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Regulation of Nonribosomal Peptide Synthesis: *bis*-Thiomethylation Attenuates Gliotoxin Biosynthesis in *Aspergillus fumigatus*

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SUMMARY

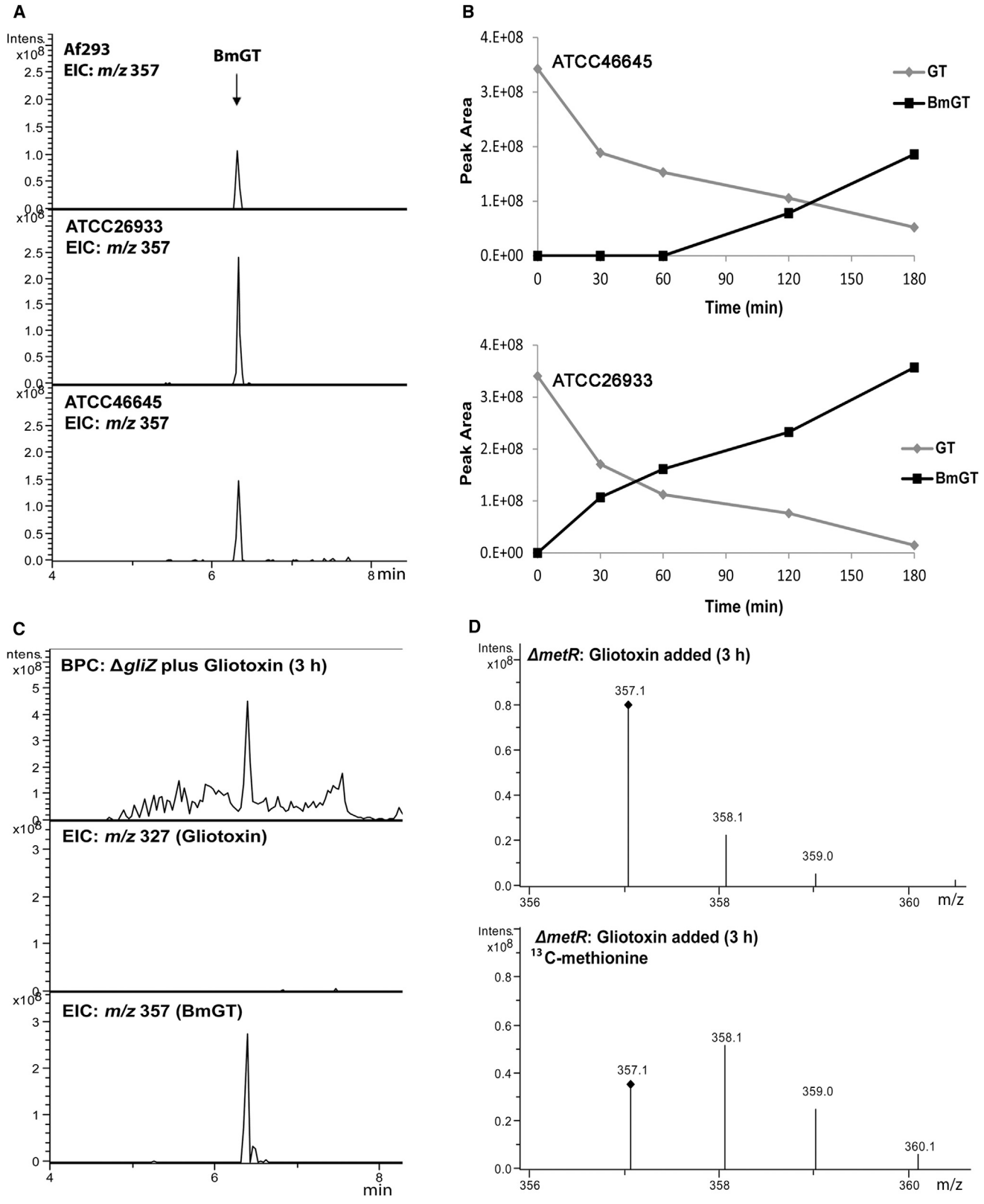
Gliotoxin is a redox-active nonribosomal peptide produced by *Aspergillus fumigatus*. Like many other disulfide-containing epipolythiodioxopiperazines, a *bis*-thiomethylated form is also produced. In the case of gliotoxin, bisdethiobis(methylthio)gliotoxin (BmGT) is formed for unknown reasons by a cryptic enzyme. Here, we identify the *S*-adenosylmethionine-dependent gliotoxin *bis*-thiomethyltransferase (GtmA), which converts dithiogliotoxin to BmGT. This activity, which is induced by exogenous gliotoxin, is only detectable in protein lysates of *A. fumigatus* deficient in the gliotoxin oxidoreductase, *gliT*. Thus, GtmA is capable of substrate *bis*-thiomethylation. Deletion of *gtmA* completely abrogates BmGT formation and we now propose that the purpose of BmGT formation is primarily to attenuate gliotoxin biosynthesis. Phylogenetic analysis reveals 124 GtmA homologs within the Ascomycota phylum. GtmA is encoded outside the gliotoxin biosynthetic cluster and primarily serves to negatively regulate gliotoxin biosynthesis. This mechanism of postbiosynthetic regulation of nonribosomal peptide synthesis appears to be quite unusual.

INTRODUCTION

Thiomethylation is deployed for the detoxification of xenobiotic thiols in bacteria, plants, and mammals, and is mediated by thiol methyltransferases (TMTs), which detoxify a broad range of reactive endogenous or foreign thiols through the formation of less reactive thiomethyl derivatives (Bremer and Greenberg, 1961; Weisiger et al., 1980). The human thiopurine *S*-methyltransferase, which participates in the detoxification of xenobiotics, is a well-characterized member of this enzyme family (Peng et al., 2008).

Several plant TMTs have been implicated in the detoxification of reactive thiol-containing metabolites that may otherwise inhibit enzymes associated with aerobic respiration and oxidative metabolism. A TMT isolated from *Brassica oleracea* L (cTMT1) was shown to methylate thiocyanate. Because thiocyanate is an extremely reactive product of glucosinolate degradation, cTMT1 is most likely responsible for enzymatic detoxification within this pathway (Attieh et al., 2000). PpSABATH1, a TMT from the moss *Physcomitrella patens*, showed a high level of activity toward thiobenzoic acid. Interestingly, the expression of *PpSABATH1* was induced by the treatment of *P. patens* with thiobenzoic acid. Additionally, the constitutive expression of *PpSABATH1* in tobacco plants resulted in an enhanced tolerance to thiobenzoic acid. These results suggest that PpSABATH1 may have a role in the detoxification of xenobiotic thiols (Zhao et al., 2012). The Madagascar periwinkle (*Catharanthus roseus*) *O*-methyltransferase CrSMT1, the true biological function of which remains unknown, has been shown to methylate a wide range of aliphatic and aryl alcohols and thiols (Coiner et al., 2006). Remarkably, *S*-methyltransferases have only been definitively classified in relation to detoxification mechanisms and primary metabolism, although the activity has long been implicated in numerous secondary metabolite pathways (Boente and Kirby, 1991; Kirby et al., 1980; Taylor, 1963). Recently, the *Streptomyces lasaliensis S*-adenosyl-L-methionine (SAM)-dependent methyltransferase *Ecm18* was shown to catalyze the conversion of a disulfide bond in the precursor antibiotic triostatin A to the thioacetal bridge of the nonribosomal dipsipeptide echinomycin (Hotta et al., 2014). This is an unusual example of a thiol-directed methyltransferase involved in secondary metabolism.

Gliotoxin is a nonribosomally synthesized, redox active metabolite secreted by several fungal species and contributes to the virulence of the human fungal pathogen *Aspergillus fumigatus* (Dagenais and Keller, 2009). Gliotoxin is an epipolythiodioxopiperazine (ETP) class fungal toxin containing a disulfide bridge responsible for the deleterious effects of this toxin (Kwon-Chung and Sugui, 2009). Modification of this rare structural motif by reduction and *S*-methylation significantly depletes the bioactivity of this metabolite (Trown and Bilello, 1972). Several mono- and *bis*-methylthio derivatives of gliotoxin have also been discovered in various fungi. Bisdethiobis(methylthio)gliotoxin (BmGT; Figure S1 available online) is the most well characterized of these gliotoxin derivatives (Forseth et al., 2011). First identified in *Gliocladium deliquescens*, BmGT appears to be formed by the irreversible disulfide bridge reduction and *S*-methylation of exogenous gliotoxin, as demonstrated by feeding [^{14}C]gliotoxin to *G. deliquescens* (Kirby et al., 1980). The enzyme that catalyzes this biotransformation has remained elusive. BmGT has also been detected in *A. fumigatus* (Amitani et al., 1995),



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Colletotrichum gloeosporioides (Guimarães et al., 2010), *Penicillium* sp. BCC16054 (Intaraudom et al., 2013), FO2047 (Van der Pyl et al., 1992), JMF034 (Sun et al., 2012), *Pseudallescheria* sp. MFB165 (Li et al., 2006), and several other fungi.

