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# 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 *elf*A; 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 $H_2O_2$, 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* Δ*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

Additional Supporting Information may be found in the online version of this article.

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except the initiator tRNA and selenocysteine tRNAs.[6] The eEF1 complex is composed of two subunits, eEF1A and eEF1B.[7] 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.[6,8] 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 eEF1Bγ.[8] In metazoans, a third subunit of eEF1B, eEF1Bβ, is present.[8,9] In *Saccharomyces cerevisiae*, the eEF1Bα subunit is the nucleotide exchange factor, while the function of eEF1Bγ has not been fully elucidated.[7]

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.[7,10] Indeed, it has been estimated that greater than 60% of eEF1A in the cell is associated with the actin cytoskeleton.[10] Actin is essential and is involved in a variety of cellular processes ranging from growth and differentiation to stress response.[11,12] 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.[13,14] *In vitro*, eEF1Bα reduces the actin bundling activity of eEF1A and may act as a regulator in directing eEF1A function to translation elongation.[7]

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.[9] GST activity, however, has been observed in eEF1Bγ proteins from different organisms (e.g., *Aspergillus fumigatus*[15]). 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,[16,17] and eEF1Bγ from both 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).[15] 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.[15–17] 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;[18,19] 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 (Δ*elfA*) was generated by homologous recombination using the bi-partite method[20] 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 $elfA^C$, and its absence in Δ*elfA* (Supporting Information Fig. 1). Expression of *elfA* was higher (1.6-fold) in $elfA^C$ than in the wild-type strain. Finally, ElfA was isolated from $elfA^C$ 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 $H_2O_2$-induced oxidative stress (2 m*M*) 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* Δ*elfA* was investigated to ascertain any altered phenotype in response to the oxidative stress-inducing agents, menadione (0–50 μ*M*), $H_2O_2$ (0–5 m*M*), diamide (0–4 m*M*), and 4,4′-dipyridyl disulfide (DPS; 0–7.5 μ*M*). *A. fumigatus* Δ*elfA* was found to be significantly more sensitive to $H_2O_2$, 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* Δ*elfA* was significantly more sensitive to 1 m*M* $H_2O_2$ ($P = 0.0006$) [Fig. 1(B)]. *A. fumigatus* Δ*elfA* was also significantly more sensitive to 0.5 m*M* diamide ($P = 0.0001$) after 72 h exposure [Fig. 1(C)]. After 96 h exposure, it was observed that *A. fumigatus* Δ*elfA* was significantly more sensitive to 7.5 μ*M* DPS ($P = 0.0007$) compared to *A. fumigatus* ATCC46645 or $elfA^C$ [Fig. 1(D)]. *A. fumigatus* Δ*elfA* displayed an increased tolerance ($P = 0.0251$) to voriconazole (0.5 μg/mL) compared to *A. fumigatus* ATCC46645 and $elfA^C$ after 72 h exposure (Supporting Information Fig. 4).

In some cases, it was observed that complementation did not restore the wild-type phenotype while

