Ergothioneine Biosynthesis and Functionality in the Opportunistic Fungal Pathogen, *Aspergillus fumigatus*

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Ergothioneine (EGT; 2-mercaptohistidine trimethylbetaine) is a trimethylated and sulphurised histidine derivative which exhibits antioxidant properties. Here we report that deletion of *Aspergillus fumigatus* egtA (AFUA_2G15650), which encodes a trimodular enzyme, abrogated EGT biosynthesis in this opportunistic pathogen. EGT biosynthetic deficiency in *A. fumigatus* significantly reduced resistance to elevated H₂O₂ and menadione, respectively, impaired gliotoxin production and resulted in attenuated conidiation. Quantitative proteomic analysis revealed substantial proteomic remodelling in ΔegtA compared to wild-type under both basal and ROS conditions, whereby the abundance of 290 proteins was altered. Specifically, the reciprocal differential abundance of cystathionine γ-synthase and β-lyase, respectively, influenced cystathionine availability to effect EGT biosynthesis. A combined deficiency in EGT biosynthesis and the oxidative stress response regulator Yap1, which led to extreme oxidative stress susceptibility, decreased resistance to heavy metals and production of the extracellular siderophore triacetylfusarinine C and increased accumulation of the intracellular siderophore ferricrocin. EGT dissipated H₂O₂ in vitro, and elevated intracellular GSH levels accompanied abrogation of EGT biosynthesis. EGT deficiency only decreased resistance to high H₂O₂ levels which suggests functionality as an auxiliary antioxidant, required for growth at elevated oxidative stress conditions. Combined, these data reveal new interactions between cellular redox homeostasis, secondary metabolism and metal ion homeostasis.

Ergothioneine (2-mercaptohistidine trimethylbetaine; EGT) is derived from histidine and exists in a tautomeric state between both the thione and thiol forms (Fig. 1a)¹. EGT exhibits a high redox potential (−0.06 V) and so is classified as a powerful antioxidant²,³. Humans cannot biosynthesize EGT and acquire it in the diet from both plant and animals sources, however it appears that EGT biosynthesis only occurs in specific bacterial and fungal species⁴. Human cells possess a receptor, termed OCTN1, which facilitates EGT uptake, and this receptor is highly abundant in trachea, ileum and kidney cells as well as CD71⁺ cells from bone marrow, cord blood and fetal liver⁵. Although its dietary antioxidant properties have been extensively studied⁶–¹¹, it remains to be conclusively proven that EGT has demonstrable health benefits in humans.

The seminal work of Seebeck¹² revealed the mechanism of EGT biosynthesis in *Mycobacterium smegmatis*, which involves five discrete enzymes (EgtA-E). It is now clear that EGT biosynthesis in bacteria requires trimethylation of the NH₂ group of histidine to generate hercynine. γ-glutamylcysteine (a thiol source) is then conjugated to the imidazole side chain of hercynine, followed by subsequent enzymatic processing to yield EGT. The crystal structures of EgtB, EgtC and EgtD have been recently determined¹³–¹⁵. In filamentous fungi, egt-1 has been demonstrated to be essential for EGT biosynthesis in *Neurospora crassa*¹⁶. This gene encodes a protein with both S-adenosyl methionine (SAM)-dependant methyltransferase and formylglycine-generating enzyme (FGE) sulphatase domains. Egt-1 is postulated to carry out the first two steps of EGT biosynthesis: trimethylation of

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Relevantly, Gallagher et al. identified the biosynthetic genes of Aspergillus fumigatus egt1 homolog of M. smegmatis, which showed domain and functional homology to EgtB and EgtD of M. smegmatis and egt2 as a possible homolog of egtE from M. smegmatis. Because fungi appear to lack the enzyme which removes the glutamyl residue from a biosynthetic intermediate in bacterial EGT biosynthetic process, N. crassa and S. pombe use cysteine rather than γ-glutamylcysteine to effect EGT formation. Deficiency of S. pombe egt1 results in the loss of EGT production. If egt2 is missing, minor amounts of EGT can be found possibly due to the spontaneous reaction of hercynylcysteine sulfoxide catalyzed by an unrelated pyridoxal phosphate (PLP)-binding enzyme. Interestingly, Pluskal et al. found no impact of EGT on the resistance against oxidative stress and argued that the antioxidant might not play a role in primary defense as loss is compensated by other mechanisms. Thus, details of the precise role of EGT in fungi are outstanding.

Although EGT presence has been studied extensively in Basidiomycetes as a source of dietary antioxidants, minimal data exist relative to the presence or function of EGT in pathogenic fungi, especially Aspergillus fumigatus. Relevantly, Gallagher et al. were the first to detect EGT in A. fumigatus, a fungal airborne pathogen which causes severe allergic or invasive diseases in immunosuppressed individuals. Gallagher et al. revealed that disruption of glutathione biosynthesis at a specific step (deletion of the γ-glutamylcyclotransferase glkI) concomitantly resulted in significant oxidative stress and significantly elevated EGT levels in A. fumigatus. Notably, apart from that work representing the first identification of EGT in A. fumigatus, it presented a novel alkylation strategy, utilizing 5′-iodoacetamidofluorescein (5′IAF), combined with either reverse-phase high-performance liquid chromatography (RP-HPLC) or LC-mass spectrometry (LC-MS), to detect EGT. An identical strategy has been subsequently deployed by others, using capillary electrophoresis, to determine EGT levels in human plasma.

Oxidative stress can also arise from altered iron metabolism in A. fumigatus. Iron is an essential trace element and is involved in various cellular processes. In excess, iron can produce oxidative stress via the Haber-Weiss/Fenton chemistry. In A. fumigatus, iron limitation leads to the formation of low molecular mass ferric iron-specific chelators (siderophores), which are either excreted as extracellular siderophores, like fusaric acid C (FsC) or triacyltuitaric acid (TAF) to import Fe³⁺ into the fungus, or intracellular siderophores ferric acid (FC) or hydroxyl-ferric acid (HFC) to store or transport iron to other parts of the hyphae or conidia. Lack of siderophores and subsequent lower uptake of iron led to attenuated virulence in A. fumigatus, which was shown in neutropenic mice. Furthermore, the inability to protect against oxidative stress by deficiency in protein phosphatase Z, PzaA, leads to defective virulence in the immunocompetent murine model of corneal infection. A key component of oxidative stress defense in A. fumigatus is Yap1. This transcriptional regulator has been shown to play an important role in coordinating the oxidative stress response and its deletion in A. fumigatus results in sensitivity to damage via elevated ROS levels.

Here we describe the identification of a key EGT biosynthetic gene in A. fumigatus and reveal new insights into systems biology of EGT biosynthesis and functionality in fungi.