Methylthio-derivatives of ETP-type fungal toxins (i.e., hyalodendrin, Boente and Kirby, 1991; sporidesmin, Taylor, 1963; rostratin, Tan et al., 2004; hematocin, Suzuki et al., 2000; leporizine, Reategui et al., 2013; epicoccin, Guo et al., 2009; glionitrin, Park et al., 2011; etc.) have been identified in the majority of ETP-producing fungi. *S*-methylation of dithiol metabolites also extends to bacteria such as *Streptomyces clavuligerus*, which has been shown to produce a *bis*-thiomethylated derivative of the dithiolpyrrolone antibiotic, holomycin (Li et al., 2012). Gliotoxin is encoded by a 13 gene cluster in *A. fumigatus* (Gardiner and Howlett, 2005) and it has been demonstrated that the gliotoxin oxidoreductase GliT, encoded within this *gli* cluster, protects *A. fumigatus* against exogenous gliotoxin and is essential for gliotoxin biosynthesis (Scharf et al., 2010; Schrettl et al., 2010). Subsequently, a similar mechanism for self-protection against holomycin was revealed in *S. clavuligerus*, where HlmI catalyzes disulfide bridge closure in holomycin (Li et al., 2012). Deletion of *hlmI* impaired holomycin biosynthesis and sensitized S. *clavuligerus* to exogenous holomycin, as had been observed for gliotoxin in *A. fumigatus*. In bacteria, thiomethylation has been posited as an additional or backup strategy, to disulfide bridge closure, for self-protection during holomycin biosynthesis, and it has been proposed that *S*-methylation of biosynthetic intermediates, or possibly shunt metabolites, protects cellular components against these reactive species (Guo et al., 2013; Li et al., 2012). Here, we conclusively identify a *bis*-thiomethyltransferase and confirm its involvement in regulating gliotoxin production, and not self-protection, in *A. fumigatus*. Interestingly, the enzyme is not encoded within the *gli* cluster.

RESULTS

In Vivo Gliotoxin Bismethylation

Data in Figure 1A show that endogenous production of BmGT (*m/z* 357) is evident in *A. fumigatus* ATCC26933, Af293, and ATCC46645. Exogenously added gliotoxin (5 μg/ml) is taken up by *A. fumigatus* ATCC26933 and 46645 within 3 hr (Figure 1B) and we demonstrate that this is converted to BmGT (Figure S1B), which is secreted into the culture medium over 3 hr. In response to the presence of exogenous gliotoxin, *A. fumigatus* Δ*gliZ* (Bok et al., 2006; a valuable gift from Prof. N. Keller, University of Wisconsin, Madison), deficient in the *gli* cluster transcriptional regulator, is capable of BmGT biosynthesis and secretion (Figure 1C). This observation indicates that the activity required for BmGT formation is not encoded within the *gli* cluster, or is independently regulated. In an effort to elucidate the origin of the methyl groups present in BmGT, *A. fumigatus* Δ*metR* (Amich et al., 2013; generously provided by Prof. S. Krappmann, Erlangen), deficient in utilization of sulfur sources other than Met, was deployed. Here, co-addition of gliotoxin and [^{13}C]-Met resulted in formation of [^{13}C]-BmGT (m/z 358, 359, respectively; Figure 1D), whereas [^{13}C]-BmGT was not detected in negative control specimens (methanol and [^{13}C]-Met). This infers that gliotoxin induces *bis*-thiomethylation activity in *A. fumigatus*.

In Vitro Enzyme-Mediated Gliotoxin Bismethylation

No enzyme has been demonstrated to catalyze gliotoxin *bis*-thiomethylation. *A. fumigatus* Δ*gliT* (Schrettl et al., 2010) lacks gliotoxin oxidase activity. Using a novel activity assay system (Figure S2A), significant ($p < 0.0013$) in vitro gliotoxin *bis*-thiomethylation was only detectable in protein lysates derived from *A. fumigatus* Δ*gliT* (Schrettl et al., 2010), following exposure to exogenous gliotoxin (5 μg/ml for 3 hr). Remarkably, this activity was effectively undetectable in *A. fumigatus* wild-type lysates, and signifies that the inability of *A. fumigatus* to affect GliT-mediated oxidation of the dithiolgliotoxin (GT-$(SH)_2$) in protein lysates (Scharf et al., 2010) results in its availability as a substrate for a putative *bis*-thiomethyltransferase. Moreover, combined anion exchange and gel filtration chromatographic fractionation of these lysates revealed that gliotoxin *bis*-thiomethyltransferase activity was coincident with detection of AFUA_2G11120 (hereinafter termed gliotoxin thiomethyltransferase; GtmA) with liquid chromatography-mass spectrometry (LC-MS; sequence coverage 23.7%; (Table S1 and Figure 2B).

Expression and Purification of Recombinant GtmA

After *A. fumigatus* ATCC26933 *gtmA* cDNA sequence confirmation (Figure S3), recombinant expression of GtmA as a soluble GST fusion protein in *Escherichia coli* (Figure 2A), along with LC-MS identity confirmation, led to the assessment of activity of pure GtmA following TEV-mediated cleavage, and removal of the GST affinity tag. As seen in Figures 2B and S1B, recombinant GtmA *bis*-thiomethylates gliotoxin using SAM as a methyl donor. Thus, this enzyme is capable of methylating two substrate-derived thiol groups. Moreover, activity analysis in the presence of limiting amounts of SAM, whereby a putative monodethiomono(methylthio)gliotoxin (MmGT) form is detectable, reveals that this reaction may proceed in a sequential manner, with MmGT formation preceding that of BmGT (Figures 2C and S2C).

GtmA Is a Non-*gli* Cluster-Encoded Methyltransferase

Transcriptomic analysis of both *A. fumigatus* ATCC46645 (wild-type) and Δ*gliT*^{46645} (Schrettl et al., 2010) in response to exposure to exogenous gliotoxin (5 μg/ml; 3 hr; G.O., S.H., T.M. Keane, D.A.F., G.W.J., and S.D., unpublished data) revealed the unexpected significant upregulated expression of GtmA (log_2 8.42 and 5.84, respectively; $p < 5 \times 10^{-5}$). This result, which was confirmed by parallel qRT-PCR (Figure 3A), led us to speculate if the cognate enzyme played a role in gliotoxin

Figure 1. Endogenous and Exogenous BmGT Formation by *A. fumigatus*

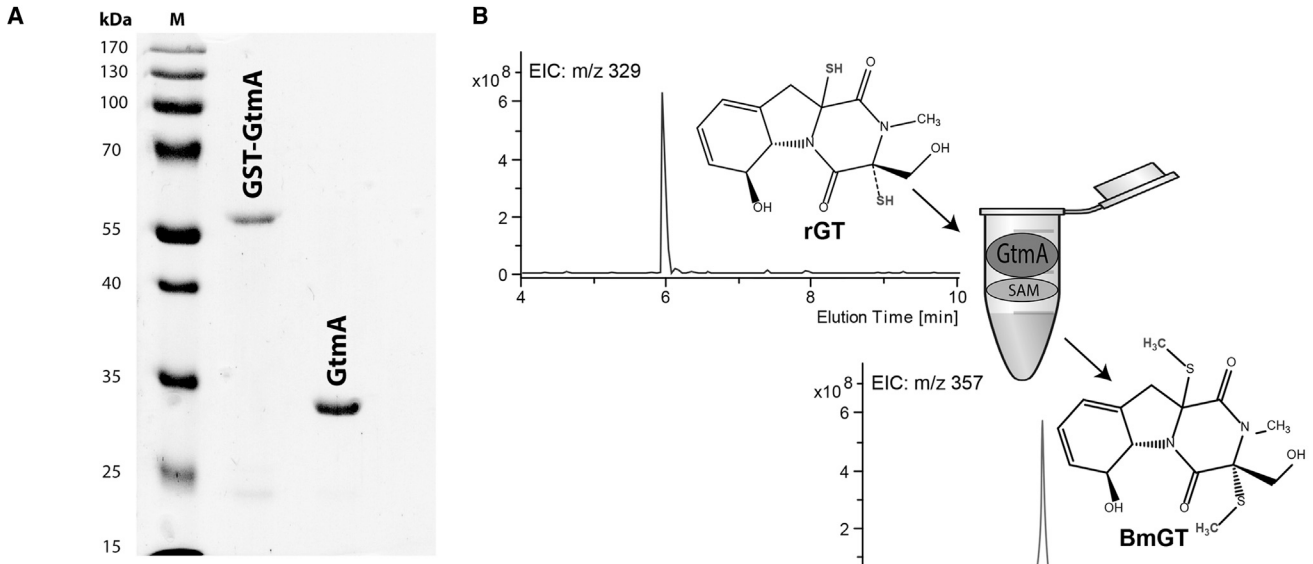
(A) BmGT (*m/z* 357) is detectable in organic extracts of *A. fumigatus* ATCC26933, Af293, and ATCC46645.

