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RNA-seq reveals the pan-transcriptomic impact of attenuating the gliotoxin self-protection mechanism in Aspergillus fumigatus

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Abstract

Background: Aspergillus fumigatus produces a number of secondary metabolites, one of which, gliotoxin, has been shown to exhibit anti-fungal activity. Thus, A. fumigatus must be able to protect itself against gliotoxin. Indeed one of the genes in the gliotoxin biosynthetic gene cluster in A. fumigatus, gliT, is required for self-protection against the toxin- however the global self-protection mechanism deployed is unclear. RNA-seq was employed to identify genes differentially regulated upon exposure to gliotoxin in A. fumigatus wild-type and A. fumigatus ΔgliT, a strain that is hypersensitive to gliotoxin.

Results: Deletion of A. fumigatus gliT resulted in altered expression of 208 genes (log2 fold change of 1.5) when compared to A. fumigatus wild-type, of which 175 genes were up-regulated and 33 genes were down-regulated. Expression of 164 genes was differentially regulated (log2 fold change of 1.5) in A. fumigatus wild-type when exposed to gliotoxin, consisting of 101 genes with up-regulated expression and 63 genes with down-regulated expression. Interestingly, a much larger number of genes, 1700, were found to be differentially regulated (log2 fold change of 1.5) in A. fumigatus ΔgliT when challenged with gliotoxin. These consisted of 508 genes with up-regulated expression, and 1192 genes with down-regulated expression. Functional Catalogue (FunCat) classification of differentially regulated genes revealed an enrichment of genes involved in both primary metabolic functions and secondary metabolism. Specifically, genes involved in gliotoxin biosynthesis, helvolic acid biosynthesis, siderophore-iron transport genes and also nitrogen metabolism genes and ribosome biogenesis genes underwent altered expression. It was confirmed that gliotoxin biosynthesis is induced upon exposure to exogenous gliotoxin, production of unrelated secondary metabolites is attenuated in A. fumigatus ΔgliT, while quantitative proteomic analysis confirmed disrupted translation in A. fumigatus ΔgliT challenged with exogenous gliotoxin.

Conclusions: This study presents the first global investigation of the transcriptional response to exogenous gliotoxin in A. fumigatus wild-type and the hyper-sensitive strain, ΔgliT. Our data highlight the global and extensive affects of exogenous gliotoxin on a sensitive strain devoid of a self-protection mechanism and infer that GliT functionality is required for the optimal biosynthesis of selected secondary metabolites in A. fumigatus.

Keywords: Gliotoxin, RNA-seq, Transcriptome, Secondary metabolism, Fungal proteomics

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Background

Gliotoxin, a non-ribosomally synthesised peptide produced by Aspergillus fumigatus and related fungi, is redox-active, depletes cellular glutathione (GSH), causes DNA damage and protein modification, and is consequently capable of inhibiting mammalian, fungal and bacterial cell growth [1-5]. Conversely, it has also been demonstrated that gliotoxin presence protects against \( \text{H}_2\text{O}_2 \)-induced oxidative stress in \( A. \text{fumigatus} \) and that gliotoxin can substitute for peroxiredoxin in mammalian cells to protect against similar oxidative stresses [6,7]. In \( A. \text{fumigatus} \), gliotoxin biosynthesis is encoded by a 13-gene cluster, \( \text{gli} \), and it has been demonstrated that gliotoxin effects induction of many genes within the \( \text{gli} \) cluster in a positive feedback manner [2,8,9]. Thus, gliotoxin presence induces \( \text{gli} \) cluster activation via \( \text{gliZ} \), a \( \text{Zn}2\text{Cys}6 \) binuclear transcription factor, and deletion of \( \text{gliZ} \) abolishes gliotoxin biosynthesis [10]. Moreover, Forseth et al. [11] revealed that an additional nine metabolites, dependent on \( \text{gliZ} \) presence, are produced consequent to gliotoxin biosynthetic pathway functionality in \( A. \text{fumigatus} \).

Gliotoxin exposure has been shown by qRT-PCR to either activate or induce increased expression of all genes in the \( \text{gli} \) cluster, as especially observed for \( A. \text{fumigatus} \) \( \Delta\text{gliP} \) deficient in the non-ribosomal peptide synthetase which mediates cyclo-L-Phe-L-Ser formation [9,12]. Others have shown induction of \( \text{gliG} \) (a glutathione \( \text{S} \)-transferase), \( \text{gliA} \) (an MFS transporter) and \( \text{gliT} \) upon exposure of \( A. \text{fumigatus} \) wild-type to gliotoxin, by Northern analysis [2]. However, definitive evidence of concomitant increased \( \text{de novo} \) gliotoxin production has not been forthcoming. Relatedly, it has been shown that transformation with \( A. \text{fumigatus} \) \( \text{gliA} \) confers resistance against exogenous gliotoxin upon \( \text{Leptosphaeria maculans} \) [13] while deletion of \( \text{gliA} \) in \( A. \text{fumigatus} \) renders it less resistant to exogenous gliotoxin [14], and Schrettl et al. [2] were the first to demonstrate increased \( \text{GliT} \) abundance by 2D-PAGE/MALDI-ToF analysis in \( A. \text{fumigatus} \) upon exposure to exogenous gliotoxin. However, within \( A. \text{fumigatus} \), gliotoxin biosynthesis must be controlled to avoid manifestation of the deleterious affects of this reactive metabolite.

Since the original observations that self-protection against gliotoxin was largely mediated by the enzyme \( \text{GliT} \), a gliotoxin oxidoreductase [2,15], it has subsequently been found that other organisms contain similar enzymes which facilitate self-protection against related epipolythiodioxopiperazines [16,17]. Indeed, in the bacterium, \( \text{Streptomyces clavuligerus} \), it has been demonstrated that an oxidoreductase, \( \text{HmlI} \), confers self-protection against the disulfide-bridge-containing, non-ribosomal peptide, holomycin [17]. Interestingly, an RNA methyltransferase, \( \text{Hom12} \) in \( \text{Yersinia ruckeri} \), also enables self-protection against holomycin, since \( \text{Hom12} \) deletion results in acquisition of a holomycin-sensitive phenotype [16]. This clearly infers that self-protection against redox-active non-ribosomal peptides is a multi-faceted process, yet few studies have attempted molecular dissection of the process. Carberry et al. [5] revealed significantly elevated GSH levels in \( A. \text{fumigatus} \) \( \Delta\text{gliT} \), and speculated about exacerbation of gliotoxin toxicity, resulting from formation of the dithiol form of gliotoxin, consequent to this apparent dysregulation in the level of an important cellular reductant. Indeed, the apparent resistance of \( \text{Saccharomyces cerevisiae} \) \( \Delta\text{gsh1} \), which exhibits significantly attenuated GSH levels, to exogenous gliotoxin supported this hypothesis. Interestingly, these authors also observed that \( S. \text{cerevisiae} \) \( \Delta\text{sod1} \) and \( \Delta\text{yap1} \) were hypersensitive to exogenous gliotoxin, suggesting that a deficient oxidative stress response sensitizes this organism to gliotoxin. Coleman et al. [4] further revealed that both \( \text{Candida albicans} \) and \( \text{Cryptococcus neoformans} \) were sensitive to gliotoxin exposure, however apart from an elegant demonstration of membrane damage consequent to gliotoxin exposure, no mechanistic basis of the anti-fungal effect of gliotoxin was forthcoming.

It is somewhat surprising that the concept of self-protection against gliotoxin, in fungi capable of its biosynthesis, has received scant attention since the discovery of gliotoxin in 1936 - given the reactive nature of this disulfide-containing metabolite. However the availability of powerful new technologies such as RNA-seq [18-21], provides us with a tool to address this information deficient. Consequently, we present here the first exploration of the global transcriptomic response of both \( A. \text{fumigatus} \) wild-type and \( \Delta\text{gliT} \) to exogenous gliotoxin, which illuminates the important role played by \( \text{gliT} \) in mediating control of the cellular systems in the presence of this reactive metabolite.

Results

Deletion of \( \text{gliT} \) results in altered expression of over 200 genes involved in many functions in \( A. \text{fumigatus} \)

As deletion of \( A. \text{fumigatus} \) \( \text{gliT} \) renders the strain sensitive to exogenous gliotoxin [2,15], to achieve a better understanding of the self-protection against gliotoxin provided by \( A. \text{fumigatus} \) \( \text{gliT} \), high throughput RNA sequencing analysis was carried out. An average of 9312 transcripts were expressed in \( A. \text{fumigatus} \) wild-type and \( \Delta\text{gliT} \) (available from the European Nucleotide Archive under accession ERP001382), which is in accordance with other RNA-seq investigations of the \( A. \text{fumigatus} \) transcriptome [18,22]. A comparison of \( A. \text{fumigatus} \) wild-type and \( \Delta\text{gliT} \) revealed that the deletion of \( \text{gliT} \) resulted in the significant \((p < 0.05)\) dysregulated expression of 208 genes, consisting of 175 up-regulated genes while 33 genes were significantly down-regulated \((p < 0.05)\) (Figure 1A, Additional file 1). The differentially regulated genes were classified according to the Functional Catalogue (FunCat)
[23] and KEGG pathways [24] to identify the functions of these genes. Following deletion of gliT, 13 2nd level, 19 3rd level and 12 4th level FunCat categories were over-represented in the up-regulated gene set, compared to 3 2nd level, 10 3rd level and 3 4th level FunCat categories enriched in the down-regulated gene set (Additional file 2: Tables S1 and S2). Categories over-represented in the up-regulated gene set include; secondary metabolism, transport and detoxification, while genes involved in C-compound and carbohydrate metabolism were enriched in the down-regulated gene set (Figure 2). Additionally, 11 KEGG pathways were over-represented in the up-regulated gene set, while genes involved in 13 KEGG pathways were enriched in the down-regulated gene set (Additional file 2: Tables S3 and S4). Carbohydrate and lipid metabolism were over-represented in the up-regulated gene set, while amino acid metabolism was enriched in the down-regulated gene set. Genes involved in secondary metabolite biosynthesis were enriched in both the up- and down-regulated gene sets.

Gliotoxin exposure alters the regulation of hundreds of genes in both A. fumigatus wild-type and ΔgliT

Following A. fumigatus wild-type exposure to exogenous gliotoxin, 164 genes were significantly differentially regulated (p < 0.05) compared to control conditions (Figure 1B). Of these 164 genes, 101 genes were up-regulated when A. fumigatus wild-type was exposed to gliotoxin, while 63 genes were down-regulated (Additional file 1). In contrast, when A. fumigatus ΔgliT was exposed to exogenous gliotoxin, 1,700 genes were significantly differentially regulated (p < 0.05) (Figure 1C), whereby expression of 508 genes was up-regulated in response to gliotoxin exposure and 1192 genes down-regulated (Additional file 1). Given the large transcriptomic remodelling observed in A. fumigatus ΔgliT following exogenous gliotoxin exposure, cell viability was assessed at 85%. Although a significant decrease (p = 0.0019) in the cell viability of A. fumigatus ΔgliT exposed to exogenous gliotoxin, compared with the methanol control (97%) (Additional file 2: Figure S1), gross cell death was not observed.