**Results**

**Characterization and bioinformatic analysis of EgtA in A. fumigatus.** The biosynthesis of EGT was first described in the prokaryote M. smegmatis and recent genetic comparative studies and homology searches identified the biosynthetic genes in N. crassa and S. pombe. Blast searches revealed possible homologous enzymes to M. smegmatis EgtB and EgtD, which are involved in the EGT biosynthesis annotated as DUF323 (AFUA_2G15650; egtA) in A. fumigatus, NCU04343 (NeEgt-1) in N. crassa, and SPBC1604.01 (egt1) in S. pombe. Alignments and genome-wide in-depth phylogenetic analyses showed
that a gene fusion occurred possibly during the evolution in bacterial species and that EgTA from *A. fumigatus* also exhibited the fusion product of the two enzymes of *M. smegmatis* as seen in the aforementioned fungal species. In *A. fumigatus*, EgTA comprises 844 amino acids, has domains of a histidine-specific SAM-dependent methyltransferase at the N-terminal end, a sulphatase-modifying factor enzyme 1 at the C-terminal end and an intervening 5-histidylcysteine sulphoxide synthase domain (Fig. 1b). The 5′UTR comprises 902 nucleotides, the 3′UTR extends 265 nucleotides and the coding sequence is interrupted by six introns, whereby the genomic DNA is 2890 nucleotides long (http://www.aspergillusgenome.org/cgi-bin/locus.pl?locus=AFUA_2g15650&organism=A_fumigatus_Af293).

**egtA deletion and complementation.** *A. fumigatus* egtA was deleted from strains AF77 (a ΔakuA::loxp strain derived from ATCC46645 lacking non-homologous recombination) and ATCC26933 using a split marker strategy. Both strains were deployed as they produce low and high amounts of gliotoxin, respectively, and the impact of egtA deletion on gliotoxin biosynthesis was of relevance, since intracellular EGT levels significantly increase in a gliotoxin-deficient strain of *A. fumigatus*.

Deletion of egtA was confirmed via Southern analysis (Supplementary Figures S1 and S2) and abolition of gene expression was confirmed via RT-PCR (Supplementary Figure S3). Complementation of egtA in ΔegtA was achieved using an alternative resistance marker as confirmed by Southern analysis (Supplementary Figure S4), and egtA expression was restored in *A. fumigatus* ΔegtA, as confirmed via RT-PCR (Supplementary Figure S3). egtA deletion was also successfully achieved in *A. fumigatus* Δyap1, to yield ΔegtAΔyap1 and complementation of egtA in this double mutant was demonstrated (Supplementary Figure S2).

**Absence of EGT biosynthesis in *A. fumigatus* ΔegtA.** Analysis of EGT biosynthesis was undertaken via RP-HPLC and LC-MS. 5′-IAF-alkylated mycelial lysates of wild-type, ΔegtA26933 and egtA26933 were compared to commercially available EGT and revealed alkylated EGT in the wild-type and complemented strains at a retention time of 12.4 min. EGT was absent in ΔegtA mycelial lysates (Fig. 2a). EGT levels in egtA26933 were elevated compared to wild-type. LC-MS analysis of TCA-precipitated 5′-IAF labelled mycelial lysates further confirmed the presence of EGT in the wild-type and complemented strains and its absence from *A. fumigatus* ΔegtA (Fig. 2b). Consistent with the ATCC26933, egtA deletion blocked EGT production also in *A. fumigatus* strain AF77 strain (data not shown).

H₂O₂ and menadione significantly impair growth of ΔegtA and exacerbate Δyap1 phenotype in ΔegtAΔyap1 during oxidative and heavy metal stress. Plate assays to assess H₂O₂, menadione and diamide sensitivity in ATCC26933, ΔegtA26933 and egtA26933 revealed that ΔegtA26933 displayed increased sensitivity to H₂O₂ and menadione, but not diamide. After 72 h growth on Aspergillus minimal media (AMM) agar containing 3 mM H₂O₂, radial growth of ΔegtA26933 was significantly (P=0.0081) reduced compared to wild-type and complemented strains. At 1 mM and 2 mM H₂O₂ however, ΔegtA26933 radial growth was unaffected compared to the wild-type (Fig. 3a). Wild-type growth was abolished at 4 mM H₂O₂, indicating EGT loss only effects protection from H₂O₂ at near lethal doses in *A. fumigatus* and positions it as an ‘antioxidant of last resort’.

A significant decrease in radial growth was seen in ΔegtA26933 when exposed to menadione, at 40μM (P=0.0043) and 60μM (P=0.0013) (Fig. 3b). No sensitivity to diamide was observed within a range of 1.25 mM–1.75 mM (P=0.0043) (Fig. 3b). To further analyze the function of EGT against oxidative burden and metal toxicity, ΔegtA was disrupted in ΔegtAΔyap1 at low and high concentrations of zinc, iron and copper (Fig. 4). The complemented strain egtA::Δyap1 showed the same phenotype as the Δyap1 strain.

**Comparative Label Free Quantitative (LFQ) Proteomics Reveals Dysregulation of Redox-related Proteins in ΔegtA in response to H₂O₂.** LFQ proteomic analysis revealed significant differences between ATCC26933 and ΔegtA26933. A comparison of ΔegtA26933 and ATCC26933 under basal conditions showed that absence of EGT biosynthesis resulted in a major proteomic adjustment in *A. fumigatus*. Specifically, the abundance of 26 proteins was increased or unique in ΔegtA26933 compared to wild-type, while 121 proteins were decreased in abundance or absent (Supplementary Table S1). Comparison of the proteomic profiles of ΔegtA26933 and ATCC26933 under H₂O₂-induced stress revealed an exacerbation of the differences between the mutant and wild-type. When exposed to 3 mM H₂O₂, the abundance of 290 proteins was dysregulated in the mutant compared to the wild-type, with 250 proteins increased or unique in ΔegtA26933 compared to ATCC26933, while 40 underwent reduced abundance or were absent (Supplementary Table S2). Many proteins which underwent dysregulated abundance under both basal and ROS conditions were reductases, oxidases, stress response proteins and enzymes with oxidising products (Supplementary Tables S3 and S4). This strongly suggests a disruption of redox homeostasis and oxidative stress defence in the ΔegtA26933, compared to ATCC26933, particularly when exposed to 3 mM H₂O₂. Of particular interest is redox sensitive subunit of the CCAAT-binding complex (CBC), HapC. Previously, the CCAAT-binding complex has been shown to be involved in oxidative stress response. Therefore the absence of HapC in ΔegtA26933 compared to wild-type under basal conditions could be indicative of a defective oxidative defence system.

Cystathionine is a sulphur-containing metabolite involved in cysteine and methionine metabolism (Fig. 5), and the abundance of two proteins with activities related to cystathionine metabolism was dysregulated when the
proteomic profiles of ΔegtA26933 and ATCC26933 were compared under both basal and H2O2-stressed conditions. Under basal conditions, cystathionine γ-synthase (CGS; AFUA_7G01590) was found to be absent in ΔegtA26933 compared to the wild-type. CGS catalyzes the formation of cystathionine from homoserine and cysteine32 and its absence in ΔegtA26933 suggests that cystathionine production is attenuated when EGT biosynthesis cannot occur, perhaps to provide cysteine in order to increase GSH production. Comparing ΔegtA26933 and ATCC26933 upon addition of H2O2, there was a significant increase (log2 3.1-fold) in cystathionine β-lyase (CBL; AFUA_4G03950). CBL catalyses the conversion of cystathionine to homocysteine, ammonia and pyruvate32. Homocysteine can be converted into methionine, required for SAM biosynthesis. EGT biosynthesis requires SAM for the tri-methylation step via EgtA. Thus, a shift towards increased homocysteine formation, via CBL, in response to H2O2 addition could be part of an overall transition towards increasing SAM availability for EGT biosynthesis.

