Reducing *Aspergillus fumigatus* Virulence Through Targeted Dysregulation of the Conidiation Pathway

James Stewart

Department of Experimental Medicine

McGill University, Montreal, Canada

March 2019

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Masters of Science

Table of Contents

List of Figures

List of Tables

Abstract

The incidence of invasive fungal infections has risen dramatically in recent decades, with *Aspergillus fumigatus* being the most common cause of invasive mould disease in humans. This infection, termed invasive aspergillosis (IA), occurs when the conidia of *A. fumigatus* enter the lower airways of the human lung and germinate to form filamentous hyphae that invade lung tissues. IA occurs primarily in immunocompromised individuals and is associated with mortality rates of 30 to 95%, despite antifungal therapy. There exists an inverse relationship between conidiation and vegetative growth in *A. fumigatus*, where conidiation is rarely observed during an invasive infection of the human host, permitting the bulk of the fungal metabolic energy to remain directed towards vegetative growth. We therefore hypothesize that forced induction of conidiation during an infection will suppress *A. fumigatus* vegetative growth, impairing the ability of this organism to cause disease.

To test this hypothesis, we expressed a key regulator of conidiation in *A. fumigatus*, *brlA*, under the control of a doxycycline-inducible promoter. Inducing expression of *brlA* was sufficient to force the inappropriate conidiation of *A. fumigatus in vitro* and high levels of *brlA* overexpression inhibited *A. fumigatus* vegetative growth. In an invertebrate model of IA, *Galleria mellonella* larvae infected with the inducible *brlA* overexpression ($brlA^{I\text{-}OE}$) strain survived significantly longer in the presence of the inducer molecule doxycycline compared to untreated larvae. Similarly, in a mouse model of IA, mice treated with doxycycline were protected from infection with the $brlA^{I\text{-}OE}$ mutant, with reduced pulmonary fungal burden and a dose-dependent decline in mortality relative to untreated, infected controls.

These findings provide proof-of-concept that targeted upregulation of *brlA* reduces *A. fumigatus* virulence *in vivo*, and that manipulation of the fungal life cycle is a potentially viable new antifungal target.

Résumé

Au cours des dernières décennies, la prévalence des infections fongiques à caractère invasif a considérablement augmenté, *Aspergillus fumigatus* étant à l'origine de la plupart d'entre elles chez l'humain. Cette infection, nommée aspergillose invasive (AI), survient quand une spore issue de la reproduction asexuée (ou conidie) pénètre dans les voies aériennes inférieures du poumon et germe en un hyphe filamenteux, lequel envahit ensuite le reste des tissus pulmonaires. L'AI se déclare principalement chez des individus immunodéficients; le taux de mortalité est alors de 30 à 95 %, malgré le recours à la thérapie antifongique disponible.

Chez *A. fumigatus* il existe une relation inverse entre conidiation et croissance végétative: la conidiation étant rarement observée durant une AI, il est admis que cela permet au champignon de diriger la plus grande partie de son énergie métabolique vers sa croissance végétative.

Nous avons donc formulé l'hypothèse selon laquelle forcer les hyphes végétatifs d'*A. fumigatus* à conidier durant une infection bloquera leur croissance, entravant ainsi le potentiel pathogénique de cet organisme.

Afin de valider cette hypothèse, nous avons expressé *brlA*, un régulateur-clé de la conidiation dans *A. fumigatus*, sous le contrôle d'un promoteur inductible par la doxycycline. Ainsi qu'attendu, nous avons observé que l'induction de l'expression de *brlA* est suffisante pour induire la conidiation d'*A. fumigatus in vitro* dans des conditions normalement inhibitrices, et que la haute

surexpression du gène inhibe complètement la croissance végétative. Dans un modèle invertébré d'AI, les larves de *Galleria mellonella* qui ont été infectées par notre souche inductible d'*A. fumigatus* (*brlA*$^{I\text{-}OE}$) et traitées à la doxycycline (inductrice du promoteur Tet-ON) ont survécu significativement plus longtemps que les larves infectées mais non traitées. Dans un modèle murin d'AI, les souris traitées avec doxycycline ont également survécu plus longtemps au mutant *brlA*$^{I\text{-}OE}$ que les souris infectées mais non traitées; dans ce modèle, nous avons pu observer que la baisse de mortalité s'est faite conjointement à une réduction de la charge fongique pulmonaire, toutes deux proportionnelles à la dose de doxycycline injectée.

Ces observations apportent une preuve-de-concept selon laquelle la surexpression de *brlA* réduit la virulence d'*A. fumigatus* et que le manipulation du cycle de vie fongique pourrait servir de base au développement d'une stratégie thérapeutique antifongique dirigée contre l'AI.

Acknowledgements

I would first and foremost like to thank my supervisor, Dr. Don Sheppard for accepting me into his research group and teaching me the skills needed to succeed in the competitive world of scientific research. You taught me how to think critically, to face problems head-on and to learn from every experience, no matter what the outcome. These lessons will stay with me for the rest of my life. The passion and enthusiasm you hold for your work is truly inspiring, and I hope to one day find my calling, just as you have.

I would also like to thank Dr. Fabrice Gravelat who has been a wonderful friend and mentor, both in the lab and outside of it. From the fundamentals of molecular biology to the interconnectedness between all life forms, you have taught me to be a better scientist and a more grounded and compassionate person. I will always cherish our time together and look forward to the many good times to come!

Thank you to all the present and past members of the Sheppard lab for making my time here so special. I would especially like to thank Josée Chabot and Brendan Snarr who have provided guidance, support, and answered my incessant questions on a daily basis. My time over these last few years would not have been the same without you.

To the members of my committee, Dr. Marcel Behr, Dr. Salman Qureshi and Dr. Suhad Ali, thank you for your support. Your guidance and encouragement have been very helpful and always appreciated.

Lastly, I would like to thank my family and friends for their ongoing support and encouragement throughout my time at McGill. You helped me to understand the importance of a work-life balance, and some of my fondest memories are of our time spent together.

Contribution of authors

James Stewart wrote this thesis, which was reviewed by Dr. Don C. Sheppard and Dr. Margo M. Moore. James Stewart performed all experiments described in chapter two of this thesis, with assistance from the following individuals: Fabrice N. Gravelat developed the idea for the project, translated the abstract and provided support for *in vitro* studies. Melanie Lehoux assisted with animal studies and Vinicius Fava performed bioinformatic processing of the RNA deep-sequencing data. Carine Bourguet performed the *ex vivo* uHPLC-MS analyses.

Chapter 1: Introduction and Literature Review

1.1 *Aspergillus* historical background and taxonomy

The genus *Aspergillus* was discovered in 1729 by Pier Antonio Micheli, an Italian catholic priest who noted the resemblance between the conidia-bearing structure of *Aspergillus* and a catholic ceremonial device, knows as an *aspergillum*, used to sprinkle holy water onto the congregation (1). In his work, Micheli described 9 mould species as *Aspergilli* but since then the genus has rapidly increased, now comprising between 260 (2, 3) and 837 (2, 4) different *Aspergillus* species, depending on the criteria used for speciation. *Aspergilli* are one of the most well-studied and economically-exploited groups of filamentous moulds to date, due to their ubiquity in nature, the ease with which they are cultured *in vitro*, and their extensive production of diverse secondary metabolites that are used in the fields of agriculture, biodegradation, fermentation and pharmaceutical development (1, 5, 6, 7, 8). Some examples of the economic use of *Aspergilli* include, but are not limited to, the production of rice wine (sake), soybean paste (miso) and soy sauce (shoyu) through the fermentation processes of *Aspergillus oryzae* (5) and solid-state fermentation by *Aspergillus niger* for the synthesis of organic acids, particularly citric acid which is used extensively for food production, preservation and flavouring, as well as a common additive in cosmetics, pharmaceuticals and cleaning products (6, 7). Despite the many beneficial roles *Aspergilli* play in our society, some members have adapted to survive within the human body, now acting as opportunistic pathogens. The most prevalent species to cause disease in humans is *A. fumigatus*, accounting for almost 90% of *Aspergillus* infections (9).

1.2 The life cycle of *A. fumigatus*

The life cycle of *A. fumigatus* begins when airborne microscopic conidia (1.3 to 1.8 μm in diameter (10, 11)) come in contact with a source of both nutrients and water. These favourable growth conditions act as a signal for conidial germination and a transition out of dormancy. Within the first 30 minutes of germination, hundreds of genes are up-regulated, the majority of which play a role in RNA metabolism and the biosynthesis of amino acids, proteins and protein assembly complexes (12). Over the next 8 hours a series of dramatic changes occur at the conidial cell surface. Beginning after 2 hours of germination, the outermost conidial cell layer, composed of a tight array of hydrophobin proteins RodA and RodB, begins to be shed (13, 14). The shedding of rodlets is associated with a loss of hydrophobicity and corresponds with the onset of conidial swelling (14). Between 4 and 8 hours of germination, radial growth is replaced by polarized vegetative outgrowth, typically in one or two directions, thus generating the first filamentous structure of *A. fumigatus*, known as the germ tube (11, 15, 16). Germ tube growth proceeds at the apical tip with occasional branching that results in the production of elongated hyphae that form a complex "web", collectively known as the mycelium (11, 17). The morphology of *A. fumigatus* filamentous hyphae is one of undifferentiated cells separated by perforate septae, many of which containing a single intact nucleus. This structural organization permits the free flow of molecules between cells and facilitates rapid growth (17).

Vegetative growth continues for a period of between 12 to 16 hours, during which time the fungus is considered developmentally immature or "pre-competent". The period of pre-competency is genetically determined in *A. fumigatus*, therefore any stimuli typically associated with the activation of asexual reproduction or "conidiation"; such as starvation, desiccation, or exposure to light, have no effect on pre-competent hyphae. Once the fungus has transitioned into

developmental maturity or “competency”, these same stimuli rapidly trigger the onset of conidiation through a tightly regulated and well-conserved genetic pathway of conidiation (2, 18, 19), to be discussed in greater detail in section 1.2.2.

Upon activation of the conidiation pathway, vegetative growth slows and energy is instead directed towards the formation of a conidiophore; the asexual reproductive structure of *A. fumigatus*. First, aerial hyphae extend vertically away from the foot cell; a specialized structure characterized by a thickened cell wall which provides additional support for the conidiophore (20). Aerial hyphae make up the conidophore “stalk’, and at the stalk tip hyphal extension gives way to swelling and the formation of a multinucleated vesicle. Multiple single-nucleated buds protrude from the vesicle resulting in a single layer of sterigmata cells known as phialides. Phialides undergo repeated asymmetric mitotic division to form chains of conidia, the asexual reproductive cell of *A. fumigatus*. A single conidiophore can give rise to as many as 50,000 conidia (18). The final step in the asexual life cycle of *A. fumigatus* is conidial cell wall maturation, which includes melanisation and rodlet biosynthesis and assembly. Finally, mature conidia are dispersed via wind currents for the colonization of new habitats and completion of the asexual life cycle.

A sexual cycle has recently been described for *A. fumigatus in vitro* (21), however little is currently known about this process. This is due in part to its relatively recent discovery as well as the extremely strict conditions required for sexual reproduction to occur *in vitro*, suggesting that this process plays a minor role in the natural life cycle of *A. fumigatus*. Conversely, asexual reproduction of *A. fumigatus* occurs readily in the environment as well as the laboratory, further supporting its role as the principle means of propagation for this species. Conidia are also the primary infectious agent of *A. fumigatus.* For these reasons the bulk of research into *Aspergillus*

reproduction to date has focussed on conidiation and the molecular pathways that govern this process.

1.2.1 The conidiation pathway

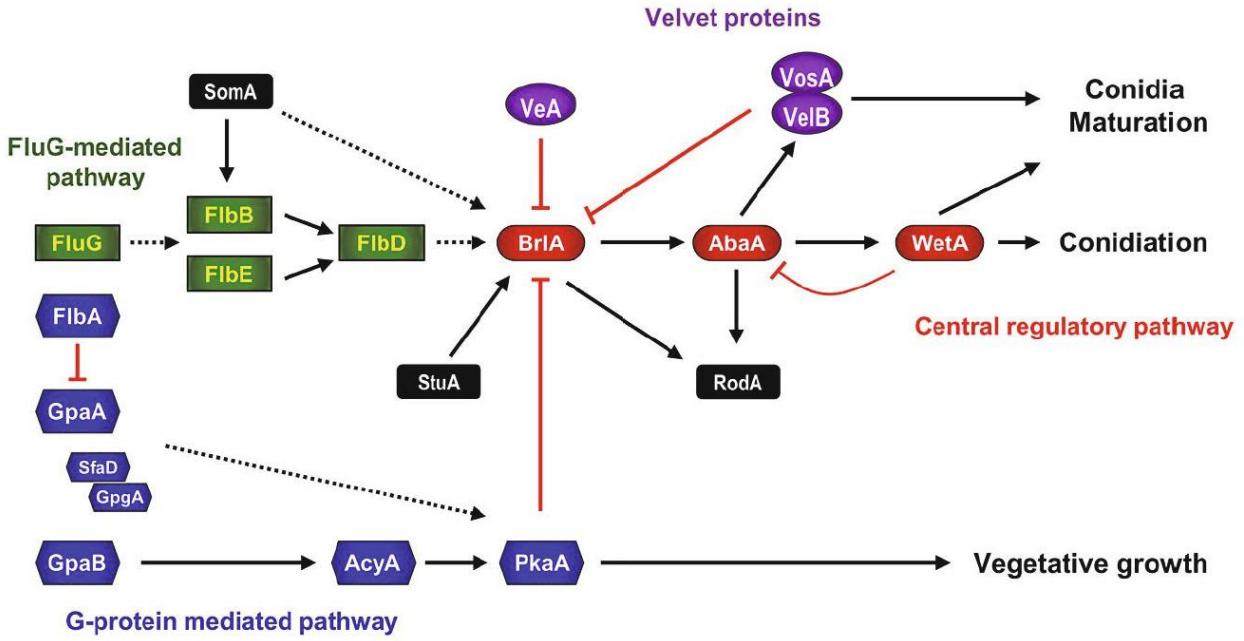


Figure 1: Genetic model of the conidiation pathway regulators in *A. fumigatus*. Adapted from *Park & Yu, Journal of Microbiology, 2016.*

Control of conidiation in *A. fumigatus* involves a complex network of genes and regulatory pathways, which respond to both genetically determined spatial and temporal switches, and environmental stimuli. Over 35 genes have been identified as encoding regulators of the conidiation pathway of *A. fumigatus* (18) and these have been separated into several distinct subsets of developmental regulation. These groups include the highly conserved central regulators; the upstream regulators, also known as the FluG-mediated regulatory pathway; the downstream regulators, consisting of the velvet family proteins; the heterotrimeric G-protein signaling

pathways; the Ras-mediated signaling pathway; the calcium-calcineurin pathway; and a group of "other factors", which are critical for appropriate conidiation, but do not fit well into these other categories (18). Though each pathway plays a distinct role in the conidiation program of *A. fumigatus*, cross-talk is common and often necessary for appropriate conidiation to occur. Given the breadth of genetic pathways implicated in the control of conidiation in *A. fumigatus*, the scope of background information in this literature review will be restricted to pathways that directly influence the expression of the central regulatory gene *brlA*, an essential activator of conidiation.

The central regulators of conidiation in *A. fumigatus* include three key players: *brlA*, *abaA* and w*etA*, all of which are necessary for the coordinated spatiotemporal expression of conidiation-specific genes during conidiophore formation and conidia maturation (18, 22, 23), and as such are highly conserved among members of the genus *Aspergillus*.

Acting first in the central regulatory pathway is *brlA*, which encodes a C_2H_2 dual zinc-finger transcription factor. Often referred to as the "master regulator" of conidiation in *A. fumigatus* (22, 24, 25), BrlA is an essential activator of conidiation, regulating a vast number of conidiation-specific genes through both indirect and direct pathways. Gene disruption studies have demonstrated that *brlA* is essential for conidiophore development. Strains deficient in *brlA* cannot progress past conidiophore stalk formation, producing pure white fungal colonies lacking green-pigmented conidia and a resulting in a "bristle-like" appearance when observed under the microscope (24, 26), which gave rise to the gene name *brlA*. Inducible overexpression of *brlA* is also sufficient to force the inappropriate conidiation of *Aspergillus nidulans in vitro* (22), though the effects of *brlA* overexpression in *A. fumigatus* have yet to be assessed.

Evidence for direct gene regulation by BrlA comes from heterologous expression studies performed in the yeast *Saccharomyces cerevisiae* (27). In this study, *brlA* expression in an *S. cerevisiae* mutant containing the *A. nidulans* conidial rodlet gene (*rodA*) promoter fused to the *Escherichia coli lacZ* gene led to the induction of β-galactosidase, the protein product of *lacZ*, suggesting that BrlA interacts with the *rodA* promoter to activate *lacZ* expression (27). The authors hypothesized the existence of a putative BrlA-recognition site (5'-[C/A][G/A]AGGG[G/A]-3'), termed the BrlA-response element (BRE), within the *rodA* promoter (27). Since then numerous studies have identified BREs within the promoters of BrlA-activated conidiation-specific genes, including the central regulators *abaA* and *wetA* (10, 18, 27), though direct observation of this interaction has yet to be confirmed.

Following *brlA* in the *A. fumigatus* central regulatory pathway of conidiation is *abaA*, encoding a transcription factor that is involved in the formation of phialides as well as their terminal differentiation to produce immature conidia (10, 20). *A. fumigatus abaA*-deficient strains produce elongated terminal phialides lacking conidia (10). The expression of *abaA* is dependent upon *brlA* expression and occurs shortly after stalk formation is complete, leading to phialide differentiation and the expression of many genes involved in the mid-to-late phases of conidiation, such as *wetA* (20, 28).

The w*etA* gene encodes an essential regulator of conidia maturation which plays diverse roles in the late phases of conidiation. A *wetA*-deficient strain of *A. fumigatus* produces conidia lacking melanin, and with abnormal cell wall morphology often resulting in failed conidial separation and the production of long chains of atypical conidia (10). These conidia also lack trehalose, a sugar that is essential for stress resistance and long-term conidia viability in *A.*

fumigatus (10, 28, 29). As a result, Δ*wetA* conidia are highly susceptible to oxidative, osmotic and thermal stressors, and have reduced long-term viability (10).

Both Δ*abaA* and Δ*wetA* strains of *A. fumigatus* exhibit increased and prolonged expression of *brlA* during the late phase of conidiation, suggesting that these regulators also function in a negative feedback loop to restrict *brlA* expression following conidiation (10, 18), likely through their activation of the velvet family of conidiation regulators. The velvet family of conidiation pathway regulators include *veA*, *vosA* and *velB*, each encoding a fungi-specific transcription factor with a "velvet" DNA-binding domain (18, 30, 31). Velvet family proteins function largely as repressors of conidiation in *A. fumigatus*, though each is required for appropriate conidiation to occur (32). Two studies involving a *veA*-deficient strain of *A. fumigatus* demonstrate dysregulated conidiophore production but with opposing phenotypes. Dhingra et al. (33) observed lower levels of *brlA* mRNA and reduced conidiation in both *veA*-deficient and *veA*-overexpressing strains of *A. fumigatus*, while Park et al. (32) observed higher levels of *brlA* mRNA and increased conidiation in a Δ*veA* strain. Loss of function mutations in either *vosA* or *velB* result in the accumulation of *brlA* mRNA *in vitro*. The accumulation of *brlA* under these conditions leads to increased or early onset conidiation in conidiating and non-conidiating conditions, respectively (18, 32), confirming the role of these two velvet proteins as repressors of conidiation. The maturation of conidia also requires *velB* and *vosA*, as *A. fumigatus* strains that are deficient in either gene produce conidia with lower trehalose content, reduced viability and stress tolerance, and hyperactive germination (18, 20, 32), closely phenocopying Δ*wetA* conidia (10). Taken together, these findings demonstrate the essential and highly coordinated role of velvet proteins and central regulators in the final phases of conidiation in *A. fumigatus* (18).

