Extensive proteomic remodeling is induced by eukaryotic translation elongation factor 1Bγ deletion in *Aspergillus fumigatus*

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**Abstract:** The opportunistic pathogen *Aspergillus fumigatus* is ubiquitous in the environment and predominantly infects immunocompromised patients. The functions of many genes remain unknown despite sequencing of the fungal genome. A putative translation elongation factor 1Bγ (*eEF1Bγ*, termed *elfA*; 750 bp) is expressed, and exhibits glutathione S-transferase activity, in *A. fumigatus*. Here, we demonstrate the role of *ElfA* in the oxidative stress response, as well as a possible involvement in translation and actin cytoskeleton organization, respectively. Comparative proteomics, in addition to phenotypic analysis, under basal and oxidative stress conditions, demonstrated a role for *A. fumigatus elfA* in the oxidative stress response. An *elfA*-deficient strain (*A. fumigatus* Δ*elfA*) was significantly more sensitive to the oxidants H2O2, diamide, and 4,4'-dipyridyl disulfide (DPS) than the wild-type. This was further supported with the identification of differentially expressed proteins of the oxidative stress response, including; mitochondrial peroxiredoxin Prx1, molecular chaperone Hsp70 and mitochondrial glycerol-3-phosphate dehydrogenase. Phenotypic analysis also revealed that *A. fumigatus elfA* was significantly more tolerant to voriconazole than the wild-type. The differential expression of two aminoacyl-tRNA synthetases suggests a role for *A. fumigatus elfA* in translation, while the identification of actin-bundling protein Sac6 and vacuolar dynamin-like GTPase VpsA link *A. fumigatus elfA* to the actin cytoskeleton. Overall, this work highlights the diverse roles of *A. fumigatus elfA*, with respect to translation, oxidative stress and actin cytoskeleton organization. In addition to this, the strategy of combining targeted gene deletion with comparative proteomics for elucidating the role of proteins of unknown function is further revealed.

**Keywords:** proteomics; translation; GST; redox; elongation factor; glutathione

**Introduction**

Translation is regulated in response to intracellular growth, extracellular signals and stress conditions.1–3 In response to stress, protein synthesis is usually reduced to prevent translation errors, and molecular chaperones are often upregulated to deal with any increase in denatured or misfolded proteins.1,4 This regulation of protein synthesis primarily occurs at the initiation stage; however, regulation has been observed to occur during elongation.1,4,5

The eukaryotic elongation factor 1 (eEF1) complex delivers all aminoacyl-tRNAs to the ribosome...
except the initiator tRNA and selenocysteine tRNAs. The eEF1 complex is composed of two subunits, eEF1A and eEF1B. eEF1A binds and recruits aminoacyl-tRNA to the ribosomal A site where, once a codon/anti-codon match is detected, it deposits the aminoacyl-tRNA. eEF1A is a G-protein and requires a guanine nucleotide exchange factor (GEF). eEF1B is the GEF for eEF1A and is composed of two subunits; eEF1Bα and eEF1Bβ. In metazoans, a third subunit of eEF1B, eEF1Bβγ, is present. In Saccharomyces cerevisiae, the eEF1B subunit is the nucleotide exchange factor, while the function of eEF1Bγ has not been fully elucidated.

In addition to delivering aminoacyl-tRNA to the elongating ribosome, eEF1A is also an actin binding and bundling protein, an interaction that is conserved from yeast to mammals. Indeed, it has been estimated that greater than 60% of eEF1A in the cell is associated with the actin cytoskeleton. Actin is essential and is involved in a variety of cellular processes ranging from growth and differentiation to stress response. The actin binding and bundling activity of eEF1A is independent of GTP and does not take place in the presence of aminoacyl-tRNA, suggesting mutual exclusivity of these two binding factors. In vitro, eEF1Bα reduces the actin bundling activity of eEF1A and may act as a regulator in directing eEF1A function to translation elongation.

The crystal structure of the N-terminal region of S. cerevisiae eEF1Bγ contains a glutathione S-transferase (GST)-like domain, although GST activity was not observed for this domain. GST activity, however, has been observed in eEF1Bγ proteins from different organisms (e.g., Aspergillus fumigatus). In addition to A. fumigatus eEF1Bγ, orthologs from rice and the silk worm Bombyx mori, expressed in Escherichia coli, were also amenable to glutathione (GSH)-affinity purification, and eEF1Bγ from both of these organisms exhibited GST activity.