Functional characterisation of differentially regulated genes in A. fumigatus wild-type and ΔgliT following exogenous gliotoxin exposure

In classifying the differentially regulated genes in A. fumigatus wild-type upon exposure to exogenous gliotoxin, 9 2nd level, 17 3rd level and 11 4th level FunCat categories, respectively, were over-represented in the up-regulated gene set compared to 12 2nd level, 17 3rd level and 8 4th level categories in the down-regulated gene set (Additional file 2: Tables S5 and S6). Comparatively, in A. fumigatus ΔgliT exposed to exogenous gliotoxin, a greater number of FunCat categories were over-represented. Here, 24 2nd level, 23 3rd level and 12 4th level FunCat
categories, respectively, were over-represented in the up-regulated gene set compared to 42 2nd level, 84 3rd level and 39 4th level categories in the down-regulated gene set (Additional file 2: Tables S7 and S8). A. fumigatus wild-type exposure to exogenous gliotoxin results in an over-representation of up-regulated expression of genes involved in secondary metabolism, transport (particularly siderophore-iron transport), detoxification processes and homeostasis processes as well as others (Figure 3). Of the down-regulated genes, some of the categories which were enriched included amino acid metabolism, carbohydrate metabolism and complex cofactor/cosubstrate/vitamin binding (Figure 3). Loss of gliT in combination with exogenous gliotoxin exposure results in the dysregulation of a large number of processes within the cell. It resulted in an over-representation of up-regulated genes involved in stress response, ribosome biogenesis and translation, and of down-regulated genes involved in metabolism of cysteine, nitrogen, sulphur and selenium metabolism, RNA synthesis, transport (including siderophore-iron transport), homeostasis and cellular import (Figure 4).

In combination with the identified FunCat categories, 2 KEGG pathways were over-represented in the up-regulated gene set, and 13 KEGG pathways in the down-regulated gene set of A. fumigatus wild-type upon exogenous gliotoxin exposure were over-represented (Additional file 2: Tables S9 and S10). Pathways involved in xenobiotic biodegradation and metabolism were enriched in the up-regulated gene set, while in the down-regulated gene set, pathways involved in the biosynthesis of secondary metabolites, glycolysis/gluconeogenesis and amino acid metabolism are over-represented. In A. fumigatus ΔgliT exposed to exogenous gliotoxin, 18 KEGG pathways were over-represented in the up-regulated gene set compared to 38 KEGG pathways in the down-regulated gene set (Additional file 2: Tables S11 and S12). Among the KEGG pathways over-represented in the up-regulated gene set were pathways involved in carbohydrate metabolism, translation and selenoamino acid metabolism. In the down-regulated gene set, some of the pathways over-represented included; biosynthesis of secondary metabolites, starch and sucrose metabolism, and amino acid metabolism.
Exogenous gliotoxin causes dysregulation of gliotoxin biosynthesis cluster gene expression in both A. fumigatus wild-type and ΔgliT

Close inspection of the 13-gene gliotoxin biosynthesis cluster [2,3], revealed that exogenous gliotoxin caused the dysregulated expression of a number of gliotoxin biosynthetic genes in both A. fumigatus wild-type and ΔgliT (Table 1). Upon exposure to exogenous gliotoxin, five out of the 13 genes in the cluster were significantly up-regulated in A. fumigatus wild-type. Expression of A. fumigatus gliZ was up-regulated log_2 3.2-fold, gliP log_2 6.2 fold, while gliA and gliF were up-regulated log_2 10- and log_2 6.7-fold, respectively. Expression of gliT, which confers protection against exogenous gliotoxin [2,15], was up-regulated log_2 9.2-fold. Increased expression of the remaining genes in the gliotoxin biosynthetic cluster was also observed in A. fumigatus wild-type upon exogenous gliotoxin exposure, however altered expression was not significant.

Upon A. fumigatus ΔgliT exposure to exogenous gliotoxin, dysregulated expression of gliotoxin biosynthetic genes was also observed (Table 1). As in A. fumigatus wild-type, gliP, gliA and gliF expression was also up-regulated in ΔgliT upon exogenous gliotoxin addition. A. fumigatus gliP was up-regulated log_2 3.4-fold, gliA was up-regulated log_2 8.5-fold and A. fumigatus gliF was up-regulated log_2 4.7-fold. Additionally, A. fumigatus gliM was up-regulated log_2 6.9-fold in A. fumigatus ΔgliT exposed to exogenous gliotoxin, which was not observed in A. fumigatus wild-type upon gliotoxin exposure. Of the remaining gliotoxin biosynthetic genes, while not significant, with the exception of A. fumigatus gliZ and gliI which did not exhibit altered expression, and gliF which appeared to undergo down-regulated expression, increased expression of the remaining genes was observed in A. fumigatus ΔgliT upon exposure to exogenous gliotoxin. Quantitative real-time PCR (qRT-PCR) analysis of the expression of A. fumigatus gliZ confirmed the up-regulation in A. fumigatus wild-type exposed to exogenous gliotoxin compared to the control, however it also showed increased gliZ expression in ΔgliT in response to gliotoxin (Figure 5),
which was not observed in the RNA-seq analysis, possibly due to the sensitivities of the different techniques. qRT-PCR analysis of the expression of \textit{A. fumigatus} \textit{gliA} in both \textit{A. fumigatus} wild-type and \textit{A. fumigatus ΔgliT} in both the absence and presence of exogenous gliotoxin confirmed the up-regulation in response to exogenous gliotoxin (Figure 5).

The impact of gliotoxin on the expression of the gliotoxin biosynthetic genes is in accordance with that observed in other studies using different techniques [2,9]. Moreover, feeding experiments with [\textsuperscript{13}C]-phenylalanine herein confirm, for the first time, that gliotoxin biosynthesis is actually induced by exogenous gliotoxin. A significant increase ($p = 0.0295$) was observed in the amount of [\textsuperscript{13}C]-gliotoxin detectable in the wild-type \textit{A. fumigatus} Af293 culture supernatants following exogenous gliotoxin addition compared to the methanol control (Figure 6).

\textbf{Exogenous gliotoxin results in down-regulation of secondary metabolite gene cluster expression in \textit{A. fumigatus ΔgliT}}

FunCat classification of the altered gene expression in \textit{A. fumigatus} wild-type and ΔgliT, respectively, revealed an enrichment of genes involved in secondary metabolism upon exogenous gliotoxin exposure. In \textit{A. fumigatus} wild-type, expression of 16 genes which was up-regulated, and that of 3 genes down-regulated in response to exogenous gliotoxin, were classified by 2\textsuperscript{nd} level of FunCat as being involved in secondary metabolism (Additional file 2:

![Image](Figure 4 Functional classification into FunCat 2\textsuperscript{nd} level categories of significantly A. up-regulated genes in \textit{A. fumigatus ΔgliT} exposed to gliotoxin compared to the MeOH control and B. down-regulated genes in \textit{A. fumigatus ΔgliT} exposed to gliotoxin compared to the MeOH control.)
Tables S5 and S6). A larger number of genes were classified in this category in *A. fumigatus ΔgliT* in response to exogenous gliotoxin presence, where 22 up-regulated genes and 103 down-regulated genes were observed (Additional file 2: Tables S7 and S8). A closer inspection of some of the secondary metabolite genes clusters revealed significant alterations in the expression of genes in the helvolic biosynthesis cluster [25] and the “supercluster” on chromosome 8 which encodes the biosynthetic pathways of a number of secondary metabolites, including fumitremorin B, fumagillin and pseurotin A [26-29] (Table 2).

Helvolic acid, a triterpene, is encoded by a 9-gene cluster on chromosome 4 [25]. In *A. fumigatus* wild-type, exogenous gliotoxin results in the significant up-regulation of 2 genes, *A. fumigatus osc3* (log2 3.28-fold) and *sdr1* (log2 3.22-fold) (Table 2). Conversely, in *A. fumigatus ΔgliT* exposed to exogenous gliotoxin, osc3 was significantly down-regulated (log2 6.3-fold) along with a predicted O-methyltransferase, AFUA_4G14580, which is significantly down-regulated (log2 2.7-fold) (Table 2).

In addition to this, expression of *cyp5081BJ* is completely abrogated in *A. fumigatus ΔgliT* exposed to exogenous gliotoxin (Table 2). qRT-PCR analysis of *A. fumigatus osc3* confirmed the observed down-regulation in *A. fumigatus ΔgliT* and up-regulation of *A. fumigatus osc3* in *A. fumigatus* wild-type exposed to exogenous gliotoxin (Figure 5).

Of the 69 genes in the “supercluster” on chromosome 8, expression of two genes is significantly down-regulated in *A. fumigatus* wild-type in response to exogenous gliotoxin (Table 2). However, in *A. fumigatus ΔgliT*, when exposed to exogenous gliotoxin, expression of 26 genes from the “supercluster” was down-regulated (Table 2). Closer inspection of the fumitremorin B biosynthetic genes revealed that expression of the non-ribosomal peptide synthetase (NRPS), *A. fumigatus ftmA* [29], was significantly down-regulated (log2 2.78-fold) in *A. fumigatus* wild-type in exogenous gliotoxin presence (Table 2). While in *A. fumigatus ΔgliT*, *ftma* (log2 6.65-fold), *ftmC* (log2 5.68-fold), *ftmD* (log2 5.87-fold), *ftmg* (log2 6.52-fold) and *ftml* (log2 1.45-fold) expression was significantly down-regulated, and *ftme* expression was completely inhibited in response to exogenous gliotoxin (Table 2). qRT-PCR analysis of *A. fumigatus ftmA* confirmed decreased expression in both wild-type and *ΔgliT* following exposure to exogenous gliotoxin (Figure 5). Determination of the levels of the fumitremorins and associated compounds in *A. fumigatus* wild-type and *ΔgliT* cultured in secondary metabolite-inducing conditions (96 h in Czapeks-Dox Broth) revealed significant alterations in the levels of a number of cognate metabolites. Specifically, brevianamide F levels were significantly increased in *A. fumigatus ΔgliT* compared to wild-type (*p* =0.0243), while the levels of both tryprostatin A and tryprostatin B were significantly decreased (*p* =0.008 and *p* =0.0453) in *A. fumigatus ΔgliT* compared to wild-type (Figure 7). There was no significant difference determined in the level of fumitremorin C between *A. fumigatus* wild-type and *ΔgliT* (Figure 7).