(B) Both *A. fumigatus* ATCC46645 and ATCC26933 convert exogenously added gliotoxin to BmGT in a time-dependent manner.

(C) BmGT is formed within 3 hr following gliotoxin addition to *A. fumigatus* Δ*gliZ* cultures.

(D) Gliotoxin and [^{13}C]-L-methionine co-addition to *A. fumigatus* Δ*metR* results in heavy BmGT (*m/z* 358, 359) formation.

See Figure S1.



AFUA_2G11120 PROTEIN SEQUENCE COVERAGE

MSKSDYIQNM FQTKSFVDRY KYTEKLTGLY AQTLVDYSGV ANTSQKPLVV
LDNACGIGAV SSVLNHTLQD EAKKTWKLTC GDLSEGMLET TKRRLQDEGW
VNAETKIVNA LPELSRRQRR LERILASGGI LASSTWQNFN WIPIMKAAIE
TIPGNLPFPT QKEFIALHNA GWDSESYIQS ELEKLGFRDV KVIPVPKETS
IPIDEFFEVC MMIIPYLLPK FWTEEQRESH EKDVPMVLRQ YLQDTYGANG
QVPLEAVALI TTGLKP

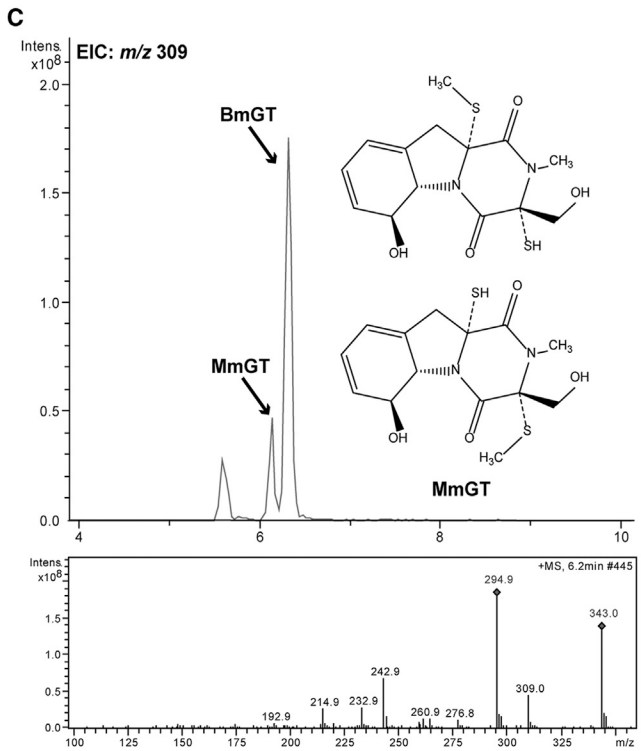


Figure 2. Expression and Activity Analysis of Recombinant GtmA

(A) SDS-PAGE analysis of GST-GtmA before and after TEV cleavage. Identity was confirmed with LC-MS analysis (15% sequence coverage).

(B) rGtmA *bis*-thiomethylates reduced gliotoxin (GT-$(SH)_2$) using SAM as a methyl donor.

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self-protection via *bis*-thiomethylation and prompted us to undertake and confirm *gtmA* deletion from *A. fumigatus* ATCC26933 (Figure S3). Indeed, previous work had posited that thiomethylation could be a back-up plan for self-protection against dithiols. So, after confirming the absence of *gtmA* expression by RT-PCR (Figure S4), surprisingly, deletion mutant analysis did not reveal acquisition of a gliotoxin- or H_2O_2-sensitive phenotype, even though *gtmA* deletion completely abrogated the ability of the mutant to biosynthesize and secrete BmGT (Figures 3B and 3C; Figure S5).

In addition to deletion of *gtmA* completely abrogating endogenous BmGT formation and secretion, conversion of exogenously added gliotoxin to BmGT was abolished (Figure 4A). Reconstitution of *gtmA* in *A. fumigatus* Δ*gtmA* restored endogenous and exogenously added gliotoxin *bis*-thiomethylation functionality (Figure 4A), thus the same enzyme catalyzes both reactions. Interestingly, a significant increase ($p < 0.0056$) in gliotoxin production was evident in *A. fumigatus* Δ*gtmA* compared to wild-type following culture in Czapek-Dox media for 3 days (Figure 4B). A further increase in gliotoxin production was evident in the Δ*gtmA* strain when the incubation time was increased to 7 days ($p < 0.0001$; Figure 4B). Sporidesmin is not converted to the *bis*-thiomethyl form by *A. fumigatus* (Figure 4C), which indicates the specificity of GtmA for GT-$(SH)_2$ *bis*-thiomethylation. From this data it is apparent that in the absence of GtmA activity, gliotoxin biosynthesis increases, which positions BmGT formation as a mechanism to attenuate *gli* cluster activity—most likely by preventing gliotoxin-mediated cluster induction (Cramer et al., 2006; Schrettl et al., 2010). If this is the case, then increased abundance of *gli* cluster enzymes would be expected to be present in *A. fumigatus* Δ*gtmA*. The inability to detect BmGT formation by *A. fumigatus* Δ*gtmA* also effectively excludes a role for either GliM or GliN [29] in gliotoxin *bis*-thiomethylation, and suggests that these enzymes are responsible for unrelated functionalities during gliotoxin biosynthesis.

Label-free Quantitative Proteomics Reveals Elevated Abundance of *gli*-Encoded Enzymes in *A. fumigatus* Δ*gtmA*

Label-free quantitative (LFQ) proteomic analysis of *A. fumigatus* wild-type, Δ*gtmA*, and *gtmA*^{C} cultured in Czapek-Dox media (gliotoxin-producing conditions), revealed significantly increased abundance ($p < 0.05$) of GliM (log_2 1.467-fold), GliP (log_2 1.399-fold), and GliF (log_2 1.297-fold) in Δ*gtmA* compared to wild-type (Table 1). This leads us to conclude that in the absence of GtmA and abrogated BmGT formation, expression of the *gli* cluster is significantly induced by endogenous gliotoxin. Notably, the unique presence of seven proteins and significantly increased abundance of 18 additional proteins was observed in *A. fumigatus* Δ*gtmA* protein lysates (Tables 1 and S2A). As can be seen in Table S2B, GtmA presence is restored in *A. fumigatus* *gtmA*^{c}, which confirms the result observed following Southern analysis of the complementation of *gtmA* deletion (Figure S3). Importantly, when the proteome of wild-type versus *gtmA*^{c} was analyzed, GliM, GliP, or GliF were not differentially abundant, which unambiguously confirms that *gtmA* complementation restored *gli* cluster regulation to that of *A. fumigatus* wild-type (Tables S3 and S4). Moreover, GliM, GliN, and GliF abundance was significantly higher (log_2 1.324, 1.115, and 1.475, respectively) in *A. fumigatus* Δ*gtmA* than in *gtmA*^{c}, which convincingly demonstrates that *gtmA* loss augments *gli* cluster-encoded enzyme levels.

LFQ Proteomics Reveals that Exogenous BmGT and Gliotoxin Induce Proteome Remodelling in *A. fumigatus* Wild-Type and Δ*gtmA*

LFQ proteomics analysis revealed that exogenous BmGT exposure resulted in significantly increased abundance of GliT (log_2 1.985-fold increase) and GtmA (log_2 1.933-fold increase) in *A. fumigatus*, and that abundance of only four other proteins were altered (unique or increased; Table S5). Of all the proteins to undergo differential abundance (BmGT vs. methanol exposure), GliT and GtmA abundance, respectively, were observed to be the most significantly altered. Moreover, no other *gli* cluster-encoded proteins were found to exhibit altered abundance following BmGT exposure, compared to negative control (methanol), exposure. Following exposure to exogenous gliotoxin, *A. fumigatus* wild-type (Table S6) and Δ*gtmA* (Table S7) each exhibit significantly increased abundance of mycelial catalase Cat1 (wild-type/Δ*gtmA*: log_2 3.552/ log_2 3.435), GliT (log_2 5.125/ log_2 4.869) and the bifunctional catalase-peroxidase Cat2 (log_2 1.947/ log_2 1.205). This confirms that both the wild-type and Δ*gtmA* strains respond almost identically to gliotoxin exposure. If BmGT formation played a role in the detoxification of intracellular gliotoxin, the Δ*gtmA* strain would be expected to be more sensitive to gliotoxin than the wild-type. Because this is not observed, it further underpins our hypothesis that GtmA activity is not directly involved in self-protection against gliotoxin.