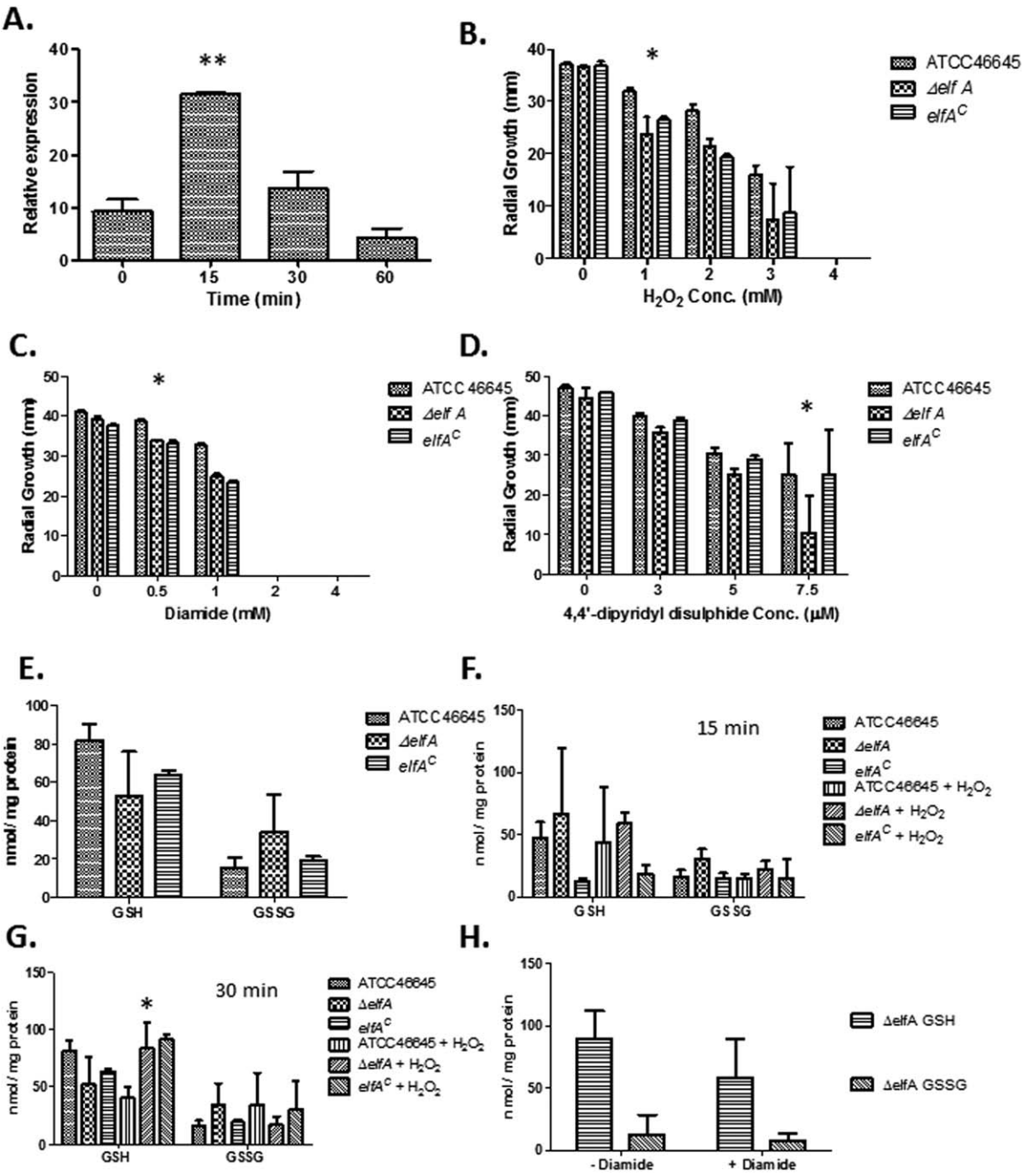


**Figure 1.** (A) $H_2O_2$-induced oxidative stress increases *elfA* expression, particularly following 15 min exposure (**$P = 0.0009$). (B) Impact of $H_2O_2$ (0–4 m*M*) on the growth of *A. fumigatus* ATCC46645, Δ*elfA*, and *elfA*$^C$, respectively, after 72 h. (C) Growth of *A. fumigatus* ATCC46645, Δ*elfA*, and *elfA*$^C$ in response to diamide (0–4 m*M*) exposure. (D) The impact of 4,4′-dipyridyl disulfide (0–7.5 μ*M*) on the growth of *A. fumigatus* ATCC46645, Δ*elfA*, and *elfA*$^C$. (E) GSH and GSSG determination in *A. fumigatus* ATCC46645, Δ*elfA*, and *elfA*$^C$ under normal growth conditions. (F) Relative amounts of GSH and GSSG in *A. fumigatus* ATCC46645, Δ*elfA*, and *elfA*$^C$ in the absence and presence of 2 m*M* $H_2O_2$ for 15 min. (G) GSH and GSSG amounts in *A. fumigatus* ATCC46645, Δ*elfA*, and *elfA*$^C$ in the absence and presence of 2 m*M* $H_2O_2$ for 30 min. (H) Exposure to diamide causes a reduction in GSH levels in *A. fumigatus* Δ*elfA*.