In A. fumigatus, eEF1Bγ is encoded by A. fumigatus elfA, first identified following GSH-Sepharose affinity chromatography and two-dimensional electrophoresis (2DE). Further analysis determined that ElfA was present as a monomer in the cell at 20 kDa. GST activity of native ElfA represented the first demonstration of such activity in native eEF1Bγ, as previously this activity had only been determined in recombinant eEF1Bγ. The relevance, and presence, of a GST domain in an elongation factor requires further investigation with respect to a possible role in redox control and oxidative stress. In other organisms (e.g., S. cerevisiae and mammalian epithelial cells), eEF1Bγ has been shown to interact with the membranes and the cytoskeleton; however, it is not known whether A. fumigatus elfA interacts with either the membrane network or the actin cytoskeleton.

Comparative proteomics is a powerful tool for investigating the impact a gene deletion has on the cell as a whole, that is, for studying fungal systems biology. Thus, we employed targeted gene deletion in conjunction with comparative proteomics to gain a better understanding of A. fumigatus elfA.

Results

Deletion and complementation of elfA in A. fumigatus

An A. fumigatus elfA deletion strain (ΔelfA) was generated by homologous recombination using the bi-partite method to facilitate functional analysis of elfA. Gene deletion, and subsequent complementation was confirmed by Southern blot analysis (Supporting Information Fig. 1). qRT-PCR confirmed the presence of elfA transcripts in A. fumigatus ATCC46645 and elfAΔ, and its absence in ΔelfA (Supporting Information Fig. 1). Expression of elfA was higher (1.6-fold) in elfAΔ than in the wild-type strain. Finally, ElfA was isolated from elfAΔ thereby confirming expression of the protein in the complemented strain (Supporting Information Fig. 2).

A. fumigatus elfA is involved in the oxidative stress response and redox control

qRT-PCR analysis of elfA expression in A. fumigatus subjected to H2O2-induced oxidative stress (2 mM) for 15–60 min revealed that elfA expression was significantly increased (P = 0.0009) after 15 min [Fig. 1(A)]. After 30 min, expression of elfA was still higher than basal levels, and decreased to below basal levels at 60-min post-exposure.

A. fumigatus ΔelfA was investigated to ascertain any altered phenotype in response to the oxidative stress-inducing agents, menadione (0–50 μM), H2O2 (0–5 mM), diamide (0–4 mM), and 4,4’-dipyridyl disulfide (DPS; 0–7.5 μM). A. fumigatus ΔelfA was found to be significantly more sensitive to H2O2, diamide and DPS (Supporting Information Fig. 3), but not to menadione (data not shown), indicating a possible role for elfA in the oxidative stress response to specific oxidizers. After 72 h exposure, A. fumigatus ΔelfA was significantly more sensitive to 1 mM H2O2 (P = 0.0006) [Fig. 1(B)]. A. fumigatus ΔelfA was also significantly more sensitive to 0.5 mM diamide (P = 0.0001) after 72 h exposure [Fig. 1(C)]. After 96 h exposure, it was observed that A. fumigatus ΔelfA was significantly more sensitive to 7.5 μM DPS (P = 0.0007) compared to A. fumigatus ATCC46645 or elfA+ [Fig. 1(D)]. A. fumigatus ΔelfA displayed an increased tolerance (P = 0.0251) to voriconazole (0.5 μg/mL) compared to A. fumigatus ATCC46645 and elfA+ after 72 h exposure (Supporting Information Fig. 4).