There was no significant dysregulation of the fumagillin biosynthetic genes in *A. fumigatus* wild-type in exogenous gliotoxin presence. However, when *A. fumigatus ΔgliT* was exposed to exogenous gliotoxin, expression of 12 fumagillin biosynthetic genes in the cluster was significantly down-regulated (*p* <0.05) (Table 2). Some of the down-regulated genes include a polyketide synthase (PKS) (*A. fumigatus fma-PKS*) [30] and a putative PKS (AFUA_8G00490) [27] which were down-regulated log2 7.20- and log2 5.65-fold,
Figure 5 qRT-PCR analysis of *A. fumigatus* gliZ (A), gliA (B), osc3 (C), ftmA (D), fma-pks (E), psoA (F), laeA (G), AFUA_3G13700 (H), sidH (I) and optB (J) expression in *A. fumigatus* wild-type and *A. fumigatus* ΔgliT in the presence and absence of exogenous gliotoxin.
Figure 6 (See legend on next page.)
respectively, *A. fumigatus* metabolite expression was down-regulated \( \log_2 5.20 \)-fold and \( \log_2 8.21 \)-fold. The decreased expression of *A. fumigatus* fma-PKS in *A. fumigatus* \( \Delta \)gliT exposed to exogenous gliotoxin was confirmed by qRT-PCR analysis (Figure 5). As was the case for fumitremorgin B and fumagillin, none of the peptidin A biosynthetic genes were differentially regulated in *A. fumigatus* wild-type in exogenous gliotoxin presence (Table 2). However, in *A. fumigatus* \( \Delta \)gliT exposed to exogenous gliotoxin, with the exception of AFUA_8G00570, expression of all of the peptidin A biosynthetic genes was significantly down-regulated (Table 2). Expression of AFUA_8G00530 and *A. fumigatus* psoA/nrps14, the PKS-NRPS hybrid [28], were down-regulated \( \log_2 7.45 \) and \( \log_2 5.79 \)-fold respectively, while *A. fumigatus* psoC, psoD and elfB [31] expression was down-regulated \( \log_2 7.41 \), \( \log_2 6.78 \) and \( \log_2 8.21 \)-fold respectively. qRT-PCR confirmed the decreased expression of *A. fumigatus* psoA/nrps14 in *A. fumigatus* \( \Delta \)gliT upon exogenous gliotoxin exposure (Figure 5). Relevant, under secondary metabolite inducing growth conditions, production of fumagillin and peptidin A was significantly reduced (\( p = 0.0471 \) and \( p = 0.0297 \), respectively) in *A. fumigatus* \( \Delta \)gliT compared to wild-type (Figure 7).

The methyltransferase, laeA, is a global regulator of secondary metabolism in *A. fumigatus*, which fully or partially regulates expression of multiple secondary metabolite gene clusters, including those encoding gliotoxin, helvolic acid, peptidin A, fumagillin and fumitremorgin biosynthesis [26,27,32,33]. Although dysregulation of secondary metabolite gene expression in *A. fumigatus* \( \Delta \)gliT compared to *A. fumigatus* was observed after exposure to gliotoxin, laeA expression was not differentially regulated in *A. fumigatus* wild-type in exogenous gliotoxin presence, but was significantly down-regulated (\( p = 0.015 \); \( \log_2 1.31 \)-fold) in *A. fumigatus* \( \Delta \)gliT under identical conditions. Although significantly down-regulated, it was outside the cut-off of \( \log_2 1.5 \)-fold change. qRT-PCR analysis for *A. fumigatus* laeA did not show any differential regulation in either *A. fumigatus* wild-type or *A. fumigatus* \( \Delta \)gliT when challenged with exogenous gliotoxin (Figure 5). This suggests that laeA expression is not solely responsible for the altered expression of secondary metabolite genes, particularly in *A. fumigatus* \( \Delta \)gliT when it is exposed to exogenous gliotoxin. Expression of *A. fumigatus* gliT in \( \Delta \)laeA [32] exposed to exogenous gliotoxin for 3 h was analysed. *A. fumigatus* gliT expression increased following exogenous gliotoxin exposure in \( \Delta \)laeA (Figure 8) indicating that loss of laeA does not affect gliT expression in the presence of exogenous gliotoxin. Recently, the global regulatory velvet gene, *A. fumigatus* veA, has been implicated in secondary metabolite biosynthesis regulation, particularly with respect to gliotoxin [20] and fumagillin [22]. However, from the data presented here, *A. fumigatus* veA is not differentially regulated in either *A. fumigatus* wild-type or *A. fumigatus* \( \Delta \)gliT in exogenous gliotoxin presence. *A. fumigatus* gliT expression in *A. fumigatus* \( \Delta \)veA [34] was assessed following exposure to exogenous gliotoxin and was increased in \( \Delta \)veA in the presence of exogenous gliotoxin (Figure 8). *A. fumigatus* gliT expression was higher in the methanol control (Figure 8) compared to the other mutants under the same conditions. Expression of *A. fumigatus* gliT in \( \Delta \)veA grown in media only was lower than the methanol control (data not shown), suggesting that methanol induced increased gliT expression in *A. fumigatus* \( \Delta \)veA. The increased gliT expression in *A. fumigatus* \( \Delta \)veA following methanol addition was only observed in this strain, and was not observed in other deletion strains generated in this background (e.g. \( \Delta \)laeA).

The expression of *A. fumigatus* gliT was also investigated in deletion mutants with abrogated fumitremorgin B, fumagillin and peptidin A production namely, *A. fumigatus* \( \Delta \)fapR, \( \Delta \)fmaA and \( \Delta \)psoA, respectively [27]. As was the case in *A. fumigatus* laeA, expression of *A. fumigatus* gliT increased in these mutants following exogenous gliotoxin exposure (Figure 8) indicating that loss of production of these metabolites does not affect gliT expression.

Siderophore-iron transport and siderophore biosynthesis

**is affected by exogenous gliotoxin and to a greater extent in *A. fumigatus* **

Functional classification of the differentially regulated gene set revealed an enrichment of genes involved in siderophore-iron transport in both *A. fumigatus* wild-type and *A. fumigatus* \( \Delta \)gliT when exposed to exogenous gliotoxin. In *A. fumigatus* wild-type exposed to exogenous gliotoxin, expression of six siderophore-iron transport genes was up-regulated, while in *A. fumigatus*...
Table 2 Log$_2$ (fold change) in helvolic acid, fumitremorgin, fumagillin and pseurotin A biosynthetic gene cluster expression in *A. fumigatus* wild-type and ΔgliT exposed to exogenous gliotoxin

| Gene          | Gene name | Log$_2$(fold change) | q value | Log$_2$(fold change) | q value |
|---------------|-----------|----------------------|---------|----------------------|---------|
| **Helvolic acid** |           |                      |         |                      |         |
| AFUA_4G14770  | osc3      | 3.279                | 0.005   | −6.296               | 0.010   |
| AFUA_4G14780  | cyp5081A1 | 2.425                | 0.076   | −6.515               | 0.092   |
| AFUA_4G14790  | cyp5081B1 | 1.993                | 0.228   | −6.515               | 0.092   |
| AFUA_4G14800  | sdr1      | 3.219                | 0.049   | −7.163               | 0.293   |
| AFUA_4G14810  | cyp5081D1 | 2.633                | 0.200   | −3.952               | 0.053   |
| AFUA_4G14820  |          | 2.701                | 0.057   | −5.964               | 0.063   |
| AFUA_4G14830  | cyp5081C1 | 0.470                | 1.000   | −4.229               | 1.000   |
| AFUA_4G14840  |          | 0.960                | 0.761   | −2.702               | 0.021   |
| **Fumitremorgin B** |           |                      |         |                      |         |
| AFUA_8G00170  | ftmA      | −2.777               | 0.008   | −6.649               | 0.028   |
| AFUA_8G00190  | ftmC      | −1.278               | 0.205   | −5.681               | 0.0005  |
| AFUA_8G00200  | ftmD      | −1.263               | 0.181   | −5.870               | 0.0005  |
| AFUA_8G00210  | ftmPT1    | −1.456               | 0.310   | −8.843               | 0.092   |
| AFUA_8G00220  | ftmE      | −0.614               | 1.000   | Absent$^a$           | 0.0005  |
| AFUA_8G00230  | ftmF      | −0.474               | 0.901   | −10.867              | 0.293   |
| AFUA_8G00240  | ftmG      | 0.611                | 0.844   | −6.519               | 0.019   |
| AFUA_8G00250  | ftmPT2    | 0.581                | 0.844   | −7.300               | 0.074   |
| AFUA_8G00260  | ftmI      | 0.031                | 0.997   | −1.455               | 0.039   |
| **Fumagillin** |           |                      |         |                      |         |
| AFUA_8G00370  | fma-PKS   | 0.396                | 0.946   | −7.287               | 0.0005  |
| AFUA_8G00380  | fma-AT    | 0.264                | 0.968   | −9.364               | 0.074   |
| AFUA_8G00390  |          | 0.220                | 0.976   | −7.902               | 0.0005  |
| AFUA_8G00400  |          | −0.025               | 0.997   | −8.094               | 0.022   |
| AFUA_8G00410  | metAP/fpall| −0.113               | 0.987   | −5.196               | 0.0005  |
| AFUA_8G00420  | fapR/fumR | −0.284               | 0.957   | −5.826               | 0.0005  |
| AFUA_8G00430  |          | 0.265                | 0.969   | −7.093               | 0.0005  |
| AFUA_8G00440  | psoF      | 0.005                | 0.998   | −5.426               | 0.0005  |
| AFUA_8G00460  | fpal      | −0.301               | 0.919   | −0.282               | 0.633   |
| AFUA_8G00470  | fmaE      | −0.176               | 0.968   | −0.271               | 0.636   |
| AFUA_8G00480  | fmaF      | 0.427                | 0.919   | −7.258               | 0.0005  |
| AFUA_8G00490  |          | 0.306                | 0.959   | −5.649               | 0.007   |
| AFUA_8G00500  |          | −0.047               | 0.997   | −6.780               | 0.002   |
| AFUA_8G00510  | fmaG      | 0.793                | 0.820   | −8.101               | 0.0005  |
| AFUA_8G00520  | fma-TC/fmaA| 0.528               | 0.904   | −8.369               | 0.022   |
| **Pseurotin A** |           |                      |         |                      |         |
| AFUA_8G00530  | psoB      | 0.415                | 0.946   | −7.450               | 0.0005  |
| AFUA_8G00540  | psoA/NRPS14| 0.768               | 0.776   | −5.792               | 0.0005  |
| AFUA_8G00550  | psoC      | 0.292                | 0.964   | −7.413               | 0.0005  |
| AFUA_8G00560  | psoD      | 0.808                | 0.735   | −6.778               | 0.0005  |
ΔgliT, expression of two siderophore-iron transport genes was up-regulated and 12 genes are down-regulated (Table 3). *A. fumigatus* sidF was de novo expressed in *A. fumigatus* wild-type upon exposure to exogenous gliotoxin. Expression of *A. fumigatus* mirD was up-regulated in both *A. fumigatus* wild-type (log₂ 2.57-fold) and *A. fumigatus* ΔgliT (log₂ 3.07-fold) in exogenous gliotoxin presence. Interestingly, three genes that were significantly up-regulated in *A. fumigatus* wild-type exposed to exogenous gliotoxin were significantly down-regulated in *A. fumigatus* ΔgliT, when challenged with exogenous gliotoxin.