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 *elfA*$^C$ (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 Δ*elfA* 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

**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*

| Spot no. | Annotation name | CADRE ID[a] | Fold change | tpI | tMW | % Sequence coverage |
|---|---|---|---|---|---|---|
| 16 | Tyrosyl-tRNA synthetase | AFUA_5G10640 | +2.7 | 6.18 | 43588 | 20 |
| 25 | Regulatory protein SUAPRGA1 | AFUA_3G09030 | +2.4 | 4.88 | 40070 | 32 |
| 31 | Mitochondrial peroxiredoxin Prx1 | AFUA_4G08580 | +2.2 | 5.38 | 23378 | 33 |
| 43 | Polysaccharide deacetylase family protein | AFUA_5G09130 | +2.0 | 5.39 | 35188 | 42 |
| 48 | Actin-bundling protein Sac6 | AFUA_2G07420 | +1.9 | 5.78 | 72434 | 12 |
| 56 | Nuclear pore complex subunit SEC13 | AFUA_4G06090 | +1.7 | 6.17 | 33856 | 28 |
| 57 | Molecular chaperone Hsp70 | AFUA_1G07440 | +1.7 | 5.09 | 69618 | 56 |
| 70 | MRS7 family protein | AFUA_3G08230 | +1.5 | 6.42 | 68745 | 18 |
| 5 | Ketol-acid reductoisomerase | AFUA_3G14490 | −4.9 | 9.32 | 56318 | 21 |
| 14 | Phoshoribosylaminoimidazole carboxamide formyltransferase/IMP cyclohydrolase | AFUA_4G07690 | −3.0 | 6.39 | 64987 | 33 |
| 59 | Transcription factor RfeF | AFUA_4G10200 | −1.7 | 4.91 | 71649 | 18 |
| 60 | Hsc70 cochaperone (SGT) | AFUA_1G09830 | −1.7 | 4.74 | 35721 | 38 |
| 68 | Short chain dehydrogenase | AFUA_4G08710 | −1.6 | 5.28 | 30909 | 38 |

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

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 Δ*elfA* under normal growth conditions compared to the wild-type and $elfA^C$ (wild-type; 5.6, Δ*elfA*; 3.3, $elfA^C$; 6.0), indicative of cells under oxidative stress (Supporting Information Table 1). This resulted from decreased GSH levels and increased GSSG levels in Δ*elfA* [Fig. 1(E)]. When exposed to $H_2O_2$ for 15 and 30 min, the effect on the GSH/GSSG ratios in wild-type and Δ*elfA* 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 Δ*elfA*, the GSH/GSSG ratio increased following 15 min exposure to $H_2O_2$ (from 3.33 to 4.61), and subsequently decreased (to 1.90) following exposure for 30 min. The increased GSH/GSSG ratio following 15 min exposure of *A. fumigatus* Δ*elfA* to $H_2O_2$ resulted from decreased GSH and GSSG levels [Fig. 1(F)]. While after 30 min exposure to $H_2O_2$, an increase in the amount of GSH in *A. fumigatus* Δ*elfA* and a decrease in the amount of GSSG resulted in the decreased GSH/GSSG ratio [Fig. 1(G)]. Significantly increased levels of GSH ($P < 0.05$) were present in Δ*elfA* compared to wild-type following exposure to $H_2O_2$ for 30 min, while the other trends, although not significant were reproducible ($n = 3$).

Measurement of GSH levels in *A. fumigatus* Δ*elfA* cultured for 24 h prior to exposure to diamide (1 m*M*) for 2 h, confirmed that diamide decreases cellular GSH levels [Fig. 1(H)].

### *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 Δ*elfA* were cultured for 24 h under basal conditions; eight proteins had a fold increase >1.5 ($P < 0.05$) in *A. fumigatus* Δ*elfA* 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 Hsc70 co-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* Δ*elfA*

*A. fumigatus* ATCC46645 and Δ*elfA* were cultured for 24 h in AMM before $H_2O_2$ (2 m*M* 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* Δ*elfA* [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 m*M* $H_2O_2$ exposure for 1 h. Vacuolar dynamin-like GTPase VpsA was identified from three different spots with fold increases of 7.8, 4.3, and 3.0 in *A. fumigatus* Δ*elfA*, while ATP citrate lyase, subunit 1 was identified from two spots which were upregulated 1.9- and 1.5-fold in *A. fumigatus* Δ*elfA*. The isoforms of both vacuolar dynamin-like GTPase VpsA and ATP citrate lyase, subunit 1 had the same molecular mass but displayed slight changes in p*I*. 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* Δ*elfA*.