The upstream regulatory pathway of *A. fumigatus* conidiation comprises four genes, *fluG*, *flbB*, *flbE*, and *flbD*, the first three of which have been characterized to encode positive regulators of conidiation. Gene disruption mutations in any of the upstream regulators results in decreased expression of *brlA* and a *"*fluffy*"* phenotype characterized by delayed, reduced, or in some cases absent conidiation (24, 34, 35). FluG acts first in the upstream regulatory pathway and is necessary for conidiation specifically in liquid submerged conditions (24). In low nutrient liquid culture, a *fluG*-deficient strain is unable to produce conidiophores and has increased hyphal mass whereas the wild type (WT) parent at the same time point conidiates extensively (24). When the Δ*fluG* strain is transferred to conidiation-inducing solid media, *brlA* expression and conidiation is delayed relative to WT (24). Together, these findings demonstrate an essential role for FluG in *A. fumigatus* conidiation under liquid submerged condition, while on solid media FluG influences *brlA* expression but is not absolutely required for conidiation. These findings led to the hypothesis that alternative pathways exist that can control *brlA*-expression and the activation of conidiation in *A. fumigatus* (18, 20, 24). Downstream of *fluG* are the three *fluffy* genes *flbB*, *flbE*, and *flbD*, encoding transcription factors that function together to activate *brlA* and conidiation (20, 34, 35). FlbB and FlbE are proposed to function interdependently and upstream of FlbD as both are required for *flbD* expression, and in turn the appropriately timed expression of *brlA* and conidiation following transfer to conidiation-inducing conditions (34, 35). Similar to their role in *A. nidulans*, FlbB and FlbE of *A. fumigatus* may interact to form a functional complex that induces *flbD* expression and FlbD in turn activates *brlA* leading to the onset of conidiation (35, 36).

Though not involved in the upstream regulatory pathway of conidiation in *A. fumigatus*, another member from the *fluffy* gene family FlbA indirectly promotes conidiation by inhibiting GpaA, a member of the heterotrimeric G protein regulatory pathway that functions largely in

maintaining vegetative growth (18, 24). Typical heterotrimeric G protein signaling involves the coordination of a G protein complex with α, β, and γ subunits, a G-protein coupled receptor (GPCR), a regulator of G-protein signaling (RGS) and various effectors. The ability of each subunit to activate multiple effectors permits the regulation of multiple processes from a single stimulus (37). The heterotrimeric G-protein signaling cascade of *A. fumigatus* fits this description well, playing a role in the maintenance of vegetative growth, the indirect repression of conidiation and the process of conidia maturation.

The Gα subunit GpaA promotes vegetative growth while simultaneously inhibiting conidiation through uncharacterized pathways (24). Conversely, another Gα subunit, GpaB, is required for normal conidiation in *A. fumigatus*, as strains deficient in *gpaB* produce significantly fewer conidia than the WT parent *in vitro.* The conidiation defects observed in the *gpaB*-deficient strain were reversed following treatment with cyclic-adenosine mono-phosphate (cAMP), suggesting that the cAMP-protein kinase A (cAMP-PKA) pathway functions downstream of GpaB to mediate these effects (38). cAMP-PKA signaling in *A. fumigatus* governs conidial pigmentation through regulated expression of the polyketide synthase gene *pksP* (also known as *alb1*) that is involved in melanin biosynthesis (39). Consistent with the role for GpaB in cAMP signaling, a *gpaB*-deficient *pksP*-luciferin tagged strain exhibits attenuated luminescence relative to the *pksP*-tagged WT (38).

The Gβ and Gγ subunits (SfaD and GpgA respectively) are important negative regulators of conidiation in *A. fumigatus* (40). *A. fumigatus* strains deficient in either gene exhibit higher levels of *brlA* mRNA in non-conidiating conditions leading to early onset and increased conidiation *in vitro*. *A. fumigatus* strains which constitutively overexpress *sfaD* or *gpgA* produce significantly fewer conidia in liquid culture when compared to WT (40). SfaD and GpgA are also

critical for vegetative growth as the *sfaD*- and *gpgA*-deficient mutants exhibit growth rates of approximately 1% that of WT (40). Taken together, these findings suggest SfaD and GpgA function as a heterodimer (40) to suppress *brlA* during the vegetative growth stage, thereby permitting the metabolism of *A. fumigatus* to remain shifted towards hyphal proliferation. Down-regulation of these Gβ and Gγ subunits is likely critical for mediating *brlA* transcript accumulation and thus the transition from vegetative growth to conidiation (40).

In addition to the major pathways governing conidiation in *A. fumigatus*, several other factors play an important role in regulating *A. fumigatus* asexual development. The SomA transcription factor is essential for conidiation and important for vegetative growth in *A. fumigatus*. Strains deficient in *somA* cannot proceed past stalk formation, lack conidia and demonstrate significantly stunted growth relative to WT (41). Transcriptional analysis of the Δ*somA* strain demonstrated the absence of *brlA* expression and significantly reduced expression of both *stuA* and *medA*, two additional transcription factors required for appropriate conidiation in *A. fumigatus*, discussed below (41). Taken together, these findings illustrate an essential role for SomA in the conidiation process of *A. fumigatus*, mediated through the central regulatory gene *brlA* as well as the regulatory proteins StuA and MedA.

The regulatory proteins StuA and MedA are both required for appropriate conidiation in *A. fumigatus* (19, 42). Strains deficient in *stuA* or *medA* produce morphologically abnormal conidiophores with significantly fewer conidia relative to WT (19, 42). A predicted StuA-binding site has been identified in the promoter region of *brlA* (41), suggesting a direct role for StuA in the regulation of *brlA*, however direct observation of this interaction remains to be verified. Strains deficient in *medA* are deficient in conidiation, but exhibit no significant alterations in central

regulatory gene expression throughout the fungal life cycle, suggesting that MedA regulates *A. fumigatus* conidiation through some alternative and unknown pathway (42).

The number of genes and regulatory pathways governing *A. fumigatus* conidiation, as well as the degree to which they are conserved across *Aspergillus* species, highlights the importance of balanced asexual development and vegetative growth to the fitness of *Aspergilli.*

1.2.2 The balance between conidiation and vegetative growth

There exists an inverse relationship between the two major developmental stages of *A. fumigatus* – conidiation and vegetative growth. When conidiation begins, vegetative growth slows, and conversely, during vegetative growth, conidiation is not observed (24). Vegetative growth and conidiation are inextricably linked through the activity of the master developmental regulator BrlA. BrlA is essential for conidiation in *A. fumigatus* (24, 26), and in the close relative *A. nidulans, brlA*-overexpression induces conidiation and inhibits vegetative growth *in vitro (22).* Tightly regulated and appropriately timed conidiation, through BrlA, permits fungal propagation and the survival of *A. fumigatus* in hostile conditions, thus contributing to its ubiquity in the environment. Unfortunately, these properties also contribute to the role of *A. fumigatus* as an opportunistic human pathogen.

1.3 Human exposure to *A. fumigatus* conidia

The production of high numbers of airborne conidia by *A. fumigatus* results in repeated human exposure to these fungal elements. Humans will typically inhale up to 1000 *A. fumigatus* conidia each day (8), yet relatively few will develop disease due to the presence of effective host defence mechanisms against *A. fumigatus*. The first line of defence against *A. fumigatus* is the

trachobronchial mucociliary elevator, which functions as a physical barrier to inhaled conidia (43, 44). The bulk of inhaled conidia become trapped within the mucous layer lining the airways and through the beating action of the underlying cilia, the conidia are easily excreted (43, 44). The conidia that evade physical removal and reach the lower airways are met by resident alveolar macrophages which rapidly phagocytose and destroy the conidia through the production of reactive oxygen species and acidification within the phagolysosome (45, 46). Alveolar macrophages are also involved in the recruitment of neutrophils to the lungs (47, 48), in response to the presence of germinating conidia and young hyphae. Neutrophils are effective at killing these more mature fungal forms through various cell-mediated mechanisms (47, 49, 50), providing a second line of defence against *A. fumigatus* infection.

1.4 Invasive aspergillosis

If the host defences against *A. fumigatus* are compromised, filamentous hyphae can invade lung tissues, to produce a severe pulmonary infection known as invasive aspergillosis (IA). IA is a severe *Aspergillus* infection characterized by hyphal invasion of epithelial barriers, tissue destruction and poor patient outcomes, even despite antifungal therapy. Though rare instances of non-pulmonary IA can occur at sites such as the skin, gastrointestinal tract or sinuses (45, 51, 52), the majority of IA cases (approximately 80-90%) originate within the lungs (53) and as such the focus of this thesis will be that of pulmonary disease. IA occurs predominantly in individuals with compromised immune defences, though the relative risk, the rate of disease progression and degree of host pathology vary greatly depending on the form and extent of host immune dysfunction.

1.4.1 Risk factors

The majority of IA cases occur in patients with compromised immune function. The population of at risk individuals for IA is increasing along with the rising use of immunosuppressive therapies in the clinic (54).

Neutrophils play a key role in the immune defence against *A. fumigatus* infection (55, 56), and as such neutropenia is a major risk factor for IA (57, 58). The probability of developing IA correlates with the degree and duration of neutropenia, with the highest risk groups demonstrating neutrophil counts of 500 cells/ml of blood or less, and an estimated 1% to 4 % increase in the probability of developing IA for each successive day of neutropenia (45, 59). In addition to reduced neutrophil number, neutrophil dysfunction is also a risk factor for IA. The most notable example is chronic granulomatous disease (CGD), characterized by impaired ROS production by neutrophils and other phagocytes (60).

Patients that have received hematopoietic stem cell or solid organ transplants must be given potent and prolonged immunosuppressive therapy to avoid transplant rejection and graft versus host disease (GVHD) (61), and as such are at high risk for developing IA. Some common therapies that increase the risk of IA in transplant patients include corticosteroids, cyclosporine A and other immunomodulatory molecules, such as anti-TNF antibodies, which reduce T-cell numbers or function (45, 61-64).

There are several other patient groups that are susceptible to developing IA, yet do not have the more classical risk factors previously described. These include patients with severe chronic obstructive pulmonary disease (COPD), patients suffering from leukemia or other non-

haematological malignancies (45), as well as patients that have experienced hepatic failure, renal failure, diabetes mellitus or severe influenza infection (58, 65-67).

1.4.2 Disease progression and pathology

During IA, *A. fumigatus* conidia that reach the lower airways and evade innate immune defences can germinate to form hyphae that invade pulmonary tissues. The rate of disease progression and degree of host pathology depends largely on host immune status.

Within the neutropenic host lung, the absence of an effective antifungal neutrophil response permits the rapid and largely unrestricted growth of *A. fumigatus* hyphae. *A. fumigatus* hyphae penetrate the lung epithelium and grow extensively within pulmonary tissues, and can invade blood vessels leading to thrombosis, hemorrhage (57, 68, 69) and fungal dissemination throughout the body (57). In non-neutropenic patients, the dysfunctional neutrophils that are recruited to the site of infection are still able limit the growth of *A. fumigatus* hyphae within the lungs, reducing fungus-driven damage to host tissues, angioinvasion and dissemination via the blood (57). However, continued neutrophil recruitment to, and activity within the lung can lead to host lung tissue damage and necrosis (57). The cause of death in many non-neutropenic cases of IA is therefore believed to result from a hyper-inflammatory response, distinct from neutropenic cases of IA where death is largely attributed to unrestricted fungal growth and destruction of tissues (57).

1.5 Antifungals

Overall, the antifungal options available to treat IA are relatively limited. Several antifungal classes exist, each targeting a biological structure or functional component of *A.*

fumigatus that is critical for the growth and survival of this opportunistic pathogen yet are absent from mammalian cells.

1.5.1 The azoles

The azole class of antifungals consists of four molecules which have been approved for clinical use in cases of IA: itraconazole (ITZ), voriconazole (VCZ), posaconazole (PCZ) and isavuconazole (ISV) (70-74). Azoles are fungistatic to *A. fumigatus*, functioning as inhibitors of lanosterol 14-α-demethylase, a critical component of the ergosterol biosynthesis pathway of *A. fumigatus* (71, 75). Azole-mediated inhibition of the lanosterol 14-α-de-methylase enzyme results in an accumulation of the substrate, 14-α-methyl sterol, which destabilizes the fungal cell membrane and impairs *A. fumigatus* vegetative growth (71, 76). ITZ was the first azole to be used clinically for the treatment of IA in 1990 (72, 77), demonstrating high efficacy *in vitro* against the majority of clinical *A. fumigatus* isolates tested, however voriconazole is now the most commonly used of these agents for treatment of IA (72). The extensive use of these compounds as environmental fungicides and preservatives in the agriculture and food industries respectively has led to the emergence of azole and multi-azole resistant *A. fumigatus* isolates, thereby limiting the efficacy of azoles against IA (78, 79).

1.5.2 The polyenes

The polyene antifungals which have been approved for use in cases of IA include amphotericin B (AmB) deoxycholate and its lipid-associated derivatives (45). Similar to the azoles, polyenes target ergosterol within the fungal cell membrane; however, they act by binding directly to ergosterol and forming a transmembrane pore that leads to membrane destabilization,

leakage of cellular contents and in some cases fungal death (76). For many years polyenes were used as the first line of therapy against IA, demonstrating particularly good efficacy against *A. fumigatus* (80-82). However, polyenes have been replaced by the azoles due to the relatively high toxicity (due to the structural similarities of ergosterol to mammalian cholesterol) and lower treatment success rate of AmB when compared to voriconazole (83). While there is no known polyene resistance mechanism in *A. fumigatus* (84), susceptibility to AmB deoxycholate appears to be decreasing (80, 81).

1.5.3 The echinocandins

The echinocandin class of antifungals consist of caspofungin, micafungin and anidulafungin, which have been approved as second line agents in cases of IA (85). The echinocandins inhibit fungal cell wall biosynthesis by targeting β-1,3-glucan synthase, an essential enzyme involved in the production of β-1,3-glucan, which is a major cell wall polysaccharide of pathogenic fungi (85, 86). β-1,3-glucan synthase is unique to fungi and as such echinocandins demonstrate relatively few off-target effects and low toxicity in humans (85, 87). Despite these benefits, the echinocandins demonstrate relatively poor efficacy against *A. fumigatus* as a monotherapy and are therefore limited for use in IA cases that are refractory to the azoles (70, 88). However, due to the targeting of a unique fungal pathway, caspofungin has recently shown promise as combination therapy with either an azole or polyene (89).

1.5.4 Need for new drug targets

Currently the efficacy of antifungals against *A. fumigatus* remain limited and with each passing year the performance of our antifungal arsenal is diminishing. The antifungal pipeline is

stagnant, with nearly 20 years since the introduction of the most recent antifungal class, the echinocandins (70, 89). Since that time, antifungal resistance in *A. fumigatus* has been observed for nearly every available antifungal compound (71, 77, 79, 80, 84, 85). Together, these facts highlight the urgent need for the identification of new and unique antifungal drug targets. One strategy that has been largely overlooked, is the manipulation of the fungal life cycle to reduce the growth and virulence of *A. fumigatus*.

1.6 Hypothesis

We hypothesize that targeted upregulation of the central regulatory gene *brlA* to induce activation of the conidiation pathway during IA will suppress *A. fumigatus* vegetative growth, impairing the ability of this organism to cause disease.

1.7 Research goals

1. Develop a strain of *A. fumigatus* in which the conidiation process can be artificially induced.

2. Determine the impact of conidiation pathway dysregulation on *A. fumigatus* fitness *in vitro*.

3. Evaluate the effects of conidiation pathway dysregulation on *A. fumigatus* virulence *in vivo*.

Preface to chapter 2

A. fumigatus is the most common cause of invasive mould disease in humans, and infections caused by this species have been historically challenging to treat (45, 53, 72, 78, 90). Few classes of antifungals have been developed with activity against *A. fumigatus*, and new antifungal targets are urgently needed. A balance exists in *A. fumigatus* between the two major developmental stages – vegetative growth and conidiation. *A. fumigatus* vegetative growth is suppressed during conidiation, and conversely, conidiation is not observed during vegetative growth (24). During an invasive infection, *A. fumigatus* rarely undergoes conidiation, permitting the bulk of its metabolic energy to remain directed towards vegetative growth. This study aimed to exploit this observation, using an inducible gene overexpression system to test the capacity of induced activation of the conidiation pathway to reduce *A. fumigatus* growth *in vitro*. The therapeutic potential of this strategy was then evaluated in an invertebrate and a neutropenic mouse model of IA. Finally, the effects of induced activation of the conidiation pathway on the *A. fumigatus* transcriptome were assessed through RNA deep-sequencing.

Chapter 2: Reducing *Aspergillus fumigatus* Virulence Through Targeted Dysregulation of the Conidiation Pathway

Author list

James Stewart[1,2], Fabrice N. Gravelat[2], Melanie Lehoux[2], Vinicius Fava[2,3], Donald C. Sheppard[1,2,4]

[1] Department of Experimental Medicine, McGill University, Glen site, Research Institute of the McGill University Health Centre, Montreal, Canada

[2] Infectious Diseases and Immunity in Global Health Program, Glen site, Research Institute of the McGill University Health Centre, Montreal, Canada

[3] McGill International TB Centre, Glen site, Research Institute of the McGill University Health Centre, Montreal, Canada

[4] Departments of Medicine and of Microbiology and Immunology, McGill University, Glen site, Research Institute of the McGill University Health Centre, Montreal, Canada

Abstract

Aspergillus fumigatus is the most common cause of invasive mould disease in humans. This infection, termed invasive aspergillosis (IA) occurs when the conidia of *A. fumigatus* enter the lower airways of the human lung and germinate to form filamentous hyphae that invade lung tissues. IA is associated with mortality rates of 30% to 95%, even despite antifungal therapy. There exists an inverse relationship between the formation of conidia (conidiation) and hyphal proliferation (vegetative growth) in *A. fumigatus* whereby vegetative growth is inhibited during conidiation, and conversely, conidiation is not observed during vegetative growth. Interestingly, *A. fumigatus* rarely undergoes conidiation during invasive infection of the human host, allowing the bulk of its energy to be directed towards vegetative growth. We therefore hypothesize that forced induction of conidiation during an infection will suppress *A. fumigatus* vegetative growth, impairing the ability of this organism to cause disease.

To study conidiation pathway dysregulation on *A. fumigatus* virulence, a key regulator of conidiation – *brlA* – was placed under the control of a tetracycline-inducible promoter (Tet-ON) and incorporated into the *A. fumigatus* genome. Functionality of the Tet-ON system *in vitro* was determined via qPCR and the effects of induced *brlA* overexpression on *A. fumigatus* virulence *in vivo* were then assessed in two distinct animal models of IA.

Time- and dose-dependent *brlA* overexpression was observed in response to the inducer molecule doxycycline, confirming functionality of the Tet-ON system within *A. fumigatus.* Low doses of doxycycline induced conidiation while high doses arrested growth of the inducible *brlA* overexpression (*brlA*$^{\text{I-OE}}$) strain *in vitro*. In an invertebrate model of IA, *Galleria mellonella* larvae infected with the *brlA*$^{\text{I-OE}}$ strain survived significantly longer in the presence of doxycycline compared to untreated larvae. Similarly, in a mouse model of IA, mice that received doxycycline

were significantly protected from infection with the *brlA*$^{I\text{-}OE}$ mutant, demonstrating decreased pulmonary fungal burden and a dose-dependent decline in mortality relative to doxycycline free controls.