*ΔgliT* expression in helvolic acid, fumitremorgin, fumagillin and pseurotin A biosynthetic gene cluster expression in *A. fumigatus* wild-type and ΔgliT exposed to exogenous gliotoxin (Continued)

| Gene       | Log₂ (fold change) | Relative expression |
|------------|--------------------|---------------------|
| AFUA_8G00570 | −0.271             | 0.965               |
| AFUA_8G00580 | 0.306              | 0.964               |
| AFUA_8G00590 | −0.982             | 0.239               |

*Gene expression absent in ΔgliT_Glio.*

Figure 7 Levels of fumagillin, pseurotin A, fumitremorgin C, tryprostatin B and tryprostatin A in *A. fumigatus* wild-type and ΔgliT following 96 h growth under secondary metabolite inducing conditions. Fumagillin (A) and pseurotin A (B) production was significantly reduced in *A. fumigatus* ΔgliT compared to wild-type (*p*=0.0471 and *p*=0.0297, respectively). There was no significant difference in fumitremorgin C (C) levels, while brevianamide F (D) levels were significantly increased (*p*=0.0243) in ΔgliT compared to wild-type. Tryprostatin B (E) and tryprostatin A (F) levels were significantly reduced in ΔgliT compared to wild-type (*p*=0.0453 and **p*=0.008, respectively).
exposure log2 7.06- and log2 7.82-fold in \textit{A. fumigatus} \textit{ΔgliT}. Expression of AFUA_3G13700 in both \textit{A. fumigatus} wild-type and \textit{ΔgliT} exposed to exogenous gliotoxin, was confirmed by qRT-PCR (Figure 5).

The observed differential regulation of genes involved in siderophore-iron transport prompted us to review whether expression of siderophore biosynthetic genes \cite{35} was affected by exogenous gliotoxin in \textit{A. fumigatus} wild-type and \textit{A. fumigatus} \textit{ΔgliT}. Indeed we have already noted that \textit{sidF} expression, a transacylase that transfers anhydromevalonyl to hydroxyornithine during extracellular siderophore biosynthesis \cite{36}, was activated in \textit{A. fumigatus} wild-type following exogenous gliotoxin exposure. In \textit{A. fumigatus} \textit{ΔgliT}, expression of two genes involved in siderophore biosynthesis was differentially regulated in response to exogenous gliotoxin (Table 3). \textit{A. fumigatus} \textit{sidH}, involved in providing the anhydromevalonyl-CoA moiety for extracellular siderophore biosynthesis \cite{37}, was \textit{de novo} expressed while \textit{A. fumigatus} \textit{sidA}, which is required for the first step of siderophore biosynthesis \cite{38}, was significantly up-regulated log2 2.16-fold (p =0.0005). qRT-PCR analysis of the expression of \textit{A. fumigatus} \textit{sidH} confirmed the increased expression in \textit{A. fumigatus} \textit{ΔgliT} following exogenous gliotoxin exposure (Figure 5).

**Nitrogen metabolism is down-regulated in \textit{A. fumigatus} \textit{ΔgliT} in response to exogenous gliotoxin**

In \textit{A. fumigatus} \textit{ΔgliT} exposed to exogenous gliotoxin, expression of 55 genes involved in nitrogen metabolism was down-regulated, while expression of 4 genes was
up-regulated (Table 4). In contrast, 3 genes were up-regulated in *A. fumigatus* wild-type and 8 genes were down-regulated in response to exogenous gliotoxin (Table 4). In *A. fumigatus ΔgliT*, expression of two genes, *cyp5081B1*, a putative cytochrome P450 monooxygenase already mentioned in helvolic acid biosynthesis [25], and *AFUA_6G00412*, predicted to have amino acid transmembrane transporter activity [39] was completely abrogated in response to exogenous gliotoxin. Expression of *A. fumigatus optB* and *cpsI*, which are induced when BSA is the sole nitrogen source [40], was downregulated log2 5.399- and log2 3.98-fold, respectively, in *A. fumigatus ΔgliT* exposed to exogenous gliotoxin. The decreased expression of *A. fumigatus optB* in *A. fumigatus ΔgliT* exposed to exogenous gliotoxin was confirmed by qRT-PCR (Figure 5). When utilising complex nitrogen sources, *A. fumigatus* secretes proteases, and increases expression of amidase, aminotransferase, and amino acid and peptide transporter genes [40,41]. These secreted proteases are regulated by a conserved regulatory factor, *prtT* [40,41], which is decreased log2 3.66-fold in expression in ΔgliT but unchanged in wild-type following exogenous gliotoxin exposure. Indeed, further examination revealed decreased expression of 14 proteases, 3 amidases and 7 amino acid and peptide transporters in ΔgliT exposed to exogenous gliotoxin, while

### Table 3 Log2 (fold change) in siderophore-iron transport gene and siderophore biosynthetic gene expression in *A. fumigatus* wild-type and ΔgliT exposed to exogenous gliotoxin

| Gene            | Gene name | Wild-type_Glio v Wild-type_MeOH | ΔgliT_Glio v ΔgliT_MeOH |
|-----------------|-----------|---------------------------------|------------------------|
|                 |           | Log2(fold change) q value        | Log2(fold change) q value |
| Siderophore biosynthesis genes |           |                                 |                        |
| AFUA_1G17190    | sidI      | 2.393 1.000                      | 0.859 1.000            |
| AFUA_1G04450    | sidL      | −0.156 0.969                     | −0.148 0.798           |
| AFUA_1G17200    | sidC      | 1.247 0.105                      | −0.232 0.694           |
| AFUA_2G07680    | sidA      | 1.066 0.155                      | 2.157 0.0005           |
| AFUA_2G08590    | pptA      | 0.534 0.738                      | 0.854 0.143            |
| AFUA_3G03400    | sidF      | Present# 0.005                   | 4.968 0.227            |
| AFUA_3G03410    | sidH      | 3.013 0.172                      | Presentb 0.0005        |
| AFUA_3G03420    | sidD      | 4.657 1.000                      | 2.815 1.000            |
| AFUA_3G03650    | sidG      | Present# 1.000                   | Presentb 1.000         |
| AFUA_3G03660    | estB      | 1.113 1.000                      | 5.852 0.308            |
| AFUA_5G11260    | sreA      | −0.740 0.510                     | −1.152 0.014           |
| Siderophore-iron transport genes |           |                                 |                        |
| AFUA_1G01430    | -         | −1.371 0.080                     | −2.561 0.0005          |
| AFUA_1G14340    | -         | 0.206 0.957                      | −3.197 0.0005          |
| AFUA_1G16040    | -         | 0.081 0.990                      | −2.115 0.004           |
| AFUA_2G01270    | -         | 0.011 0.997                      | −1.605 0.031           |
| AFUA_3G01360    | -         | 0.251 0.969                      | −2.778 0.045           |
| AFUA_3G02670    | -         | −1.506 0.054                     | −2.514 0.0005          |
| AFUA_3G03400    | sidF      | Present# 0.005                   | 4.968 0.227            |
| AFUA_3G03440    | mirD      | 2.569 0.005                      | 3.075 0.0005           |
| AFUA_3G13670    | -         | 7.033 0.005                      | −7.058 0.0005          |
| AFUA_3G13700    | -         | 7.619 0.005                      | −7.824 0.0005          |
| AFUA_4G03940    | fre7      | 2.803 0.005                      | −2.765 0.005           |
| AFUA_6G02170    | -         | −1.026 0.189                     | −3.820 0.0005          |
| AFUA_6G02820    | -         | 0.290 0.926                      | −2.396 0.0005          |
| AFUA_6G13750    | -         | 2.256 0.005                      | −0.998 0.146           |
| AFUA_7G06060    | sirI      | 0.927 0.306                      | 2.785 0.0005           |
| AFUA_8G06210    | -         | Present# 1.000                   | Absentc 0.0005         |

* Switched on in ATCC46645_Glio, b switched on in ΔgliT_Glio, c Gene expression absent in ΔgliT_Glio.
Table 4 Log₂ (fold change) in nitrogen metabolic gene, protease gene, amidase gene and amino acid transporter gene expression in *A. fumigatus* wild-type and ΔgliT exposed to exogenous gliotoxin

| Gene   | Gene name | Wild-type_Glio v Wild-type_MeOH | ΔgliT_Glio v ΔgliT_MeOH |
|--------|-----------|---------------------------------|--------------------------|
|        |           | Log₂(fold_change) | q_value | Log₂(fold_change) | q_value |
| AFUA_1G02780 | -         | -2.005             | 0.064 | -1.839          | 0.004  |
| AFUA_1G04160 | -         | -1.796             | 0.015 | -3.834          | 0.0005 |
| AFUA_1G04560 | -         | -0.364             | 0.922 | -2.161          | 0.0005 |
| AFUA_1G11250 | -         | -0.610             | 0.695 | -3.325          | 0.0005 |
| AFUA_1G12850 | crnA      | 0.276              | 0.963 | -2.689          | 0.002  |
| AFUA_1G13220 | -         | 0.455              | 0.835 | -3.209          | 0.0005 |
| AFUA_1G17470 | ornB      | -0.098             | 0.990 | -4.504          | 0.013  |
| AFUA_2G02020 | -         | -0.366             | 0.962 | -4.403          | 0.0005 |
| AFUA_2G02250 | -         | -0.518             | 0.895 | -2.276          | 0.007  |
| AFUA_2G03900 | -         | -0.355             | 0.910 | -3.894          | 0.0005 |
| AFUA_2G10520 | uaZ       | -0.780             | 0.834 | -2.793          | 0.002  |
| AFUA_2G10560 | -         | -0.135             | 0.988 | -4.450          | 0.0005 |
| AFUA_2G12900 | -         | -0.822             | 0.556 | -1.577          | 0.003  |
| AFUA_2G15240 | optB      | 0.002              | 0.999 | -5.399          | 0.0005 |
| AFUA_2G17430 | -         | -0.680             | 0.736 | -4.444          | 0.005  |
| AFUA_3G07040 | cps1      | -0.120             | 0.984 | -3.980          | 0.0005 |
| AFUA_4G01230 | -         | -0.149             | 0.973 | -4.195          | 0.0005 |
| AFUA_4G03770 | -         | -1.008             | 0.311 | -2.329          | 0.0005 |
| AFUA_4G04160 | -         | -1.926             | 0.033 | -0.593          | 0.319  |
| AFUA_4G04170 | -         | -2.622             | 0.005 | -0.820          | 0.144  |
| AFUA_4G07940 | -         | -0.943             | 0.527 | -2.304          | 0.008  |
| AFUA_4G14790 | cyp5081B1 | 1.993              | 0.228 | Absent*         | 0.0005 |
| AFUA_5G00710 | -         | 0.605              | 0.691 | -3.021          | 0.0005 |
| AFUA_5G01360 | -         | -0.498             | 0.891 | -3.701          | 0.024  |
| AFUA_5G0230 | -         | -1.355             | 0.503 | -4.916          | 0.0005 |
| AFUA_5G07520 | -         | -0.378             | 0.922 | -2.654          | 0.014  |
| AFUA_5G13810 | -         | -0.423             | 0.895 | -1.715          | 0.029  |
| AFUA_5G15050 | -         | -1.577             | 0.362 | -3.783          | 0.010  |
| AFUA_6G00412 | -         | 0.915              | 1.000 | Absent*         | 0.0005 |
| AFUA_6G01920 | -         | -1.011             | 0.433 | -1.887          | 0.004  |
| AFUA_6G02030 | -         | -0.313             | 0.926 | -4.194          | 0.0005 |
| AFUA_6G02210 | -         | -1.136             | 0.161 | -3.475          | 0.0005 |
| AFUA_6G06020 | -         | -0.816             | 0.339 | -1.510          | 0.002  |
| AFUA_6G08000 | gmdA      | -1.332             | 0.293 | -2.252          | 0.0005 |
| AFUA_6G10210 | -         | -0.664             | 0.708 | -2.153          | 0.0005 |
| AFUA_7G00910 | optH      | -0.132             | 0.986 | -2.089          | 0.007  |
| AFUA_7G01690 | -         | -1.322             | 0.269 | -1.753          | 0.0005 |
| AFUA_7G02070 | nfr1      | -0.453             | 0.839 | -3.250          | 0.0005 |
| AFUA_7G03850 | -         | -1.776             | 0.018 | -3.643          | 0.0005 |
| AFUA_8G00190 | tmrC      | -1.278             | 0.205 | -5.681          | 0.0005 |
| AFUA_8G01570 | -         | -1.158             | 0.561 | -3.979          | 0.003  |
| AFUA_8G01780 | -         | -1.171             | 0.639 | -2.758          | 0.036  |
| AFUA_8G04340 | -         | -0.308             | 0.910 | -1.530          | 0.001  |
Table 4 Log₂ (fold change) in nitrogen metabolic gene, protease gene, amidase gene and amino acid transporter gene expression in *A. fumigatus* wild-type and ΔgliT exposed to exogenous gliotoxin (Continued)