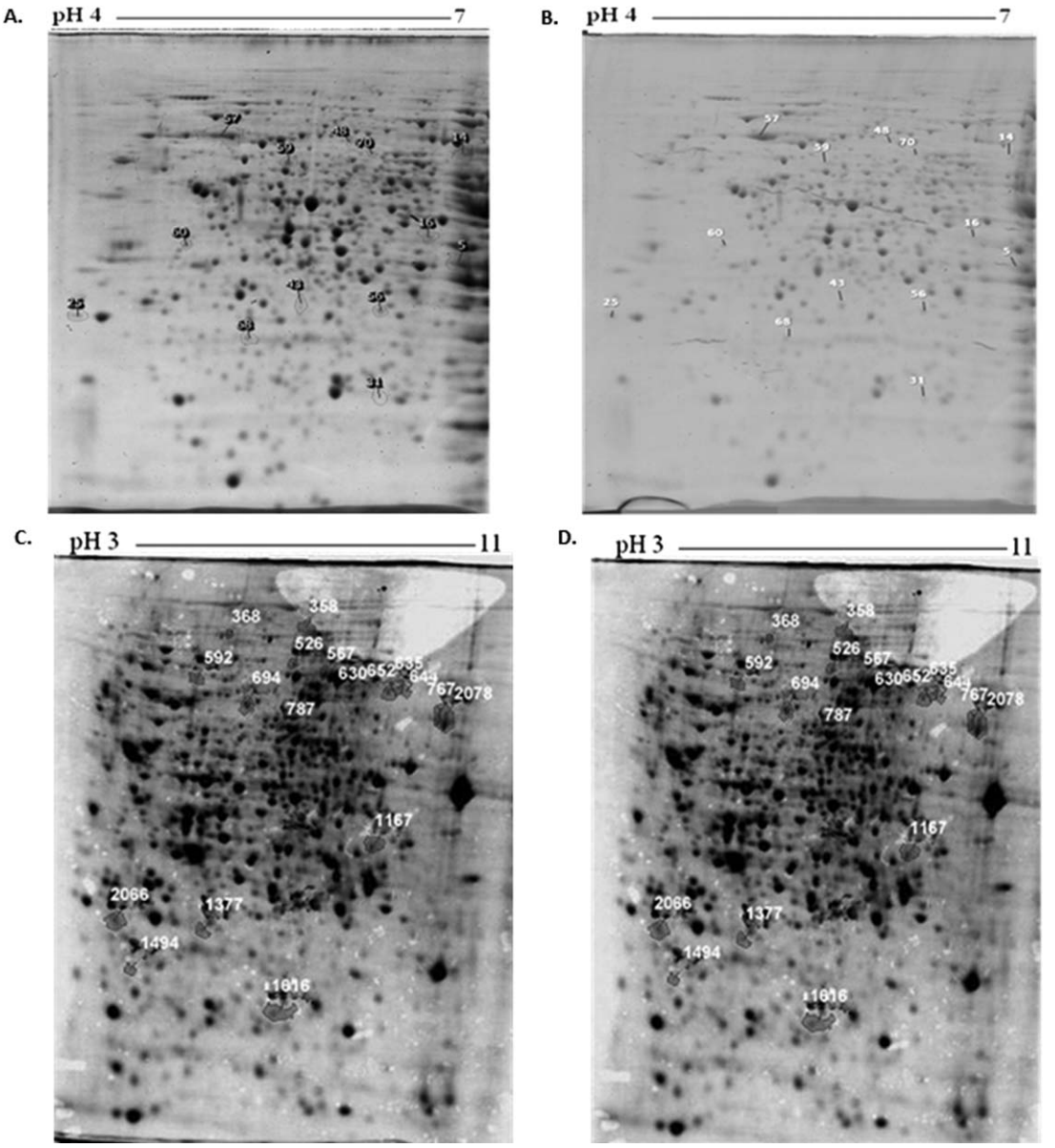


**Figure 2.** Proteomic remodeling in *A. fumigatus* Δ*elfA* in the absence and presence of oxidative stress. 2DE analysis of *A. fumigatus* ATCC46645 (A) and Δ*elfA* (B) under normal growth conditions. 2DE analysis of *A. fumigatus* ATCC46645 (C) and Δ*elfA* (D) following exposure to 2 m*M* $H_2O_2$ for 1 h. The proteins found to be differentially expressed after analysis using Progenesis™ SameSpot software are numbered.