RT-qPCR analysis of CGS and CBL gene expression revealed a similar pattern to proteomic abundance alteration (Supplementary Figure S5). The CGS gene showed a non-significant reduction in expression in ΔegtA26933 compared to wild-type under basal conditions. A significant (P = 0.003) increase in the CBL gene was seen in ΔegtA26933 compared to wild-type under ROS conditions.

Figure 2. EGT detection via RP-HPLC and LC-MS in ATCC26933, ΔegtA26933 and egtA26933C. (a) RP-HPLC Chromatograms showing the detection of 5′-IAF labelled EGT. EGT was detected at a retention time of 12.4 min. EGT was detected in the wild-type and complemented sample at 12.4 min, but is absent from ΔegtA. (b) Extracted Ion Chromatographs (m/z: 617) following LC-MS analysis of TCA precipitated 5′-IAF-labelled protein extracts from ATCC26933, ΔegtA26933 and egtA26933C in addition to an EGT standard. A peak at 5.3 min was confirmed to be EGT. This peak was absent from the ΔegtA26933 fraction, confirming the absence of EGT from the mutant. (c) Signature Ion breakdown corresponding to 5′-IAF labelled EGT.
It was noted that no significant change in abundance of these proteins (CGS and CBL) was observed when comparing ΔegtA^26933 under basal conditions to ΔegtA^26933 under ROS conditions (Supplementary Table S5). This suggests that these changes are only significant when compared to the ATCC26933 response when exposed to 3 mM H₂O₂. This further illustrates the proteomic remodeling A. fumigatus undergoes when EGT biosynthesis is impeded and the mutant is forced to deal with oxidative stress.

H₂O₂ addition to ΔegtA^26933 resulted in increased abundance of imidazole glycerol phosphate synthase subunit HisF (AFUA_2G06230) of log 2 1.11-fold compared to ATCC26933 under the same conditions. HisF is involved in histidine biosynthesis catalyzing the closure of the imidazole ring. The observation of an increased

Figure 3. Plate assays performed on AMM agar (containing 5 mM ammonium tartrate as nitrogen source) for 72 h to test for sensitivity to various ROS inducing agents. (a) Plate assay with H₂O₂ ranging from 0 to 3 mM. ΔegtA^26933 shows a significant (P = 0.0081) reduction in growth compared to ATCC26933 and egtA^C at 3 mM H₂O₂. (b) Plate assay with menadione ranging from 0 to 60 μM. ΔegtA^26933 shows significantly reduced growth compared to ATCC26933 and egtA^C at both 40 μM (p = 0.0043) and 60 μM (P = 0.0013) menadione. (c) Plate assays with diamide ranging from 1.25 to 1.75 mM. ΔegtA^26933 shows no significant difference in growth compared to ATCC26933 or egtA^C.
The abundance of imidazole glycerol phosphate synthase subunit HisF in ΔegtA26933 compared to wild-type under oxidative stress suggests that histidine production is increased when *A. fumigatus* is exposed to H$_2$O$_2$ in the absence of EGT biosynthesis. This could further indicate a shift towards attempting to synthesize EGT in the mutant, given that histidine is a key biosynthetic precursor of EGT.

A log$_2$ 2.22-fold increase in a putative sulphite reductase (AFUA_2G15590; Supplementary Table S2) in ΔegtA26933 under oxidative stress compared to wild-type indicates an increased need for sulphur in ΔegtA26933, which could reflect attempted EGT production or an increase in GSH biosynthesis.

**egtA expression, but not EGT levels, increases in response to H$_2$O$_2$ exposure.** Quantitative RT-PCR of ATCC26933 cultures exposed to 3 mM H$_2$O$_2$ for 1 h in Sabouraud Dextrose broth showed a significant (P = 0.0002) increase in egtA expression in response to H$_2$O$_2$ compared to the control (Fig. 6a). This indicates H$_2$O$_2$ induces egtA expression. Unexpectedly, no corresponding increase in EGT levels was observed in ATCC26933 mycelial lysates following H$_2$O$_2$ exposure, which suggested that EGT may react to dissipate H$_2$O$_2$ (Fig. 6b). It was observed that treatment of purified EGT with H$_2$O$_2$ caused a significant decrease (~50%; P = 0.0029) in levels of the antioxidant (Fig. 6c), which is in accordance with EGT consumption by reaction with H$_2$O$_2$, and infers that egtA expression is increased to maintain intracellular EGT homeostasis.

**GSH levels in ΔegtA26933 are increased compared to wild-type and complement.** Supernatants from mycelial lysates following 5′-IAF alkylation and TCA precipitation, from 72 h cultures of ATCC26933,
ΔegtA<sup>26933</sup> and egtA<sup>C26933</sup>, were analysed via LC-MS. Extracted Ion Chromatograms at m/z 695 revealed a significantly (P = 0.0016) increased abundance of total cellular glutathione (GSH) in ΔegtA<sup>26933</sup> compared to both the wild-type and complemented strains (Fig. 7 and Supplementary Figure S6). This indicates a significant increase in the level of GSH in response to, or as a result of, abrogation of EGT biosynthesis. LFQ proteomics revealed that cystathionine γ-synthase (CGS; AFUA_7G01590) is missing from ΔegtA<sup>26933</sup>. This enzyme converts cysteine to cystathionine and its absence in ΔegtA<sup>26933</sup> may be to ensure cysteine flux is available to facilitate increased GSH biosynthesis (Supplementary Table S1). The ratio of free GSH:GSSG pools increases when egtA is deleted, and drops again upon complementation (Supplementary Table S7, Supplementary Figure S6). Thus, absence of EGT biosynthesis is accompanied by an increased relative GSH content in mycelia.

EGT deficiency increased ferricrocin in wild-type and reduced triacetylfusarinine C but increased fusarinine C content in a ∆yap1 background. To analyze a possible role of EGT in adaptation to iron starvation, production of biomass and siderophores of strains lacking egtA, yap1 or both was compared to the wild-type during iron sufficiency and starvation. The biomass production of the strains did not differ significantly (Fig. 8a). Biomass production during iron depletion was about 30% compared to iron sufficiency confirming iron starvation conditions. During iron starvation, A. fumigatus produces the intracellular siderophore ferricrocin (FC) and the two extracellular siderophores fusarinine C (FsC) and triacetylfusarinine C (TAFC)<sup>34</sup>. In the wild-type, EGT deficiency increased the cellular accumulation of FC by about 15% (Fig. 8c). In A. fumigatus ∆egtA∆yap1, EGT deficiency increased production of FsC by 60% and decreased production of TAFC by 53%, while the total extracellular siderophore production was not significantly altered. Figure 8d shows an exemplar RP-HPLC analysis of culture supernatants, demonstrating the increase of FsC and decrease of TAFC in the ∆egtA∆yap1 strain compared to ∆yap1.