| Gene   | log₂ Fold Change | p-value | fold change | p-value |
|--------|------------------|---------|-------------|---------|
| AFUA_BG05220 | - | 0.048 | 0.996 | -2.941 | 0.0005 |
| AFUA_BG06580 | - | 0.118 | 0.988 | -4.540 | 0.0005 |
| AFUA_1G13440 | - | 0.520 | 0.792 | -1.692 | 0.010 |
| AFUA_3G0680 | - | -4.122 | 0.005 | -1.910 | 0.0005 |
| AFUA_3G14590 | - | -2.555 | 0.005 | -2.518 | 0.0005 |
| AFUA_4G0630 | - | -1.078 | 0.246 | -3.158 | 0.0005 |
| AFUA_4G09840 | - | -1.815 | 0.028 | -3.253 | 0.0005 |
| AFUA_5G01470 | - | -0.769 | 0.739 | -4.125 | 0.001 |
| AFUA_5G07630 | - | -1.768 | 0.138 | -3.948 | 0.0005 |
| AFUA_7G04180 | - | -3.796 | 0.005 | -0.913 | 0.156 |
| AFUA_8G01580 | - | -4.122 | 0.0005 | -1.910 | 0.0005 |
| AFUA_8G06470 | - | -0.077 | 0.990 | -4.663 | 0.0005 |
| AFUA_5G01450 | - | -1.259 | 0.122 | -3.491 | 0.0005 |
| AFUA_5G08910 | - | -1.162 | 0.102 | -3.491 | 0.0005 |
| AFUA_7G06270 | - | -1.038 | 0.309 | -2.581 | 0.0005 |
| AFUA_1G10820 | - | 0.846 | 0.311 | 1.730 | 0.0005 |
| AFUA_2G15590 | - | 1.173 | 0.130 | 2.559 | 0.0005 |
| AFUA_2G15590 | met16 | -0.276 | 0.942 | 3.029 | 0.0005 |
| AFUA_5G0820 | - | -0.570 | 0.716 | -2.019 | 0.0005 |
| AFUA_4G01440 | - | 1.259 | 0.716 | 2.019 | 0.0005 |
| AFUA_2G09300 | dppV | 1.020 | 0.347 | -6.695 | 0.0005 |
| AFUA_3G00650 | lat2 | -0.065 | 0.990 | -1.608 | 0.008 |
| AFUA_2G07500 | pepP | -0.790 | 0.447 | -1.933 | 0.0005 |
| AFUA_4G03490 | - | -0.845 | 0.649 | -2.546 | 0.0005 |
| AFUA_2G17330 | - | -0.543 | 0.707 | -1.719 | 0.001 |
| AFUA_2G02500 | - | -0.683 | 0.745 | -1.989 | 0.0005 |
| AFUA_6G13540 | cp3 | -0.474 | 0.839 | -1.549 | 0.008 |
| AFUA_6G00310 | cpdS | -0.748 | 0.531 | -3.976 | 0.0005 |
| AFUA_5G13300 | pep1 | -0.833 | 0.678 | -4.357 | 0.0005 |
| AFUA_3G02970 | gprA | -0.228 | 0.979 | -5.119 | 0.0005 |
| AFUA_5G01200 | cp6 | -0.621 | 0.834 | -4.932 | 0.0005 |
| AFUA_8G00410 | metAP | -0.113 | 0.987 | -5.196 | 0.0005 |
| AFUA_5G09140 | - | -0.443 | 0.910 | 3.372 | 0.002 |
| AFUA_5G09990 | - | -0.805 | 0.512 | -4.047 | 0.0005 |
| AFUA_1G09120 | - | -0.272 | 0.962 | -2.985 | 0.002 |
| AFUA_8G00800 | - | 0.641 | 0.899 | -6.795 | 0.0005 |
| AFUA_1G72240 | - | 0.547 | 0.964 | -5.161 | 0.0005 |
| AFUA_8G02550 | - | 0.564 | 0.819 | -8.305 | 0.003 |
| AFUA_7G04200 | - | -0.464 | 0.910 | -2.355 | 0.0005 |
| AFUA_7G05880 | - | -0.463 | 0.861 | -2.099 | 0.003 |

*Gene expression absent in ΔgliT_Glio.*
expression of one amidase, AFUA_5G09140, was increased log2 3.37-fold (Table 4). Expression of both nitrate transporters, crnA and nrtB, which are predicted gene pairs [42], was significantly down-regulated (log2 2.689- and log2 4.504-fold) in A. fumigatus ΔgliT upon exogenous gliotoxin exposure. Expression of a number of genes with predicted involvement in the oxidation-reduction process was down-regulated in A. fumigatus ΔgliT only following exogenous gliotoxin exposure. Among these genes were nitrate monooxygenases (AFUA_2G17430 and AFUA_4G07940 down-regulated log2 4.444- and log2 2.304-fold) and copper ion binding domain genes (AFUA_1G13440, AFUA_5G01470 and AFUA_5G07360; down-regulated log2 1.692-, log2 4.125- and log2 3.948-fold, respectively). In A. fumigatus wild-type exposed to exogenous gliotoxin, two predicted carbon-nitrogen ligases, AFUA_4G04160 and AFUA_4G04170, were down-regulated log2 1.926- and log2 2.622-fold, respectively. These carbon-nitrogen ligases are not differentially regulated in A. fumigatus ΔgliT upon exogenous gliotoxin exposure, suggesting that loss of gliT may hinder the down-regulation of these genes in response to gliotoxin.

**Exogenous gliotoxin exposure alters gene expression involved in ribosome biogenesis and translation in A. fumigatus ΔgliT while transcription related genes are down-regulated**

Dysregulation of ribosome biogenesis and translation was observed in A. fumigatus ΔgliT following exogenous gliotoxin exposure, whereby expression of 20 genes was up-regulated and two genes down-regulated (Table 5). Of the 20 genes up-regulated in A. fumigatus ΔgliT in response to exogenous gliotoxin, 7 genes encode 60S ribosomal proteins and two encode 40S ribosomal proteins, all of which are increased log2 1.5-1.7-fold in expression (Table 5). The two genes down-regulated in response to exogenous gliotoxin in A. fumigatus ΔgliT have already been mentioned as secondary metabolite biosynthetic genes. A. fumigatus metAP in the fumagillin biosynthetic gene cluster [30] was down-regulated log2 4.340-fold and A. fumigatus elfB in the pseurotin A biosynthetic gene cluster was down-regulated log2 7.265-fold [27,31]. In contrast to A. fumigatus ΔgliT, only two genes involved in ribosome biogenesis and translation were up-regulated in A. fumigatus wild-type exposed to exogenous gliotoxin (Table 5). AFUA_5G07340 was up-regulated log2 1.909-fold while AFUA_2G12150 was up-regulated log2 2.417-fold. In addition to the dysregulation of ribosome biogenesis and translation, 44 genes involved in transcription were also down-regulated in A. fumigatus ΔgliT in response to exogenous gliotoxin, compared to 3 down-regulated genes in wild-type exposed to exogenous gliotoxin (Table 6). A proteomic investigation of A. fumigatus ΔgliT ATCC26933 reflected this alteration in translation whereby 6 proteins were uniquely expressed or increased in abundance and 15 proteins were absent or decreased in abundance when exposed to exogenous gliotoxin (Tables 7 and 8). Loss of GlIT negatively impacts expression of genes involved in transcription processes and results in significantly decreased transcription of genes in the presence of exogenous gliotoxin, culminating in disruption of translation.

**Discussion**

To dissect the role played by the gliotoxin oxidoreductase, gliT, in self-protection against gliotoxin, a comparative transcriptomic analysis of the impact of exogenous gliotoxin on A. fumigatus wild-type and ΔgliT was carried out via RNA-seq analysis. To reduce the affects of endogenous gliotoxin, a low gliotoxin-producing background strain, A. fumigatus ATCC46645 [2] was employed, along with Sabouraud-Dextrose media which is non-permissive for gliotoxin biosynthesis [5]. Exogenous gliotoxin exposure resulted in changed expression of 164 genes in A. fumigatus wild-type. However, altered expression of over 1700 genes was observed in A. fumigatus ΔgliT. Closer inspection revealed alterations in expression of clusters encoding secondary metabolites, particularly gliotoxin, helvolic acid, fumitremorin, fumagillin and pseudotin A biosynthesis genes, siderophore biosynthesis genes, ribosome biogenesis genes and genes involved in translation and nitrogen metabolism.