In some cases, it was observed that complementation did not restore the wild-type phenotype while
in others, i.e., in the presence of DPS and voriconazole, the wild-type phenotype was restored. As described earlier, Southern blot analysis determined a single targeted integration of the complementation construct (Supporting Information Fig. 1) and ElfA was isolated from elfAC (Supporting Information Fig. 2). In addition to this, sequencing of elfA in the complemented strain did not reveal any mutations (data not shown). Importantly, qRT-PCR for the genes flanking elfA showed the identical expression levels in wild-type and ΔelfA confirming that the observed phenotypes were not due to a disruption of the flanking genes (Supporting Information Fig. 5). Conceivably, the genomic architecture of the

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**Figure 1.** (A) H₂O₂-induced oxidative stress increases elfA expression, particularly following 15 min exposure (**P < 0.0009**). (B) Impact of H₂O₂ (0–4 mM) on the growth of *A. fumigatus* ATCC46645, ΔelfA, and elfAC², respectively, after 72 h. (C) Growth of *A. fumigatus* ATCC46645, ΔelfA, and elfAC² in response to diamide (0–4 mM) exposure. (D) The impact of 4,4′-dipyridyl disulfide (0–7.5 μM) on the growth of *A. fumigatus* ATCC46645, ΔelfA, and elfAC². (E) GSH and GSSG determination in *A. fumigatus* ATCC46645, ΔelfA, and elfAC² under normal growth conditions. (F) Relative amounts of GSH and GSSG in *A. fumigatus* ATCC46645, ΔelfA, and elfAC² in the absence and presence of 2 mM H₂O₂ for 15 min. (G) GSH and GSSG amounts in *A. fumigatus* ATCC46645, ΔelfA, and elfAC² in the absence and presence of 2 mM H₂O₂ for 30 min. (H) Exposure to diamide causes a reduction in GSH levels in *A. fumigatus* ΔelfA.
Complemented locus may be altered whereby the hygromycin resistance cassette has a positional effect on elfA expression.

A reduced GSH/GSSG ratio was observed in ΔelfA under normal growth conditions compared to the wild-type and elfAΔC (wild-type; 5.6, ΔelfA; 3.3, elfAΔC; 6.0), indicative of cells under oxidative stress (Supporting Information Table 1). This resulted from decreased GSH levels and increased GSSG levels in ΔelfA (Fig. 1E). When exposed to H2O2 for 15 and 30 min, the effect on the GSH/GSSG ratios in wild-type and ΔelfA differed. The ratios decreased in wild-type which is indicative of an oxidative stress response (0 min; 5.66, 15 min; 4.51, 30 min; 2.05) (Supporting Information Table 1). However, in ΔelfA, the GSH/GSSG ratio increased following 15 min exposure to H2O2 from 3.33 to 4.61), and subsequently decreased (to 1.90) following exposure for 30 min. The increased GSH/GSSG following 15 min exposure of A. fumigatus ΔelfA and ΔelfAΔC were cultured for 24 h under basal conditions; eight proteins had a fold increase >1.5 (P < 0.05) in A. fumigatus ΔelfA while five proteins had a fold decrease >1.5 (P < 0.05) when compared to A. fumigatus ATCC46645 [Table I; Fig. 2(A,B)]. Specifically, mitochondrial peroxiredoxin Prx1 and molecular chaperone Hsp70 exhibited increased expression of 2.2- and 1.7-fold, respectively, while Hsp70 chaperone (SGT) was decreased 1.7-fold in expression. Tyrosyl-tRNA synthetase was upregulated 2.7-fold. In addition to this, actin-bundling protein Sac6 and Sec13 displayed a 1.9- and 1.7-fold increase in expression.

**Oxidative stress induces differential protein expression in A. fumigatus ΔelfA**

A. fumigatus ATCC46645 and ΔelfA were cultured for 24 h in AMM before H2O2 (2 mM final concentration) was added for 1 h. Following LC-MS/MS of 18 protein spots that were found to be differentially expressed, 10 proteins were identified as having a fold increase ≥1.5 (P < 0.05) in A. fumigatus ΔelfA [Table II; Fig. 2(C,D)] while five proteins exhibited a fold decrease >1.5 (P < 0.05) compared to A. fumigatus ATCC46645 in response to 2 mM H2O2 exposure for 1 h. Vacular dynamin-like GTPase VpsA was identified from three different spots with fold increases of 7.8, 4.3, and 3.0 in A. fumigatus ΔelfA, while ATP citrate lyase, subunit 1 was identified from two spots which were upregulated 1.9- and 1.5-fold in A. fumigatus ΔelfA. The isoforms of both vacular dynamin-like GTPase VpsA and ATP citrate lyase, subunit 1 had the same molecular mass but displayed slight changes in pI. Cobalamin-independent methionine synthase Met H/D was upregulated 1.7-fold, while cysteinyl-tRNA synthetase and glycerol-3-phosphate dehydrogenase were upregulated 1.5-fold in A. fumigatus ΔelfA.