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 Δ*elfA*

Due to the altered redox environment in *A. fumigatus* Δ*elfA*, 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.[23] Increased protein glutathionylation in *A. fumigatus* Δ*elfA* 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* Δ*elfA* 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

**Table II.** *Proteins (n = 15) with a Fold Change in* A. fumigatus Δ*elfA Compared with A. fumigatus After Exposure to 2 m*M $H_2O_2$ *Following Identification by 2DE and LC-MS/MS*

| Spot no. | Annotation name | CADRE ID[a] | Fold change | tpI | tMW | % Sequence coverage |
|---|---|---|---|---|---|---|
| 644 | Vacuolar dynamin-like GTPase VpsA | AFUA_5G02360 | +7.8 | 8.30 | 78,425 | 18 |
| 635 | Vacuolar dynamin-like GTPase VpsA | AFUA_5G02360 | +4.3 | 8.30 | 78,425 | 3 |
| 652 | Vacuolar dynamin-like GTPase VpsA | AFUA_5G02360 | + 3.0 | 8.30 | 78,425 | 19 |
| 592 | Mitochondrial outer membrane translocase receptor (TOM70) putative | AFUA_2G01660 | +1.9 | 5.40 | 70,009 | 10 |
| 767 | ATP citrate lyase, subunit 1, putative | AFUA_6G10650 | +1.9 | 8.60 | 78,828 | 33 |
| 368 | Carbamoyl-phosphate synthase, large subunit | AFUA_2G10070 | +1.7 | 6.10 | 129,214 | 25 |
| 567 | Cobalamin-independent methionine synthase Met H/D | AFUA_4G07360 | +1.7 | 6.33 | 87,072 | 31 |
| 1377 | Metallo-β-lactamase family protein | AFUA_5G12770 | +1.6 | 5.60 | 34,197 | 30 |
| 526 | Cysteinyl-tRNA synthetase | AFUA_5G09610 | +1.5 | 5.95 | 91,041 | 25 |
| 630 | Glycerol-3-phosphate dehydrogenase, mitochondrial | AFUA_1G08810 | +1.5 | 6.71 | 76,899 | 31 |
| 694 | Vacuolar ATP synthase catalytic subunit A | AFUA_5G02370 | +1.5 | 5.83 | 75,329 | 43 |
| 787 | Pyruvate decarboxylase PdcA | AFUA_3G11070 | +1.5 | 6.08 | 63,307 | 33 |
| 2078 | ATP citrate lyase, subunit 1 | AFUA_6G10650 | +1.5 | 8.66 | 79,359 | 42 |
| 1494 | RAS small monomeric GTPase RasA | AFUA_5G11230 | −2.2 | 4.97 | 24,292 | 61 |
| 1167 | Nuclear pore complex protein (SonA) | AFUA_1G09020 | −2.1 | 7.61 | 40,220 | 32 |
| 2066 | Proteasome regulatory Particle Subunit (RpnL) | AFUA_3G08940 | −1.8 | 4.9 | 31,201 | 55 |
| 358 | Pyruvate carboxylase | AFUA_4G07710 | −1.6 | 6.23 | 132,003 | 27 |
| 1616 | Haloalkanoic acid dehalogenase | AFUA_6G14460 | −1.6 | 6.19 | 26,716 | 39 |

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

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.[7,8] 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 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* Δ*elfA* where, under normal growth conditions the GSH/GSSG ratio of *A. fumigatus* Δ*elfA* was decreased compared to that of *A. fumigatus* ATCC46645, strongly indicative of oxidative stress.[24] Further evidence of an altered redox state is the increased level of protein glutathionylation in *A. fumigatus* Δ*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* Δ*elfA* was especially interesting because in *S. cerevisiae*, mitochondrial peroxiredoxin Prx1 requires GSH-mediated reduction for antioxidant activity.[25] 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.[26] The increased expression of mitochondrial peroxiredoxin Prx1 in *A. fumigatus* Δ*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 $H_2O_2$ confirmed our initial hypothesis that *elfA* is involved in the oxidative stress response in *A. fumigatus*. Consolidating this hypothesis, *A. fumigatus* Δ*elfA* was found to be significantly more sensitive than wild-type to the oxidants $H_2O_2$, diamide, and DPS. Both diamide and DPS are thiol-specific oxidants which react directly with GSH.[26] The reduced GSH levels in *A. fumigatus* Δ*elfA* compared to those in *A. fumigatus* ATCC46645 confirm that GSH levels are depleted to a greater extent in *A. fumigatus* Δ*elfA* in the presence of these oxidants resulting in the increased sensitivity of *A. fumigatus* Δ*elfA*. Conversely, in *S. cerevisiae*, an eEF1Bγ deletion strain was more resistant to the oxidants; $H_2O_2$, menadione and $CdSO_4$.[27] However, in *S. cerevisiae*, the GST