This study has provided proof-of-concept that activation of the conidiation pathway in *A. fumigatus* is attainable through induction of a single transcriptional regulator – *brlA* – and that targeted up-regulation of this gene reduces *A. fumigatus* vegetative growth *in vitro* as well as virulence in two *in vivo* models of IA. Our group has therefore identified a novel target for antifungal therapeutics, opening the door to further research for *brlA*-inducing small molecules as a potentially viable new class of therapeutics against invasive *A. fumigatus* infection.

Introduction:

Aspergillus fumigatus is a ubiquitous mould that produces large numbers of airborne conidia during growth in the environment to facilitate colonization of new environments (1). Due to their small size, the conidia of *A. fumigatus* can enter the lower airways and alveoli of the human lung, where in healthy individuals they are rapidly cleared via innate immune defences (2). Immunocompromised patients are unable to effectively clear these conidia and as a result uncleared conidia can germinate to form filamentous hyphae that invade lung tissues. This infection, termed invasive aspergillosis (IA), is associated with mortality rates of between 30% and 95%, even despite antifungal therapy (3-5).

The formation of conidia (conidiation) and hyphal proliferation (vegetative growth) are inversely related in *A. fumigatus* as vegetative growth is inhibited during conidiation *in vitro* (6). During invasive infection *A. fumigatus* rarely undergoes conidiation (7), allowing the majority of its energy to be directed towards vegetative growth. We hypothesize that the converse is also true, and that forced induction of the conidiation pathway during an infection will suppress *A. fumigatus* vegetative growth, impairing the ability of this organism to cause disease.

Control of conidiation in *Aspergillus spp.* has been well studied in the model organism *Aspergillus nidulans*. These studies have identified the product of one gene, *brlA*, as a master regulator of conidiation. Disruption of *brlA* renders *A. nidulans* unable to form conidia, and its overexpression rapidly induces conidiation and inhibits vegetative growth *in vitro* (8, 9). Similarly, disruption of *brlA* (Δ*brlA*) in *A. fumigatus* results in an absence of conidiation, however, the effects of overexpressing *brlA* have yet to be determined in *A. fumigatus*.

In this study, we demonstrate that targeted upregulation of *brlA* is sufficient to induce conidiation and inhibit *A. fumigatus* vegetative growth *in vitro*. We also show that the inducer molecule doxycycline significantly reduces the virulence of an inducible *brlA* overexpression strain of *A. fumigatus* in two distinct *in vivo* models of IA. These results provide strong evidence that conidiation pathway dysregulation, through the activation of *brlA*, is a novel approach to reduce *A. fumigatus* virulence *in vivo*, and thus provide the foundation for further studies to identify *brlA*-inducing small molecules that may one day be used as prophylaxis against invasive *A. fumigatus* infection.

Methods:

Fungal strains and growth conditions.

A. fumigatus strain Af293 was used to generate the inducible *brlA* overexpression strain (*brlA*$^{I\text{-}OE}$) and is referred to as the wild type strain throughout this article. All strains were routinely cultured at 37°C on yeast peptone dextrose (YPD) media (BD Difco) for 6 days with 12-hour light/dark cycles to generate conidia stocks for use in all downstream assays. Conidia were harvested by gentle washing of the mycelia with PBS supplemented with 0.01% v/v Tween 80 (PBS-T). All *in vitro* assays were performed at 37°C in either YPD media or *Aspergillus* minimal media (AMM)(10) supplemented with 3x trace elements and 1.5 % agar for solid media conditions. Liquid shaking cultures were agitated at 200 RPM and liquid static cultures were grown in the presence of 5% CO_2.

Modification of tetracycline-inducible gene expression vectors.

The optimized Tet-ON plasmid pJW128 used to clone an inducible *brlA* overexpression strain was a generous gift from Dr. Robert A. Cramer, Geisel School of Medicine in New Hampshire. Plasmid pJW128 contains both the Tet-ON system, consisting of a doxycycline-responsive reverse transactivator gene (*rtTA*) fused to a strong viral activation domain and a transactivator-response element ($TetO_7$) embedded within a minimal *oliC* promoter (*Pmin*) upstream of the gene of interest (11), as well as a resistance cassette for pyrithiamine. To place *brlA* under the control of $TetO_7$, the *brlA* ORF was amplified from wild type *A. fumigatus* gDNA and fused to the terminator from an *A. nidulans* tryptophan biosynthesis gene (*trpC*) via PCR, generating *brlA-TtrpC*. The *brlA-TtrpC* PCR product was purified from agarose gel using the QIAquick Gel Extraction kit (Qiagen) as per the manufacturer's instructions and subcloned into the blunt cloning vector pCR-Blunt-II-TOPO (Invitrogen) as per the manufacturer's instructions, to generate pCR-*brlA-TtrpC*. Next, pCR-*brlA-TtrpC* and pJW128 were digested using restriction enzymes PmeI and Bsu361 and *brlA-TtrpC* was ligated into empty pJW128, generating the final vector pJW128-*brlA* (Tet-ON-*brlA*). Final plasmids were validated using Sanger sequencing, confirming correct orientation and sequence within the Tet-ON plasmid.

Transformation of *A. fumigatus* wild type strain (Af293).

Transformation of the *A. fumigatus* wild type strain (Af293) was performed via protoplasting, as previously described (12). Antifungal-resistant transformants were selected using pyrithiamine (Sigma) at a concentration of 0.5 µg/ml. Verification of the presence of the linear Tet-ON-*brlA* construct and the absence of a circular autonomously replicating Tet-ON-*brlA*

plasmid within pyrithiamine-resistant transformants was accomplished using a PCR screen of DNA extracted from 18 hr liquid YPD shaking cultures.

Quantitative real-time PCR analysis.

Quantification of mRNA expression was performed using SsoAdvanced Universal SYBR Green supermix (Bio-Rad) and a 7300 Real Time PCR System (Applied Biosystems). Strains of *A. fumigatus* were grown at 37°C in AMM broth for 18 hours with shaking. Doxycycline was then administered at final concentrations of 1.5 µg/ml or 20 µg/ml for 30 minutes or 3 hours for gene induction. Non-induced controls were treated with an equal volume of dH_2O for the same time intervals. The fungal biomass was collected by filtration and crushed under liquid nitrogen before extracting RNA using a Biolynx RNA extraction kit (MJS Biolynx inc.) as per the manufacturer's instructions. Genomic DNA (gDNA) was digested and RNA was reverse transcribed into complementary DNA (cDNA) using the QuantiTect Reverse Transcription Kit (Qiagen). Gene-specific cDNA was quantified via RT-qPCR and normalized to the endogenous reference gene translation elongation factor 1 (*tef1*).

Growth kinetic assays *in vitro.*

All growth kinetic assays were performed at 37°C using AMM or YPD media in the presence or absence of doxycycline, as indicated. For minimum inhibitory concentration (MIC) analyses of pre-competent *A. fumigatus*, conidia were inoculated at 1×10^4 conidia per well in 96-well plates and cultured for 20 hours in AMM containing doxycycline at the indicated concentrations. Growth inhibition was assessed by staining the resulting biomass with crystal violet and quantifying the optical density at 600 nm as previously described (13). Changes to pre-competent fungal morphology were assessed by inoculating *A. fumigatus* conidia at 1×10^4 conidia

per well in 24-well plates containing sterile coverslips and AMM supplemented with indicated concentrations of doxycycline. At 20 hours of growth coverslips were washed and fixed in 4% paraformaldehyde, then mounted and imaged using a LSM880 ElyraPS1 laser scanning confocal microscope (Zeiss) with a 63x oil-objective lens. Images were processed using ZEN blue edition software (Zeiss). For pre-competent dynamic cultures, *A. fumigatus* conidia were inoculated at 1×10^6 conidia/ml into 50 ml liquid AMM containing indicated concentrations of doxycycline and incubated for 48 hours with agitation. Solid media pre-competent growth assays were performed on AMM and YPD media supplemented with indicated concentrations of doxycycline. One hundred *A. fumigatus* conidia were point inoculated onto solid media and the diameter of fungal colonies measured for 7 days.

For assays of competent hyphae, *A. fumigatus* conidia were inoculated at 1×10^6 conidia/ml into 50 ml AMM broth and incubated for 18 hours with agitation. Doxycycline was then administered at the indicated concentrations and cultures were incubated for another 22 hours. Images were acquired prior to removing supernatant and fungal biomass was then frozen at -80°C and lyophilized. Dry biomass was weighed and normalized to cultures harvested and dried at the time of doxycycline treatment. Solid media competent growth assays were performed on AMM and YPD media. One hundred *A. fumigatus* conidia were point inoculated onto solid media and incubated for 24 hours. Doxycycline was then administered at the indicated concentrations and growth assessed daily by measuring the diameter of fungal colonies for 7 days. The morphology of competent hyphae was assessed by inoculating *A. fumigatus* conidia at 1×10^4 conidia per well in 24-well plates containing sterile coverslips and AMM broth. At 18 hours of growth, cultures were treated with doxycycline at the indicated concentrations and incubated for another 4 hours. Coverslips were treated, imaged and images processed as described above.

***Galleria mellonella* larvae infection model.**

Larvae of the *Galleria mellonella* moth were purchased from Magazoo (Montreal, QC) and maintained in the dark at 23°C in cedar wood shavings. Groups of 13 larvae were separated into petri dishes and incubated at 37°C for 24 hours prior to infection. Conidia were resuspended at a concentration of 2×10^8 conidia/ml in PBS with or without 400 µg/ml doxycycline. 7.5 µl of this conidial suspension was then injected into the larvae's last proleg using a Hamilton® 25µl glass gas-tight syringe (1702RN) with 33G gas chromatography needle (33/1.5'/3), resulting in a dose of 1.5×10^6 conidia to each larva and 3 µg of doxycycline per larva as indicated. Uninfected controls received PBS with doxycycline. Larvae were incubated in the dark at 37°C for 7 days and monitored every 24 hours. Death was confirmed through a combination of melanisation and lack of movement.

Mouse model of invasive pulmonary aspergillosis.

All procedures involving mice were approved by the McGill University Animal Care Committee and followed the guidelines established by the Canadian Council on Animal Care. 8-10-week-old female BALB/c mice were purchased from Charles River (Senneville, QC). For the low dose doxycycline model, beginning on day -3 relative to infection and for the duration of the experiment drinking water was supplemented with 500 µg/ml doxycycline (Sigma) and 5% sucrose and mouse chow was supplemented with 625 mg/kg doxycycline (Envigo-Teklad). Doxycycline-untreated controls received water supplemented with 500 µg/ml Enrofloxacin (Baytril) and unsupplemented chow. On day -1 and every 48 hours thereafter, mice were given 200 µg anti-Ly6G antibody (clone 1A8, BioXcell) via intraperitoneal (i.p.) injection to induce neutropenia. On day -1 and every 24 hours thereafter, mice were also given 10 mg/kg doxycycline

via i.p. injection. Mice were infected intratracheally with 1×10^7 *A. fumigatus* conidia in 50 µl PBS-T or PBS-T lacking conidia for uninfected controls. Mice were monitored every 12 hours for signs of illness and moribund animals euthanized. For the high dose doxycycline model, doxycycline was omitted from drinking water and mouse chow, and instead the drinking water was supplemented with 500 µg/ml Enrofloxacin (Baytril) to prevent bacterial infection. On day -1 mice were given 25 mg/kg doxycycline via i.p. injection and 100 mg/kg doxycycline via oral gavage every 12 hours thereafter. Doxycycline-free control mice were given equal volumes of PBS.

Quantification of doxycycline in mouse serum.

All ultra high-performance liquid chromatography coupled to mass-spectrometry (uHPLC-MS/MS) analyses were performed at the Drug Discovery Platform of the Research Institute of the McGill University Health Centre (Montreal, Canada). LC-MS grade solvent, acetonitrile and water were obtained from EMD Millipore, and formic acid from Fisher Scientific. 50 µl of serum was extracted with 200 µl (4 x sample volume) of cold acetonitrile with 1% formic acid. The mixture was vortexed for 5 min and centrifuged (5 min, 15000 rpm, 10°C) to separate the precipitate. Supernatant was transferred to a new tube and evaporated under vacuum. The resulting dry pellets were re-suspended in 100 µl of acetonitrile-0.1%formic acid/water-0.1% formic acid (v/v; 5:95) and 10 µl injected into the LC-MS. Results were collected in positive mode on a triple quadrupole MS system (EVOQ Elite, Bruker, Billerica, MA) coupled with an ultrahigh-performance liquid chromatography pump (Advance, Bruker) and equipped with a reversed-phase Agilent ZORBAX Eclipse Plus C18 column (50 x 2.1 mm, 1.8um). Mobile phases were water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). Doxycycline was eluted at 3.17 min with the following LC method: 0 to 1 min at 5% B, then from 1 to 4 min with a gradient to 55% B, followed

by 4 min to 5 min with a gradient to 85% and 5 min to 6 min with a plateau at 85% B and returning to initial conditions with an equilibration until 10 min. Column temperature was set at 40°C and the flow rate was 0.35 ml/min. Each sample was injected three times. The operating parameters of the mass spectrometer were: positive spray voltage 4500V, cone temperature 350°C, cone gas flow 20 (arbitrary units), heated probe temperature 400°C, probe gas flow 40 (arbitrary units), nebulizer gas flow 60 (arbitrary units). The mass spectrometer was used in multiple reaction monitoring mode (MRM). Two transitions were followed for doxycycline: 445.2→428.1 (CE 13 eV) and 445.2→154.1 (CE 25 eV). Quantification of doxycycline, based on peak areas, was performed following an external calibration curve. Seven points of calibration were used to produce standard curve and the linearity was assessed by the correlation coefficient, R2.

RNA deep-sequencing analysis.

A. fumigatus hyphae were pre-grown in liquid AMM at a concentration of 1×10^6 conidia/ml and cultured for 10 hours at 37°C. Doxycycline was then administered at a concentration of 20 µg/ml for gene induction. Non-induced controls were treated with an equal volume of dH_2O. After 30 minutes, the supernatant was removed by filtration and the fungal biomass crushed under liquid nitrogen before extracting RNA using a RNeasy minikit (Qiagen) as per the manufacturer's instructions. cDNA libraries were constructed and sequenced at the McGill University and Genome Quebec Innovation Centre (Montreal, Canada) following Illumina specifications, generating over 25 million paired-end reads for each sample. FASTQ read files were assessed for quality using the FASTQC software version 0.11.5 and reads further assessed for quality and bam indexing using SAMTOOLS software, version 1.9. For each dataset, FASTQ sequence read files were mapped to the *A. fumigatus* Af293 reference genome ASM265 version 1.40 (14) by using the STAR alignment software, version 2.6.1a in 2-pass mode (15). Aligned

reads were then quantified and transformed to obtain fragments per kilobase per million (FPKM) values using RSEM software version 1.3.0. Genes with an FPKM value below 30 were excluded from analysis.

Results:

Doxycycline-mediated induction of *brlA* in *A. fumigatus* is both time- and dose-dependent.

To investigate the effects of *brlA* overexpression on the growth and conidiation of *A. fumigatus*, an inducible *brlA* overexpression (*brlA*$^{I\text{-}OE}$) strain was constructed in which *brlA* was placed under the control of a doxycycline-inducible promotor. PCR screening of DNA extracted from 18-hour liquid shaking cultures of *A. fumigatus* confirmed the presence of the linear Tet-ON system controlling *brlA* as well as the absence of a circular autonomously replicating Tet-ON-*brlA* plasmid within the *brlA*$^{I\text{-}OE}$ mutant (Supplemental figure S1). As has been reported previously with this promotor system (16), higher levels of *brlA* expression were observed in vegetative hyphae of the *brlA*$^{I\text{-}OE}$ mutant, even in the absence of doxycycline treatment (Figure 1). This level of basal *brlA* expression in the *brlA*$^{I\text{-}OE}$ strain was similar to that of the conidiating wild-type *A. fumigatus*. Exposure of the *brlA*$^{I\text{-}OE}$ mutant to doxycycline resulted in a dose and time-dependent overexpression of *brlA* (Figure 1), reaching levels 2391 fold higher than non-conidiating wild type *A. fumigatus* following 3 hours of exposure to 20 µg/ml doxycycline. This strain was therefore selected for further analyses to determine the effects of *brlA* overexpression on *A. fumigatus* fitness *in vitro* and *in vivo*.

Overexpression of *brlA* inhibits the growth of pre-competent *A. fumigatus* hyphae *in vitro*.

The development of *A. fumigatus* hyphae begins with a genetically defined period of pre-competence, during which hyphae are not able to respond to appropriate stimuli and undergo conidiation (9, 17-21). To determine the effect of *brlA* overexpression on the growth of pre-competent *A. fumigatus* in static culture, conidia were inoculated into liquid AMM containing various concentrations of doxycycline and incubated for 18 hours and the resulting biomass quantified by crystal violet staining. Under these conditions, the growth of the *brlA*$^{\text{I-OE}}$ mutant was reduced in a doxycycline dose-dependent manner, with a minimum inhibitory concentration (MIC) of 0.4 µg/ml doxycycline (Figure 2a). Under the same conditions, the growth of wild type *A. fumigatus* was unaffected at concentrations of doxycycline as high as 1 µg/ml (Figure 2a). Using confocal microscopy, the effects of *brlA* overexpression on fungal morphology were assessed after 18 hours of growth in liquid AMM containing doxycycline (Figure 2b). At doxycycline concentrations of 0.54 µg/ml and above, *brlA*$^{\text{I-OE}}$ conidia germinated but could not proceed with hyphal extension. At the lower doxycycline concentrations of 0.06 µg/ml and 0.18 µg/ml, *brlA*$^{\text{I-OE}}$ cultures conidiated extensively although at 0.18 µg/ml of doxycycline, *brlA*$^{\text{I-OE}}$ conidiophore morphology was atypical, with reduced vesicule size and elongated phialides (Figure 2b). Wild type *A. fumigatus* germinated and produced morphologically normal hyphae only under these conditions, as did the *brlA*$^{\text{I-OE}}$ mutant in the absence of doxycyline. Taken together, these findings demonstrate that lower levels of *brlA* induction in pre-competent *A. fumigatus* hyphae reduces vegetative growth and induces conidiation while higher levels result in the complete inhibition of vegetative growth *in vitro*.

Due to overgrowth of the wild type strain under liquid static conditions (data not shown), analysis of the effects of *brlA* overexpression on *A. fumigatus* growth and conidiation were limited

to 24 hours. To assess the effects of induced *brlA* expression on precompetent hyphae at a later time point, *A. fumigatus* conidia were grown in liquid shaking culture containing low (1.5 µg/ml) and high (20 µg/ml) concentrations of doxycycline for 48 hours (Figure 2c). Under these conditions, growth inhibition of the *brlA*$^{I\text{-}OE}$ strain was observed at both concentrations of doxycycline tested and persisted for 24 hours (Figure 2c). At 48 hours of growth, several breakthrough clusters of *brlA*$^{I\text{-}OE}$ hyphae were observed. The growth rate and colouring of these hyphal masses were abnormal with reduced growth rate and darkening of fungal biomass consistent with the production of conidia (Supplemental figure S2). The reduced growth rate and darkening of fungal biomass was more apparent in cultures containing the high concentration of doxycycline. Normal hyphal growth of wild type *A. fumigatus* in the presence of doxycycline and the *brlA*$^{I\text{-}OE}$ mutant in the absence of doxycycline were observed under these conditions (Figure 2c and Supplemental figure S2). These findings indicate that high levels of *brlA* overexpression also inhibit the growth of pre-competent *A. fumigatus* in shaking culture and suggest that the level of *brlA* overexpression may influence the duration of growth inhibition as well as the rate of vegetative growth and induction of conidiation thereafter.