### Table I. Proteins (n = 13) with a Fold Change in A. fumigatus ΔelfA Compared with A. fumigatus Under Normal Growth Conditions Following Identification by 2DE and LC-MS/MS

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* CADRE ID: A. fumigatus gene annotation nomenclature.21,22

## Proteome remodelling occurs as a consequence of elfA deletion in A. fumigatus

Thirteen proteins were found to be differentially expressed when A. fumigatus ATCC46645 and ΔelfA were exposed to H2O2 for 30 min, while the other trends, although increased levels of GSH ([Fig. 1(G)]. Significantly decrease in the amount of GSSG resulted in the...
Meanwhile, Ras small monomeric GTPase RasA and proteosome regulatory particle Subunit (RpnL) underwent decreased expression of 2.2- and 1.8-fold, respectively.

Increased protein glutathionylation was observed in A. fumigatus ΔelfA. Due to the altered redox environment in A. fumigatus ΔelfA, it was hypothesized that global protein glutathionylation may be increased as a protective measure. Biotinylation of GSSG was confirmed by both HPLC and MALDI-ToF analysis (Supporting Information Fig. 6) and was subsequently used as a probe to detect proteins susceptible to glutathionylation as the addition of GSSG leads to thiol-disulfide exchange reactions with these proteins, while the biotin moiety allows for their detection by Western analysis. Increased protein glutathionylation in A. fumigatus ΔelfA compared to A. fumigatus ATCC46645 was observed [Fig. 3(A,B)], which was supported by image analysis that determined a 24% increase in chemiluminescent signal intensity in A. fumigatus ΔelfA compared to wild-type signals [Fig. 3(C)]. Addition of DTT and treatment of whole cell lysates with water acted as controls to ensure that any proteins detected were as a direct result of
glutathionylation by biotin-GSSG (Fig. 3(B)), while equal protein loading (Fig. 3(A)) confirmed observed signal intensity differences between the strains.

**Discussion**

The work described here reveals the functional characterization of ElfA, an eEF1Bγ from *A. fumigatus*. eEF1Bγ is a member of the eEF1 complex required for translation and, along with eEF1Bα, forms the eEF1B component of the eEF1 complex which provides nucleotide exchange factor activity to the eEF1A subunit.\(^2\)\(^1\),\(^2\)\(^2\) Comparative proteomics highlighted that under normal growth conditions, proteins involved in stress responses (i.e., mitochondrial peroxiredoxin Prx1, molecular chaperone Hsp70, and Hsc70 co-chaperone) were differentially expressed, with Prx1 and Hsp70 expression increased while the expression of Hsc70 was depleted to a greater extent in *A. fumigatus*. Combined, these data confirmed our initial hypothesis that *A. fumigatus elfA* was more resistant to the oxidants; H₂O₂, menadione, and DPS. Both diamide and DPS are thiol-specific oxidants which react directly with GSH.\(^2\)\(^6\) The reduced GSH levels observed indicated that under normal growth conditions, proteins involved in stress responses (i.e., mitochondrial peroxiredoxin Prx1, molecular chaperone Hsp70, and Hsc70 co-chaperone) were differentially expressed, with Prx1 and Hsp70 expression increased while the expression of Hsc70 was decreased. The observation of these differentially expressed proteins indicates that in the absence of *A. fumigatus elfA*, a stressed intracellular environment exists. This is confirmed by altered redox homeostasis in *A. fumigatus elfA* where, under normal growth conditions the GSH/GSSG ratio of *A. fumigatus elfA* was decreased compared to that of *A. fumigatus* ATCC46645, strongly indicative of oxidative stress.\(^2\)\(^4\) Further evidence of an altered redox state is the increased level of protein glutathionylation in *A. fumigatus elfA*. Combined, these data suggest that in the absence of ElfA, translation is partially disrupted (as evidenced by altered levels of certain tRNA synthetases and enzymes dealing with protein aggregation), either resulting in, or consequent to, oxidative stress, allied to dysregulation of GSH/GSSG in *A. fumigatus*.