GtmA Phylogeny

Using *A. fumigatus* GtmA as a query sequence, 124 homologs in 103 fungal genomes were located. Interestingly, all homologs were from the Ascomycota phylum. We did not locate any homologs in the Basidiomycota, Chytridmycota, or Zygomycota phyla (Figure 5). Database searches show that species belonging to the Taphrinomycotina subphylum and CTG clade (*Candida albicans* and close relatives; Fitzpatrick et al., 2006) contain GtmA homologs; however, no homologs were located in species that have undergone a whole genome duplication or close relatives of whole genome duplication species such as *Kluyveromyces lactis* and *Ashbya gossypii* (Figure 5).

All species belonging to the filamentous ascomycetes (Pezizomycotina subphylum) with the exceptions of *Uniocarpus reesii*, *Verticillium dahliae*, and *Verticillium alboatrum*, contain a homolog of GtmA (Figure 5). Furthermore, most Pezizomycotina

(C) rGtmA-mediated *bis*-thiomethylation proceeds in a sequential manner, whereby mono-thiomethylgliotoxin (MmGT; *m/z* 343)) formation precedes BmGT formation, as determined with RP-HPLC and LC-MS analysis. As both BmGT and MmGT fragment in a similar manner, an extracted ion chromatogram displaying both metabolites was obtained by searching for the *m/z* 309 ion.
See Figure S2.

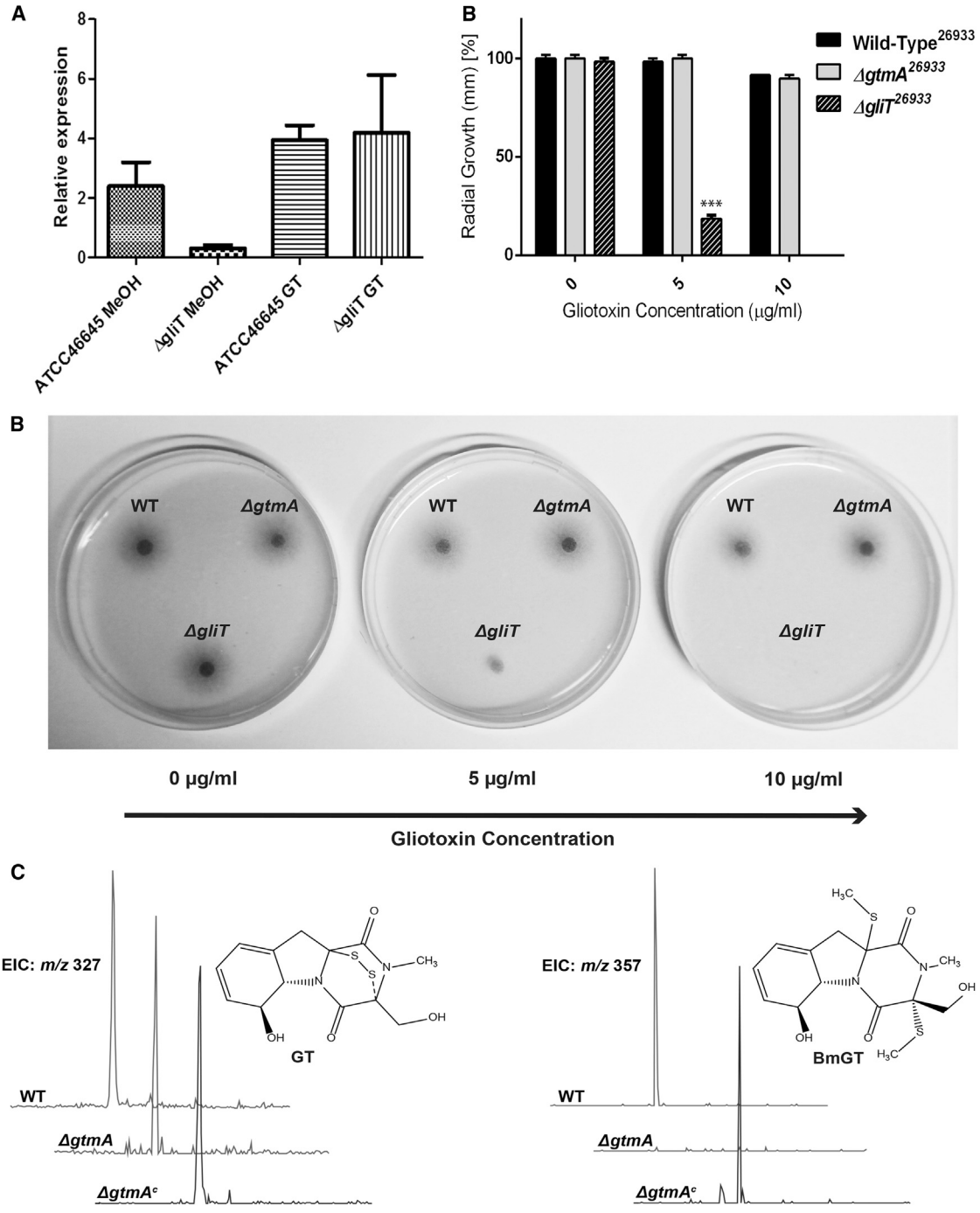


Figure 3. *A. fumigatus gtmA* Expression, Deletion, and Complementation

(A) qRT-PCR analysis confirms increased *gtmA* expression in *A. fumigatus* ATCC46645 (wild-type) and Δ*gliT*^{46645} in response to exposure to exogenous gliotoxin (5 μg/ml; 3 hr). Error bars represent the SD of two biological replicates.

(B) Exogenous gliotoxin (up to 10 μg/ml) does not inhibit growth of *A. fumigatus* Δ*gtmA*, even though growth of Δ*gliT* is completely inhibited. The depicted values are from three independent samples. Error bars represent SDs from the mean.

(C) High sensitivity LC-MS analysis confirms complete abrogation, and restoration, of BmGT biosynthesis in *A. fumigatus* Δ*gtmA* and *gtmA*^{c}, respectively. See Figures S3–S5.

species contain multiple GtmA homologs. For example, *A. fumigatus* has two homologs, AFUA_4G14510 and AFUA_6G08850. According to AspGD (http://www.aspgd.org/), AFUA_4G14510 is annotated as an uncharacterized protein with predicted methyltransferase activity, whereas AFUA_6G08850 is a ubiquinone biosynthesis methyltransferase (Vödisch et al., 2011).