To determine the relationship between *brlA* expression and the duration of growth inhibition, the effects of doxycycline induction on the radial growth rate of the *brlA*$^{I\text{-}OE}$ mutant was measured over 7 days (Figure 3). In the absence of doxycycline, the mean growth rate of the *brlA*$^{I\text{-}OE}$ strain was slightly lower than that of wild type *A. fumigatus* at 10.3 mm/day and 11.4 mm/day respectively (91%) (Figure 3). Exposure to 1.5 µg/ml of doxycycline inhibited the growth of the *brlA*$^{I\text{-}OE}$ mutant for 2 days, after which the radial growth rate was reduced at 40.8% relative to wild type *A. fumigatus* (Figure 3). Exposure to 20 µg/ml of doxycycline, inhibited the growth of the

brlA$^{I\text{-}OE}$ strain for 6 days, followed by a reduced radial growth rate of 13.5% relative to wild type (Figure 3).

To determine if nutrient availability would influence the growth inhibitory effects of high-level *brlA* overexpression on pre-competent hyphae, the effects of doxycycline on growth inhibition of the *brlA*$^{I\text{-}OE}$ mutant were determined on nutrient-rich solid YPD media (Supplemental figure S3). In the absence of doxycycline, the growth rate of the *brlA*$^{I\text{-}OE}$ strain was indistinguishable from wild type *A. fumigatus* under the same conditions (Supplemental figure S3). At the low dose of doxycycline, growth inhibition of the *brlA*$^{I\text{-}OE}$ strain persisted for 2 days with a reduced radial growth rate of 33.1% relative to wild type following breakthrough. At the high dose of doxycycline, growth inhibition of the *brlA*$^{I\text{-}OE}$ strain persisted for 4 days, followed by a mean radial growth rate of 22.4% relative to wild type *A. fumigatus* (Supplemental figure S3).

Taken together, these findings demonstrate that high levels of *brlA* overexpression inhibit the growth of pre-competent *A. fumigatus* hyphae *in vitro* and that at early time points this effect is independent of culture conditions or nutrient availability. These data also suggest that at later time points, the level of *brlA* overexpression and the type of culture conditions influence the duration of pre-competent *A. fumigatus* growth inhibition as well as the resulting growth rate following breakthrough.

Overexpression of *brlA* arrests growth of competent *A. fumigatus* hyphae and induces conidiation in a dose-dependent manner.

To analyze the effects of *brlA* overexpression on developmentally competent hyphae, 18-hour liquid AMM shaking cultures of *A. fumigatus* were exposed to a range of concentrations of doxycycline to induce *brlA* overexpression and cultured for a further 22 hours before determining

fungal growth by measuring the dry weight of fungal biomass. The growth of wild type *A. fumigatus* was unaffected at all concentrations of doxycycline tested, up to 14.58 µg/ml (Figure 4a). In contrast, the growth of competent hyphae of the *brlA*$^{I\text{-}OE}$ mutant was reduced in a doxycycline dose-dependent manner, with a MIC of 0.54 µg/ml doxycycline (Figure 4a). Visual inspection of liquid cultures demonstrated that at this time point, hyphae of *brlA*$^{I\text{-}OE}$ cultures treated with sub-MIC concentrations of doxycycline had developed a light green pigment. All wild type cultures of *A. fumigatus* remained white under these same conditions (Figure 4b). To determine if this colour change was a consequence of the production of conidia, strains were examined by confocal microscopy after 4 hours of doxycycline exposure (Figure 4c). Under these conditions, *brlA*$^{I\text{-}OE}$ cultures contained an abundance of conidiophores while untreated *brlA*$^{I\text{-}OE}$ cultures and wild type cultures contained hyphae only (Figure 4c). As was observed with pre-competent hyphae, competent *brlA*$^{I\text{-}OE}$ cultures exposed to 0.18 µg/ml of doxycycline exhibited atypical conidiophore morphology with reduced vesicule size and elongated phialides. However, at 0.54 µg/ml and 1.62 µg/ml, competent *brlA*$^{I\text{-}OE}$ cultures contained conidiophores with both normal and atypical morphology as well as hyphal tips that differentiated into a spherical structure, similar in appearance to a conidium but notably larger in size. At 1.62 µg/ml, regions of the *brlA*$^{I\text{-}OE}$ mutant hyphae also appeared to have undergone autolysis, resulting in the leakage of cytoplasmic contents into the surroundings. Wild type cultures treated with doxycycline displayed no changes in fungal morphology at this time point (Figure 4c). These results suggest that lower levels of *brlA* overexpression reduce growth and induce conidiation of competent *A. fumigatus* hyphae in a dose-dependent manner. These findings also indicate that in competent *A. fumigatus* hyphae, higher levels of *brlA* overexpression induce the formation of normal and atypical conidiophores, cause

changes to hyphal morphology including the formation of apical "budding" structures and potentially autolysis, and lead to arrested vegetative growth *in vitro*.

As the level of *brlA* overexpression correlated with the duration of pre-competent *A. fumigatus* growth inhibition and the rate of growth after breakthrough, we next sought to determine the ability of competent hyphae to escape *brlA*-induced growth inhibition (Figure 5). Consistent with previous findings, growth arrest of the *brlA*$^{\text{I-OE}}$ strain was observed at both low (1.5 µg/ml) and high (20 µg/ml) concentrations of doxycycline, while no effect on wild type *A. fumigatus* growth was observed (Figure 5). The duration of competent *brlA*$^{\text{I-OE}}$ growth arrest at both concentrations of doxycycline tested was similar and lasted for 3 days. Following breakthrough, the mean radial growth rate of the *brlA*$^{\text{I-OE}}$ strain returned to a wild type level at both concentrations of doxycycline, although some variability between replicates was observed (Figure 5). The addition of 20 µg/ml doxycycline had no effect on the growth of the competent *brlA*$^{\text{I-OE}}$ strain following breakthrough (data not shown), suggesting that fungal breakthrough was not a result of doxycycline degradation over time. When grown on YPD media, exposure to both low and high doxycycline concentrations resulted in growth inhibition of the competent *brlA*$^{\text{I-OE}}$ mutant for 2 days, after which the mean radial growth rate returned to a wild type level (Supplemental figure S4). The growth rate of wild type *A. fumigatus* was unaffected under these same conditions (Supplemental figure S4).

Taken together, these findings suggest that, consistent with our findings in pre-competent hyphae, nutrient availability influences the duration of competent *A. fumigatus* growth arrest in response to *brlA* overexpression. However, unlike pre-competent hyphae, increasing the level of *brlA* overexpression above the growth inhibitory threshold does not influence the rate of competent fungal breakthrough or growth rate thereafter.

BrlA-mediated growth inhibition requires a functional *brlA* allele.

To probe the mechanism by which competent hyphae escape growth arrest, a competent *brlA*$^{\text{I-OE}}$ colony was isolated following breakthrough on solid YPD media (*brlA*$^{\text{BT}}$). This breakthrough mutant, *brlA*$^{\text{BT}}$ was able to grow on solid AMM in the presence of inhibitory doxycycline concentrations as high as 20 µg/ml although at a reduced rate of growth as compared to the no doxycycline control condition (9.5 mm/day vs 12.2 mm/day; Figure 6a). Similar findings were observed on nutrient-rich solid YPD media, with a radial growth rate of 17.5 mm/day vs 25.3 mm/day in the presence or absence of doxycycline, respectively (Supplemental figure S5a). After three days of growth on AMM, *brlA*$^{\text{BT}}$ colonies were more compact and contained markedly less green pigmentation in the presence of doxycycline as compared to doxycycline-free conditions and wild type controls (Figure 6b). Collectively these data confirm that escape from doxycycline-mediated growth inhibition was not a consequence of doxycycline degradation within media, but is rather due to intrinsic changes within the fungus.

We therefore hypothesized that a spontaneous mutation had occurred within the *brlA* open-reading frame (ORF) or the Tet-ON system of the *brlA*$^{\text{BT}}$ mutant resulting in the loss of *brlA* overexpression or the expression of a dysfunctional *brlA* in response to doxycycline. RT-qPCR analysis confirmed that the *brlA*$^{\text{BT}}$ mutant retained normal overexpression of *brlA* in response to doxycycline (Figure 6c), suggesting that the Tet-ON system remained functional in this mutant. Sequencing of the *brlA* ORF identified a nucleotide insertion at position 1083, leading to a frameshift mutation and the production of an altered BrlA protein, predicted to contain 24 altered amino acid residues at the c-terminus and truncated by 42 amino acid residues (Supplemental figure S5b). These findings add strength to the hypothesis that doxycycline-induced growth

inhibition of the *brlA*$^{I\text{-}OE}$ mutant is a direct consequence of *brlA* overexpression and not due to secondary effects of the Tet-ON system.

Doxycycline reduces the virulence of an inducible *brlA* overexpression strain of *A. fumigatus* in an invertebrate model of *Aspergillus* infection.

To determine if doxycycline could reduce the virulence of the *brlA*$^{I\text{-}OE}$ strain *in vivo*, larvae of the *Galleria mellonella* moth were infected with wild type or *brlA*$^{I\text{-}OE}$ mutant *A. fumigatus* conidia with, or without 400 µg/ml doxycycline. Due to early pupation of uninfected larvae by day 5 post-infection, these experiments were restricted to 5 days. Five days after infection 47 % of *brlA*$^{I\text{-}OE}$-infected, doxycycline-treated larvae had succumbed to infection as compared to 84 % of *brlA*$^{I\text{-}OE}$-infected, untreated larvae (Figure 7). Larvae infected with wild type *A. fumigatus* displayed similarly high mortality of 100 % and 94 % for doxycycline-treated and untreated larvae, respectively (Figure 7). These findings suggest that targeted upregulation of *brlA* significantly reduces *A. fumigatus* virulence in an invertebrate model of IA.

Doxycycline attenuates the virulence of an inducible *brlA* overexpression strain of *A. fumigatus* in a mouse model of invasive aspergillosis.

To determine if doxycycline could reduce the virulence of the *brlA*$^{I\text{-}OE}$ strain in an animal model that more closely mimics a human infection, a neutropenic mouse model of invasive aspergillosis was used (22, 23). As doxycycline-induced *brlA* overexpression was most effective at inhibiting the growth of pre-competent *A. fumigatus* hyphae *in vitro*, mice were treated with doxycycline prior to infection and throughout the course of the experiment as a prophylactic strategy. Mice infected with the *brlA*$^{I\text{-}OE}$ strain that received doxycycline survived significantly longer than doxycycline-free mice (median survival times of 108 hours and 60 hours respectively;

Figure 8a). The mortality rate of *brlA*$^{I\text{-}OE}$-infected mice that received doxycycline was also reduced as compared *brlA*$^{I\text{-}OE}$-infected doxycycline-free mice (67 % vs 100 % respectively; Figure 8a). Doxycycline had no effect on the survival of wild type-infected mice, with both groups exhibiting a median survival time of 48 hours and a mortality rate of 94 % (Figure 8a). Pulmonary fungal burden levels as measured by galactomannan (GM) quantification mirrored the results of these survival studies. At 36 hours post-infection, pulmonary fungal burden of *brlA*$^{I\text{-}OE}$-infected mice that received doxycycline was significantly lower than those not receiving doxycycline or mice infected with wild type *A. fumigatus* with or without doxycycline treatment (Figure 8b). Pulmonary histopathology examination was consistent with the survival and pulmonary fungal burden determination studies. Gomori methenamine-silver staining of lung sections at 36 hours after infection revealed that the lungs of *brlA*$^{I\text{-}OE}$-infected mice that received doxycycline contained fewer fungal lesions than the other experimental groups, and these lesions were largely composed of swollen conidia and short hyphae (Figure 8c). In contrast, *brlA*$^{I\text{-}OE}$-infected mice that did not receive doxycycline or mice that were infected with wild type *A. fumigatus* with or without doxycycline treatment had more and larger pulmonary lesions that consisted mainly of long hyphae (Figure 8c). Collectively these results suggest that overexpression of *brlA* early in fungal infection attenuates fungal growth and virulence in a mouse model of invasive aspergillosis.

Although treatment with doxycycline increased the survival of *brlA*$^{I\text{-}OE}$-infected mice, the majority of mice eventually succumbed to the infection. We therefore hypothesized that the concentrations of doxycycline at the site of infection in these mice may be below the *brlA*$^{I\text{-}OE}$ strain MIC of 400 ng/ml, permitting fungal growth. To address this hypothesis, serum was collected from infected mice at 36 hours post-infection, at the mid-point between the 3rd and 4th dose of doxycycline, and the concentration of doxycycline was quantified using uHPLC-MS/MS. At this

time point, the median serum concentrations of doxycycline in *brlA*$^{I\text{-}OE}$-infected and wild type-infected mice were 290 ng/ml and 392 ng/ml, respectively (Supplemental figure S6a), suggesting that the doxycycline concentrations were below the *brlA*$^{I\text{-}OE}$ strain MIC of 400 ng/ml during much of the infection course, likely allowing continued fungal growth.

To determine if increasing the serum doxycycline concentration of mice would improve survival of mice infected with the *brlA*$^{I\text{-}OE}$ mutant, the survival experiment was repeated with a 10-fold higher dose of doxycycline administered every 12 hours via oral gavage. This dosage regimen was chosen as it has been shown to effectively induce gene expression in a Tet-ON subcutaneous xenograft mouse model (24) and the selected dosage can result in an accumulation of doxycycline in mouse serum at concentrations over 10,000 ng/ml (25), well above the *brlA*$^{I\text{-}OE}$ strain MIC of 400 ng/ml. Serum doxycycline concentrations in infected mice were quantified, and the median doxycycline concentrations in *brlA*$^{I\text{-}OE}$- and wild type-infected mice were significantly higher at 5564 ng/ml and 4194 ng/ml, respectively (Supplemental figure S6b). With this higher doxycycline exposure level, doxycycline treatment reduced the mortality rate of mice infected with the *brlA*$^{I\text{-}OE}$ mutant from 100 % to 25 % (Figure 8d). Taken together, these results illustrate a dose-dependent relationship between doxycycline exposure and mortality *in vivo*, supporting the hypothesis that overexpression of *brlA* reduces *A. fumigatus* virulence.

RNA deep-sequencing of pre-competent *A. fumigatus* hyphae exposed to high-level *brlA* overexpression reveals altered expression of genes associated with metabolism, development and virulence.

To explore the mechanisms by which high-level *brlA* overexpression mediates growth inhibition of pre-competent hyphae and attenuates virulence, we performed a transcriptomic analysis of pre-competent *A. fumigatus* hyphae exposed to doxycycline using RNA deep-

sequencing. Expression analysis identified 143 genes that were highly downregulated and 165 genes that were highly upregulated in the *brlA*$^{I\text{-}OE}$ cultures exposed to doxycycline as compared to wild type *A. fumigatus* cultures. Genes involved in several metabolic processes were strongly downregulated in the *brlA*$^{I\text{-}OE}$ mutant treated with doxycycline. These processes include amino acid metabolism, particularly lysine, glutamate and aspartate; plasma membrane (PM) structure and transport; the synthesis of cell wall polysaccharides including galactosaminogalactan (GAG) and chitin; as well as genes associated with the hypoxia stress response (Table 1). As predicted, genes involved in conidiation were strongly upregulated in the *brlA*$^{I\text{-}OE}$ mutant following exposure to doxycycline (Table 2). Additionally, genes involved in cell wall biosynthesis, oxidative phosphorylation and stress responses including reactive oxygen species detoxification, metal resistance and toxin efflux were also induced in response to doxycycline treatment (Table 2). These results indicate that *brlA* overexpression in *A. fumigatus* leads to marked downregulation in the metabolism of amino acids and a shift in *A. fumigatus* metabolism towards oxidative phosphorylation, as well as alterations in cell wall composition with the reduced synthesis of the immunosuppressive polysaccharide GAG.

Discussion:

In the present study, we have demonstrated that the expression of a single transcriptional regulator, *brlA*, is sufficient to mediate activation of the conidiation pathway in *A. fumigatus*. Overexpression of *brlA* inhibits vegetative growth *in vitro* and reduces virulence of this opportunistic pathogen in two *in vivo* models of invasive *Aspergillus* infection.

The results of this study highlight important similarities between the conidiation programs of *A. fumigatus* and *A. nidulans*. Consistent with what has been observed in *A. nidulans* (8), forcing

brlA overexpression in competent *A. fumigatus* hyphae leads to inappropriate conidiation, induces marked changes to hyphal and conidiophore morphology, and reduces vegetative growth *in vitro*. The hyphal tip "budding" structures observed in *brlA* overexpressing *A. fumigatus* closely resemble those reported in *brlA*-overexpressing *A. nidulans* (8). Induced *brlA* overexpression also inhibits the growth of both *Aspergillus spp.* prior to their reaching developmental competency, though the developmental phase at which growth arrest takes place differs between them. In *A. nidulans, brlA* overexpression inhibits germination (8), while in *A. fumigatus brlA* overexpression permits germination but rapidly arrests hyphal proliferation. These findings support a highly conserved role for *brlA* in the conidiation programs *A. fumigatus* and *A. nidulans* and may suggest that therapies that induce *brlA* overexpression could also reduce the virulence of other pathogenic *Aspergillus spp.*

The effects of high-level *brlA* overexpression on competent *A. fumigatus* hyphae were distinct from that of pre-competent hyphae. In competent hyphae, the rapid induction of both normal and atypical conidiophores prior to growth arrest was observed. In addition, breakthrough of *brlA*-mediated growth inhibition was more rapid, and the growth rate following escape was similar to that of the wild type organism. It is possible that competent hyphae, when faced with the selective pressure of *brlA* overexpression-induced growth arrest, are more prone to developing resistance mutations that permit vegetative growth to proceed. Further studies will therefore be required to compare the frequency and mechanism of spontaneous breakthrough mutations in competent and pre-competent hyphae exposed to prolonged high-level *brlA* overexpression. While relatively little is known about the importance of pre-competent states during the early stages of an invasive *A. fumigatus* infection, the *in vivo* gene expression profile of *A. fumigatus* at later time points of 24 hours post-infection and onwards is one of competent hyphae (26).

Therefore, if the rapid development of resistance to *brlA* overexpression-induced growth arrest in competent hyphae is confirmed *in vitro*, this may present an important obstacle in applying this strategy for the treatment of an established *A. fumigatus* infection. Targeted *brlA* overexpression may therefore be limited for use as prophylaxis against *A. fumigatus* in high-risk individuals.

Outside of the two C_2H_2 zinc-finger domains of BrlA (9, 17), relatively little is known about the functional domains of BrlA in *A. fumigatus*. The identification of a breakthrough isolate that overexpresses a spontaneously mutated and apparently dysfunctional *brlA* in response to doxycycline therefore presents a unique opportunity to address this gap in the scientific knowledge surrounding *brlA*. Future studies will therefore focus on protein modeling of both the wild type and the spontaneously mutated BrlA in order to analyze differences in both protein structure as well as conformation that may explain the observed divergent responses of *A. fumigatus* to their overexpression. *In silico* functional domain predictions would then be validated using reverse genetics combined with the Tet-ON system, testing the effects of induced overexpression of *brlA* functional domain mutants on *A. fumigatus*.

The Tet-ON system has demonstrated elevated basal expression levels in our hands as well as others (27, 28), and lower levels of *brlA* overexpression were shown to activate conidiation in our inducible *brlA* overexpression strain of *A. fumigatus in vitro*. A concern, therefore, was that basal overexpression of *brlA* could activate conidiation during an infection, leading to fungal dissemination. However, after extensive histopathological analysis, conidiophores could not be found within the lungs of any mice infected with the inducible *brlA* overexpression strain or wild type strain of *A. fumigatus*. The absence of conidiation in mice, despite serum doxycycline levels falling below the MIC of 400 ng/ml may indicate an accumulation of doxycycline within lung tissues. Evidence for this has been observed in rats where a significantly higher concentration of

doxycycline was observed in lung tissue when compared to serum (29) though to our knowledge a similar comparison has not been performed in mice. Alternatively, as the conidiation of *A. fumigatus* is rarely observed during an invasive infection (7), other host or fungal factors may actively suppress conidiation *in vivo*. Future studies will therefore focus on quantifying the level of *brlA* and other conidiation-specific genes in the inducible *brlA* overexpression strain during a mouse infection, to determine if other genes within the conidiation pathway of *A. fumigatus* are suppressed under these conditions.