Exogenous gliotoxin induces the expression of gliotoxin biosynthetic genes in both A. fumigatus wild-type and ΔgliT. Expression of gliZ was increased in expression in wild-type, along with gliP, the bimodular non-ribosomal peptide synthetase that produces the cyclo-L-Phe-L-Ser diketopiperazine intermediate [9,10,43]. Up-regulation of gliZ and gliP expression in particular, along with gliT suggests that exogenous gliotoxin induces de novo gliotoxin production in wild-type. Indeed this has been shown to be the case in A. fumigatus AF293 where addition of exogenous gliotoxin under gliotoxin-inducing culture conditions resulted in detection of de novo gliotoxin production determined by the use of [13C]-Phenylalanine and LC-MS analysis. Recently, cyclo-L-Phe-L-Ser was determined to be a major component of the metabolome and was detected in lung tissue of infected mice [11,44]. The production of this compound may be increased consequent to exogenous gliotoxin exposure in wild-type due to up-regulation of gliP expression. It has been demonstrated that A. fumigatus gliT expression is not solely under the control of gliZ [2], and so up-regulation of gliZ expression is not absolutely required to induce gliT expression for the purpose of self-protection. While gliZ expression is not significantly altered in ΔgliT following exogenous gliotoxin exposure in the RNA-seq analysis, qRT-PCR demonstrated increased expression under these conditions.
Expression of a number of gli genes was up-regulated in ΔgliT following exogenous gliotoxin exposure, including gliM, which was not altered in wild-type. *A. fumigatus* gliM is a predicted O-methyltransferase [3] and methylation has been proposed as a method of self-protection against disulfide bridge-containing metabolites. Holomycin contains a disulfide bridge similar to gliotoxin [16,17,45] and S-methylation has been proposed as an alternative method of self-protection against this antibiotic and its biosynthetic intermediates in *S. clavuligerus* upon deletion of the dithiol oxidase, HlmI, which is functionally homologous to *A. fumigatus* gliT [17,45]. In *Y. ruckeri*, an RNA methyltransferase, Hom12, methylates RNA in a proposed self-protection mechanism against the cytotoxic effects of holomycin [16]. Interestingly, in *A. fumigatus* ΔgliT following exogenous gliotoxin exposure, a tRNA methyltransferase, AFUA_4G12280, is up-regulated log2 2.56-fold, while its expression is unaltered in wild-type in the presence of exogenous gliotoxin. The increased expression of these methyltransferases, among others, in *A. fumigatus* ΔgliT following exposure to exogenous gliotoxin may suggest possible alternative functionalities, or self-protection mechanisms against gliotoxin in the absence of GliT. Interestingly, a newly-identified methyltransferase, gliotoxin methyltransferase A (GtmA), has been demonstrated to play a role in attenuating gliotoxin biosynthesis [46]. GtmA (AFUA_2G11120) expression is significantly up-regulated (p <0.00005) by gliotoxin exposure (Additional file 1).

In addition to up-regulation of the gli cluster, dysregulation of other secondary metabolite gene clusters was observed upon exogenous gliotoxin addition, particularly to *A. fumigatus* ΔgliT. Up-regulated expression of two genes; osc3 and sdrI, from the gene cluster encoding the fusidane antibiotic helvolic acid was observed upon exogenous gliotoxin addition to *A. fumigatus* wild-type [25]. However, exogenous gliotoxin addition to *A. fumigatus* ΔgliT resulted in down-regulated expression of two genes and abrogation of the expression of cyp5081D1, suggesting that gliT deletion, along with exogenous gliotoxin exposure.

| Gene   | Gene name | Wild-type_Glio v Wild-type_MeOH | ΔgliT_Glio v ΔgliT_MeOH |
|--------|-----------|--------------------------------|------------------------|
|        | Log2(fold_change) | q_value | Log2(fold_change) | q_value |
| AFUA_3G05600 | -          | 1.122  | 0.228          | 1.647     | 0.004 |
| AFUA_3G06760 | -          | 1.249  | 0.075          | 1.763     | 0.001 |
| AFUA_5G07340 | -          | 1.909  | 0.012          | 0.268     | 0.728 |
| AFUA_6G05200 | -          | 1.061  | 0.227          | 1.590     | 0.008 |
| AFUA_6G13250 | -          | 1.203  | 0.117          | 1.609     | 0.003 |
| AFUA_8G00410 | metAP     | -0.113 | 0.987          | −4.340    | 0.001 |
| AFUA_8G00580 | ellB      | 0.306  | 0.964          | −7.265    | 0.003 |
| AFUA_2G09200 | -          | 1.100  | 0.143          | 1.510     | 0.003 |
| AFUA_3G07360 | -          | 0.492  | 0.739          | 1.506     | 0.004 |
| AFUA_1G12890 | -          | 1.191  | 0.212          | 1.595     | 0.006 |
| AFUA_1G15020 | -          | 1.036  | 0.277          | 1.545     | 0.008 |
| AFUA_2G10300 | -          | 1.353  | 0.060          | 1.537     | 0.004 |
| AFUA_2G12150 | -          | 2.417  | 0.005          | 0.561     | 0.422 |
| AFUA_3G08080 | -          | 0.214  | 0.952          | 1.655     | 0.001 |
| AFUA_4G07250 | -          | 0.599  | 0.589          | 1.572     | 0.002 |
| AFUA_4G07730 | -          | 1.190  | 0.163          | 1.592     | 0.004 |
| AFUA_4G11990 | -          | 0.309  | 0.891          | 1.682     | 0.001 |
| AFUA_5G05630 | -          | 1.222  | 0.122          | 1.661     | 0.002 |
| AFUA_5G06430 | -          | 0.559  | 0.649          | 1.501     | 0.002 |
| AFUA_6G02440 | -          | 1.006  | 0.272          | 1.658     | 0.002 |
| AFUA_6G02450 | -          | 0.714  | 0.493          | 1.564     | 0.003 |
| AFUA_6G11260 | -          | 1.105  | 0.232          | 1.532     | 0.008 |
| AFUA_6G12960 | -          | 0.521  | 0.686          | 1.721     | 0.001 |
| AFUA_2G10100 | aspB      | 1.295  | 0.138          | 1.580     | 0.007 |
negatively regulates the helvolic acid gene cluster. In an A. fumigatus gliZ complemented strain, helvolic acid production was detectable at 37°C, whereas no helvolic acid was detectable in wild-type or ΔgliZ strains at an identical growth temperature [10], suggesting that gliZ may be involved in regulating helvolic acid production. Indeed in the present study in wild-type, gliZ expression was up-regulated upon gliotoxin exposure and the helvolic acid biosynthetic genes, including osc3 which encodes the protein that catalyses the first biosynthetic step of helvolic acid biosynthesis [25], are also up-regulated, whereas in ΔgliT, the helvolic acid biosynthesis genes are down-regulated. However, we cannot unambiguously assign gliZ functionality to hevolic acid production because consistently discrepant gliZ expression data was obtained by RNA-seq and qRT-PCR (Figure 5).

Table 6 Log2 (fold change) in transcription gene expression in A. fumigatus wild-type and ΔgliT exposed to exogenous gliotoxin

| Gene         | Gene name | Log2(fold change) | q_value | Log2(fold change) | q_value |
|--------------|-----------|-------------------|---------|-------------------|---------|
| AFUA_4G11480 | -         | -0.766            | 0.446   | -1.532            | 0.003   |
| AFUA_6G05160 | azf1      | -1.541            | 0.043   | -0.251            | 0.753   |
| AFUA_6G12150 | -         | 0.502             | 0.913   | -6.233            | 0.001   |
| AFUA_7G01340 | -         | 0.082             | 0.990   | -2.982            | 0.001   |
| AFUA_4G02930 | -         | 0.450             | 0.796   | -1.971            | 0.001   |
| AFUA_3G10120 | -         | -0.819            | 0.488   | -1.669            | 0.003   |
| AFUA_4G10110 | htaA      | 0.105             | 0.986   | -1.565            | 0.012   |
| AFUA_1G10080 | zosA      | -0.434            | 0.843   | -1.535            | 0.001   |
| AFUA_1G01240 | -         | -0.561            | 0.770   | -1.702            | 0.025   |
| AFUA_1G14945 | -         | -0.839            | 0.505   | -1.754            | 0.026   |
| AFUA_2G04262 | -         | -1.644            | 0.012   | -0.759            | 0.171   |
| AFUA_2G11180 | ribA      | -0.126            | 0.977   | -1.511            | 0.003   |
| AFUA_3G03330 | -         | -2.132            | 0.021   | -0.739            | 0.221   |
| AFUA_5G03780 | -         | 1.277             | 0.320   | -2.864            | 0.001   |
| AFUA_5G09720 | -         | -1.780            | 0.086   | -1.586            | 0.033   |
| AFUA_5G14530 | -         | 0.120             | 0.987   | -2.166            | 0.010   |
| AFUA_8G01150 | -         | 0.037             | 0.997   | -3.345            | 0.001   |
| AFUA_8G01940 | -         | -1.248            | 0.221   | -1.846            | 0.027   |
| AFUA_8G02720 | -         | -0.032            | 0.997   | -1.848            | 0.001   |
| AFUA_8G04130 | farB1     | -0.496            | 0.798   | -1.898            | 0.001   |
| AFUA_8G06460 | -         | -0.005            | 0.998   | -1.741            | 0.009   |
| AFUA_6G11740 | -         | -0.919            | 0.475   | -7.145            | 0.004   |
| AFUA_2G05180 | -         | -0.361            | 0.891   | -3.819            | 0.001   |
| AFUA_1G16600 | -         | -0.343            | 0.964   | -4.481            | 0.001   |
| AFUA_8G00280 | -         | -0.720            | 0.574   | -1.813            | 0.007   |

Table 7 Proteins with increased abundance in, or unique to, A. fumigatus ΔgliT ATCC26933 with gliotoxin compared to the methanol control

| Protein IDs | Log2 (fold increase) | p_value | Peptides | Sequence coverage [%] |
|-------------|----------------------|---------|----------|----------------------|
| AFUA_6G06470 | Unique               | N/A     | 6        | 58.5                 |
| AFUA_1G09510 | Unique               | N/A     | 2        | 34.5                 |
| AFUA_4G03140 | Unique               | N/A     | 4        | 12.5                 |
| AFUA_3G14540 | 2.279                | 1.52E-02| 6        | 38.3                 |
| AFUA_1G15270 | 1.135                | 2.05E-02| 33       | 43.1                 |
| AFUA_7G0350* | 1.119                | 1.49E-02| 6        | 23.2                 |

*Gene expression also significantly up-regulated in RNA-seq analysis.
PKS in the fumagillin cluster and the PKS-NRPS hybrid psoA/nrps14 in the pseudotin A cluster were all significantly down-regulated suggesting production of these secondary metabolites is also decreased. A. fumigatus fmaA encodes the enzyme required for the first biosynthetic step of fumitremorgin synthesis, the synthesis of the diketopiperazine, brevianamide F [29], and is significantly down-regulated log2 6.640-fold in A. fumigatus ΔgliT following exogenous gliotoxin addition. Expression of the cytochrome P450 A. fumigatus ftmA, which was absent in ΔgliT exposed to exogenous gliotoxin, encodes the enzyme responsible for formation of fumitremorgin C [48], while another cytochrome P450, ftmG, which encodes the enzyme that dihydroxylates the fumitremorgin B intermediate was significantly down-regulated log2 6.519-fold [48]. We hypothesise that fumitremorgin B biosynthesis is decreased, if not abrogated, in A. fumigatus ΔgliT when exposed to exogenous gliotoxin given the decreased expression of genes essential for synthesis. While determination of fumitremorgin B levels was not successful, measurement of fumitremorgin C and related compounds [48] was carried out in A. fumigatus wild-type and ΔgliT cultured under secondary metabolite-inducing conditions. Significant reductions in the levels of tryprostatin A and tryprostatin B were observed in A. fumigatus ΔgliT compared to wild-type, while there was no significant difference in levels of fumitremorgin C between the two strains. Therefore, GliT loss disrupts the production of brevianamide F, tryprostatin A and tryprostatin B, from the fumitremorgin biosynthetic pathway in A. fumigatus. The PKS, fma-PKS, down-regulated log2 7.287-fold in A. fumigatus ΔgliTS, is essential for fumagillin biosynthesis [30]. Additionally, the C6 type transcription factor fapR/fumR, which controls the expression of the other fumagillin cluster genes [22], was down-regulated log2 5.826-fold in A. fumigatus ΔgliT following exogenous gliotoxin addition. Decreased expression of these two genes, in addition to others in the biosynthetic gene cluster, suggests down-regulation of fumagillin biosynthesis as a direct consequence of A. fumigatus gliT loss combined with exogenous gliotoxin stress. Indeed supporting the hypothesis of A. fumigatus gliT involvement in facilitating fumagillin biosynthesis, under secondary metabolite-inducing conditions we observed a significant decrease in the production of fumagillin in A. fumigatus ΔgliT compared to wild-type. It has been determined that the biosynthetic genes for fumagillin and pseudotin A are physically intertwined [27] and these authors also revealed that fapR, which controls expression of the fumagillin biosynthesis genes, also controls the expression of pseudotin A biosynthesis genes [27]. Indeed, with the exception of one gene, expression of the pseudotin A biosynthetic cluster was significantly down-regulated in A. fumigatus ΔgliT upon exogenous gliotoxin addition. psoA/nrps14 is essential for pseudotin A biosynthesis, as demonstrated by Maiya et al. [28], while over-expression of this gene increased pseudotin A accumulation. In A. fumigatus ΔgliT exposed to exogenous gliotoxin, psoA/nrps14 expression was down-regulated log2 5.826-fold, while in the proteomic investigation, PsoA/nrps14 was decreased log2 3.032-fold in abundance. In addition to PsoA/nrps14, two other proteins required for pseudotin A biosynthesis [27] were decreased in abundance upon exogenous gliotoxin exposure in A. fumigatus ΔgliT. PsoF (AFUA_8G00440) and PsoC (AFUA_8G00550) were decreased log2 2.461- and log2 2.016-fold providing further support to the hypothesis that pseudotin A biosynthesis is down-regulated in the absence of A. fumigatus gliT when challenged with exogenous gliotoxin. As was the case with fumagillin, pseudotin A levels were significantly decreased in A. fumigatus ΔgliT compared to wild-type when cultured under secondary metabolite-inducing conditions again suggesting possible GliT involvement in enabling pseudotin A biosynthesis.