The increased expression of mitochondrial peroxiredoxin Prx1 in *A. fumigatus elfA* was especially interesting because in *S. cerevisiae*, mitochondrial peroxiredoxin Prx1 requires GSH-mediated reduction for antioxidant activity.\(^2\)\(^5\) Indeed, both GSH and a thioredoxin were required to maintain the mitochondrial peroxiredoxin Prx1 in its reduced, active state. Specifically, the catalytic cysteine of peroxiredoxin is reactivated by glutathionylation and subsequent reduction by thioredoxin reductase coupled with GSH.\(^2\)\(^6\) The increased expression of mitochondrial peroxiredoxin Prx1 in *A. fumigatus elfA* may be consequent to the decreased GSH levels observed.

The significant increase in expression of *A. fumigatus elfA* after 15 min of exposure to H₂O₂ confirmed our initial hypothesis that elfA is involved in the oxidative stress response in *A. fumigatus*. Consolidating this hypothesis, *A. fumigatus elfA* was found to be significantly more sensitive than wild-type to the oxidants H₂O₂, diamide, and DPS. Both diamide and DPS are thiol-specific oxidants which react directly with GSH.\(^2\)\(^6\) The reduced GSH levels in *A. fumigatus elfA* compared to those in *A. fumigatus* ATCC46645 confirm that GSH levels are depleted to a greater extent in *A. fumigatus elfA* in the presence of these oxidants resulting in the increased sensitivity of *A. fumigatus elfA*. Conversely, in *S. cerevisiae*, an eEF1Bγ deletion strain was more resistant to the oxidants; H₂O₂, menadione and CdSO₄.\(^2\)\(^7\) However, in *S. cerevisiae*, the GST

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**Table II.** Proteins (n = 15) with a Fold Change in *A. fumigatus ΔelfA* Compared with *A. fumigatus* After Exposure to 2 mM H₂O₂ Following Identification by 2DE and LC-MS/MS

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<td>652</td>
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* CADRE ID: *A. fumigatus* gene annotation nomenclature.\(^2\)\(^1\),\(^2\)\(^2\)
domain of eEF1Bγ is not active, unlike in A. fumigatus. Thus, the data presented here lead to the conclusion that the eEF1Bγ proteins in A. fumigatus and S. cerevisiae, respectively, mediate differential responses consequent to oxidative stress which may be due to the presence of an active GST domain in ElfA.

The effects of H₂O₂-induced oxidative stress on the proteome of A. fumigatus ΔelfA was investigated and compared to that of A. fumigatus ATCC46645. Proteins which participate in the oxidative stress response such as cobalamin-independent methionine synthase and mitochondrial glycerol-3-phosphate dehydrogenase exhibited increased expression, while Ras small monomeric GTPase RasA and the proteasome regulatory particle underwent decreased expression.

Cobalamin-independent methionine synthase, which exhibited increased expression in A. fumigatus ΔelfA, is essential for S-adenosylmethionine (SAM) cycle occurrence and homocysteine, an intermediate of the SAM cycle, can be converted to glutathione. This is particularly relevant since GSH/GSSG determination in A. fumigatus ΔelfA, following exposure to H₂O₂, revealed an increase in GSH levels. Thus, a clear systems link is established between the proteomic and phenotypic analyses. Because GSH levels in A. fumigatus ΔelfA were lower than those in the wild-type due to the stressed environment, we speculate that cobalamin-independent methionine synthase expression was upregulated to increase the GSH levels following exposure to H₂O₂. Interestingly, it has been observed that addition of methionine to Penicillium chrysogenum cultures also resulted in increased intracellular GSH levels, and if this is also the case in A. fumigatus, it may represent a universal response to GSH depletion consequent to oxidative stress in filamentous fungi.

Further analysis of differentially expressed proteins identified in A. fumigatus ΔelfA under both normal growth conditions and following exposure to H₂O₂, revealed a number of proteins involved in protein folding and protein degradation were differentially expressed. Under normal growth conditions, molecular chaperone Hsp70 and nuclear pore complex Sec13 exhibited increased expression in A. fumigatus ΔelfA. The Hsp protein family is required for the disassembly of protein aggregates, protein folding, and the degradation of misfolded proteins, while Sec13 functions in the coat protein complex II (COPII), vesicular trafficking, nuclear pore function, and endoplasmic reticulum-associated degradation (ERAD). The COPII complex also transports misfolded proteins to the ERAD, which in turn is involved in the retrotranslocation of misfolded proteins from the ER to the cytosol for degradation by proteasomes. Previously, Sec13 was found to be upregulated in S. cerevisiae by the unfolded protein response (UPR), which has been shown to target genes involved in the ERAD. The increased expression of these proteins under normal growth conditions is indicative of a greater requirement for protein degradation in A. fumigatus ΔelfA which is in accordance with the stressed environment.