The results of our RNA deep-sequencing analysis provide important insights into the possible mechanisms of *brlA*-overexpression induced growth inhibition and virulence attenuation of pre-competent *A. fumigatus* hyphae.

Evidence for dysregulation of the mitotic cell cycle was observed in response to *brlA* overexpression. Strong overexpression of the genes encoding a protein tyrosine phosphatase NimT/Mih1 as well as the 14-3-3 family protein ArtA. NimT is a Cdc25-type phosphatase that is an essential regulator of cell cycle progression and growth in *A. nidulans*, in which a *nimT*-deficient strain undergoes late G2- and M-phase arrest shortly after germination (30, 31). ArtA plays a role in regulating germ tube formation and hyphal morphogenesis in *A. nidulans.* Overexpression of *artA* in *A. nidulans* results in abnormal germ tube formation and hyphal branching, as well as significantly reduced colony size (30). Strong upregulation of these two genes in *A. fumigatus* in response to *brlA* overexpression likely indicates dysfunctional cell cycle control, leading to defects in mitosis and, in turn, growth inhibition.

The observed downregulation of a putative phosphatidyl synthase gene combined with the upregulation of a GPI-anchored serine-threonine rich protein gene with predicted lysophospholipase activity may indicate an imbalance in the phospholipid plasma membrane

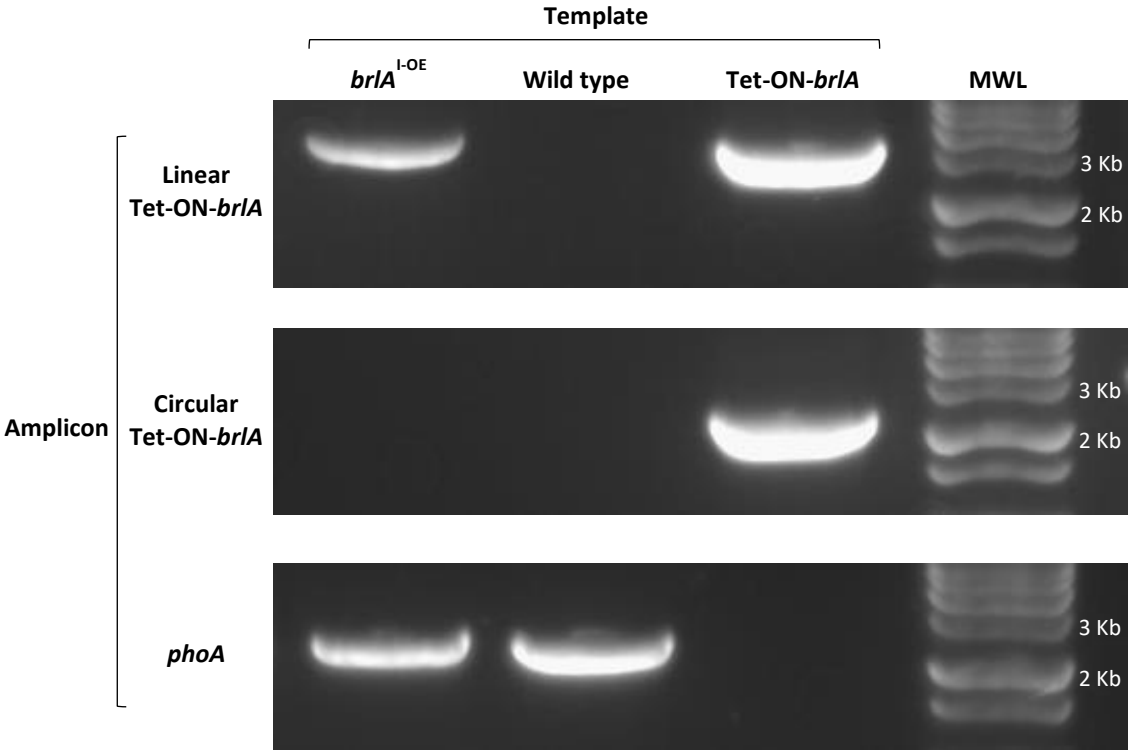
composition of *brlA*-overexpressing *A. fumigatus*. Phosphatidyl synthases are involved in phosphatidylcholine biosynthesis (32, 33), a major constituent of the *Aspergillus niger* plasma membrane (34, 35), while lysophospholipases function to cleave plasma membrane phospholipids (36). Secreted phospholipases have been identified in various pathogenic fungi including *Candida albicans* and *Cryptococcus neoformans* (37) and have been implicated as virulence factors by cleaving host phospholipids and thereby destabilizing the host plasma membrane and permitting fungal penetration (36, 38). However, marked upregulation of this putative GPI-anchored phospholipase could result in its accumulation within the *A. fumigatus* cytoplasm, leading to cleavage of fungal plasma membrane phospholipids and an imbalance in the phospholipid composition of the *A. fumigatus* plasma membrane.

Finally, the results of our RNA deep-sequencing analysis may also indicate a role for *brlA* in shifting *A. fumigatus* metabolism towards oxidative phosphorylation and away from hypoxic metabolism. In *brlA*-overexpressing pre-competent hyphae, strong upregulation of genes encoding putative enzymes involved in oxidative phosphorylation, including a cytochrome c peroxidase Ccp1, a cytochrome P450 and an NAD-binding Rossman fold oxidoreductase were observed (39-42). Similarly, downregulation of genes encoding glutamate dehydrogenase and glutamate decarboxylase was observed. These two enzymes are upregulated in *A. nidulans* under hypoxic conditions and are believed to contribute to the assimilation and/or detoxification of excess ammonia via the γ-aminobutyric acid (GABA) shunt (43). Downregulation of *srbB*, encoding a sterol regulatory element binding protein (SREBP) SrbB that is critical for the adaptation of *A. fumigatus* to hypoxic growth conditions (44), as well as several previously identified SrbB-dependent hypoxia response genes including *hem13*, *alcC*, *rcfB*, *fhpA*, *frdA*, *pdcA* (44, 45) were also observed. Taken together, these findings indicate that during conidiation in *A. fumigatus*, BrlA

may function to skew fungal metabolism towards oxidative respiration and repress metabolic pathways important for hypoxic metabolism, potentially through the downregulation of SrbB. This observation is consistent with the fact that conidiation is commonly observed in response to an air-water or air-solid interphase, when *A. fumigatus* is exposed to increases in oxygen availability. This shift away from adaptation to hypoxia may also contribute to attenuation of virulence, as adaptation to the hypoxic pulmonary microenvironment during infection has been identified as a critical virulence trait in *A. fumigatus* (44, 46, 47).

One important limitation of this study is that the RNA-deep sequencing was performed on only a single sample, limiting statistical analyses and confidence in these results. Further studies will therefore be required to validate these findings, including repeating the RNA deep-sequencing experiment with a minimum of 2 more biological replicates.

The results of this study provide strong evidence that conidiation pathway dysregulation, through the activation of *brlA*, is novel approach to reduce *A. fumigatus* vegetative growth *in vitro* and virulence *in vivo*. We have therefore identified a novel target for antifungal therapeutics, emphasizing the need for further studies to identify *brlA*-inducing small molecules that may one day be used as prophylaxis against invasive *A. fumigatus* infection.



Supplemental figure S1. Confirmation of the presence of the linear Tet-ON-*brlA* construct and the absence of a circular autonomously replicating Tet-ON-*brlA* plasmid within the *brlA*$^{\text{I-OE}}$ strain of *A. fumigatus*. PCR screening of DNA extracted from one transformant demonstrating resistance to the Tet-ON-*brlA* system's drug selection marker pyrithiamine (*brlA*$^{\text{I-OE}}$), of DNA extracted from the parental Af293 (Wild type), and of the circular Tet-ON-*brlA* plasmid (Tet-ON-*brlA*). Target amplicons include the essential components of the linear Tet-ON-*brlA* construct used for the transformation of Wild type *A. fumigatus*, with an expected length of 2.6 Kb (top), the non-essential components of the circular Tet-ON-*brlA* plasmid that were excluded during the transformation of Wild type *A. fumigatus*, with an expected length of 2.1 Kb (middle), and for the *phoA* gene of *A. fumigatus*, encoding for an acid phosphatase and functioning as a DNA integrity control, with an expected length of 2.6 Kb (bottom). MWL = Molecular weight ladder

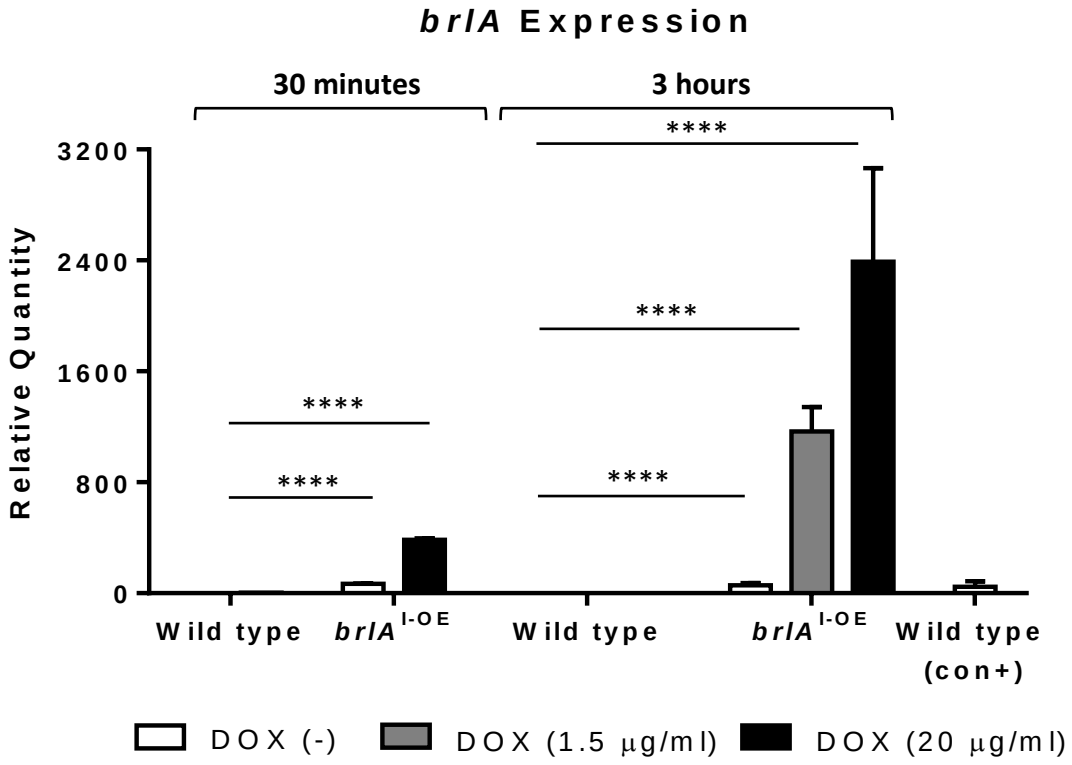


Figure 1. Doxycycline induces *brlA* overexpression in a time- and dose-dependent manner. *brlA* expression levels in the parental Af293 (Wild type), inducible *brlA*-overexpressing ($brlA^{I\text{-}OE}$), and conidiating Af293 (Wild type con+) strains of *A. fumigatus* after 30 minutes or 3 hours exposure to the indicated concentrations of doxycycline as measured by RT-qPCR. Gene expression was normalized to the endogenous reference gene *tef1* and presented as fold change relative to the wild type strain in the absence of doxycycline. Combined data of four independent experiments is presented as the mean and range (as defined by the standard error of the delta Ct) of four biological replicates, each in technical triplicate. ****: $p < 0.0001$; two-tailed student's t-test. $brlA^{I\text{-}OE}$ DOX (-), $brlA^{I\text{-}OE}$ DOX (1.5 µg/ml) and $brlA^{I\text{-}OE}$ DOX (20 µg/ml) compared to wild type DOX (-) at the same time point.

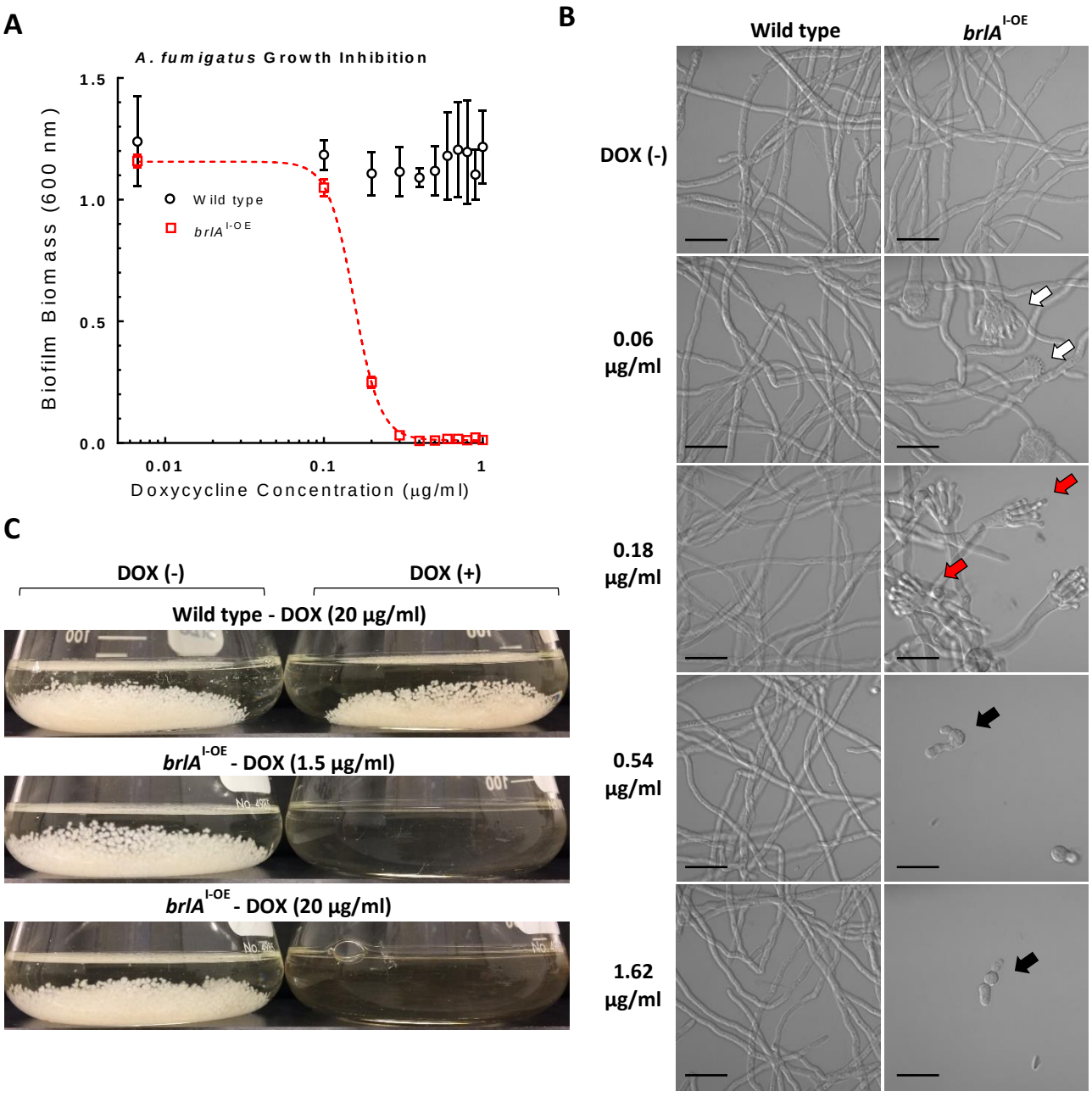
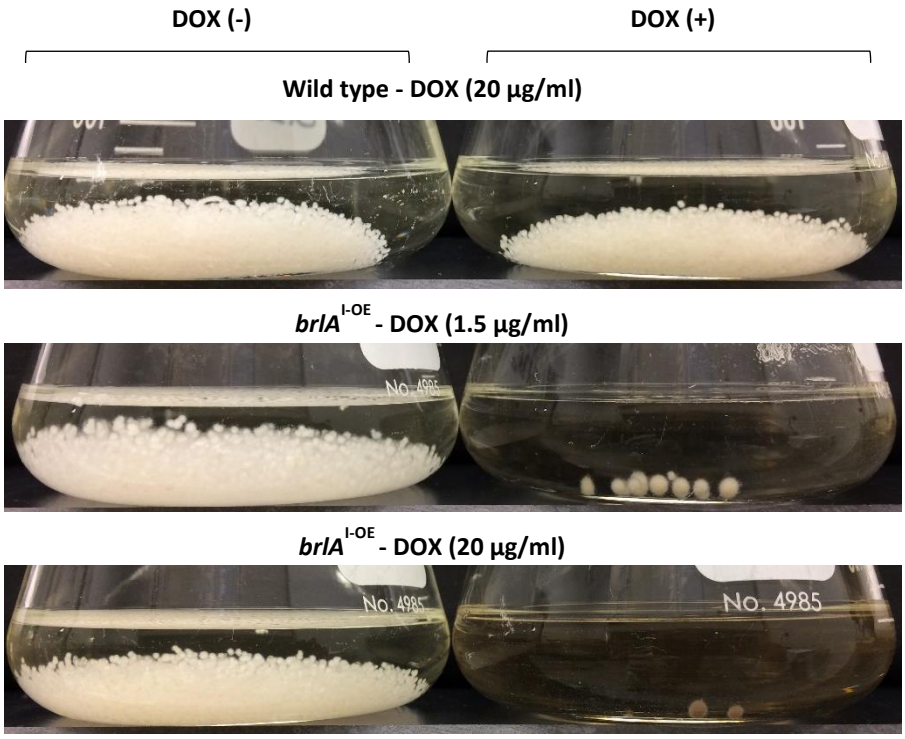

A
A. fumigatus Growth Inhibition
Biofilm Biomass (600 nm)
1.5
1.0
0.5
0.0
Wild type
brlA I-OE
0.01
0.1
1
Doxycycline Concentration (µg/ml)
B
Wild type
brlA I-OE
DOX (-)
0.06 µg/ml
0.18 µg/ml
0.54 µg/ml
1.62 µg/ml
C
DOX (-)
DOX (+)
Wild type - DOX (20 µg/ml)
brlA I-OE - DOX (1.5 µg/ml)
brlA I-OE - DOX (20 µg/ml)

Figure 2. *brlA* overexpression inhibits the growth of pre-competent *A. fumigatus* hyphae in a dose-dependent manner. Effects of *brlA* overexpression on the growth and conidiation of precompetent *A. fumigatus* in liquid culture. **(A)** Crystal violet staining of the biomass of the parental Af293 (Wild type) and inducible *brlA* overexpressing strain ($brlA^{I\text{-}OE}$) after 20 hours of exposure to the indicated concentrations of doxycycline. Each data point represents the mean and standard deviation of 4 technical replicates. **(B)** Differential interference contrast imaging of wild type and $brlA^{I\text{-}OE}$ strains after 20 hours exposure to the indicated concentrations of doxycycline. White arrows indicate normal conidiophore formation, red arrows indicate stunted atypical conidiophore formation and black arrows indicate hyphae that have undergone growth arrest. Scale bars: 20 μm. **(C)** Liquid shaking cultures of the wild type and $brlA^{I\text{-}OE}$ strains after 24 hours of exposure to indicated concentrations of doxycycline.