The biosynthetic gene clusters of fumitremorgin, fumagillin and pseudotin are under the control of the global regulator, A. fumigatus laeA [26,27]. A. fumigatus laeA is a methyltransferase and regulates chromatin remodelling through this methyltransferase activity [26,32,49]. In A. fumigatus ΔgliT treated with exogenous gliotoxin, laeA expression was not significantly altered, suggesting that it is not responsible for the down-regulation of these gene clusters. In A. nidulans, LaeA forms a trimeric complex with two members of the

### Table 8 Proteins with decreased abundance in, or absent from, ΔgliTΔATCC26933 with gliotoxin compared to the methanol control

| Protein IDs | Log2(fold decrease) | p value | Peptides | Sequence coverage [%] |
|-------------|----------------------|---------|----------|----------------------|
| AFUA_3G00330* | Unique | N/A | 6 | 38.2 |
| AFUA_5G14000* | Unique | N/A | 7 | 61.3 |
| AFUA_1G10960 | Unique | N/A | 2 | 18.2 |
| AFUA_8G00540* | 3.032 | 1.72E-02 | 37 | 18.7 |
| AFUA_2G15290 | 2.788 | 6.36E-04 | 6 | 81.6 |
| AFUA_8G00440* | 2.461 | 7.98E-04 | 12 | 21.2 |
| AFUA_7G06420 | 2.451 | 3.18E-03 | 18 | 63.5 |
| AFUA_8G00550* | 2.016 | 1.03E-02 | 12 | 63.8 |
| AFUA_2G04060* | 1.799 | 5.33E-03 | 12 | 58.7 |
| AFUA_3G03350* | 1.603 | 2.31E-03 | 50 | 38.6 |
| AFUA_5G07170* | 1.361 | 3.93E-02 | 7 | 44.9 |
| AFUB_044910 | 1.234 | 4.74E-02 | 33 | 26.4 |
| AFUA_1G01010 | 1.128 | 8.60E-02 | 33 | 26.4 |
| AFUA_8G05580 | 1.091 | 3.18E-03 | 18 | 63.5 |
| AFUA_6G10120 | 1.072 | 2.60E-02 | 9 | 46.4 |

*Gene expression also significantly down-regulated in RNA-seq analysis.
velvet protein family; VeA and VelB, and this complex up-regulates secondary metabolism and sexual development [50]. The complex was subsequently identified and characterised in *A. fumigatus* [34]. *A. fumigatus veA* has been demonstrated to regulate fumagillin and gliotoxin production [20,22], however veA expression was not significantly altered in *A. fumigatus ΔgliT* following exogenous gliotoxin production. The bZip transcriptional enhancer RsmA has been shown to positively regulate gliotoxin biosynthesis [44], however the expression of *A. fumigatus rsmA* was not altered significantly in *A. fumigatus ΔgliT*, in the absence or presence of exogenous gliotoxin. Overall, our findings lead us to postulate that the gliotoxin biosynthetic/self-protection capacity (i.e., GliT functionality) is necessary for optimal biosynthesis of selected secondary metabolites in *A. fumigatus*.

Altered expression of siderophore-iron transport and siderophore biosynthetic genes in both *A. fumigatus* wild-type and ΔgliT following exogenous gliotoxin exposure suggests a disruption in iron homeostasis or iron sensing. Iron is an essential nutrient that is required for many cellular processes, including as a cofactor for numerous enzymes [35]. In both wild-type and ΔgliT, siderophore biosynthesis gene expression was up-regulated in response to exogenous gliotoxin indicating that consequent to exogenous gliotoxin exposure, there is an increased requirement for iron despite sufficient iron availability in the culture media, or else a deficit in iron-sensing. *A. fumigatus sidF* expression was activated in wild-type, while in ΔgliT, sidH expression was activated and that of sidA was up-regulated [35]. Interestingly, up-regulation of sidA expression has also been observed in ΔmetR, an *A. fumigatus* mutant deficient in the transcription factor that regulates sulfur assimilation, under iron sufficient but sulfur deficient conditions [21]. Regulatory cross-talk between secondary metabolism and iron requirement has been reported whereby in a laeA mutant deficient in gliotoxin production, decreased expression of a number of the siderophore biosynthesis genes was observed under high iron conditions [26]. These authors concluded that laeA was also involved in regulating expression of the siderophore biosynthetic genes and in particular, sidD. As discussed, we have observed that the combined effect of exogenous gliotoxin exposure and gliT deletion has a significant impact on secondary metabolism, despite laeA expression being unaffected, and therefore it is interesting that siderophore biosynthesis is also affected. In *A. fumigatus* wild-type, expression of six siderophore-iron transport genes was up-regulated in response to exogenous gliotoxin, in contrast to up-regulation of two siderophore-iron transport genes in ΔgliT. Interestingly, in ΔgliT, expression of 12 siderophore-iron transport genes was down-regulated. Taken together, this suggests that deletion of gliT in combination with exogenous gliotoxin exposure results in a disruption of, or decrease in, siderophore-iron transport. Amich et al. [21] noted up-regulation of siderophore-iron transport genes in ΔmetR under sulfur-limited but iron replete conditions. Despite observing increased expression of both siderophore biosynthetic genes and siderophore-iron transport genes in ΔmetR, suggesting iron starvation, the authors noted increased levels of ferricocin, the intracellular siderophore that is used for transport and storage [38,51]. Iron is utilised in many processes one of which is iron-sulfur cluster biosynthesis. Iron-sulfur clusters are inorganic cofactors involved in cellular processes including enzyme activity regulation, mitochondrial respiration, ribosome biosynthesis and cofactor biosynthesis [52]. Translocation of iron-sulfur clusters requires glutathione, and depletions have been shown to induce an iron starvation-like response in *Saccharomyces cerevisiae* [53-55]. It has previously been shown that exogenous gliotoxin exposure to both *A. fumigatus* wild-type and ΔgliT results in decreased GSH levels [5] and so it is conceivable that this decrease in cellular GSH could impact on iron homeostasis or iron sensing. *A. fumigatus* encodes two transcription factors, hapX and sreA, that maintain iron homeostasis whereby hapX represses sreA expression and subsequently iron-consuming pathways, and activates siderophore biosynthesis during iron-starvation, while sreA represses hapX during iron-sufficient conditions in a negative feedback loop [35,56]. Interestingly, induction of gliT has been shown before when cultures were shifted from iron-limited to replete conditions in both wild-type and a sreA deletion strain [57]. Although expression of hapX and sreA is unchanged in both wild-type and ΔgliT upon exogenous gliotoxin exposure, there is significant interplay between gliT, sulfur and iron as demonstrated by the altered expression of siderophore biosynthesis and siderophore-iron transport genes and the decreased GSH levels [5] in *A. fumigatus* ΔgliT.

FunCat analysis identified significant enrichment of genes involved in nitrogen metabolism in the down-regulated gene set in ΔgliT exposed to exogenous gliotoxin. Fungi can utilise various sources of nitrogen, from easily assimilated sources (e.g., ammonium and glutamate), to more complex secondary sources including amino acids and proteins [40,58]. Following exposure to exogenous gliotoxin, the expression of *prtT*, the conserved regulator of secreted proteases, was decreased and consequently, a number of proteases previously identified to be under its control were also decreased in expression, including *alp1* and *pep1* [41]. Additionally, a number of other proteases underwent decreased expression in ΔgliT when exposed to exogenous gliotoxin. These proteases, some of which include *dppV*, *dppVI*, *cpdS* and *gprA*, have been shown to be induced when BSA is the sole nitrogen source [41]. In
order to conserve energy, fungi will preferentially utilise nitrogen sources that are easily assimilated over complex nitrogen sources [59]. Sabouraud-Dextrose medium contains a pancreatic digest of casein and a peptic digest of animal tissue. As the media already contained digested proteins, we conclude that expression of these secreted proteases is significantly down-regulated in ΔgliT exposed to exogenous gliotoxin in order to conserve energy. Interestingly, a number of genes involved in amino acid and peptide transport are also decreased in expression with the exception of one amidase, AFUA_5G09140, the expression of which is up-regulated in ΔgliT following exogenous gliotoxin exposure. This was surprising as, despite the availability of amino acids and peptides in the media, the expression of genes encoding enzymes required for uptake of these amino acids and peptides are down-regulated. It is possible that sufficient enzymes are present for adequate uptake of nitrogen sources to compensate for the decreased expression of these genes, or that the down-regulation of these genes is a consequence of the down-regulation of prtT and the other secreted proteases suggesting that there is a decreased nitrogen requirement in ΔgliT, when exposed to exogenous gliotoxin.

Loss of A. fumigatus gliT had a significant impact on the transcriptome when challenged with exogenous gliotoxin whereby expression of 1,700 genes was altered, of which 1192 were down-regulated, that was not observed in wild-type where 164 genes had altered expression. This suggests that consequent to GliT absence, transcription is suppressed in the presence of exogenous gliotoxin. This is further supported by the decreased expression of 44 genes involved in transcription processes, many of which are transcription factors. Further to the effects of gliT deletion on transcription, translation is also disrupted in A. fumigatus ΔgliT following exposure to exogenous gliotoxin. Altered expression of genes required for ribosome biogenesis and translation processes was observed in ΔgliT. To support the hypothesis that translation is disrupted, the LFQ proteomic investigation of A. fumigatus ΔgliT did not reflect the large transcriptome changes, as only 6 proteins with increased abundance or uniquely present, and 15 proteins absent or with decreased abundance were identified. It is important to note that despite the low number of proteins altered in abundance, there is agreement between the RNA-seq analysis and the proteomic analysis of A. fumigatus ΔgliT exposed to exogenous gliotoxin, whereby the genes encoding one of the proteins increased in abundance and 8 of the proteins decreased in abundance were up-regulated and down-regulated, respectively.