EGT deficiency leads to transcriptional downregulation of sidG. To further analyze the role of EGT in siderophore production, the transcript levels of selected genes were analysed by Northern analysis during iron starvation and sufficiency with and without H<sub>2</sub>O<sub>2</sub> stress (Fig. 9). The absence of egtA transcripts in ∆egtA<sup>ATCC77</sup> confirmed deletion of the gene. Slight up-regulation of egtA expression was seen after wild-type treatment with H<sub>2</sub>O<sub>2</sub>, which is in accordance with the results for ATCC26933 exposed to 3 mM H<sub>2</sub>O<sub>2</sub>. The up-regulation of the catalase-peroxidase gene cat2 (AFUA_8G01670)<sup>35</sup> in response to H<sub>2</sub>O<sub>2</sub> treatment in wild-type and ∆egtA confirms oxidative stress. The lack of cat2 up-regulation in ∆yap1 is consistent with the function of Yap1 as an activator of oxidative stress response<sup>28</sup>. The conversion of FsC to TAFC is mediated by the acetyltransferase SidG (AFUA_3G03650)<sup>34</sup>. As shown in Fig. 9, sidG is transcriptionally up-regulated during iron starvation, as
previously shown\(^3^4\), and down-regulated in \(\Delta\text{egtA}\), \(\Delta\text{yap1}\) and nearly undetectable in \(\Delta\text{egtA}\Delta\text{yap1}\). Notably, \(\text{sidG}\) expression is completely absent after treatment with H\(_2\)O\(_2\). The down-regulation of \(\text{sidG}\) is consistent with increased FsC and decreased TAFC production.

**Gliotoxin production in \(\Delta\text{egtA}^{26933}\) is significantly reduced compared to wild-type.** Organic extracts from 72 h culture supernatants of ATCC26933 and \(\Delta\text{egtA}^{26933}\) were analysed via RP-HPLC, gliotoxin was detected at a retention time of 14.9 min in both wild-type and \(\Delta\text{egtA}^{26933}\) and subsequent analysis via LC-MS confirmed the presence of gliotoxin (Fig. 10). However, gliotoxin levels in \(A.\text{fumigatus}\ \Delta\text{egtA}^{26933}\) were significantly reduced compared to the wild-type (\(P = 0.0003\)) (Fig. 10b). Thus, an inability to biosynthesize EGT appears to lead to attenuated gliotoxin production compared to wild-type. Relevantly, gliotoxin oxidoreductase GliT (AFUA_6G09740), essential for gliotoxin production\(^3^6\), was absent from \(\Delta\text{egtA}^{26933}\) through LFQ proteomic analysis (Supplementary Table S1), which could, at least in part, explain the significant diminution of gliotoxin biosynthesis in \(A.\text{fumigatus}\ \Delta\text{egtA}^{26933}\). GliT absence in \(\Delta\text{egtA}^{26933}\) could point towards potential sensitivity as per Schrettl et al.\(^3^6\), however this was not found to be the case (data not shown). LFQ proteomic comparison of \(\Delta\text{egtA}^{26933}\) with and without gliotoxin revealed GliT is still induced by gliotoxin exposure, thus no sensitivity is observed (Supplementary Figure S7).

**\(\Delta\text{egtA}^{26933}\) colonies produce paler conidia and show lower levels of conidiation.** Colonies from \(\Delta\text{egtA}^{26933}\) grown on AMM for 72 h exhibited visibly paler conidia compared to those of ATCC26933. Subsequently, levels of conidia were measured by haemocytometry which revealed that \(\Delta\text{egtA}^{26933}\) produced significantly (\(P < 0.05\)) lower levels of conidia compared to the wild-type (Fig. 11). This suggests a link between EGT and conidial health, as has been previously observed\(^1^6\). Relevantly, LFQ proteomics revealed that proteins important for conidiation and conidial health are absent in \(\Delta\text{egtA}^{26933}\) (Supplementary Table S1). These include mannosyltransferases PMT2 (AFUA_1G07690) and PMT4 (AFUA_8G04500), conidial hydrophobin RodB (AFUA_1G17250) and FluG (AFUA_3G07140), an extracellular developmental signal biosynthesis protein\(^3^5\)\(^–^3^9\). In addition, abundance of the LaeA-like protein VipC (AFUA_8G01930) was shown to be increased under basal conditions (log 2 1.23 fold increase) and further enhanced when exposed to 3 mM H\(_2\)O\(_2\) (log 2 2.5 fold increase) (Supplementary Tables S1 and S2). VipC is part of the Velvet complex and dysregulation could have consequences for conidiation and has been shown to control the switch between sexual and asexual reproduction in \(A.\text{nidulans}\)\(^4^0\). An \(A.\text{nidulans}\) mutant with over-abundant VipC protein showed reduced asexual development\(^4^0\).
Figure 7. GSH detection via LC-MS in ATCC26933, ΔegtA\textsuperscript{26933} and egtA\textsuperscript{C26933}. (a) Extracted Ion Chromatographs (m/z: 695) following LC-MS analysis of TCA precipitated 5′-IAF-labelled mycelial extracts from ATCC26933, ΔegtA\textsuperscript{26933} and egtA\textsuperscript{C26933}, in addition to a GSH standard. A peak at 5.9 min was confirmed to be GSH. (b) Peak height data from LC-MS analysis comparing GSH levels in ATCC26933, ΔegtA\textsuperscript{26933} and egtA\textsuperscript{C26933}. GSH levels are significantly (P = 0.0016) increased in ΔegtA\textsuperscript{26933} compared to the wild-type and complemented samples. (c) Signature Ion breakdown corresponding to 5-IAF labelled GSH.

Figure 8. Biomass levels and siderophore production of AF577, ΔegtA\textsuperscript{AF577}, Δyap1 and ΔegtAΔyap1 under iron-deplete conditions (containing 20 mM L-glutamine as nitrogen source). (a) Biomass production of A. fumigatus strains cultivated for 24 h at 37 °C during iron starvation (-Fe) and iron sufficiency (+Fe) show no remarkable differences. (b) EGT-deficiency (strain ΔegtA) caused higher levels of FC. (c) Combined with Yap1 deficiency, EGT deficiency increased FsC and decreased TAFC production (d) Exemplar RP-HPLC chromatogram (detection at 435 nm to detect red siderophores after iron saturation of the culture supernatants) confirming increased FsC and decreased TAFC production in ΔegtAΔyap1 (red line) in comparison to Δyap1 (black line).
Fe) at 37 °C for 16 h plus 45 min with or without addition of H2O2 to a final concentration of
+ sufficiency (AMM cultures (containing 20 mM L-glutamine as nitrogen source) grown under iron starvation (−Fe) or
sufficiency (+ Fe) at 37 °C for 16 h plus 45 min with or without addition of H2O2, to a final concentration of
1 mM. See Supplementary Figure S8.