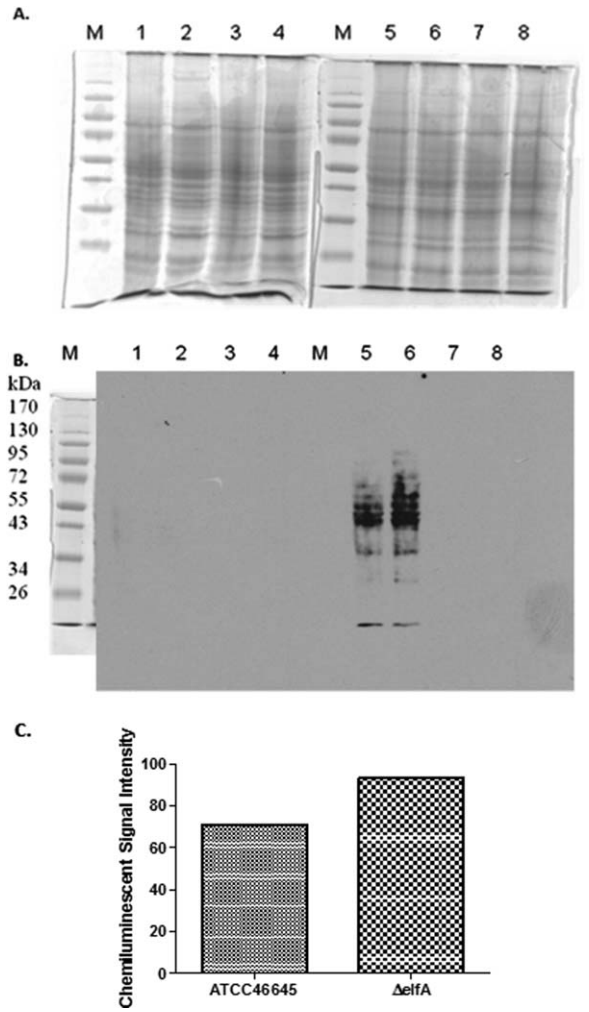


**Figure 3.** Western blot analysis of protein glutathionylation in *A. fumigatus* ATCC46645 and Δ*elfA*. (A) SDS-PAGE of *A. fumigatus* ATCC46645 (Lanes 1, 3, 5, 7) and Δ*elfA* (Lanes 2, 4, 6, 8) whole cell lysates (50 μg) under reducing (Lanes 1–4) and nonreducing conditions (Lanes 5–8). The samples 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 Δ*elfA* (Lane 6). There were more glutathionylated proteins detected in *A. fumigatus* Δ*elfA* (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* Δ*elfA* compared to *A. fumigatus* ATCC46645 supporting the observation that there was a higher level of protein glutathionylation in *A. fumigatus* Δ*elfA*.

domain of eEF1Bγ is not active,[9] unlike in *A. fumigatus*.[15] 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 $H_2O_2$-induced oxidative stress on the proteome of *A. fumigatus* Δ*elfA* 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.[28–33]