Supplemental figure S2. Pre-competent *A. fumigatus* hyphae break through *brlA* overexpression-induced growth inhibition in a dose-dependent manner. Effects of *brlA* overexpression on the growth and conidiation of pre-competent *A. fumigatus* in liquid culture. Visual inspection of liquid shaking cultures of the parental strain Af293 (Wild type) and the inducible *brlA* overexpressing strain (*brlA*$^{\text{I-OE}}$) after exposure to indicated concentrations of doxycycline for 48 hours.

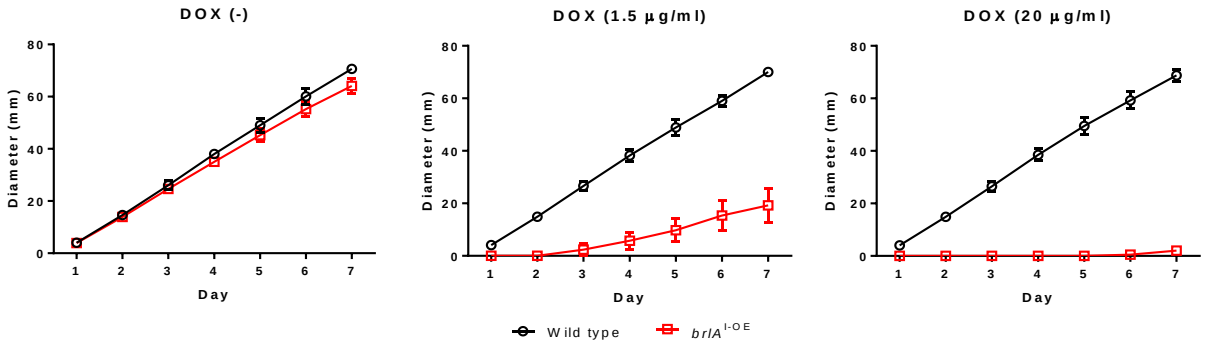
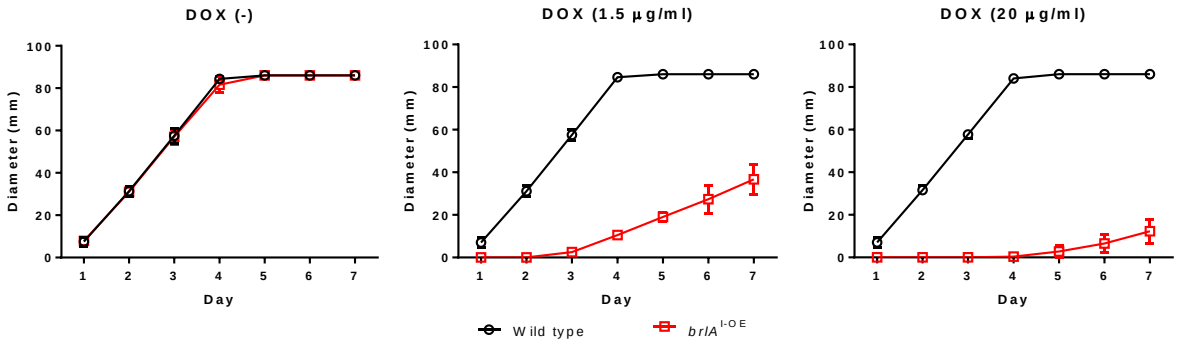


Figure 3. Pre-competent *A. fumigatus* hyphae exhibit dose-dependent rates of breakthrough and growth following *brlA* overexpression-induced growth inhibition. Radial growth of the parental Af293 (Wild type) and inducible *brlA* overexpressing ($brlA^{I\text{-}OE}$) strains over 7 days of exposure to the indicated concentrations of doxycycline on solid AMM media. Growth was assessed by measuring the diameter of fungal colonies daily. Each data point represents the mean and standard deviation of 3 biological replicates each with 1-2 technical replicates.



Supplemental figure S3. Pre-competent *A. fumigatus* hyphae exhibit dose-dependent rates of breakthrough and growth following *brlA* overexpression-induced growth inhibition on nutrient-rich media. Radial growth of the parental Af293 (Wild type) and inducible *brlA* overexpressing ($brlA^{I\text{-}OE}$) strains over 7 days of exposure to the indicated concentrations of doxycycline on solid YPD media. Growth was assessed by measuring the diameter of fungal colonies daily. Each data point represents the mean and standard deviation of 2 biological replicates each with 1-2 technical replicates.

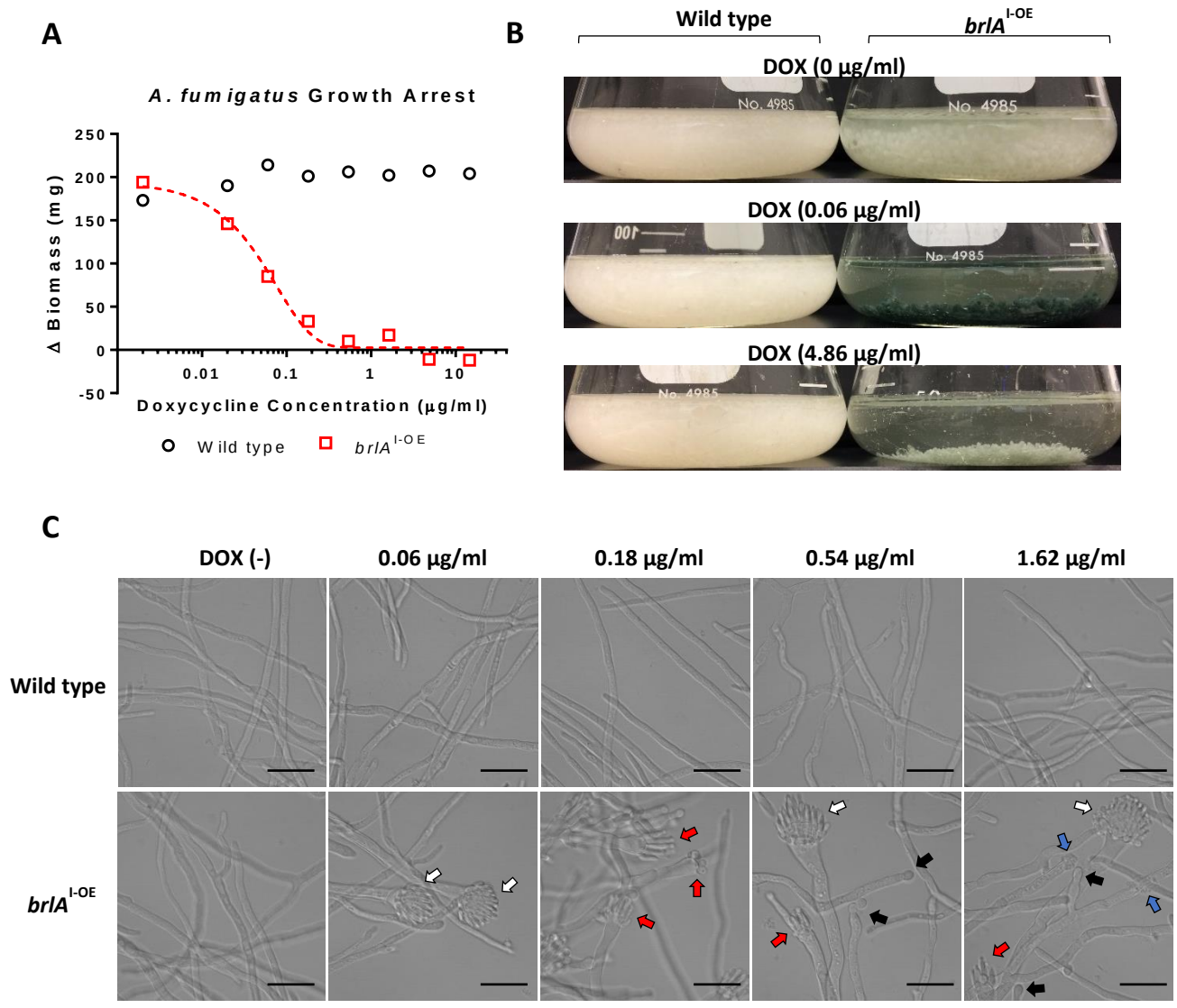

A
A. fumigatus Growth Arrest
Δ Biomass (mg)
250
200
150
100
50
0
-50
0.01
0.1
1
10
Doxycycline Concentration (µg/ml)
Wild type
brlA I-OE
B
Wild type
brlA I-OE
DOX (0 µg/ml)
DOX (0.06 µg/ml)
DOX (4.86 µg/ml)
C
DOX (-)
0.06 µg/ml
0.18 µg/ml
0.54 µg/ml
1.62 µg/ml
Wild type
brlA I-OE

Figure 4. *brlA* overexpression induces conidiation and arrests growth of competent *A. fumigatus* hyphae in a dose-dependent manner. Effects of *brlA* overexpression on the growth and conidiation of competent *A. fumigatus* hyphae in liquid culture. **(A)** Biomass dry weight of the parental Af293 (Wild type) and inducible *brlA* overexpressing ($brlA^{I\text{-}OE}$) strains was quantified after treating pre-grown hyphae with the indicated concentrations of doxycycline. Each data point represents the change in dry weight normalized to dry weight of the same strain prior to doxycycline treatment. **(B)** Appearance of cultures described in (A) at the indicated concentrations of doxycycline prior to harvesting fungal biomass. **(C)** Differential interference contrast imaging of pre-grown wild type and $brlA^{I\text{-}OE}$ hyphae after 4 hours of exposure to the indicated concentrations of doxycycline in liquid AMM static cultures. White arrows indicate conidiophore formation, red arrows indicate atypical conidiophore formation, black arrows indicate apical and subapical budding and blue arrows indicate rupturing of the hyphal cell wall and leakage of cytoplasmic content into the surrounding media. Scale bars: 20 μm.

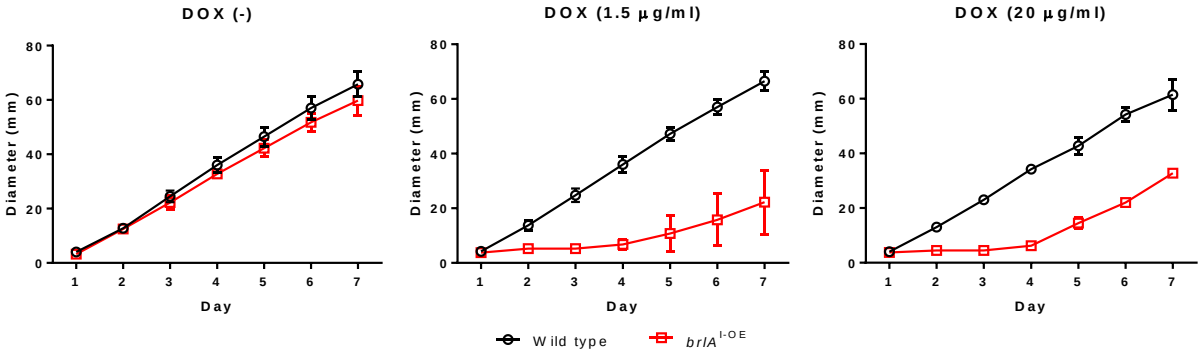
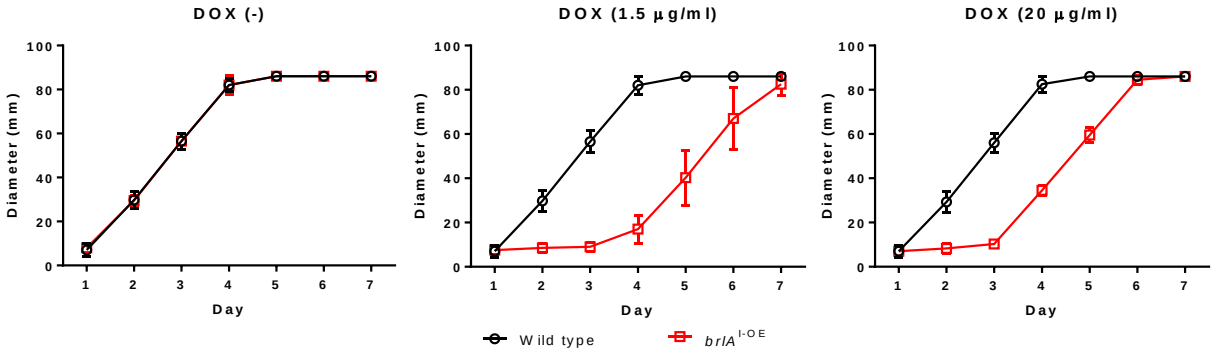
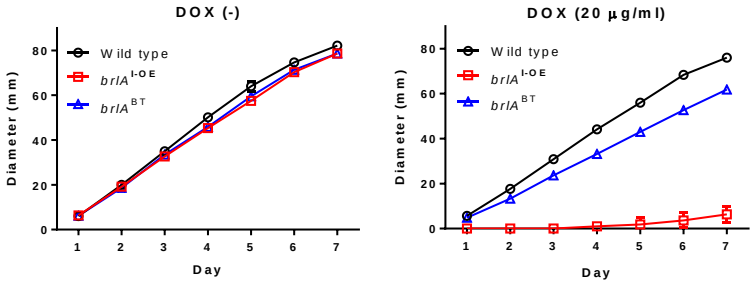


Figure 5. The growth rate of competent *A. fumigatus* hyphae following *brlA* overexpression-induced growth arrest is similar to wild type. Radial growth of the parental Af293 (Wild type) and inducible *brlA* overexpressing ($brlA^{\text{I-OE}}$) strains over 6 days following treatment with the indicated concentrations of doxycycline on solid AMM media. Growth was assessed by measuring the diameter of fungal colonies daily. Each data point represents the mean and standard deviation of 2 biological replicates each with 1-2 technical replicates.

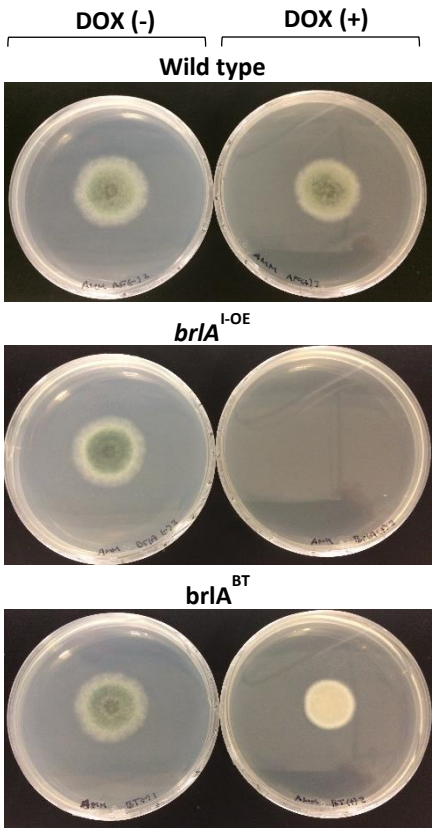


Supplemental figure S4. The growth rate of competent *A. fumigatus* hyphae following *brlA* overexpression-induced growth arrest is similar to wild type on nutrient-rich media. Radial growth of the parental Af293 (Wild type) and inducible *brlA* overexpressing ($brlA^{\text{I-OE}}$) strains over 6 days following treatment with indicated concentrations of doxycycline on solid YPD media. Growth was assessed by measuring the diameter of fungal colonies daily. Each data point represents the mean and standard deviation of 2 biological replicates each with 1-2 technical replicates.

A


DOX (-)
DOX (20 μg/ml)
Wild type
brlA I-OE
brlA BT
Diameter (mm)
Day

B


DOX (-)
DOX (+)
Wild type
brlA I-OE
brlA BT

C

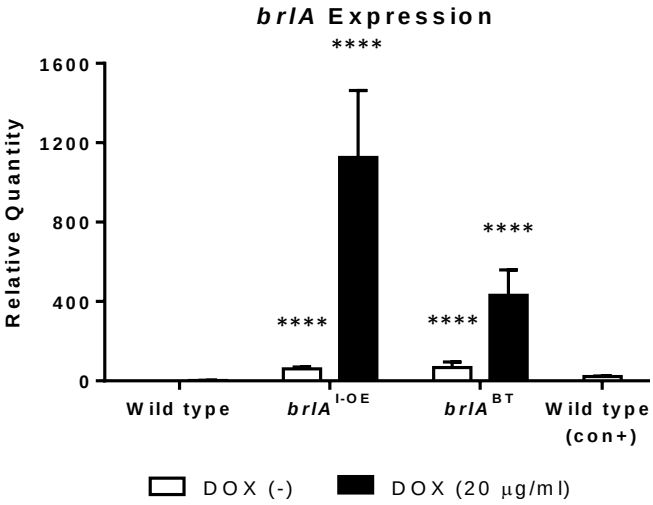
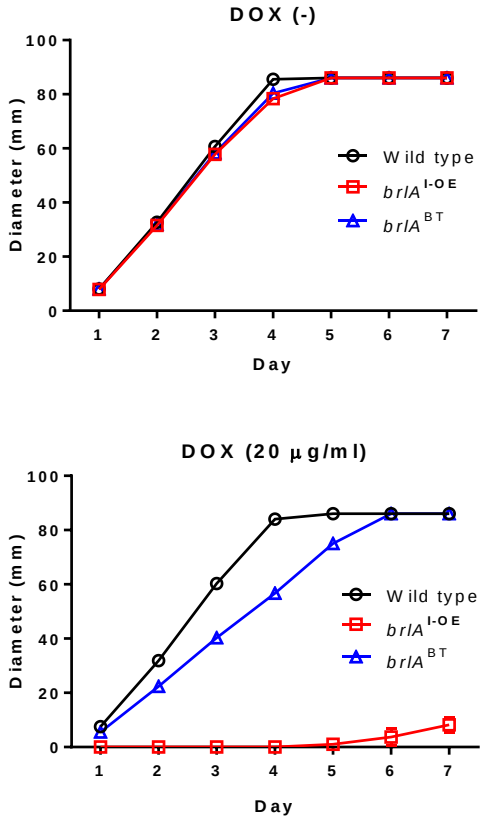

brlA Expression
Relative Quantity
****
Wild type
brlA I-OE
brlA BT
Wild type (con+)
DOX (-)
DOX (20 μg/ml)

Figure 6. Overexpression of *brlA* containing a spontaneously generated mutation reduces *A. fumigatus* growth and conidiation *in vitro*. (A) Radial growth of the parental Af293 (Wild type), the inducible *brlA* overexpressing ($brlA^{I\text{-}OE}$), and the spontaneous $brlA^{I\text{-}OE}$ breakthrough isolate ($brlA^{BT}$) strains over 6 days following treatment with 20 µg/ml doxycycline on solid AMM media. Growth was assessed by measuring the diameter of fungal colonies daily. Each data point represents the mean and standard deviation of 3 technical replicates. **(B)** Appearance of the wild type, $brlA^{I\text{-}OE}$ and $brlA^{BT}$ strains after 3 days of growth under the conditions described in (A). **(C)** Gene expression levels of *brlA* in the wild type, $brlA^{I\text{-}OE}$, $brlA^{BT}$ and conidiating parental Af293 (Wild type con+) strains after exposure for 3 hours to 20 µg/ml doxycycline as measured by RT-qPCR. Gene expression was normalized to the endogenous reference gene *tef1* and presented as fold change relative to wild type in the absence of doxycycline. Data are presented as the mean and range (as defined by the standard deviation of the delta Ct) of three technical replicates. ****: $p < 0.0001$; two-tailed student's t-test. $brlA^{I\text{-}OE}$ DOX (-), $brlA^{I\text{-}OE}$ DOX (1.5 µg/ml) and $brlA^{I\text{-}OE}$ DOX (20 µg/ml) compared to wild type DOX (-) at the same time point.

