase as development proceeds. The graph represents mean values of results from 2 biological and 3 technical replicates with standard errors. (B) Conidia production was examined by the overlay method on GMM. A *perA* null mutant produces significantly reduced numbers of conidia (8% of wild type, $P = 0.003$ by the t test). Conidia production in wild type and the *perA*-reconstituted strain was not significantly different ($P = 0.38$ by the t test). (C) Germination rate was studied in liquid shaking cultures. *perA* initiates germination one hour earlier (at 4 h after inoculation) than wild type, and reaches the stationary stage similar to wild type (at 8 h after inoculation). At 5, 6, and 7 hour time points, germination rates of *perA* are significantly different from wild type or *perA*-recon ($P < 0.001$ by two-way ANOVA followed by Bonferroni post-test). (D) Fungal biomass was measured in liquid shaking cultures using liquid GMM. There are no differences among all strains tested. ($P > 0.05$ by one-way ANOVA followed by the Dunnett's multiple comparison test) Experiments for conidia production, germination, and biomass were performed in biological triplicate, and the results are displayed as mean values with standard errors.

Figure 5. *perA* has increased susceptibility to the antifungal triazole drug, voriconazole (VCZ)
 (A) One million conidia of each strain were overlaid on GMM and E-test strips of VCZ were placed on the plates. Minimum inhibitory concentrations (MICs) were recorded based on cleared areas that represented inhibited fungal growth by VCZ treatment. Arrows indicate where the MICs were measured. This experiment was performed in triplicate, and MICs were shown by mean values $\pm$ standard errors. Compared to wild type (MIC = $0.272 \pm 0.022 \mu\text{g ml}^{-1}$) and the *perA*-reconstituted (*perA*-recon, MIC = $0.293 \pm 0.022 \mu\text{g ml}^{-1}$) strain, *perA* (MIC = $0.064 \pm 0.000 \mu\text{g ml}^{-1}$) is more susceptible to VCZ. *perA* is significantly more susceptible to VCZ compared to wild type and *perA*-recon (one-way ANOVA, $P < 0.0001$). A Dunnett's multiple comparison test followed by one-way ANOVA shows that *perA* is significantly more susceptible to VCZ compared to wild type ($P < 0.05$), and there is no difference in susceptibility to VCZ between wild type and *perA*-recon ($P > 0.05$). (B) Sterols were extracted from fungal mycelia and ergosterol was quantified using high performance liquid chromatography (HPLC). Ergosterol production (ergosterol dry weight⁻¹) in each strain was normalized to the ergosterol level of wild type (wild type = 1). *perA* ergosterol production is not significantly different from wild type and the *perA*-reconstituted strain. (C) Fungal cultures grown at 250 rpm at 37 °C for 18 hours were treated with either DMSO or DMSO containing VCZ. Expression levels were normalized to actin (*actA*). The degree of induction of *erg11A* transcription in response to VCZ treatment compared to the control (DMSO) is significantly higher in *perA* compared to wild type or the reconstituted strain. These experiments were performed in triplicate, and the results are displayed as mean values with standard errors.

Figure 6. Virulence of *perA* in a murine model of invasive pulmonary aspergillosis
 (A) CD-1 mice were immunocompromised with triamcinolone (40 mg kg^{-1}), and inoculated in 24 hours with 2×10^6 conidia intranasally. Compared to wild type and the *perA*-reconstituted (*perA*-recon) strain, *perA* showed a significant attenuation in virulence as measured by murine survival ($P < 0.0001$ by log-rank analysis). Survival difference between wild type and *perA*-recon was not statistically significant ($P > 0.1$ by log-rank analyses). (B) Histopathological analyses were conducted using lung tissue collected from mice inoculated with each strain at day 3 post-inoculation. Hematoxylin and eosin (H&E) and Gomori methenamine silver (GMS) stains were utilized to visualize immune cells and fungal growth, respectively. The lungs from mice inoculated with wild type or the reconstituted strain show invasive fungal growth (indicated by arrowheads) and inflammatory cellular infiltrate in areas where fungal growth is present. In contrast, no apparent fungal growth and inflammatory response was detected in the lungs inoculated with *perA*. Scale bar = $100 \mu\text{m}$. (C) Selected areas indicated by red boxes in (B) were enlarged to highlight fungal growth in the lungs from mice inoculated with wild type or the *perA* reconstituted strain.