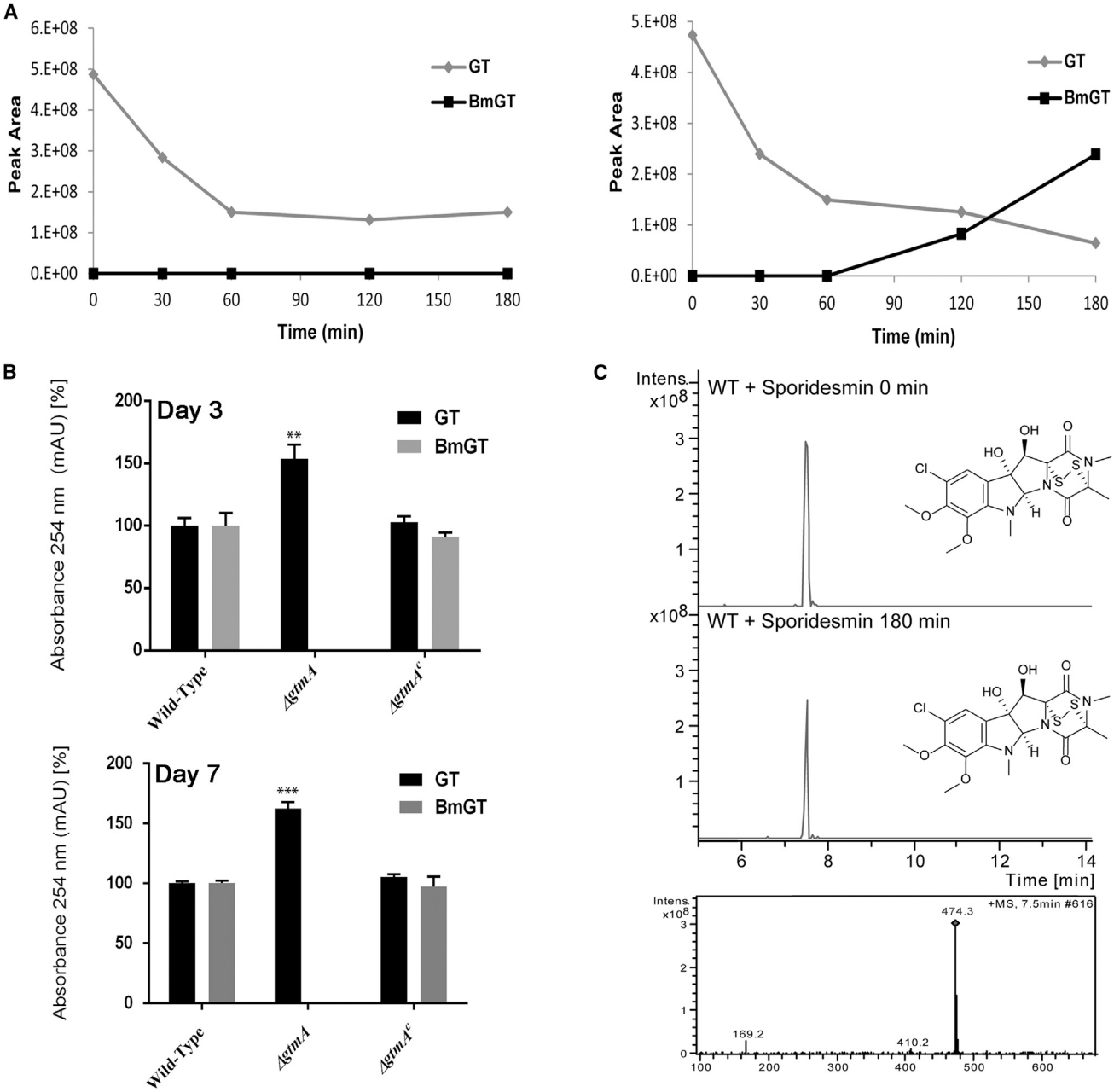


Figure 4. BmGT Formation Attenuates Gliotoxin Biosynthesis

(A) Exogenously-added gliotoxin (5 μg/ml) is taken up but not converted to BmGT in *A. fumigatus* Δ*gtmA*, whereas *gtmA* complementation restores the ability to biosynthesize BmGT from exogenous gliotoxin.

(B) Deletion of *gtmA* significantly increases gliotoxin biosynthesis at day 3 ($p < 0.0056$) and day 7 ($p < 0.0001$) culture. *gtmA* complementation restores wild-type gliotoxin biosynthesis levels. The depicted values are from three independent samples. Error bars represents SDs from the mean.

(C) Sporidesmin (*m/z* 474) is not converted to *bis*-thiomethylsporidesmin by *A. fumigatus* wild-type under conditions (3 hr exposure) that promote BmGT formation.

DISCUSSION

This work comprises a characterization of an enzyme responsible for nonribosomal peptide *bis*-thiomethylation in microbial secondary metabolism, whereby GtmA catalyzes BmGT biosynthesis in *A. fumigatus*. We unambiguously demonstrate that BmGT production acts as a negative regulatory mechanism for *gli* cluster expression by disrupting the gliotoxin-mediated *gli* cluster positive feedback system (Figure 6)—a model that may be applicable to all ETPs synthesized by various filamentous fungi. Surprisingly, *gtmA* is located outside the *gli* cluster despite the fact that BmGT production is conserved across all gliotoxin-producing

Table 1. Proteins Encoded by the *gli* Cluster with Increased Abundance in *A. fumigatus* ΔgtmA Grown for 72 hr in Czapek-Dox Media Compared to *A. fumigatus* Wild-Type

Protein Description	Log_2 (Fold Increase)	p Value	Peptides	Sequence Coverage (%)	Protein IDs
Predicted O-methyltransferase, encoded in the putative gliotoxin biosynthetic gene cluster. GliM.	1.467	1.97×10^{-3}	4	11.6	AFUA_6G09680
Nonribosomal peptide synthetase encoded in the gliotoxin biosynthetic gene cluster; catalyzes the first step in gliotoxin biosynthesis; regulated by the transcription factor StuA; expression increases in vivo. GliP.	1.399	4.42×10^{-3}	5	3.5	AFUA_6G09660
Predicted cytochrome P450 monooxygenase, encoded in the putative gliotoxin biosynthetic gene cluster. GliF.	1.297	1.67×10^{-3}	6	16.3	AFUA_6G09730

Data are sorted by fold change, in descending order. For a complete list of proteins that underwent increased abundance in, or were unique to, *A. fumigatus* Δ*gtmA*, see Table S2A.

species. We show that unlike the gliotoxin oxidoreductase GliT, GtmA is not essential for gliotoxin biosynthesis or self-protection in *A. fumigatus*.

Thus, exogenous gliotoxin can be converted to BmGT by *A. fumigatus* and methionine-derived methyl groups facilitate this bioconversion. Previously, it has been demonstrated that *G. deliquescens* could convert exogenous gliotoxin to BmGT (Kirby et al., 1980), indeed this metabolite has been identified to be endogenously produced by many fungal species (Amitani et al., 1995; Guimarães et al., 2010; Intaraudom et al., 2013; Li et al., 2006; Van der Pyl et al., 1992; Sun et al., 2012). It has been proposed that *bis*-thiomethylation represents a back-up detoxification strategy to the GliT-mediated gliotoxin self-protection system in *A. fumigatus*. Further, it has been speculated, not unreasonably, that the *gli* cluster-encoded GliN may be responsible for gliotoxin *bis*-thiomethylation (Li and Walsh, 2011). However, although endogenous or exogenous gliotoxin clearly underwent conversion to BmGT in *A. fumigatus*, our initial attempts to detect *bis*-thiomethylation activity in wild-type lysates were unsuccessful. Extensive experimentation effected resolution of this issue whereby in vitro BmGT formation was readily detectable by LC-MS in reaction mixtures containing Tris(2-carboxyethyl)phosphine hydrochloride (TCEP)-reduced gliotoxin, SAM, and *A. fumigatus* Δ*gliT* mycelial lysates. Thus, GliT absence facilitated GtmA-mediated gliotoxin *bis*-thiomethylation, which infers that dithiogliotoxin is a cosubstrate for both enzymes (Figure 6). Fractionation of *A. fumigatus* Δ*gliT* mycelial lysates via a combination of anion exchange and gel filtration chromatography resulted in a semi-pure protein preparation which retained gliotoxin *bis*-thiomethylation activity. Subsequent high-sensitivity shotgun mass spectrometric analysis of this fraction revealed the presence of a putative methyltransferase (AFUA_2G11120; GtmA), which had also been observed to undergo significantly increased expression in transcriptomic analysis of *A. fumigatus* ATCC46645 and Δ$gliT^{46645}$ (G.O., S.H., T.M. Keane, D.A.F., G.W.J., and S.D., unpublished data; Figure 3A).

Localization of *gtmA* on chromosome 2, distal to the *gli* cluster on chromosome 6 (Cramer et al., 2006), is highly relevant to assigning a main role for *gtmA* in either self-protection against a toxic biosynthetic intermediate, or as a regulator of gliotoxin production. It has been proposed that the colocalization of genes within metabolite-encoding clusters has evolved to protect against toxic biosynthetic intermediates (McGary et al., 2013). Our demonstration that *gtmA* deletion completely abrogates BmGT formation, but does not enhance sensitivity to exogenous gliotoxin, is in complete accordance with this proposal- and strongly underpins our conclusion that GtmA is primarily a negative regulator of gliotoxin biosynthesis. Moreover, our data endorse the key role of GliT, located within the *gli* cluster, in mediating self-protection against reactive dithiogliotoxin (Figure 6), and increased *gli* cluster expression consequent to gliotoxin exposure.

Gliotoxin *bis*-thiomethylation appears to be controlled by transcription factors involved in regulating *A. fumigatus* secondary metabolism, whereby as an uncharacterized gene, *gtmA* expression was found to be significantly attenuated in *A. fumigatus* Δ*laeA* (Perrin et al., 2007). *veA* is a global regulator of secondary metabolism in *A. fumigatus* and *gtmA* was noted as significantly downregulated in a *veA* overexpression mutant (and also downregulated in Δ*veA*; Dhingra et al., 2013). Indeed, differentially regulated genes in these mutants were dramatically enriched for secondary metabolism-associated processes. In addition, *stuA* encodes a developmental transcription factor and regulates the expression of six secondary metabolite gene clusters in *A. fumigatus* (including gliotoxin) and *gtmA* was also differentially regulated in Δ*stuA* (Twumasi-Boateng et al., 2009) and downregulated during co-incubation of *A. fumigatus* with human immature dendritic cells (Morton et al., 2011).