**Figure 3.** Western blot analysis of protein glutathionylation in A. fumigatus ATCC46645 and ΔelfA. (A) SDS-PAGE of A. fumigatus ATCC46645 (Lanes 1, 3, 5, 7) and ΔelfA (Lanes 2, 4, 6, 8) whole cell lysates (50 μg) under reducing (Lanes 1–4) and nonreducing conditions (Lanes 5–8). The samples in Lanes 1, 2, 5, and 6 were incubated with biotin-GSSG, while the samples in Lanes 3, 4, 7, and 8 were incubated with water as a control. (B) Western blot probed with streptavidin-HRP. Proteins glutathionylated upon addition of biotin-GSSG were detected in A. fumigatus ATCC46645 (Lane 5) and ΔelfA (Lane 6). There were more glutathionylated proteins detected in A. fumigatus ΔelfA (Lane 6) than in A. fumigatus ATCC46645 (Lane 5). No glutathionylated proteins were detected on the reduced blot (Lanes 1–4) or in the samples treated with water (Lanes 7–8). (C) Image analysis indicates a 24% increase in chemiluminescent signal intensity in A. fumigatus ΔelfA compared to A. fumigatus ATCC46645 supporting the observation that there was a higher level of protein glutathionylation in A. fumigatus ΔelfA.
environment evident due to aberrant translation and decreased GSH/GSSG ratio.

One of the consequences of oxidative stress is an increase in misfolded and irreversibly damaged proteins which, unless removed from the cell, are highly toxic. Following exposure to H\textsubscript{2}O\textsubscript{2}, the increased expression of vacuolar dynamin-like GTPase VpsA and the decreased expression of the proteasome regulatory particle subunit (RpnL) pointed toward an increased requirement for protein degradation in *A. fumigatus*. One of the functions of vacuolar dynamin-like GTPase VpsA is in vacuole fission. Vacuoles, along with proteasomes, degrade proteins exported from the ER by the ERAD. The proteasome regulatory particle subunit forms the lid of the 19S regulatory subunit which is required for degradation of ubiquitinylated proteins but inhibits degradation of oxidized proteins. This decreased expression of the proteasome regulatory particle subunit in *A. fumigatus* is indicative of an attenuation of proteasome regulation to facilitate degradation of the increased levels of misfolded or oxidized proteins present in the cell, consequent to loss of ElfA. Interestingly, in *S. cerevisiae*, increased accumulation of oxidized proteins was observed in an eEF1B\textsubscript{y} deletion strain which also exhibited altered vacuole morphology and altered expression of Hsp proteins. Thus, the identification of a number of proteins involved in protein folding and degradation of misfolded proteins in *A. fumigatus* under both normal growth conditions and following exposure to H\textsubscript{2}O\textsubscript{2}, suggests that in the absence of ElfA, the cellular protein degradation systems are activated. We hypothesize that in the absence of ElfA, a stressed environment ensues resulting in increased levels of misfolded and irreversibly damaged proteins, which must be removed from the cell to prevent manifestation of toxic effects.

Tyrosyl-tRNA synthetase exhibited increased expression in *A. fumigatus* ElfA under normal growth conditions, while cysteinyll-tRNA synthetase exhibited increased expression upon exposure to H\textsubscript{2}O\textsubscript{2}. We postulate that the increased expression of these aminoacyl-tRNA synthetases is indicative of altered protein synthesis in *A. fumigatus* ElfA. In particular, the increased expression of cysteinyl-tRNA suggests an increased requirement for cysteine-containing proteins, such as peroxiredoxins, which are essential for attenuation of oxidative stress within the cell.