Figure 7. TNF secretion from bone marrow derived macrophages (BMDMs) and fungal damage by macrophages (MΦs) in *perA*

(A) To study inflammatory responses induced by *perA*, TNF secretion from BMDMs was measured by ELISA. *perA* hyphae trigger significantly decreased release of TNF from BMDMs compared to wild type and the reconstituted strain ($P < 0.01$ by the t-test). (B) J774.1 murine macrophage-like cell lines were incubated with hyphae of each strain in a 9:1 (effector:target) ratio. The percent fungal damage was calculated by ODs at 450 nm measured after 1h incubation with XTT solution with coenzyme Q. Fungal damage of *perA* by MΦs is not significantly distinct from that of wild type or the reconstituted strain ($P = 0.1739$ by one-way ANOVA followed by the Dunnett's multiple comparison test). These experiments were performed in triplicate and the results are displayed as mean values with standard errors.

Figure 8. Quantification of the β -glucan and chitin content on the cell surface

Conidia were cultured in liquid media to the hyphal stage, UV-killed, and stained with soluble dectin-1 or Calcofluor white to quantitate the content of exposed β -glucan (A) or chitin (B), respectively. The intensity of staining was calculated by averaging the amount of staining to the total area of each fungal cell using ImageJ software. *perA* shows increased exposure of both β -glucan and chitin on the cell surface compared to wild type and the reconstituted strain (perA-recon). These experiments were performed in triplicate, and the results are displayed as mean values with standard errors ($P < 0.05$ by t-tests). (C) Surface proteins of respective fungal strains were extracted from conidia using 0.5 M NaCl solution for 2 hours at room temperature. The supernatant was collected and loaded to a 10% SDS-PAGE gel. Proteins were visualized by GelCodeTM Blue protein stain. Compared to wild type and perA-recon, *perA* shows more abundant proteins extracted by NaCl treatment from the cell surface. This suggests that loss of PerA alters the protein component of the cell surface. This gel image represents 3 individual experiments with similar results.

Figure 9. Heterologous expression of *S. cerevisiae* *PER1* using its native promoter partially restores resistance to Calcofluor white (CFW) in *perA*

(A) Illustration of *S. cerevisiae* *PER1* expression constructs. The *PER1* promoter (*ScPER1(p)*) or the *A. fumigatus perA* promoter (*AfperA(p)*) was used to express *PER1*. ‘*PER1 CDS*’ and ‘*ScPER1(t)*’ indicates a *PER1* coding sequence and a putative transcriptional termination region of *PER1*, respectively. ‘*AfperA(t)*’ indicates a putative transcriptional termination region of *A. fumigatus perA*. Each construct resulted in a *ScPER1(p):PER1* (top) and a *AfperA(p):PER1* strain (bottom), respectively. (B) Expression of *PER1* using the *PER1* or *perA* promoter was examined using quantitative real-time PCR in the resulting strains from (A). mRNA levels of *PER1* and *perA* were normalized to a mRNA level of actin (*actA*). Both promoters induced expression of *PER1* although the expression level was lower in the *ScPER1(p):PER1* strain compared to the *AfperA(p):PER1* strain. *PerA* was not expressed in *ScPER1(p):PER1* and *AfperA(p):PER1* because those strains were generated in *perA* background. *PER1* was not expressed in wild type, which supports the specificity of primers for the yeast *PER1* sequence. (C) Susceptibility to CFW was tested on GMM plates. A thousand conidia were inoculated in each plate, and cultured at 37 °C for 3 days. Compared to wild type and the *perA*-reconstituted strain (*perA*-recon), *perA* is not able to grow in GMM containing CFW (2.5 μg ml⁻¹). In contrast, *ScPER1(p):PER1* showed improved growth under the same condition compared to *perA*.