Figure 9. Northern analysis revealing downregulation of sidG expression in ΔegtAΔyap1. Total RNA
(10 μg) from AF77 (wt), ΔegtAΔyap1 (Δe), Δyap1 (Δy) and ΔegtAΔyap1 (ΔeΔy) was isolated from submersed
AMM cultures (containing 20 mM L-glutamine as nitrogen source) grown under iron starvation (− Fe) or
sufficiency (+ Fe) at 37 °C for 16 h plus 45 min with or without addition of H2O2, to a final concentration of
1 mM. See Supplementary Figure S8.

Discussion

Here we present the first identification of the key gene, egtA, which encodes EGT biosynthesis in the opportunistic
pathogen, A. fumigatus. EGT significantly contributes to resistance to high level oxidative stress induced by
H2O2, superoxide and metal ions, and its presence also influences siderophore and glutoxin biosynthesis.
Conidial proteome remodeling occurs in the absence of EGT, which is associated with attenuated conidiation.
Overall, we introduce a new player into the sulphur and iron interactome of A. fumigatus.

Through Blast searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi), it was shown that the first two enzymes of
M. smegmatis, EgdT and EgdB, show sequence or functional homologies to a single enzyme in A. fumigatus,
encoded by AFUA_2G15650, herein termed egtA. EgtA from A. fumigatus encodes putative sulphotase and
methyltransferase activities; additionally it contains an OvoA-domain (5-histidylcysteine sulphone synthase
domain). In marine organisms and some human pathogens, this enzyme fulfills the function of a sulphone synthase
catalyzing the first step in ovothiol A biosynthesis to generate an unmethylated sulphur-containing thiocysteine
precursor of this metabolite45,46. The same enzymatic reaction is carried out by EgtB in M. smegmatis
and by EgtA in A. fumigatus. Ovothiol A originates from L-cysteine, L-histidine, O2 and SAM, as does EGT43,44.
The iron-dependent step catalyzed by OvoA was described to mediate the oxidative sulphone transfer in Erwiniata
smaniensis45. Ovothiol A is thought to play a role in cellular redox homeostasis in the fertilization of sea urchin
eggs46, during infection by Leishmania sp., Trypanosoma sp.47,48 and it also induced autophagy in the human
hepatic cancer cell line Hep-G249. In vitro studies revealed a potent antioxidative function of ovothiols against
various radicals via ovothiol-promoted NAD(P)H-O2 oxidoreductase activity49.

The biosynthetic pathway for EGT was recently studied in non-pathogenic fungi. In S. pombe the first step is
catalyzed by Egt1 leading to hercynylcysteine sulphone formation16,17. The next and last step for the generation
of EGT remains still elusive and was not well characterized in eukaryotes. However S. pombe likely contains
a two-step biosynthetic pathway to generate EGT from L-histidine and utilizes L-cysteine rather than
γ-glutamylcysteine as in M. smegmatis, since no homolog of egtC was found in S. pombe which could encode
an enzyme capable of glutamyl residue removal17. No hits have been found in A. fumigatus when searching for
homologs of M. smegmatis EgtC. However, Pluskal et al.17 showed that there is a possible homolog of EgtE, a
pyridoxal-phosphate (PLP)-binding enzyme in S. pombe by screening for EGT production in deletion mutants
of possible homologs obtained from a stock center (Bioneer haploid deletion library). They found one mutant
strain defective in the gene designated as SPBC660.12c, renamed to egt2, which showed lower EGT but raised
amounts of the precursor hercynylcysteine sulphone. A minimal amount of EGT was still found in the Egt2
strain defective in the gene designated as SPBC660.12c, renamed to egt2, which showed lower EGT but raised
amounts of the precursor hercynylcysteine sulphone. A minimal amount of EGT was still found in the Egt2
deletion strain, explained by a spontaneous conversion of hercynylcysteine sulphone to EGT by an unre-
lated PLP-binding enzyme50. While no homologs have been reported for N. crassa, a potential homolog to the
M. smegmatis EgtE and Egt2 from S. pombe could be the gene annotated as lolT (AFUA_2G13295) in A. fumigatus.
Further studies will validate this result of sequence homology analysis.

EGT production was abolished in A. fumigatus ΔegtAΔyap1 and ΔegtAΔyap2, and complementation with egtA
restored EGT production in ΔegtAΔyap1, thereby confirming the role of the gene in EGT biosynthesis. Both
A. fumigatus ΔegtA deletion strains exhibited a redox-sensitive phenotype at 3 mM H2O2, with no affects seen at
lower H2O2 concentrations, while wild-type growth was completely inhibited at 4 mM H2O2. This suggests EGT
may act as an auxiliary, or ‘anti-oxidant of last resort’ against oxidative stress in A. fumigatus. Sensitivity to mena-
dione was also observed, at both 40 μM and 60 μM, though the mutant matched wild-type growth levels at 20 μM.
This reveals that while not essential for primary protection, EGT is important for protection against elevated
levels of superoxide radicals in A. fumigatus.

Previously, no phenotypic or protective effect of EGT could be shown in S. pombe when tested against oxida-
tive stress-inducing agents like H2O2 and tert-butylhydroperoxide17 as was demonstrated in N. crassa, where
EGT contributes to the antioxidative defense against peroxides in conidia and plays a role in conidiogenesis and conidial longevity, but does not protect against UV-induced mutation rate\(^{16,50}\). However, herein we show that endogenous EGT confers significant resistance against \(\text{H}_2\text{O}_2\) and menadione in the \(A.\ fumigatus\). It was also shown that the key regulator of oxidative response Yap1\(^{28}\) is very important to protect \(A.\ fumigatus\) against oxidative burden. Additionally, it was demonstrated that EGT deficiency exacerbates the phenotype of the extremely sensitive mutant strain lacking Yap1 too (\(\Delta\text{egtA}\Delta\text{yap1}\)) during treatment with oxidative stressors or metals. The fact that Yap1 deficiency extends the \(\Delta\text{egtA}\) phenotype indicates that in wild-type, EGT limitation is partially compensated by other detoxification mechanisms that require activation by Yap1.

The role of EGT in protection against oxidative stress and maintenance of redox homeostasis is further underlined by the results of LFQ proteomics. The abundance of a number of proteins related to redox homeostasis and oxidative stress is dysregulated in \(\Delta\text{egtA}\)\(^{26933}\) (Supplementary Tables S3 and S4). This apparent proteomic remodelling when \(\Delta\text{egtA}\)\(^{26933}\) is exposed to \(\text{H}_2\text{O}_2\) underpins the important role played by EGT in maintaining redox homeostasis in the presence of specific oxidants. Loss of EGT results in altered abundance of redox-related proteins, possibly due to an increased level of cellular \(\text{H}_2\text{O}_2\) consequent to abrogation of cellular EGT presence.