**Conclusions**

We present the first global investigation of the transcriptional response to exogenous gliotoxin in A. fumigatus wild-type and ΔgliT employing RNA-seq analysis. While exogenous gliotoxin elicits some transcriptome remodelling in wild-type, in ΔgliT, the transcriptional response is 10-fold that of wild-type, with approximately 70% of these altered genes decreased in expression. We found that the combined loss of gliT and exogenous gliotoxin exposure results in decreased expression of a number of secondary metabolite genes from the biosynthetic clusters of helvolic acid, fumitremorgin B, pseurotin A and fumagillin despite the unchanged expression of laeA, the global regulator of secondary metabolism. Thus, GliT functionality may extend to enhancing the biosynthesis of selected secondary metabolites in A. fumigatus. In addition to this, the decreased expression of many transcription factor genes, along with genes involved in siderophore-iron transport and siderophore biosynthesis and nitrogen metabolism indicates that exogenous gliotoxin induces a starvation-like response despite the use of a rich media. Furthermore, the combined RNA-seq and proteomic analysis revealed deletion of gliT abrogated transcription and disrupts translation when exposed to exogenous gliotoxin. Overall, this study provides a detailed overview of the response to exogenous gliotoxin in resistant and sensitive A. fumigatus strains, enhances our understanding of the manner in which gliotoxin exerts its affects as a toxin, and provides a unique glimpse into cross-talk between apparently unrelated secondary metabolite gene clusters.

**Methods**

*A. fumigatus* strain information and growth conditions

Conidia were maintained on malt extract agar plates. *A. fumigatus* wild-type and mutant strains (1 × 10⁶ cfu/ml) were cultured in Sabouraud-Dextrose media at 37°C with shaking at 200 rpm for 21 h in duplicate or triplicate. Gliotoxin (5 μg/ml final) or MeOH (solvent control) was added and the cultures were incubated for a further 3 h. The mycelia were harvested through miracloth, washed with DEPC water and snap frozen in liquid N₂.

RNA extraction and mRNA isolation

RNA was isolated from mycelia, ground to a fine powder in liquid N₂, using the RNeasy™ Plant Mini Kit (Qiagen), according to the manufacturer’s instructions. RNA integrity was analysed using an Agilent 2100 Bioanalyzer™ and an Agilent RNA 6000 Nano Kit following the manufacturer’s recommendation.

Library preparation and sequencing

A library was independently prepared for each biological replicate. Two protocols (TruSeq and Illumina mRNA-seq kit) were used for preparing the Illumina transcriptome libraries. For both protocols, polyadenylated mRNA was
purified from total RNA using oligo-dT dynabead selection followed by metal ion hydrolysis fragmentation with an RNA fragmentation solution supplied in kits. First strand synthesis, primed using random oligonucleotides, was followed by second strand synthesis with RNaseH and DNA poll to produce double-stranded cDNA using the Illumina mRNA Seq kit or the TruSeq Illumina kit. Template DNA fragments were end-repaired with T4 and Klenow DNA polymerases and blunt-ended with T4 polynucleotide kinase. A single 3’ adenosine was added to the repaired ends using Klenow exon- and dATP to reduce template concatemerization and adapter dimer formation, and to increase the efficiency of adapter ligation. Adapters (containing primer sites for sequencing, and index sequences when using the TruSeq protocol) were then ligated. Libraries made with the TruSeq protocol were amplified by PCR using KAPA HiFi Polymerase (to enrich for properly ligated template strands, to generate enough DNA, and to add primers for flowcell surface annealing). AMPure SPRI beads were used to purify amplified templates before pooling based on quantification using an Agilent Bioanalyser chip. Pooled TruSeq libraries were then pooled and size selected using the Caliper. After adapter ligation, individual libraries made with the Illumina mRNA-seq kit were size selected using the Caliper-LabChip before PCR amplification followed by AMPure SPRI bead clean up and removal of adapters with a second Caliper run. KAPA Illumina SYBR Fast qPCR kit was used to quantify the Illumina mRNA-seq libraries before pooling. No qPCR was necessary with the TruSeq libraries and instead a final Agilent Bioanalyser chip analysis was run to confirm the dilution of the final pool. The libraries were sequenced on the Illumina HiSeq platform with a read length of 75 bp paired-ended according to manufacturer’s instructions.

Data processing

The RNA-seq paired-end reads for each biological replicate were aligned independently using Tophat v2.0.4 [23618408] to the A. fumigatus Af293 (CADRE 3a) reference genome sequence with default parameters. The numbers of fragments mapped per replicate is given in Additional file 2: Table S14. Differential gene expression analysis was carried out for each sample independently using Cufflinks (cuffdiff) v2.0.2 [20436464] with default parameters against the gene set in Ensembl Genomes release 14 (CADRE genebuild 3a). The sequencing data has been submitted to the European Nucleotide Archive (ENA) under accession ERP001382 (https://www.ebi.ac.uk/ena/data/view/ERP001382&display=html).

Data analysis

The differentially regulated genes were analysed using FungiFun [60] to establish association with any functions or pathways in comparison with the non-differentially regulated genes. Gene enrichment analysis was carried out on the up-regulated and down-regulated genes, respectively, for FunCat [23] categories and the A. fumigatus annotated KEGG pathways [24]. A p value cut-off of 0.05 was used.

Real time PCR (qRT-PCR)

RNA samples were DNase treated using a DNase kit supplied by Sigma-Aldrich. cDNA synthesis was performed using qScript™ cDNA SuperMix (Quanta Biosciences) following the kit instructions. Primers used in this study are listed in Additional file 2: Table S14. The constitutively expressed gene, A. fumigatus calmodulin (calm) [61] was used as a reference gene. qRT-PCR was performed on the LightCycler®2 480 Real-Time PCR System using the LightCycler®1 Sybr Green 1 Master Mix (Roche) as described previously [62]. qRT-PCR reactions for each gene were analysed in triplicate and were carried out for 40 cycles.

Feeding experiments with [13C]-phenylalanine

A. fumigatus Af293 was cultured in Czapek-Dox Broth at 37°C with shaking at 200 rpm in duplicate for 24 h before addition of gliotoxin (5 μg/ml final) or methanol (solvent control) and [13C]-phenylalanine (10 μg/ml final) or water (solvent control). Cultures were incubated again at 37°C with shaking at 200 rpm. Culture supernatant was removed after 48 h and again after 72 h. Supernatants were chloroform extracted, dried and resolubilised in methanol prior to LC-MS analysis as described previously [2].

Whole proteome analysis

A. fumigatus ΔgltT ATCC26933 was cultured in Sabouraud-Dextrose media for 21 h followed by gliotoxin (5 μg/ml final) or methanol addition for 3 h (n =4 biological replicates for all specimens). Mycelial lysates were prepared in lysis buffer (100 mM Tris–HCl, 50 mM NaCl, 20 mM EDTA, 10% (v/v) Glycerol, 1 mM PMSF, pH 7.5) with grinding, sonication and clarified using centrifugation. The resultant protein lysates were precipitated using TCA/acetone and resuspended in 8 M Urea. After DTT reduction and IAA-mediated alkylation [63], sequencing grade trypsin combined with Protease-Max surfactant was added. Digested samples were desalted prior to analysis using C18 spin columns (Thermo Scientific). All peptide mixtures were analysed via a Thermo Scientific Q-Exactive mass spectrometer coupled to a Dionex RSLCnano. LC gradients ran from 14–35% B (A: 0.1% (v/v) formic acid, B: 80% (v/v) acetonitrile, 0.1% (v/v) formic acid) over 2 h, and data was collected using a Top15 method for MS/MS scans. Comparative proteome abundance and data analysis was performed using MaxQuant software (Version 1.3.0.5) [64], with Andromeda used for database searching and
Perseus (Version 1.4.1.3) used to organise the data. Carbamidomethylation of cysteines was set as a fixed modification, while oxidation of methionines and acetylation of N-termini were set as variable modifications. The maximum peptide/protein false discovery rates (FDR) were set to 1% based on comparison to a reverse database. The Label-Free Quantification (LFQ) algorithm was used to generate normalised spectral intensities and infer relative protein abundance. Proteins that matched to a contaminants database or the reverse database were removed and proteins were only retained in final analysis if detected in at least three replicates from at least one sample. Quantitative analysis was performed using a t-test to compare pairs of samples, and proteins with significant change in abundance (p value <0.05; fold change ≥2) were included in the quantitative results. Qualitative analysis was also performed, to detect proteins that were found in at least 3 replicates of a particular sample, but undetectable in the comparator sample.

Secondary metabolite analysis

A. fumigatus wild-type and ΔgliT<sup>Δ6645</sup> were cultured in Czapeks-Dox broth for 96 h at 37°C at 200 rpm in duplicate. Culture supernatants were organically extracted using an equal volume of chloroform and the extracts were dried by rotavaporation and resuspended in methanol. Extracts were diluted 1/10 in 0.01% formic acid prior to analysis by LC-MS as described previously [2].

Cell viability analysis

Following culturing and treatment as described above, mycelia were removed from the culture supennatant and washed with water. They were resuspended in 10 mM HEPES before aliquots were plated on Sabouraud dextrose agar and incubated at 37°C for 24 h. Viability was recorded.

Additional files

Additional file 1: Gene sequencing information. Combined FPKM values for all replicates. Log2 fold changes and statistical significance data for all genes. Processed RNA-seq data. Relative expression of genes in A. fumigatus +/- gliotoxin; Relative expression of genes in A. fumigatus ΔgliT +/- gliotoxin.

Additional file 2: Additional study information. Additional tables containing the functional categorisation of differentially regulated genes, illumina RNA-seq summary statistics and primers used in the study. Additional Figure S1 is included. FunCat and KEGG categories of genes altered in expression; Summary Statistics for illumina RNA-seq; Primers used for qRT-PCR; cell viability data.

Abbreviations

GSH: Glutathione; qRT-PCR: Quantitative real-time PCR; NRPS: Non-ribosomal peptide synthetase; PKS: Polyketide synthase; DTT: Dithiothreitol; IAA: Iodoacetamide.

Competing interests

The authors declare they have no competing interests.

Authors’ contributions

GOK, SH, RAO, GWJ, and SD conceived and designed the experiments. SH and GOK extracted the RNA. TMK carried out library prep, sequencing and data mapping. Data analysis was performed by GOK, RAO, SH, TMK, DAF and SD. GOK and SH performed qPCR analysis. GOK carried out gliotoxin and secondary metabolite measurements and viability analysis. RAO performed the proteomics experimentation. GOK, GWJ and SD wrote the paper with contributions from RAO and TMK. All authors read and approved the final manuscript.

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