A



B

Wild type	*brlA*^{BT}
MRSQGNMSDRLGVEVD CHSLGSNECPSMGSSFSP LESPTPTPTSIYSQGSLASP SWPENGSYPGHAYDRGT GSTPIRGHFRLASMPSHE NMGLPPYSSLDGQDRM AVTDFLPSYDENADQFW LPSDVPKTYDHHVHGLPC PPSMHQYPPMLRSNYRH HPAPYFPESATNPCLSRPI FHHQPERLPPSLSMSHM MPWMGHTESIAPETIAP SQVAPVTPPPSYTDFSNSI NTFKTHSPDTPIRSCSLGT VSGADTPLSRLSGGAGEY MDECHQSPIYRDASGVR LQRQPSRKMARKQPSKQ SLSLENLPSIIKQVQFKCKE PGCKGRFKRQEHLKRHM KSHSKEKPHVCWVPGCH RAFSRSDNLNAHYTKTHS KRGGRNRYVATLDETSPD YNPDYRGPLTADGRPMP GGTLDESMPSREISMEW DE(STOP)	MRSQGNMSDRLGVEVD CHSLGSNECPSMGSSFSP LESPTPTPTSIYSQGSLASP SWPENGSYPGHAYDRGT GSTPIRGHFRLASMPSHE NMGLPPYSSLDGQDRM AVTDFLPSYDENADQFW LPSDVPKTYDHHVHGLPC PPSMHQYPPMLRSNYRH HPAPYFPESATNPCLSRPI FHHQPERLPPSLSMSHM MPWMGHTESIAPETIAP SQVAPVTPPPSYTDFSNSI NTFKTHSPDTPIRSCSLGT VSGADTPLSRLSGGAGEY MDECHQSPIYRDASGVR LQRQPSRKMARKQPSKQ SLSLENLPSIIKQVQFKCKE PGCKGRFKRQEHLKRHM KSHSKEKPHVCWVPGCH RAFSRSDKPQCPLHQDP QQTRRPQPLCGHLG (STOP)

Supplemental figure S5. Prolonged overexpression of *brlA* in *A. fumigatus* growing on nutrient-rich media results in a spontaneous mutation within *brlA* that permits growth but impairs conidiation. (A) Radial growth of the parental Af293 (Wild type), the inducible *brlA* overexpressing ($brlA^{I\text{-}OE}$), and the spontaneous $brlA^{I\text{-}OE}$ breakthrough isolate ($brlA^{BT}$) strains over 7 days of exposure to 20 μg/ml doxycycline on solid YPD media. Growth was assessed by measuring the diameter of fungal colonies daily. Each data point represents the mean and standard deviation of 3 technical replicates. **(B)** Amino acid sequences of the wild type *brlA* ORF and the $brlA^{BT}$ Tet-ON *brlA* ORF as determined by Sanger sequencing of genomic DNA and *in silico* translation to protein. Blue highlight indicates region unique to the wild type BrlA and yellow highlight indicates the mutated region unique to the $brlA^{BT}$ Tet-ON BrlA.

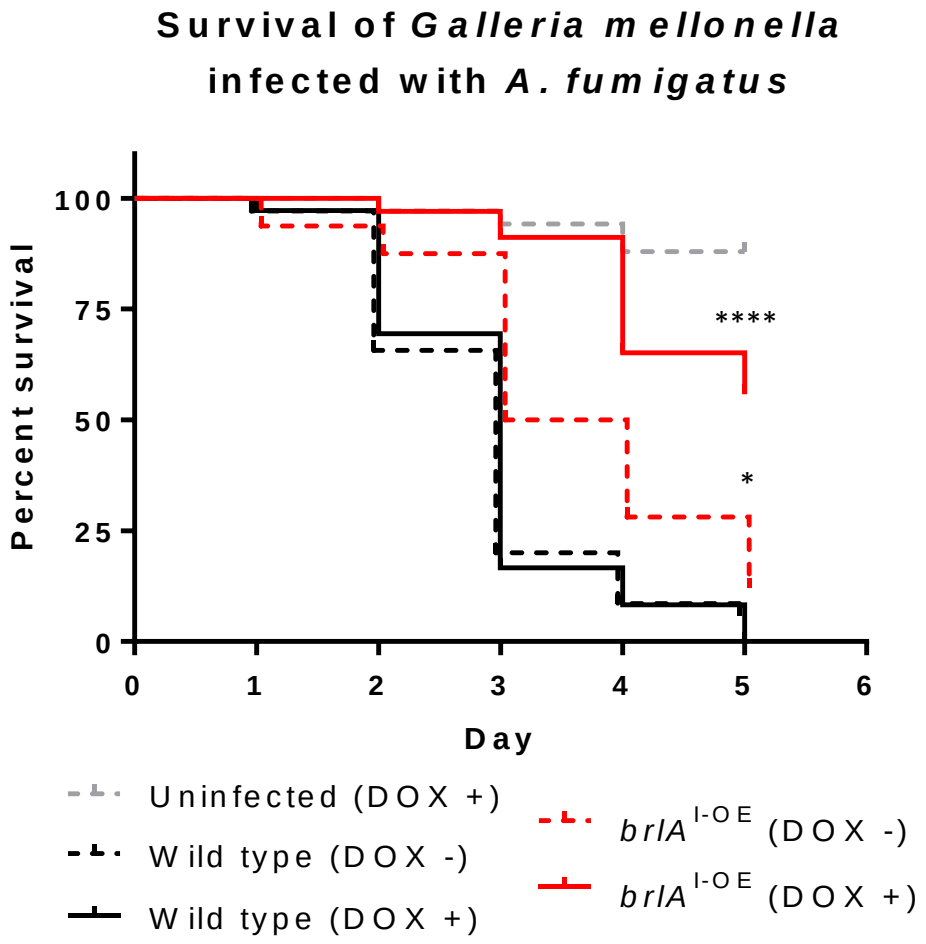


Figure 7. Doxycycline reduces the virulence of the inducible *brlA* overexpression strain of *A. fumigatus* in an invertebrate model of invasive aspergillosis. Survival of *Galleria mellonella* larvae infected with conidia of the parental Af293 (Wild type) and the inducible *brlA* overexpressing ($brlA^{I\text{-}OE}$) strains in the presence of 3 µg doxycycline. n = at least 32 larvae per group from 3 independent experiments. *: $p < 0.05$; Mantel-Cox log rank test comparing $brlA^{I\text{-}OE}$ (DOX -) to wild type (DOX -); ****: $p < 0.0001$; Mantel-Cox log rank test comparing $brlA^{I\text{-}OE}$ (DOX +) to $brlA^{I\text{-}OE}$ (DOX -).

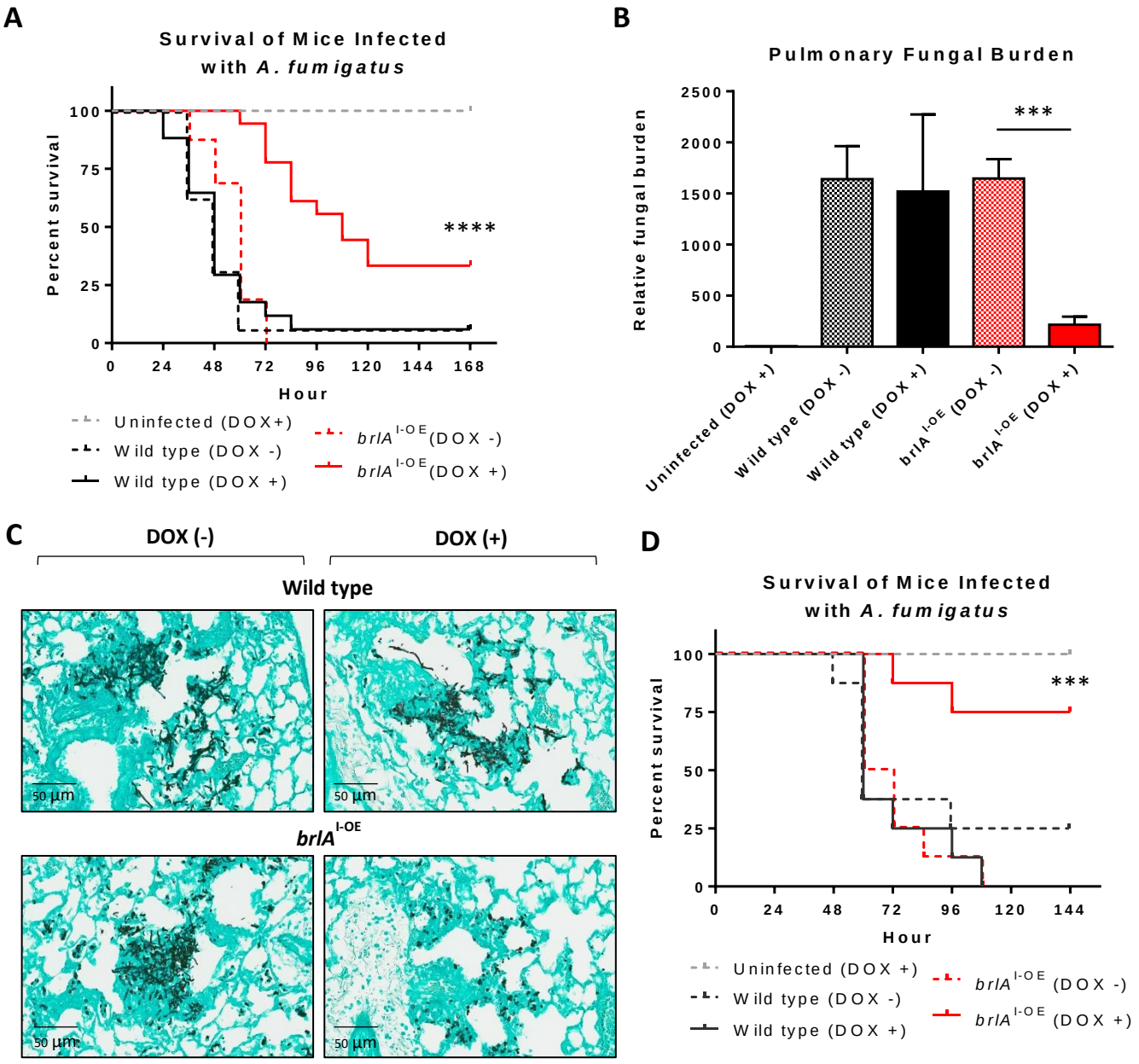
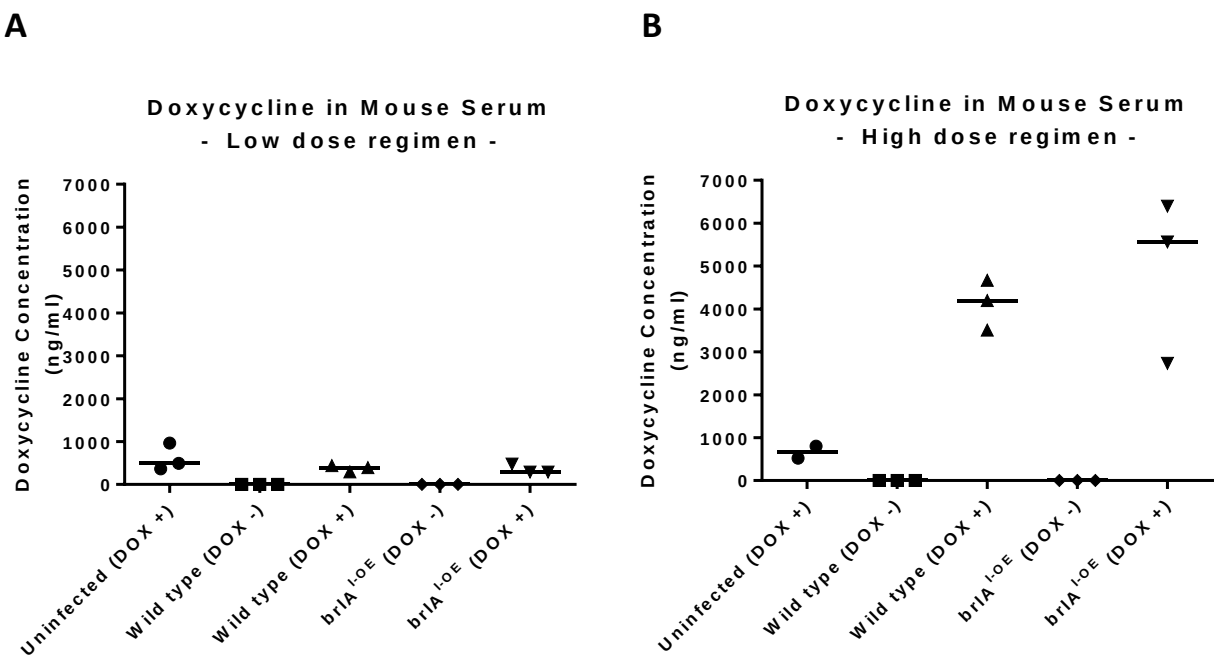

A
Survival of Mice Infected with A. fumigatus
Percent survival
Hour
****
Uninfected (DOX+)
Wild type (DOX -)
Wild type (DOX +)
brlA I-OE (DOX -)
brlA I-OE (DOX +)
B
Pulmonary Fungal Burden
Relative fungal burden
***
Uninfected (DOX +)
Wild type (DOX -)
Wild type (DOX +)
brlA I-OE (DOX -)
brlA I-OE (DOX +)
C
DOX (-)
DOX (+)
Wild type
brlA I-OE
50 µm
D
Survival of Mice Infected with A. fumigatus
Percent survival
Hour
***
Uninfected (DOX +)
Wild type (DOX -)
Wild type (DOX +)
brlA I-OE (DOX -)
brlA I-OE (DOX +)

Figure 8. Doxycycline reduces the virulence of the inducible *brlA* overexpression strain of *A. fumigatus* in a mouse model of invasive aspergillosis. (A) Survival of neutropenic BALB/c mice receiving daily intraperitoneal injections of 10 mg/kg doxycycline and infected with conidia of the parental Af293 (Wild type) or the inducible *brlA* overexpressing ($brlA^{I\text{-}OE}$) strains. n = at least 16 mice per group from 2 independent experiments. ****: $p < 0.0001$; Mantel-Cox log rank test comparing $brlA^{I\text{-}OE}$ (DOX +) to $brlA^{I\text{-}OE}$ (DOX -). **(B)** Pulmonary fungal burden of mice infected and treated as in (A) at 36 hours post-infection as determined by quantifying pulmonary galactomannan content. n = 8 mice per group. ***: $p < 0.001$; Wilcoxon rank-sum test comparing $brlA^{I\text{-}OE}$ (DOX +) to $brlA^{I\text{-}OE}$ (DOX -). **(C)** Gomori methenamine-silver stained lung sections from mice infected and treated as in (A) at 36 hours post-infection. **(D)** Survival of neutropenic BALB/c mice receiving 100 mg/kg doxycycline every 12 hours via oral gavage and infected with conidia of the wild type or $brlA^{I\text{-}OE}$ strains. n = 8 mice per group. ***: $p < 0.001$; Mantel-Cox log rank test comparing $brlA^{I\text{-}OE}$ (DOX +) to $brlA^{I\text{-}OE}$ (DOX -).



Supplemental figure S6. Doxycycline levels in infected mouse serum. Serum doxycycline concentration in neutropenic BALB/c mice receiving either **(A)** daily intraperitoneal injections of 10 mg/kg doxycycline, or **(B)** 100 mg/kg doxycycline every 12 hours via oral gavage, and infected with conidia of the parental Af293 (Wild type) or inducible *brlA* overexpressing (*brlA*$^{\text{I-OE}}$) strains, as determined using uHPLC-MS. Data represent the median of 3 mice from a single experiment.

Common name	Locus	Wild type (DOX +)	*brlA*$^{I\text{-}OE}$ (DOX -)	*brlA*$^{I\text{-}OE}$ (DOX +)
Amino acid metabolism				
Homocitrate synthase HcsA	AFUA_4G10460	1.02	1.23	0.33
Glutamate dehydrogenase, putative	AFUA_4G06620	1.06	0.56	0.20
Glutamate decarboxylase, putative	AFUA_6G13490	1.11	0.38	0.46
Aspartate aminotransferase, putative	AFUA_6G02490	0.97	1.23	0.43
Aspartate transaminase, putative	AFUA_2G09650	1.03	0.80	0.37
Plasma membrane				
Phosphatidyl synthase, putative	AFUA_4G11720	1.33	0.93	0.32
Plasma membrane H+ ATPase Pma1	AFUA_3G07640	1.12	0.93	0.48
GPR/FUN34 family protein	AFUA_2G04080	0.93	1.08	0.45
Cell wall polysaccharide metabolism				
GPI-anchored endochitinase ChiA1, putative	AFUA_5G03760	0.98	0.54	0.27
Extracellular serine-threonine rich adhesin protein, putative	AFUA_3G13110	0.90	0.61	0.19
UDP-glucose 4-epimerase Uge3	AFUA_3G07910	0.87	1.12	0.16
Extracellular serine-rich deacetylase Agd3	AFUA_3G07870	0.84	1.19	0.24
Cell wall glycoside hydrolase Aspf9/Crf1	AFUA_1G16190	0.99	0.42	0.28
Stress response				
Coproporphyrinogen III oxidase Hem13, putative	AFUA_1G07480	0.95	0.65	0.39
Flavohemoprotein FhpA, putative	AFUA_4G03410	1.03	0.62	0.32
Mitochondrial cytochrome b2, putative	AFUA_4G03120	0.87	1.10	0.38
Uncharacterized protein	AFUA_1G03610	1.05	0.61	0.33
Hypoxia responsive domain protein RcfB, putative	AFUA_1G12250	1.09	0.64	0.42
FAD-dependent oxidoreductase FrdA, putative	AFUA_7G05070	1.03	0.55	0.40
NAD-dependent formate dehydrogenase Fdh/AciA, putative	AFUA_6G04920	1.25	0.14	0.11
Uncharacterized protein	AFUA_1G13990	1.02	1.24	0.47
HLH SREBP DNA binding domain protein SrbB	AFUA_4G03460	1.20	0.85	0.48
Mannitol-1-phosphate 5-dehydrogenase MpdA	AFUA_2G10660	1.29	0.91	0.26
Zinc-dependent alcohol dehydrogenase AlcC, putative	AFUA_5G06240	0.91	0.53	0.40
Pyruvate decarboxylase PdcA, putative	AFUA_3G11070	1.04	0.63	0.48

Table 1. Downregulated genes in *brlA*-overexpressing pre-competent *A. fumigatus* hyphae. Differentially expressed genes in pre-competent parental Af293 (Wild type) as compared with the inducible *brlA* overexpressing (*brlA*$^{I\text{-}OE}$) strain after 30 minutes exposure to 20 µg/ml doxycycline, as measured by RNA deep-sequencing. Gene expression was normalized using the FPKM method and presented as fold change relative to wild type in the absence of doxycycline.