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**Figure 10.** Gliotoxin detection via RP-HPLC and LC-MS in ATCC26933 and \(\Delta\text{egtA}\)\(^{26933}\). (a) RP-HPLC analysis of organic extracts from the supernatants of 72 h cultures of ATCC26933 and \(\Delta\text{egtA}\)\(^{26933}\). Gliotoxin is present in all chromatograms for both samples at 14.9 min. (b) Comparison of gliotoxin peak area from RP-HPLC analysis for ATCC26933 and \(\Delta\text{egtA}\)\(^{26933}\) performed in triplicate. The peak area was found to be significantly (\(P = 0.0003\)) lowered in \(\Delta\text{egtA}\)\(^{26933}\). (c) Extracted Ion Chromatographs (m/z: 327) following LC-MS analysis of organic extracts of supernatants from ATCC26933 and \(\Delta\text{egtA}\)\(^{26933}\), in addition to a gliotoxin standard. A peak at 6.3 min was confirmed to be gliotoxin. (d) Signature ion breakdown corresponding to gliotoxin.
proteomic data also suggests that ΔegtAΔ26933 attempts to synthesise EGT upon H2O2 exposure, and hints at a possible degradative pathway for EGT. In addition, increased abundance of HisF (AFUA_2G06230) and cystathionine β-lyase (AFUA_4G03950) shows an increased requirement for histidine and SAM respectively, necessary for the biosynthesis of EGT. Furthermore, the increased abundance of a sulphite reductase (AFUA_2G15590) observed could indicate an increased need for sulphur in order to biosynthesise EGT. Previously it has been shown in A. nidulans that the CCAAT-binding complex (CBC, also termed Hap complex or AnCF in A. nidulans) senses the cellular redox status via oxidative modification of thiol groups within its subunit HapC31. The absence of HapC in ΔegtAΔ26933 (Supplementary Tables S3 and S4) indicates another layer of CBC regulation, which could lead to an impaired global oxidative stress response.

The changes observed in CGS and CBL in ΔegtAΔ26933 under basal and ROS conditions when compared to wild-type suggest a metabolic change caused by EGT absence centred on cystathionine usage. Under basal conditions, CGS is downregulated, which would curtail cystathionine production32. This would result in cysteine availability for GSH production. Upon addition of 3 mM H2O2, a “switch” is observed and CBL abundance was increased in ΔegtAΔ26933 compared to wild-type. This should increase conversion of cystathionine to homocysteine32 which may ultimately provide SAM, via the methyl/methionine cycle31 for EGT biosynthesis (Fig. 5) and

Figure 11. Conidiation in ATCC26933 and ΔegtAΔ26933. (a) Comparison of conidial colour and appearance in colonies from ATCC26933 and egtAΔ26933. (b) Comparison of conidiation levels from ATCC26933 and egtAΔ26933 (5 mM ammonium tartrate as nitrogen source).
suggests that ΔegtAΔegtAΔegtAΔegtAΔegtAΔegtA attempts to biosynthesise EGT under ROS conditions, thereby highlighting its role in oxidative stress response.

In correspondence, qRT-PCR analysis showed that egtA expression was up-regulated in response to H₂O₂ exposure, a further indicator that EGT is essential for attenuating oxidative stress in A. fumigatus. However, despite the increase in egtA expression, intracellular EGT levels did not increase upon addition of H₂O₂, most likely due to reactivity with ROS species as we observed for purified EGT. This suggests that intracellular EGT is dissipated consequent to its antioxidant activity and is consistent with data from Servillo et al. who reported that EGT is degraded upon oxidation, mainly into hercynine and sulphurous acid. Conversely, the observed rise in EGT levels in ΔgliK, deficient in gliotoxin biosynthesis, could be due to either a sensory deficiency in the cellular oxidative stress response, or a compensatory mechanism to replace the frontline antioxidant, GSH, utilised for gliotoxin biosynthesis but which cannot undergo replenishment due to GliKγ-glutamyl cyclotransferase deficiency.

RP-HPLC and LC-MS analysis revealed attenuated gliotoxin production, while GSH production is significantly increased, in ΔegtAΔegtAΔegtAΔegtAΔegtAΔegtA. GSH production is likely increased to deal with the increased ROS caused by EGT absence. LFOQ proteomic analysis provides revealing insight into the putative mechanisms facilitating this observation. Under basal conditions, cystathionine γ-synthase is undetectable in ΔegtAΔegtAΔegtAΔegtAΔegtAΔegtA, which would prevent the conversion of cysteine to cystathionine, therefore channelling more cysteine towards GSH biosynthesis (Fig. 5). GSH is the source of both thiols in gliotoxin, and gliotoxin biosynthesis has been shown to be greater in ATCC26933 than ATCC46645. Thus, increased GSH levels in ΔegtAΔegtAΔegtAΔegtAΔegtAΔegtA may be consequent to the observed decrease in gliotoxin biosynthesis, and the consequential absence of GSH incorporation into gliotoxin. GSH may, in turn, be diverted from gliotoxin biosynthesis to detoxify ROS, leading to reduced gliotoxin production in ΔegtAΔegtAΔegtAΔegtAΔegtAΔegtA. LFOQ proteomics also revealed accumulation of GltH in ΔegtAΔegtAΔegtAΔegtAΔegtAΔegtA (Supplementary Table S6). Deletion of gliT, which catalyzes the final step of gliotoxin biosynthesis, results in aborted gliotoxin production and its absence in ΔegtAΔegtAΔegtAΔegtAΔegtAΔegtA is entirely in accordance with the observed diminution in gliotoxin biosynthesis. Attenuated gliotoxin production was unexpected, as previous observations in ΔgliK suggested an inverse relationship between gliotoxin and EGT. Decreased gliotoxin production following the loss of EGT contradicts that view; however it nonetheless suggests that the production of these two sulphur-containing, redox-active metabolites is interlinked.

Unexpectedly, GSH biosynthesis is not altered in A. fumigatus ΔegtA (Supplementary Figure S6). Because of the interconnection of the EGT pathway to GSH biosynthesis in EGT producing bacteria such as Synechocystis sp. PCC6803 it had been argued that this interaction may be the case for other EGT-producing organisms, like A. fumigatus, resulting in an up-regulation of GSH biosynthesis as a result of a block in the EGT pathway and a higher content of intermediates, or to compensate for the loss of the enzyme annotated as EgtC in M. smegmatis biosynthesis in EGT-producing fungi does not provide any intermediate for the EGT pathway, because of the higher content of intermediates, or to compensate for the loss of EGT. However, it has been suggested that GSH biosynthesis in EGT-producing fungi does not provide any intermediate for the EGT pathway, because of the missing enzyme annotated as EgTC in M. smegmatis, which would catalyze further reactions, remove the glutamyl residue or the absent up-regulation of GSH to utilize the accumulated intermediates or to compensate for the loss of EGT. Another factor is the previously discussed link between gliotoxin and GSH, the latter essential for the biosynthesis of gliotoxin. With lower gliotoxin production in ΔegtA compared to ATCC26933, this may impact the behavior of GSH in the two EGT deficient mutants. Indeed a comparison of GSH levels (Supplementary Figure S6) in the two wild-type strains show that ATCC26933 has lower GSH levels than ΔegtA, perhaps linked to the different levels of gliotoxin production. The subsequent drop in gliotoxin production in ΔegtAΔegtAΔegtAΔegtAΔegtAΔegtA may explain the rise in GSH.