The unexpected increased expression of additional proteins (i.e., actin-bundling protein Sac6 and vacuolar dynamin-like GTPase VpsA), in *A. fumigatus* ElfA under both normal growth conditions and following oxidative stress, involved in cytoskeletal transformation, suggests the involvement of ElfA with the cytoskeleton. Under normal growth conditions, actin-bundling protein Sac6 exhibited increased expression in *A. fumigatus* ElfA. Moreover, following exposure to H\textsubscript{2}O\textsubscript{2}, increased expression of vacuolar dynamin-like GTPase VpsA, which in addition to its function in vacuole fission is also required for normal actin cytoskeleton organization in *S. cerevisiae*, was observed. The cytoskeleton plays a role in the oxidative stress response whereby it confers protection by collapsing into actin bundles that sequester actin and its associated proteins into immobile structures. In *S. cerevisiae*, Sac6 was observed to co-localize with these actin bundles during oxidative stress induced by H\textsubscript{2}O\textsubscript{2}, menadione, and diamide. Translation elongation and actin cytoskeleton modulation are linked, because eEF1A in addition to its canonical role of delivering aminoacyl-tRNA to the elongating ribosome, is also an actin binding and bundling protein. In *S. cerevisiae*, eEF1B\textsubscript{y} regulates the actin binding and bundling activities of eEF1A and has been proposed to direct eEF1A toward binding aminoacyl-tRNA. In mammalian epithelial cells, eEF1B\textsubscript{y} increased the formation of keratin intermediate filament bundles in vivo. Moreover, overexpression of eEF1B\textsubscript{y} in epithelial cells resulted in a disrupted interaction between eEF1B\textsubscript{y} and keratin, and also reduced protein synthesis suggesting a functional link between the cytoskeletal structure and translation in epithelial cells. The observation of increased expression of Sac6 in *A. fumigatus* ElfA under normal growth conditions suggests that ElfA (an EF1B\textsubscript{y}) also links the actin cytoskeleton with the translational apparatus.

We have demonstrated a diverse role for ElfA with respect to the oxidative stress response, GSH/GSSG redox homeostasis, regulation of protein degradation and antifungal resistance in *A. fumigatus*. We hypothesize that the ensuing alteration in protein synthesis, after loss of ElfA, results in increased levels of misfolded proteins which may in turn generate a stressed environment which activates the oxidative stress response. Alternatively, loss of ElfA and the subsequent deficit in translation results in a stressed environment, which not only activates the oxidative stress response, but also results in increased levels of misfolded and irreversibly damaged proteins. The result of either outcome is an increase in protein degradation and oxidative stress response pathways in *A. fumigatus*.

**Materials and Methods**

**Strains and growth conditions**

*A. fumigatus* strains were grown at 37°C in *Aspergillus* minimal media (AMM), and fungal culturing was carried out as described previously. The bacterial strain *E. coli* TOP10 (Invitrogen, The Netherlands) which was cultivated in LB (1% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl,
pH 7.5) medium was used for general cloning procedures.

Deletion and complementation of A. fumigatus elfA
An A. fumigatus ΔelfA strain was generated using the bipartite marker technique\(^5\) using overlapping fragments of a pyrithiamine resistance gene (\(ptrA\)),\(^20,52\) fused to 1.2 kb of the 5' and 3' flanking regions of the elfA coding sequence. The primers required for generating the constructs are listed in Supporting Information Table 2. A. fumigatus ATCC46645 was transformed as described previously\(^50\) and colonies picked in order to obtain homokaryotic transformants. Single genomic integration was confirmed by Southern blot analysis following DNA purification using a ZR Fungal/Bacterial DNA Kit (Zymo research). A. fumigatus ΔelfA was complemented with a construct that contained elfA and the 5' and 3' flanking regions, and also a hygromycin resistance gene (\(hph\)) for selection.\(^53\) Briefly, elfA and the 5' and 3' flanking regions were amplified using the primers elfA5 and elfA6, the resulting 3.1 kb product was cloned into TOPO\(^\text{R}\) vector. The resulting plasmid was linearized and ligated to \(hph\), which was released from pAN7-1\(^53\) by restriction digestion, and cloned into TOPO\(^\text{R}\) vector to give the complementation construct. The plasmid was linearized before transforming A. fumigatus ΔelfA protoplasts.

RNA isolation and quantitative RT-PCR
Fungal RNA isolation, DNase treatment, cDNA synthesis and qRT-PCR was performed as described previously.\(^54\) The primers used in the qRT-PCR reactions are listed in Supporting Information Table 2.