Activity analysis of purified recombinant GtmA confirmed that this enzyme catalyzes dithiogliotoxin *bis*-thiomethylation. There are few, if any, reports of fungal thiomethyltransferases, and it appears that no *bis*-thiomethyltransferase has been identified to date. The thiomethyltransferase (PpSABATH1) identified in a bryophyte *P. patens*, which can monomethylate a range of thiol-containing compounds, including thiobenzoic acid in a SAM-dependent manner, has been proposed to play a role in the detoxification of xenobiotic thiols (Zhao et al., 2012). However, deletion of *gtmA* from *A. fumigatus*, which completely abrogated BmGT production, did not result in acquisition of a gliotoxin- or H_2O_2-sensitive phenotype, which leads us to conclude that GtmA is not primarily involved in detoxification of gliotoxin, or related biosynthetic intermediates (Forseth et al., 2011) as has been previously proposed (Li et al., 2012). Although we cannot exclude the possibility that GliT activity compensates for absence of GtmA, we considered that a counter-balance for the positive regulation of *gli* cluster expression by gliotoxin (Cramer et al., 2006; Davis et al., 2011; Schrettl et al., 2010) presented an

alternative purpose for BmGT formation. In fact, no consideration has heretofore been given to a mechanism by which gliotoxin-induced *gli* cluster activity can be downregulated. Now, we propose that BmGT biosynthesis via GtmA, instead of GliT-catalyzed de novo gliotoxin formation, represents a postbiosynthetic "off-switch" for gliotoxin biosynthesis, and leads to diminution of *gli* cluster expression, and consequent diminished gliotoxin biosynthesis (Figure 6). This hypothesis is underpinned by increased abundance of key gliotoxin biosynthetic enzymes (GliP, GliM, and GliF) in *A. fumigatus* Δ*gtmA* compared to wild-type or *gtmA*^{c}, under conditions permissive for gliotoxin biosynthesis, as well as by the significant elevation ($p < 0.0056$) in gliotoxin production in *A. fumigatus* Δ*gtmA* under these conditions. Moreover, the positive regulation of *gtmA* expression and GtmA abundance by exposure to elevated gliotoxin levels further consolidates the biological role of this enzyme in attenuating *gli* cluster activity and gliotoxin biosynthesis. Importantly, significantly elevated abundance of gliotoxin-related biosynthetic enzymes exclude the possibility that increased gliotoxin biosynthesis is simply due to absence of "competing" BmGT biosynthesis (Figure 6). Thus, ETP *bis*-thiomethylation represents a strategy for suppressing ETP biosynthesis, as opposed to, or in addition to, acting as an alternative self-protection strategy. Nonetheless, removal of dithiogliotoxin by *bis*-thiomethylation likely confers some indirect element of self-protection against this reactive biosynthetic intermediate. Just as *A. fumigatus* Δ*gtmA* facilitates gliotoxin overproduction, deletion mutants of *gtmA* orthologs in other ETP-producing fungi may result in similar outcomes, in addition to attenuating the *bis*-thiomethylation of reactive biosynthetic intermediates (Forseth et al., 2011). Interestingly, overexpression of RsmA, a Yap-like bZIP transcriptional enhancer, in *A. fumigatus* (*OErsmA*) resulted in increased *gliT* expression, enhanced production of gliotoxin and resulted in the effective abolition of BmGT formation (Sekonyela et al., 2013). Our observation that GtmA is the SAM-dependent methyltransferase that *bis*-thiomethylates GT-$(SH)_2$, is in complete accordance with these observations and provides a definitive explanation for the loss of BmGT formation by *A. fumigatus OErsmA*.

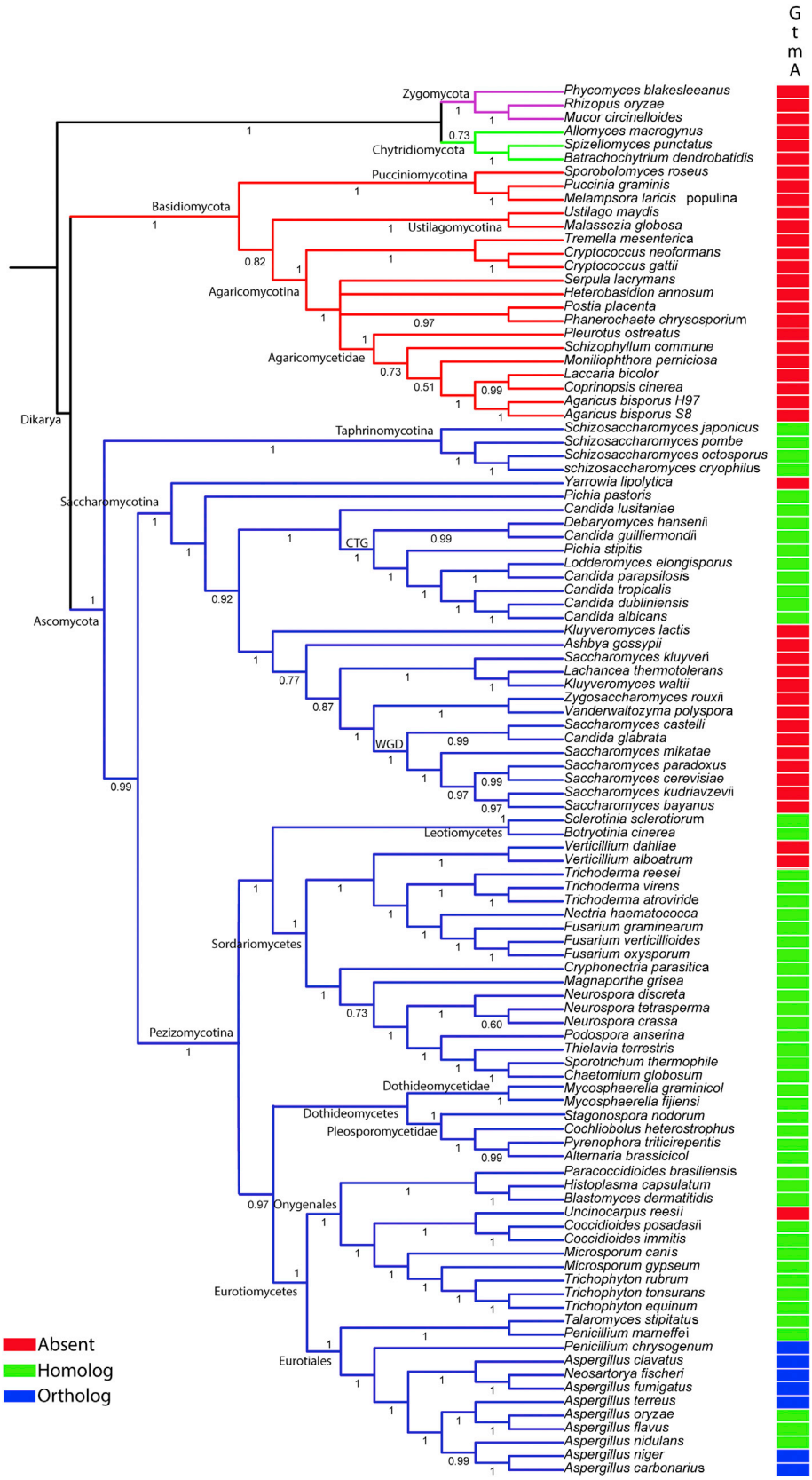
The definitive demonstration that GtmA *bis*-thiomethylates GT-$(SH)_2$ raises the question as to the function of both methyltransferases encoded within the *gli* cluster. We hypothesize that while *gliN* most likely encodes the *N*-methyltransferase that inserts the N-CH_3 in gliotoxin, *gliM* potentially encodes an *O*-methyltransferase that transiently modifies a gliotoxin biosynthetic intermediate/shunt metabolite. Indeed such an intermediate, 6-benzyl-6-hydroxy-1-methoxy-3-methylenepiperazine-2, 5-dione, has been identified by two groups following deletion of *A. fumigatus gliG* (Davis et al., 2011; Scharf et al., 2011).