EGT deficiency alters siderophore biosynthesis in A. fumigatus ΔegtAΔegtAΔegtAΔegtAΔegtAΔegtA. Whereas, FsC and TAF are produced under iron starvation to be excreted for chelation and import of ferric iron, FC and HFC act as intracellular storage and transport vehicles. Moreover, it was demonstrated that oxidative stress (H₂O₂, PQ) leads to higher levels of FC during both iron states, but most obvious at excess iron in Aspergillus nidulans through up-regulation of sidC. Siderophore analysis herein implies that EGT is involved in oxidative stress defense because of the higher amounts of FC in EGT-lacking strains. Additionally, this study supports the theory that siderophore biosynthesis is intertwined with oxidative stress response by shifting the production of extracellular siderophores towards the progenitor FsC instead of TAF in the sensitive ΔegtAΔegtAΔegtAΔegtAΔegtAΔegtA mutants. Northern analysis related the decreased amounts of TAF in ΔegtAΔegtAΔegtAΔegtAΔegtAΔegtA consequent to a down-regulated expression of sidG (AFUA_3G03650), the acetyltransferase catalyzing the triacylation of FsC to generate TAF. It was shown previously that HapX deficiency results in selective suppression of TAFC but not FsC biosynthesis, caused by lower transcript levels of sidG, but only minor impact on sidD and sidF expression. This phenotype partially matches that of the ΔegtAΔegtAΔegtAΔegtAΔegtAΔegtA mutant indicating that EGT or oxidative stress might negatively influence HapX activity, which leads to the reduction in sidG transcription. Alternatively, oxidative stress and EGT, respectively, affect sidG, sidD and sidF expression and HapX independently. Northern blot analysis indicated transcriptional upregulation of egtA during iron starvation compared to iron sufficiency (Fig. 9). In this context it is interesting to note that cellular accumulation of the EGT precursor histidine was previously also found to be significantly increased during iron starvation compared to iron sufficiency.

Bello et al. demonstrated that EGT plays an important role in conidial health in N. crassa. This seems to be shared with A. fumigatus, with paler conidia and lower levels of conidiation observed in ΔegtAΔegtAΔegtAΔegtAΔegtAΔegtA. LFQ proteomics reveals several important proteins involved in conidial health and development are missing in ΔegtAΔegtAΔegtAΔegtAΔegtAΔegtA including, conidial hydropophin RodB (AFUA_1G17250), extracellular developmental signal biosynthesis protein FluG (AFUA_3G07140) and mannosyltransferases PMT2 (AFUA_1G07690) and PMT4 (AFUA_8G04500) (Supplementary Table S1). RodB is a structural protein found in the cell wall of conidia. FluG is a signaling protein associated with asexual development, indeed deletion of FluG in A. flavus resulted in reduced conidiation. A mannosyltransferase PMT4 mutant also displayed reduced conidiation, however a deletion mutant of PMT2 was not viable suggesting the gene is essential. Fang et al. demonstrated that reducing pmt2 transcription in...
**A. fumigatus** resulted in reduced conidiation, retarded germination and impaired cell wall integrity. The absence of these conidiation-associated proteins suggests that EGT presence, or global cellular redox homeostasis play an important role in the conidiation process in **A. fumigatus**. Moreover, it has been postulated that ROS may play a role in regulating cellular differentiation and the loss of EGT and subsequent dysregulation of the redox system, may further explain the loss of these proteins and the effects on conidiation observed in **ΔegtA**. Another protein with altered expression was VipC (AFUA_8G01930), a methyltransferase that forms part of the Velvet complex (Supplementary Tables S1 and S2). Sarikaya-Bayram *et al.* demonstrated in **A. nidulans** that VipC is important for the appropriate activation of either sexual or asexual reproduction in response to darkness or light respectively. While increased abundance of VipC was associated with increased asexual reproduction, a VipC over-expression mutant showed repression of both asexual conidiation and sexual fruiting bodies.

To conclude, EGT biosynthesis is linked to multiple, apparently unrelated systems in **A. fumigatus**. Cystathionine metabolism and the transsulphuration pathway is altered in **ΔegtA**, especially under oxidative stress conditions. This 'cystathionine switch' could be an important control mechanism to deal with altered redox homeostasis in **A. fumigatus**.

**Methods**

**Strains, growth conditions, and oligonucleotides.** **A. fumigatus** strains were grown at 37 °C using *Aspergillus* minimal medium (AMM) agar. AMM contained 1% (w/v) glucose as the carbon source, 5 mM ammonium tartrate or 20 mM L-glutamine as the nitrogen source and trace elements. Liquid cultures were performed with 100 ml Czapek-Dox broth or AMM in 500 ml Erlenmeyer flasks inoculated with 10⁸ conidia. For growth assays, 5 × 10⁸ conidia of the respective strains were point inoculated on AMM agar plates containing the relevant supplements and incubated for 72 h at 37 °C. All **A. fumigatus** strains and oligonucleotides used are listed in Supplementary Tables S8 and S9 respectively.

**Deletion of egtA and yap1 and complementation of the ΔegtA and ΔegtAΔyap1 strains.** For generating egtA and yap1 deletions in **AIS77**, and the associated double mutants, the bipartite marker technique was used. Briefly, **A. fumigatus** was co-transformed with two DNA fragments, each containing overlapping but incomplete fragments of a resistance cassette (pyrithiamine, ptrA; hygromycin, hph) fused to the 5′ and 3′ flanks of egtA. The egtA 5′-flanking region (1199 bp) was PCR amplified from genomic DNA using primers oAfDUF323.1 and oAfDUF323.2. For the amplification of the 3′-flanking region (1149 bp) primers oAfDUF323.3 and oAfDUF323.4 were employed. Subsequent to gel purification, these fragments were digested with HinIII and PstI respectively. The ptrA selection marker was released from plasmid pSK275 by digestion with HindIII (5′-flanking region) and PstI (3′-flanking region) and ligated with the 5′- and 3′-flanking regions respectively. The transformation construct A (2022 bp, fusion of the egtA 5′-flanking region to ptrA split marker) was amplified from the ligation product using oAfDUF323.5 and oAoPtrA1. For amplification of transformation construct B (2476 bp, fusion of ptrA split marker with 3′-flanking region) primers oAoPtrA2 and oAfDUF323.6 were employed. For transformation of **A. fumigatus** protoplasts both constructs were simultaneously used. To generate a knockout mutant strain deficient in Yap1 in the background of **AIS77**, the respective strains were point inoculated on AMM plates containing the relevant supplements and incubated for 72 h at 37 °C. All **A. fumigatus** strains and oligonucleotides used are listed in Supplementary Tables S8 and S9 respectively.

The same strategy was used for the generation of **ΔegtA**. **A. fumigatus** strain ATCC26933 was co-transformed with two DNA constructs, both containing incomplete fragments of the pyrithiamine gene, ptrA. Fragments were generated via PCR from DNA extracted from **ΔegtA** using primers oAfDUF323.5 and oAoPtrA1 for the 5′ fragments, and oADUF323.6 and oAoPtrA2 for the 3′ fragment. The PCR-product was transformed in **A. fumigatus** ATCC26933 and **ΔegtA** protoplasts to generate **Δyap1** and **ΔegtAΔyap1** respectively.