Plate assays
Conidia (10\(^6\)), harvested aseptically from 1-week-old AMM plates, were subject to a variety of phenotypic assays by point inoculating on AMM plates containing a stressor, as shown in Supporting Information Table 3. Plates were incubated at 37°C. Colony diameter was measured periodically and statistical analysis was carried out using one-way ANOVA.

GSH/GSSG determination
A. fumigatus ATCC46645 and ΔelfA (1 × 10\(^5\) cfu/mL) were cultured for 24 h in AMM before addition of H\(_2\)O\(_2\) (2 mM final) for 15 and 30 min, respectively. A. fumigatus ATCC46645 and ΔelfA not exposed to H\(_2\)O\(_2\) were used as controls. Mycelia were harvested through miracloth and dried before glutathione reduced/glutathione disulfide (GSH/GSSG) determination.\(^55\)

Protein extraction
A. fumigatus ATCC46645 and ΔelfA (1 × 10\(^5\) cfu/mL) were grown for 24 h in AMM (100 mL cultures), at 37°C with shaking at 200 rpm. After 24 h, H\(_2\)O\(_2\) (2 mM final) was added. Following 1 h incubation, mycelia were harvested, washed with cold deionized water, dried in tissue and snap frozen in liquid N\(_2\). The mycelia were ground into a fine powder under liquid N\(_2\). Ground mycelia (250 mg) were added to 10% (w/v) TCA (1.5 mL), incubated on ice for 30 min and sonicated with a sonication probe (Bandelin Sonopuls, Bandelin electronic, Berlin) with cooling. Lysed mycelia were incubated on ice for a further 30 min before centrifugation at 12,000 g for 10 min at 4°C. Supernatants were discarded and 60 μL H\(_2\)O was added to protein and vortexed. Following acetone washes, protein was resuspended in IEF Buffer (500 μL), incubated at room temperature for 1 h before centrifugation at 13,000 g for 3 min. The supernatants were removed, protein concentration determined and analyzed by 2DE. Protein was quantified using Bradford reagent (BioRad Laboratories). ElfA isolation were carried out as described previously.\(^15\)

Two-dimensional electrophoresis and LC-MS
2DE was carried out as described previously.\(^15,54\) Gels were stained with Colloidal Coomassie® Blue G-250 (Serva Electrophoresis, Germany) and scanned using a Typhoon Trio Variable Mode Imager or ImageScanner III (GE Healthcare, Freiburg, Germany). Five replicate gels of each, wild-type or ΔelfA were used for analysis using the Progenesis™ SameSpot Software (Nonlinear Dynamics, UK) to identify differentially regulated spots. Proteins were excised from 2DE gels and digested with trypsin and analyzed as previously described.\(^54,56\) LC-MS Analysis was carried out on a 6340 Ion-trap LC Mass Spectrometer using electrospray ionization (Agilent Technologies). MS\(^\text{TM}\) analysis was carried out on the 3 most abundant peptide precursor ions in each sample, by automatic selection. Pepsides from the MS\(^\text{TM}\) spectra were compared to the NCBI nr database using MASCOT (www.matrixscience.com) for protein identification.\(^54\)

Biotinylation of glutathione disulfide (Biotin-GSSG)
Glutathione disulfide was glutathionylated following a published protocol.\(^23\) To confirm the successful biotinylation of GSSG, the Biotin-GSSG was analysed by RP–HPLC with UV detection (Agilent 1200 system), using a C18 RP–HPLC column (Agilent Zorbax Eclipse XDB-C18; 5 mm particle size; 4.6 × 15 mm) and MALDI-ToF analysis (Ettan MALDI-ToF Pro mass spectrometer (Amersham Biosciences)).

Analysis of protein glutathionylation
Biotin-GSSG was incubated with whole protein lysates, extracted under nonreducing conditions in a 1/10 ratio for 10 min at room temperature followed...
by SDS-PAGE under nonreducing conditions and Western Blot was carried out using Streptavidin-HRP and ECL for detection. The analysis was also carried out under reducing conditions and whole cell lysates were also treated with water as controls to ensure that any proteins detected were as a direct result of glutathionylation by biotin-GSSG.

References