We inferred GtmA orthology using three approaches. First proteins were located that shared a bidirectional best hit to *A. fumigatus* GtmA (AFUA_2G11120). Second, a GtmA phylogenetic tree was reconstructed (Figure S6A) and finally we viewed the synteny around GtmA homologs to confirm orthology (Figure S6B). All proteins that share a bidirectional BlastP hit with *A. fumigatus* GtmA are located in a single clade (Figure S6, clade-A). Based on the GtmA phylogeny, paralogs seem to have arisen in a species/lineage dependent manner as some species such as *A. niger* and *Nectria hematococca* contain multiple homologs (4 and 6, respectively), while others such as *A. nidulans* contain a single GtmA homolog. The most robust manner to infer orthology is by phylogenetic reconstruction and confirmation by synteny. Our phylogenetic tree inferred a GtmA ortholog clade containing *A. clavatus*, *N. fischeri*, *A. fumigatus*, *A. niger* and *A. carbonarius* (Figure S6, clade-C). These species, along with *Penicillium chrysogenum*, *A. terreus*, *A. oryzae*, *A. flavus*, and *A. nidulans* all belong to the Eurotiales order (Figure S6, clade-B). *A. terreus* and *P. chrysogenum* both contain proteins that have reciprocal best blast hits to *A. fumigatus* GtmA yet neither is found within the GtmA ortholog clade (Figure S6A). However based on our synteny analysis, we can confirm that both species possess orthologs of GtmA (Figure S6B). Based on our phylogeny and reciprocal best-hit strategy, there are two scenarios to explain the evolutionary history of GtmA. First, GtmA is the result of a duplication of the ortholog of AFUA_4G14510 in the last common ancestor of all these species. Species-specific losses of GtmA have since occurred in *A. terreus*, *A. oryzae*, *A. flavus* and *A. nidulans*. Alternatively, AFUA_4G14510 may be the result of GtmA duplication with subsequent retention of the GtmA paralog and loss of the GtmA ortholog in these species. The presence of GtmA orthologs in fungi nonpermissive for gliotoxin biosynthesis (e.g., *A. niger*) is interesting and perhaps represents a self-protection mechanism in these species.

The mechanism responsible for *bis*-thiomethyl ETP biosynthesis is of substantial interest due to the clear value of these natural products. Furthermore, SAM-dependent methyltransferases have been established as highly versatile enzymes for applications in biocatalysis, metabolic engineering, chemical biology, and other areas of biotechnology (Struck et al., 2012), because the chemospecific and regiospecific methylation of natural products using classical chemical alkylating agents requires multistep reactions that can be costly and time consuming (Wessjohann et al., 2013). However, despite the fact that a vast number of natural product methyltransferases have been characterized, TMTs are a largely unclassified group of biosynthetic enzymes (Liscombe et al., 2012).

Although initially regarded as inactive versions of toxic ETPs, there has been a renewed interest in the biological properties of ETP *bis*-thiomethyl derivatives (Watts et al., 2010). BmGT has also been shown to display anti-angiogenic activity (Lee et al., 2001), antibacterial activity against both methicillin-resistant and multidrug-resistant *Staphylococcus aureus* (Li et al., 2006), and the ability to specifically inhibit platelet-activating factor induced platelet aggregation (Okamoto et al., 1986). Interestingly, glionitrin B (the nontoxic *bis*-thiomethyl derivative of the ETP, glionitrin A) caused the suppression of DU145 cell invasion whereas the disulfide-containing glionitrin A and gliotoxin exhibited significant cytotoxicity, which eliminated their use in a clinical setting. This highlights the potential of these derivatives as antimetastatic agents (Park et al., 2011). BmGT has recently been evaluated as a highly specific diagnostic marker of invasive aspergillosis (Domingo et al., 2012). Thus, these recent studies indicate that ETP *bis*-thiomethyl derivatives have the potential to act as lead compounds in the search for new therapeutically valuable agents and effective biomarkers of fungal infection, and underpin the impact of our finding of an ETP *bis*-thiomethlytransferase.

In summary, we identified a *bis*-thiomethyltransferase, demonstrated its role in the *bis*-thiomethylation of GT-$(SH)_2$ (Figure 6), and proposed that it primarily acts as a molecular switch



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to attenuate *gli* cluster expression, and consequently gliotoxin biosynthesis, in *A. fumigatus*.

SIGNIFICANCE

It is well established that *bis*-thiomethylated forms of epipolythiodioxopiperazines (e.g., bisdethiobis(methylthio) gliotoxin (BmGT)) are secreted by fungi; however, both the reasons for, and mechanism of, biosynthesis have remained obscure. Finding gliotoxin *bis*-thiomethyltransferase (GtmA) in *A. fumigatus* reveals an "off switch" for gliotoxin biosynthesis—a negative regulatory mechanism. The switch is based on the observation that dithiolgliotoxin is now identified as a cosubstrate for GtmA and the gliotoxin oxidoreductase, GliT. Once gliotoxin levels increase in *A. fumigatus*, *gtmA* expression is induced, which in turn facilitates BmGT formation. This depletes dithiolgliotoxin and consequently reduces GliT-mediated gliotoxin biosynthesis, thereby attenuating expression of the gliotoxin biosynthetic cluster (*gli*). BmGT also increases GtmA expression, and so it potentiates its own biosynthesis. The absence of *gtmA* in *A. fumigatus* leads to significantly increased gliotoxin production. Unusually, GtmA is encoded outside the *gli* cluster which, according to nascent theories pertaining to gene cluster evolution, suggests that it is not involved in self-protection against gliotoxin. The occurrence of *gtmA* orthologs in other ETP-producing species suggests that this negative regulatory mechanism is widely used by fungi to control ETP biosynthesis and infers that attempts to increase natural product formation (e.g., by promoter switching or chromatin modification) may need to take cognizance of postbiosynthetic negative regulatory systems.

EXPERIMENTAL PROCEDURES

LC-MS Detection of Gliotoxin and BmGT

A. fumigatus wild-type, deletion, and complementation strains were generally grown for 72 hr in Czapek-Dox media followed by organic extraction and LC-MS analysis as previously described (Gallagher et al., 2012) Alternative media or culture conditions are noted, where relevant. *A. fumigatus* Δ*metR* (Amich et al., 2013) was cultured in Sabouraud-Dextrose media supplemented with either 1 mM L-methionine or [methyl-^{13}C]-L-methionine. Cultures were grown for 21 hr at 37°C before the addition of gliotoxin (5 μg/ml) and incubation for an additional 3 hr. Organic extraction and LC-MS analysis of culture supernatants was carried out to determine incorporation of methyl-^{13}C into BmGT. All strains used are given in Table S8. Gliotoxin (purity: 98%) and BmGT (purity: 99%) were obtained from Sigma-Aldrich and Enzo Life Sciences, respectively.

Gliotoxin *bis*-Thiomethyltransferase Activity and Partial Purification

A. fumigatus wild-type (ATCC26933; high-level gliotoxin producer) and Δ*gliT* (Schrettl et al., 2010) mycelial lysates were obtained following 24 hr growth in Sabouraud-Dextrose media (21 hr culture followed by supplementation with gliotoxin (5 μg/ml; Induced) or methanol (Uninduced) for 3 hr. Mycelia were snap-frozen and ground in liquid N_2, and lysed by multiple rounds of sonication in lysis buffer (Carberry et al., 2006). Protein lysates were clarified by centrifugation prior to use in assays. Reaction mixtures comprised relevant lysate (20 μμl), PBS (54 μl), SAM (25 mg/ml in PBS; 6 μl), and NADPH (60 mM; 10 μl). Negative controls used 20 μl lysis buffer in place of lysate. TCEP-reduced gliotoxin (1 mg/ml; 10 μl) was added to each reaction followed by incubation at 37°C overnight. Reactions were terminated by adjustment to 10% (v/v) TCA, vortexed briefly, incubated on ice for 30 min, followed by centrifugation at 10,000 × *g*, 10 min, 4°C. Supernatants were diluted 1/10 in 0.1% (v/v) formic acid and spin filtered prior to LC-MS analysis (Agilent Ion Trap 6340) to detect BmGT. Mycelial protein lysate (7.6 mg protein) from gliotoxin-induced *A. fumigatus* Δ*gliT* was subjected to sequential Q-Sepharose (anion-exchange) and Superose 12 (gel filtration) chromatography using an AKTA Purifier-100. A combinatorial strategy of *bis*-thiomethyltransferase activity (above) and protein mass spectrometric analysis (Supplemental Experimental Procedures and below; Thermo Fisher Q-Exactive mass spectrometer) of column fractions was used to screen for putative *bis*-thiomethyltransferase presence.

Gliotoxin Thiomethyltransferase A Cloning and Expression

A. fumigatus gtmA (CADRE; http://www.cadre-genomes.org.uk; AFUA_2G11120) was amplified from *A. fumigatus* ATCC26933 cDNA using primers (Table S9) and expressed in *Escherichia coli* (See Supplemental Experimental Procedures).