For the reconstitution of **ΔegtA** a functional copy of egtA (a 5691 bp PCR-amplified fragment generated with the primers oAegtA1.7_f and oAegtA1.8_r) was subcloned into pAN8.1 containing the phleomycin resistance cassette (ble) by a digestion with SphiI and Nhel resulting in (p)egtA:ble. The resulting 10,508 bp plasmid was linearized with SphiI and used for the transformation of **A. fumigatus** protoplasts of **ΔegtA** and **ΔegtAΔyap1**. To complement **ΔegtA**, a PCR fragment containing the egtA locus including promoter and terminator was amplified using primers oAfDUF323.5 and oAfDUF323.6 and then inserted into the pCR® 2.1-TOPO® TA vector. This vector (p egtA) was linearised with AatII and transformed into **ΔegtA** alongside the plasmid pAN 7-1, containing the hph selection marker for hygromycin resistance. Insertion of the egtA gene was confirmed via Southern analysis. Transformation of **A. fumigatus** was carried out as described previously. For selection of positive transformants 0.1 mg/ml pyrithiamine (Sigma), 0.2 mg/ml hygromycin (Sigma) or 0.02 mg/ml phleomycin (Eubio) was used. Southern blot analyses were used to screen for positive transformants. PCR primers used for generating hybridization probes are listed in Supplementary Table S9.

**RNA isolation, Reverse Transcription PCR and Quantitative PCR.** RNA was isolated and purified from **A. fumigatus** hyphae crushed in liquid N₂ using the RNeasy plant mini kit (Qiagen). RNA was treated with DNase I (Invitrogen), and cDNA synthesis from mRNA (500 ng) was performed using a qScript cDNA SuperMix kit (Quanta Biosciences). The gene encoding calmodulin (AFUA_4G10050) is, which is constitutively expressed in **A. fumigatus**, served as a control in RT-PCR and RT-qPCR experiments. RT-qPCR was performed using a Roche Lightcycler 480. Northern Analysis was performed as described elsewhere.

**Ergothioneine Analysis.** To analyze ergothioneine production, **A. fumigatus** ATCC26933, **ΔegtA** and **egtAC20633** were grown at 37 °C for 72 h in Czapek-Dox broth. Mycelia were snap frozen in liquid N₂ and lyophilised overnight. Lyophilised mycelia were bead beaten in lysis buffer, incubated on ice for 1 h and centrifuged...
(13,000 g) at 4 °C. Supernatants (50 μl) were treated with 5′-IAF (10 μl; 3 mg/ml in DMSO) and analyzed via RP-HPLC and LC-MS as described19.

Sensitivity Assays for Oxidative Stress. *A. fumigatus* strains were incubated on AMM agar at 37 °C for up to 72 h in the presence of oxidising agents. Sensitivity was determined by comparing mean radial growth from 3 replicates, significance was determined via one-way ANOVA. Oxidising agents used were *H*$_2$O$_2$ (0–3 mM), menadione (0–60 μM) and diamide (0–1.75 mM). Metal toxicity was assayed with iron (0–10 mM), copper (0–1.0 mM), zinc (0–8 mM), and cobalt (0–1 mM).

Determination of Ergothioneine reactivity with *H*$_2$O$_2$. Reactivity between EGT and *H*$_2$O$_2$ was analysed by incubating 3 mM EGT (Sigma-Aldrich) with, and without, 3 mM *H*$_2$O$_2$ for 3 h at room temperature. Triplicate specimens were then analyzed by back-titration to detect remaining EGT by labelling with 5′-IAF followed by RP-HPLC detection as described above. Residual EGT amounts were compared using unpaired t-test.

Glutathione Analysis via LC-MS. *A. fumigatus* mycelia were grown for 72 h at 37 °C in Czapek-Dox broth, snap-frozen in liquid N$_2$ and lyophilised overnight. Lyophilised mycelia were bead beaten in lysis buffer, incubated on ice for 1 h and centrifuged (13,000 g) at 4 °C. Supernatants (50 μl) were treated with 5′-IAF (10 μl; 3 mg/ml in DMSO) and analyzed using LC-MS as described19. GSH levels were compared using one-way ANOVA.

GSH/GSSG determination. *A. fumigatus* mycelia were grown for 72 h at 37 °C in Czapek-Dox Broth, harvested through miracloth and snap frozen in liquid N$_2$. GSH and GSSG levels were then determined as described previously65.

Comparative Label Free Quantitative (LFQ) Proteomic Analysis of *A. fumigatus* ATCC26933 and Δ*egtA*26933. *A. fumigatus* ATCC26933 and Δ*egtA*26933 (*n* = 4 biological replicates each) were cultured in Sabouraud-Dextrose media for 23 h followed by *H*$_2$O$_2$ addition (3 mM final) or equivalent volume of *H*$_2$O for 1 h. Mycelial lysates were prepared in lysis buffer (100 mM Tris–HCl, 50 mM NaCl, 20 mM EDTA, 10% (v/v) Glycerol, 1 mM PMSE, 1 μg/ml pepstatin A, pH 7.5) with grinding, sonication and clarified using centrifugation (13,000 g, 20 min, 4 °C). Lysates were then precipitated using trichloroacetic acid/acetone and resuspended in 100 mM Tris–HCl, 6 M urea, 2 M thiourea, pH 8.0. Samples were reduced and alkylated using DTT and iodoacetamide respectively, then treated with trypsin and ProteaseMax surfactant85,66. Resultant peptide mixtures were analyzed via a Thermo Fisher Q-Exactive mass spectrometer coupled to a Dionex RSLCnano. LC gradients operated from 4 to 45% B over 2 h, and data was collected using a Top15 method for MS/MS scans. Comparative proteome abundance and data analysis were performed using MaxQuant software (version 1.3.0.57), with Andromeda used for database searching and Perseus used to organize the data (version 1.4.1.3).

Siderophore analysis. Analysis of extracellular and intracellular siderophores was performed by RP-HPLC as described previously24.

Gliotoxin Analysis. To analyze gliotoxin production, *A. fumigatus* ATCC26933 and Δ*egtA*26933 were grown at 37 °C for 72 h in Czapek-Dox medium. Supernatants were chloroform extracted and fractions were dried to completion under vacuum. Extracts were resolubilised in methanol and analyzed using RP-HPLC and LC-MS as previously described40. Gliotoxin levels were compared via unpaired t-test.

Conidial Quantification. Colonies of ATCC26933 or Δ*egtA*26933 were grown on AMM (containing 5 mM ammonium tartrate as nitrogen source) for 72 h at 37 °C. Plugs were taken from the centre of each colony and placed into 1 ml of PBS in 1.5 ml tubes. After vigorous vortexing, 20 μl of each solution was placed on a haemocytometer and quantified. Mean conidial concentrations were computed from *n* = 3 independent colonies. Conidial concentrations were compared via unpaired t-test.

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Author Contributions
S.D., H.H. and G.W.J. conceived the study and wrote the manuscript. B.E.L., K.J.S., G.O.’K., M.A.K., E.R.W. and H.L. carried out experimental work. B.E.L. and K.J.S. also contributed to manuscript drafting.

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