Cobalamin-independent methionine synthase, which exhibited increased expression in *A. fumigatus* Δ*elfA*, is essential for *S*-adenosylmethionine (SAM) cycle occurrence and homocysteine, an intermediate of the SAM cycle, can be converted to glutathione.[34] This is particularly relevant since GSH/GSSG determination in *A. fumigatus* Δ*elfA*, following exposure to $H_2O_2$, 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* Δ*elfA* 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 $H_2O_2$. Interestingly, it has been observed that addition of methionine to *Penicillium chrysogenum* cultures also resulted in increased intracellular GSH levels,[35] 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* Δ*elfA* under both normal growth conditions and following exposure to $H_2O_2$, 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* Δ*elfA*. The Hsp protein family is required for the disassembly of protein aggregates, protein folding, and the degradation of misfolded proteins,[36,37] while Sec13 functions in the coat protein complex II (COPII), vesicular trafficking, nuclear pore function, and endoplasmic reticulum-associated degradation (ERAD).[38] The COPII complex also transports misfolded proteins to the ERAD,[39] which in turn is involved in the retrotranslocation of misfolded proteins from the ER to the cytosol for degradation by proteasomes.[40] Previously, Sec13 was found to be upregulated in *S. cerevisiae* by the unfolded protein response (UPR),[41] which has been shown to target genes involved in the ERAD.[42,43] The increased expression of these proteins under normal growth conditions is indicative of a greater requirement for protein degradation in *A. fumigatus* Δ*elfA* which is in accordance with the stressed

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.[26,44] Following exposure to $H_2O_2$, 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* Δ*elfA*. One of the functions of vacuolar dynamin-like GTPase VpsA is in vacuole fission.[28] Vacuoles, along with proteasomes, degrade proteins exported from the ER by the ERAD.[18,40,45] 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.[31,33] This decreased expression of the proteasome regulatory particle subunit in *A. fumigatus* Δ*elfA* 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 eEF1Bγ deletion strain which also exhibited altered vacuole morphology and altered expression of Hsp proteins.[18] Thus, the identification of a number of proteins involved in protein folding and degradation of misfolded proteins in *A. fumigatus* Δ*elfA*, under both normal growth conditions and following exposure to $H_2O_2$, 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* Δ*elfA* under normal growth conditions, while cysteinyl-tRNA synthetase exhibited increased expression upon exposure to $H_2O_2$. We postulate that the increased expression of these aminoacyl-tRNA synthetases is indicative of altered protein synthesis in *A. fumigatus* Δ*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.[46,47]

The unexpected increased expression of additional proteins (i.e., actin-bundling protein Sac6 and vacuolar dynamin-like GTPase VpsA), in *A. fumigatus* Δ*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* Δ*elfA*. Moreover, following exposure to $H_2O_2$, 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*,[48] 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.[11] In *S. cerevisiae*, Sac6 was observed to co-localize with these actin bundles during oxidative stress induced by $H_2O_2$, menadione, and diamide.[11] 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.[10] In *S. cerevisiae*, eEF1Bα regulates the actin binding and bundling activities of eEF1A and has been proposed to direct eEF1A toward binding aminoacyl-tRNA.[7] In mammalian epithelial cells, eEF1Bγ increased the formation of keratin intermediate filament bundles *in vivo*.[19] Moreover, overexpression of eEF1Bγ in epithelial cells resulted in a disrupted interaction between eEF1Bγ and keratin, and also reduced protein synthesis suggesting a functional link between the cytoskeletal structure and translation in epithelial cells.[19,49] The observation of increased expression of Sac6 in *A. fumigatus* Δ*elfA* under normal growth conditions suggests that ElfA (an EF1Bγ) 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.[50] The bacterial strain *E. coli* TOP10 (Invitrogen, The Netherlands) which was cultivated in LB (1% (w/v) Bactotryptone, 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* Δ*elfA* strain was generated using the bipartite marker technique[51] 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 (Zymoresearch).

*A. fumigatus* Δ*elfA* 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 oelfA5 and oelfA6, the resulting 3.1 kb product was cloned into TOPO® vector. The resulting plasmid was linearized and ligated to *hph*, which was released from pAN7-1[53] by restriction digestion, and cloned into TOPO® vector to give the complementation construct. The plasmid was linearized before transforming *A. fumigatus* Δ*elfA* 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 Δ*elfA* ($1 \times 10^5$ cfu/mL) were cultured for 24 h in AMM before addition of $H_2O_2$ (2 m*M* final) for 15 and 30 min, respectively. *A. fumigatus* ATCC46645 and Δ*elfA* not exposed to $H_2O_2$ 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 Δ*elfA* ($1 \times 10^5$ cfu/mL) were grown for 24 h in AMM (100 mL cultures), at 37°C with shaking at 200 rpm. After 24 h, $H_2O_2$ (2 m*M* 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 $H_2O$ 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 Δ*elfA* 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). $MS^n$ analysis was carried out on the 3 most abundant peptide precursor ions in each sample, by automatic selection. Peptides from the $MS^n$ 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.

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