Gene Deletion, Complementation, and Gene Expression Analysis in *A. fumigatus* ATCC26933

A. fumigatus Δ*gtmA* and a complemented strain *A. fumigatus gtmA*^{c} were generated via the bipartite marker technique using the pyrithiamine resistance gene (*ptrA*) and hygromycin (*hph*) for deletion and complementation selection, respectively (Supplemental Experimental Procedures; Davis et al., 2011). Primers used for generating all deletion and complementation constructs are given in Table S9. Fungal RNA isolation, DNase treatment, cDNA synthesis, and RT-PCR to confirm absence of *gtmA* expression in *A. fumigatus* Δ*gtmA* were performed as described previously (Davis et al., 2011; O'Keeffe et al., 2013). The primers used in RT-PCR and qRT-PCR reactions are listed in Table S9. qRT-PCR analysis was performed using a Roche Lightcycler 480.

Comparative Quantitative Proteomic Analysis of *A. fumigatus* Wild-Type, Δ*gtmA*, and *gtmA*^{c} Strains

A. fumigatus ATCC26933, Δ*gtmA*^{26933}, and *gtmA*^{c} (n = 3 biological replicates each) were cultured in Czapek-Dox media for 72 hr (gliotoxin-inducing conditions). *A. fumigatus* ATCC26933 and Δ*gtmA*^{26933} strains were also cultured in Sabouraud-Dextrose media for 21 hr followed by gliotoxin (5 μg/ml final), BmGT (5 μg/ml final), or methanol addition for 3 hr (n = 4 biological replicates for all specimens). Mycelial lysates were prepared as described in the Supplemental Experimental Procedures, and treated with trypsin and ProteaseMax surfactant was added (Collins et al., 2013). Resultant peptide mixtures were analyzed via a Thermo Fisher Q-Exactive mass spectrometer coupled to a Dionex RSLCnano. LC gradients ran from 4% to 35%B over 2 hr, and data were collected using a Top15 method for MS/MS scans. Comparative proteome abundance and data analysis were performed using MaxQuant software (version 1.3.0.5; Cox and Mann, 2008), with Andromeda used for database searching and Perseus used to organize the data (version 1.4.1.3).

Sequence Data and Phylogenetic Methods

The fungal protein data set consisted of 103 genomes and 1,001,217 individual genes. Information on the sources of genome data are presented elsewhere (Medina et al., 2011). Using BlastP (Altschul et al., 1997), with an expectation (E) value of 10^{-5}, *A. fumigatus* GtmA (AFUA_2G11120) was searched against each individual fungal genome to locate all putative fungal homologs. Top significant hits were also searched back against the *A. fumigatus* genome and reciprocal top hits to *A. fumigatus* GtmA were putatively labeled as orthologs.

All GtmA homologs were aligned using MUSCLE (Edgar, 2004), with the default settings. Obvious alignment ambiguities were manually corrected. Phylogenetic relationships were inferred using maximum likelihood methods and the appropriate protein models of substitution were selected using the Bayesian information criterion implemented in ProtTest (Darriba et al., 2011).

Figure 5. Fungal Species Phylogeny

Modified from Medina et al., 2011. The presence and absence of GtmA is indicated. Species are deemed to contain an ortholog if they share a best bidirectional hit with *A. fumigatus* GtmA (AFUA_2G11120) and are located in the GtmA monophyletic clade (clade-c) in Figure S6.

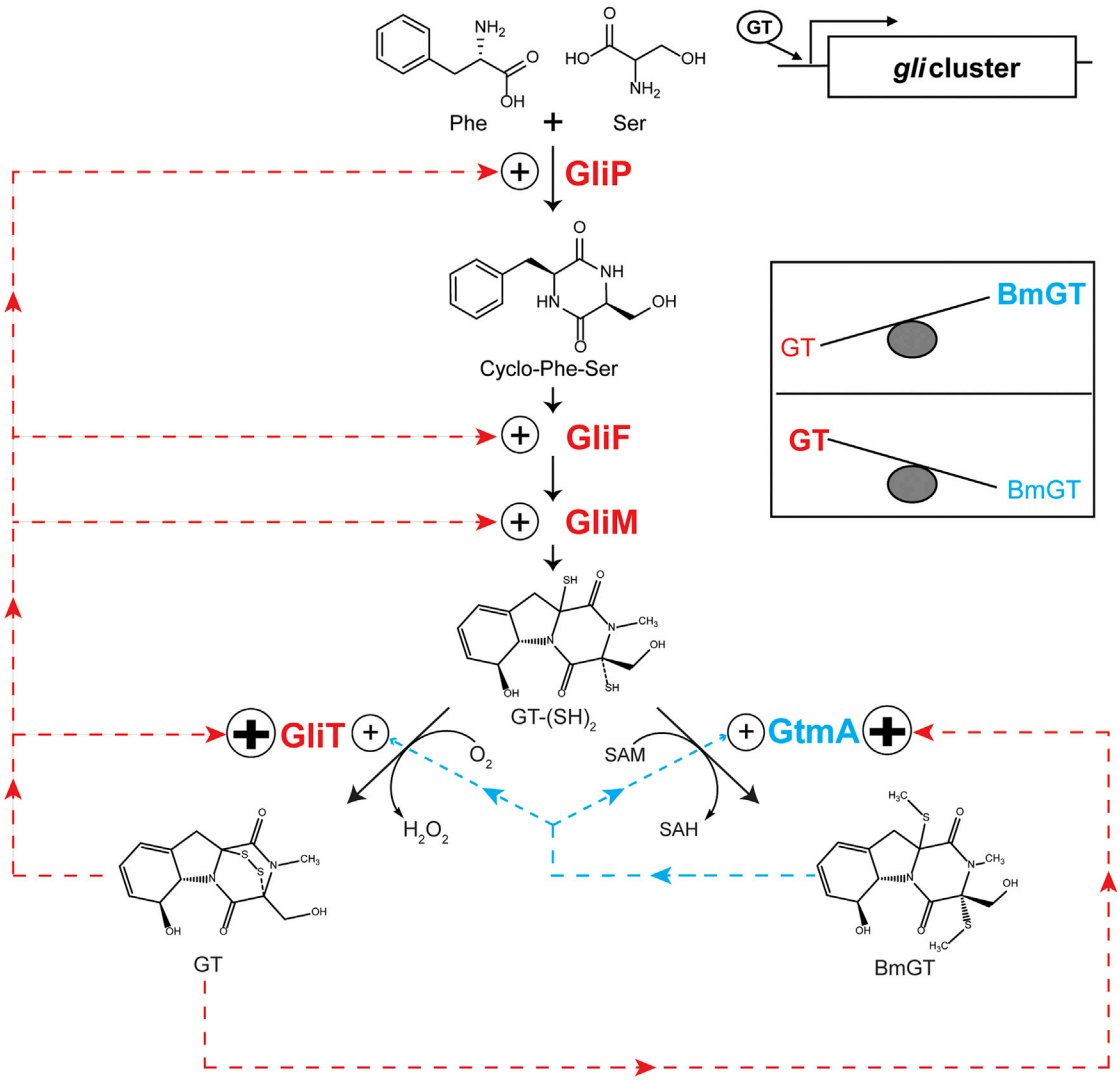

Figure 6. Model of GtmA-Mediated Regulation Gliotoxin Biosynthesis

Gliotoxin induces *gli* cluster expression and acts to potentiate its own biosynthesis. The toxic biosynthetic intermediate, GT-$(SH)_2$, is a shared substrate between GliT and GtmA, and is either converted to gliotoxin or BmGT, respectively. Gliotoxin production is suppressed by increased conversion to BmGT that occurs due to increased GtmA abundance once gliotoxin levels increase (gliotoxin significantly induces *gtmA* expression and GtmA abundance). The consequent depletion of gliotoxin leads to a reduction in *gli* cluster expression and *gli* enzyme abundance. BmGT also promotes self- and gliotoxin formation to dissipate reactive GT-$(SH)_2$.

One hundred bootstrap replicates were then carried out with the appropriate protein model using the software program PHYML (Guindon and Gascuel, 2003) and summarized using the majority-rule consensus method.

Phenotypic Assays

See the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and nine tables and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2014.07.006.

AUTHOR CONTRIBUTIONS

S.K.D., R.A.O., G.O.K., D.A.F., and S.H. carried out experimental work. S.D. and G.W.J. conceived the study. S.D., G.W.J. and D.A.F. designed the experimentation. S.K.D., R.A.O., D.A.F., G.W.J., and S.D. wrote the manuscript.

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