Common name	Locus	Wild type (DOX +)	*brlA*$^{I\text{-}OE}$ (DOX -)	*brlA*$^{I\text{-}OE}$ (DOX +)
Conidiation				
C2H2-type master regulator of conidiation BrlA	AFUA_1G16590	0.91	10.05	88.38
C2H2 transcription factor Ace2/Swi5, putative	AFUA_3G11250	1.08	1.18	8.85
Hydrophobin RodB	AFUA_1G17250	0.99	1.04	2.05
Plasma membrane				
GPI-anchored serine-threonine rich protein, putative	AFUA_1G03630	0.99	0.93	3.90
Cell wall protein biosynthesis				
Cell wall protein, putative	AFUA_5G08180	1.28	2.31	130.08
GPI anchored adhesin-like protein, putative	AFUA_1G11220	1.01	0.68	2.11
Protein glycosylation				
Alpha-1,2-mannosidase	AFUA_4G10070	0.90	1.03	4.05
Cell cycle regulation				
RNP domain protein	AFUA_6G12300	0.99	1.11	4.32
mRNA binding protein Pumilio 2, putative	AFUA_6G04310	1.00	1.13	5.17
Protein tyrosine phosphatase NimT/Mih1, putative	AFUA_6G08200	1.01	1.32	3.98
14-3-3 family protein ArtA, putative	AFUA_2G03290	1.02	1.13	2.20
Oxidative phosphorylation				
Cytochrome c peroxidase, Ccp1, putative	AFUA_4G09110	1.02	1.07	2.84
Cytochrome P450, putative	AFUA_8G00962	1.00	0.97	2.73
NAD-binding Rossman fold oxidoreductase, putative	AFUA_4G10190	1.02	0.82	15.22
Stress response				
GTP cyclohydrolase II, putative	AFUA_2G01220	1.04	0.83	3.21
Zinc/cadmium resistance protein	AFUA_2G14570	1.07	0.99	3.79
MFS toxin efflux pump, putative	AFUA_6G02220	0.92	1.22	5.03
2-methylcitrate dehydratase, putative	AFUA_6G03730	1.07	1.17	3.54
Calnexin homolog ClxA	AFUA_4G12850	0.97	1.00	2.06
Zinc-containing alcohol dehydrogenase, putative	AFUA_1G04620	1.15	1.06	2.32

Table 2. Upregulated genes in *brlA*-overexpressing pre-competent *A. fumigatus* hyphae. Differentially expressed genes in pre-competent parental Af293 (Wild type) as compared with the inducible *brlA* overexpressing (*brlA*$^{I\text{-}OE}$) strain after 30 minutes exposure to 20 μg/ml doxycycline, as measured by RNA deep-sequencing. Gene expression was normalized using the FPKM method and presented as fold change relative to wild type in the absence of doxycycline.

References

1. Alkhayyat F, Chang Kim S, Yu JH. Genetic control of asexual development in aspergillus fumigatus. Advances in applied microbiology. 2015;90:93-107.
2. Latgé J-P, Steinbach WJ. *Aspergillus fumigatus* and aspergillosis. Clinical Microbiology Reviews. 1999;12(2):310-50.
3. Brown GD, Denning DW, Gow NAR, Levitz SM, Netea MG, White TC. Hidden Killers: Human Fungal Infections. Science Translational Medicine. 2012;4(165):165rv13-rv13.
4. Kousha M, Tadi R, Soubani AO. Pulmonary aspergillosis: a clinical review. European Respiratory Review. 2011;20(121):156-74.
5. Abad A, Victoria Fernández-Molina J, Bikandi J, Ramírez A, Margareto J, Sendino J, et al. What makes Aspergillus fumigatus a successful pathogen? Genes and molecules involved in invasive aspergillosis. Revista Iberoamericana de Micología. 2010;27(4):155-82.
6. Mah JH, Yu JH. Upstream and downstream regulation of asexual development in Aspergillus fumigatus. Eukaryotic cell. 2006;5(10):1585-95.
7. Tochigi N, Okubo Y, Ando T, Wakayama M, Shinozaki M, Gocho K, et al. Histopathological implications of Aspergillus infection in lung. Mediators of inflammation. 2013;2013.
8. Adams TH, Boylan MT, Timberlake WE. brlA is necessary and sufficient to direct conidiophore development in Aspergillus nidulans. Cell. 1988;54(3):353-62.
9. Mah J-H, Yu J-H. Upstream and Downstream Regulation of Asexual Development in Aspergillus fumigatus. Eukaryotic Cell. 2006;5(10):1585-95.
10. Kaminskyj SGW. Fundamentals of growth, storage, genetics and microscopy of *Aspergillus nidulans*. Fungal Genetics Newsletter. 2001;48:25-31.
11. Urlinger S, Baron U, Thellmann M, Hasan MT, Bujard H, Hillen W. Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and

sensitivity. Proceedings of the National Academy of Sciences of the United States of America. 2000;97(14):7963-8.

12. Gravelat FN, Askew DS, Sheppard DC. Targeted gene deletion in Aspergillus fumigatus using the hygromycin-resistance split-marker approach. Methods in molecular biology (Clifton, NJ). 2012;845:119-30.

13. Snarr BD, Baker P, Bamford NC, Sato Y, Liu H, Lehoux M, et al. Microbial glycoside hydrolases as antibiofilm agents with cross-kingdom activity. Proceedings of the National Academy of Sciences. 2017;114(27):7124-9.

14. Nierman WC, Pain A, Anderson MJ, Wortman JR, Kim HS, Arroyo J, et al. Genomic sequence of the pathogenic and allergenic filamentous fungus Aspergillus fumigatus. Nature. 2005;438(7071):1151.

15. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics. 2013;29(1):15-21.

16. Helmschrott C, Sasse A, Samantaray S, Krappmann S, Wagener J. Upgrading fungal gene expression on demand: improved systems for doxycycline-dependent silencing in Aspergillus fumigatus. Appl Environ Microbiol. 2013;79(5):1751-4.

17. Park H-S, Yu J-H. Developmental regulators in Aspergillus fumigatus. Journal of Microbiology. 2016;54(3):223-31.

18. Krijgsheld P, Bleichrodt R, van Veluw GJ, Wang F, Müller WH, Dijksterhuis J, et al. Development in Aspergillus. Studies in Mycology. 2013;74:1-29.

19. Sheppard DC, Doedt T, Chiang LY, Kim HS, Chen D, Nierman WC, et al. The Aspergillus fumigatus StuA Protein Governs the Up-Regulation of a Discrete Transcriptional Program during the Acquisition of Developmental Competence. Molecular Biology of the Cell. 2005;16(12):5866-79.

20. Mirabito PM, Adams TH, Timberlake WE. Interactions of three sequentially expressed genes control temporal and spatial specificity in aspergillus development. Cell. 1989;57(5):859-68.

21. Adams TH, Wieser JK, Yu J-H. Asexual Sporulation in Aspergillus nidulans. Microbiology and Molecular Biology Reviews. 1998;62(1):35-54.

22. Daley JM, Thomay AA, Connolly MD, Reichner JS, Albina JE. Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. Journal of leukocyte biology. 2008;83(1):64-70.

23. O'Dea EM, Amarsaikhan N, Li H, Downey J, Steele E, Van Dyken SJ, et al. Eosinophils are recruited in response to chitin exposure and enhance Th2-mediated immune pathology in Aspergillus fumigatus infection. Infection and immunity. 2014;82(8):3199-205.

24. Cawthorne C, Swindell R, Stratford IJ, Dive C, Welman A. Comparison of doxycycline delivery methods for Tet-inducible gene expression in a subcutaneous xenograft model. Journal of biomolecular techniques: JBT. 2007;18(2):120.

25. Prall AK, Longo GM, Mayhan WG, Waltke EA, Fleckten B, Thompson RW, et al. Doxycycline in patients with abdominal aortic aneurysms and in mice: comparison of serum levels and effect on aneurysm growth in mice. Journal of vascular surgery. 2002;35(5):923-9.

26. Gravelat FN, Doedt T, Chiang LY, Liu H, Filler SG, Patterson TF, et al. In vivo analysis of Aspergillus fumigatus developmental gene expression determined by real-time reverse transcription-PCR. Infection and immunity. 2008;76(8):3632-9.

27. Helmschrott C, Sasse A, Samantaray S, Krappmann S, Wagener J. Upgrading Fungal Gene Expression on Demand: Improved Systems for Doxycycline-Dependent Silencing in Aspergillus fumigatus. Applied and Environmental Microbiology. 2013;79(5):1751-4.

28. Vogt K, Bhabhra R, Rhodes JC, Askew DS. Doxycycline-regulated gene expression in the opportunistic fungal pathogen Aspergillus fumigatus. BMC microbiology. 2005;5.

29. Blanchard P, Rudhardt M, Fabre J. Behaviour of doxycycline in the tissues. Chemotherapy. 1975;21(Suppl. 1):8-18.

30. Kraus PR, Hofmann AF, Harris SD. Characterization of the Aspergillus nidulans 14-3–3 homologue, ArtA. FEMS microbiology letters. 2002;210(1):61-6.

31. Son S, Osmani SA. Analysis of all protein phosphatase genes in Aspergillus nidulans identifies a new mitotic regulator, fcp1. Eukaryotic cell. 2009;8(4):573-85.

32. Bell RM, Coleman RA. Enzymes of glycerolipid synthesis in eukaryotes. Annual review of biochemistry. 1980;49(1):459-87.

33. Letts V, Klig L, Bae-Lee M, Carman G, Henry S. Isolation of the yeast structural gene for the membrane-associated enzyme phosphatidylserine synthase. Proceedings of the National Academy of Sciences. 1983;80(23):7279-83.

34. Chattopadhyay P, Banerjee SK, Sen K, Chakrabarti P. Lipid profiles of Aspergillus niger and its unsaturated fatty acid auxotroph, UFA2. Canadian journal of microbiology. 1985;31(4):352-5.

35. Meixner O, Mischak H, Kubicek C, Röhr M. Effect of manganese deficiency on plasma-membrane lipid composition and glucose uptake in Aspergillus niger. FEMS microbiology letters. 1985;26(3):271-4.

36. Ghannoum MA. Potential role of phospholipases in virulence and fungal pathogenesis. Clinical microbiology reviews. 2000;13(1):122-43.

37. Djordjevic J. Role of phospholipases in fungal fitness, pathogenicity, and drug development–lessons from Cryptococcus neoformans. Frontiers in microbiology. 2010;1:125.

38. Leidich SD, Ibrahim AS, Fu Y, Koul A, Jessup C, Vitullo J, et al. Cloning and disruption of caPLB1, a phospholipase B gene involved in the pathogenicity of Candida albicans. Journal of Biological Chemistry. 1998;273(40):26078-86.

39. Bossche HV, Koymans L. Review Article Cytochromes P450 in fungi: Cytochrome P450 bei Pilzen. Mycoses. 1998;41:32-8.

40. Hatefi Y. The mitochondrial electron transport and oxidative phosphorylation system. Annual review of biochemistry. 1985;54(1):1015-69.

41. Joseph-Horne T, Hollomon DW, Wood PM. Fungal respiration: a fusion of standard and alternative components. Biochimica et Biophysica Acta (BBA) - Bioenergetics. 2001;1504(2):179-95.

42. Kathiresan M, Martins D, English AM. Respiration triggers heme transfer from cytochrome c peroxidase to catalase in yeast mitochondria. Proceedings of the National Academy of Sciences of the United States of America. 2014;111(49):17468-73.

43. Shimizu M, Fujii T, Masuo S, Fujita K, Takaya N. Proteomic analysis of Aspergillus nidulans cultured under hypoxic conditions. Proteomics. 2009;9(1):7-19.

44. Chung D, Barker BM, Carey CC, Merriman B, Werner ER, Lechner BE, et al. ChIP-seq and in vivo transcriptome analyses of the Aspergillus fumigatus SREBP SrbA reveals a new regulator of the fungal hypoxia response and virulence. PLoS pathogens. 2014;10(11):e1004487.

45. Kroll K, Pähtz V, Hillmann F, Vaknin Y, Schmidt-Heck W, Roth M, et al. Identification of hypoxia-inducible target genes of Aspergillus fumigatus by transcriptome analysis reveals cellular respiration as important contributor to hypoxic survival. Eukaryotic cell. 2014:EC. 00084-14.

46. Grahl N, Puttikamonkul S, Macdonald JM, Gamcsik MP, Ngo LY, Hohl TM, et al. In vivo Hypoxia and a Fungal Alcohol Dehydrogenase Influence the Pathogenesis of Invasive Pulmonary Aspergillosis. PLOS Pathogens. 2011;7(7):e1002145.

47. Willger SD, Puttikamonkul S, Kim K-H, Burritt JB, Grahl N, Metzler LJ, et al. A sterol-regulatory element binding protein is required for cell polarity, hypoxia adaptation, azole drug resistance, and virulence in Aspergillus fumigatus. PLoS pathogens. 2008;4(11):e1000200.

Chapter 3: General Discussion and Conclusions

With the ever-increasing use of immunosuppressive therapies in the clinic, opportunistic fungal pathogens have become a significant threat to human health (53, 91, 92). *A. fumigatus* is the most common cause of invasive mould disease in humans, and infections caused by this species have been historically challenging to treat (45, 53, 72, 78, 90). Few classes of antifungals have been developed with activity against *A. fumigatus*, and new antifungal targets are urgently needed.

In this study, we sought to investigate a novel target for the treatment of IA. In the environment, *A. fumigatus* propagates through the abundant production of microscopic airborne conidia, which are ubiquitous in nature. Susceptible humans are infected by inhalation of these airborne conidia (8, 9, 93). A balance exists between conidiation and vegetative growth in *A. fumigatus*, whereby growth is reduced during conidiation (24). *A. fumigatus* rarely undergoes conidiation during an invasive human infection, permitting the bulk of its metabolic energy to be directed towards vegetative growth. We therefore hypothesized that induced activation of conidiation during an invasive infection would suppress *A. fumigatus* vegetative growth, impairing the ability of this organism to cause disease. To test this hypothesis, we developed a strain of *A. fumigatus* that overexpresses *brlA*, a key regulatory gene of the conidiation pathway in *Aspergillus spp.* (22, 24), in response to an inducer molecule, doxycycline. We analyzed the *in vitro* effects of induced overexpression of *brlA* on *A. fumigatus* growth and conidiation at two distinct developmental time points *in vitro* and the effects of induced *brlA* overexpression on *A. fumigatus* virulence in two *in vivo* models of IA. Finally, we used an exploratory RNA deep-sequencing analysis of pre-competent hyphae of the inducible *brlA* overexpression strain after exposure to doxycycline to investigate possible mechanisms of *brlA* overexpression-induced growth inhibition at the gene expression level.

The findings of our study, as demonstrated in chapter 2, indicate that induction of *brlA* overexpression *in vitro* is sufficient to induce conidiation in competent hyphae and reduce the growth of *A. fumigatus* at all stages of development. The level of *brlA* overexpression dictates the effects on *A. fumigatus* hyphae, with lower levels inducing conidiation and higher levels inhibiting vegetative growth. The effects of high-level *brlA* overexpression on fungal growth are most pronounced in pre-competent hyphae which exhibit a longer duration of growth inhibition and reduced rates of breakthrough and growth thereafter. Doxycycline treatment protects *G. mellonella* larvae during infection with the inducible *brlA* overexpression strain of *A. fumigatus* but not during infection with wild type *A. fumigatus*. Similarly, in a neutropenic mouse model of IA, doxycycline protects mice from infection with the inducible *brlA* overexpression strain of *A. fumigatus*. These mice exhibit reduced mortality, reduced pulmonary fungal burden and *in vivo* fungal growth inhibition as compared to untreated mice infected with the inducible *brlA* overexpression strain or mice infected with wild type *A. fumigatus.* RNA deep-sequencing of pre-competent hyphae following induced *brlA* overexpression demonstrates changes in the expression of genes associated with amino acid metabolism, plasma membrane and cell wall composition, cell cycle regulation and the balance between normoxic and hypoxic metabolism. This proof-of-concept study demonstrates that doxycycline treatment significantly reduces virulence of an inducible *brlA* overexpression strain of *A. fumigatus in vivo*, suggesting that inducing *brlA* overexpression may hold promise as a viable approach to antifungal therapy against IA.

The Tet-ON system has been reported to result in elevated basal expression levels of the gene of interest (94, 95), a finding reproduced in our studies. This overexpression been attributed to the *A. nidulans oliC* minimal promoter driving gene expression in the absence of the inducer molecule doxycycline (96). Supporting this hypothesis, replacing the *oliC* minimal promoter with

an upstream region of the *A. nidulans tpiA* gene, which encodes a triosephosphate isomerase, results in a marked reduction in basal expression levels of the Tet-ON system (96). This strategy could therefore be used optimize the Tet-ON system and generate an inducible *brlA* overexpression strain with a lower basal level of *brlA* expression.

The results of our RNA deep-sequencing analysis provide unique insight into the effects of induced *brlA* overexpression on pre-competent *A. fumigatus* hyphae at the gene expression level. Overexpression of *brlA* was associated with strong downregulation of genes involved in cell wall polysaccharide metabolism, most notably, the UDP-glucose 4-epimerase (*uge3*) and an extracellular serine-rich deacetylase (*agd3*) which are essential for the synthesis and function of the polysaccharide virulence factor GAG (97, 98). Previously, our group identified *uge3* and *agd3* as being strongly downregulated in a *stuA*-deficient strain of *A. fumigatus* (97). The product of the *stuA* gene is essential for appropriate conidiation in *A. fumigatus* through the regulation of *brlA* (19, 41). Our findings support this hypothesis, as induced overexpression of *brlA* led to marked downregulation of *uge3* and *agd3* with no effect on the expression level of *stuA*. Taken together, these findings may also indicate that an appropriate level of *brlA* expression is required to maintain normal GAG production in *A. fumigatus*, and if the level of *brlA* expression is downregulated or upregulated outside of this range, GAG production is adversely affected. GAG is a proven virulence factor of *A. fumigatus*, mediating adherence of the fungus to host cells and abiotic substrates, acting as a protective barrier to antifungals and masking fungal pathogen-associated molecular patterns (PAMPs) such as β-glucans, from detection by host immune cells (97). The ability to inhibit *A. fumigatus* growth and GAG production through the induced upregulation of a single transcriptional regulator – *brlA* – demonstrates the diverse roles *brlA* plays in the fitness and

virulence of this opportunistic pathogen, and further supports *brlA* induction as a viable new target for antifungal therapy.

Previously our group reported that *brlA* is necessary for the downregulation of ribosomal protein gene expression in *A. fumigatus* following nitrogen depletion (26). We therefore hypothesized that the observed growth inhibition of pre-competent hyphae following induced *brlA* overexpression was due to *brlA*-mediated downregulation of ribosomal protein genes, resulting in a transition to metabolic inactivity. Interestingly however, we failed to observe dysregulation of any ribosomal protein genes that were identified in our previous study. This observation may suggest that while *brlA* is essential for the down-regulation of ribosomal protein gene expression during nitrogen depletion, induced overexpression of *brlA* does not recapitulate this starvation response and an alternative mechanism must exist by which high-level *brlA* overexpression inhibits the growth of pre-competent *A. fumigatus* hyphae.

As described in chapter two, RNA deep-sequencing of *brlA* overexpressing pre-competent hyphae demonstrated strong upregulation of genes associated with oxidative phosphorylation (99-102), as well as downregulation of genes previously associated with the adaptation response to hypoxia in *A. fumigatus* and *A. nidulans* (103-105). These findings indicate that during conidiation in *A. fumigatus*, *brlA* may function to skew fungal metabolism towards oxidative respiration and repress metabolic pathways important for hypoxic metabolism. Adaptation to the hypoxic pulmonary microenvironment during infection has been identified as an important virulence factor in *A. fumigatus* (103, 106, 107). We therefore hypothesize that inducing *brlA* overexpression in *A. fumigatus* under hypoxic growth conditions would compromise the hypoxia adaptation response and impair fungal fitness. To test this hypothesis, the *in vitro* doxycycline MIC of the inducible *brlA* overexpression strain will be compared under hypoxic and normoxic culture conditions to

determine if MIC values are reduced under hypoxia. The inverse experiment could also be performed *in vivo* using hyperbaric oxygen (HBO). Previous studies have shown that HBO can be used to alter *in vivo* oxygen levels at the site of an infection (108, 109) and that HBO reduces *A. fumigatus* growth *in vitro* and virulence in a chemotherapy mouse model of IA (110). It is possible therefore that sub-inhibitory levels of *brlA* overexpression could restore *A. fumigatus* virulence in the presence of HBO by forcing *A. fumigatus* metabolism towards oxidative respiration and improving fungal fitness in an elevated oxygen environment *in vivo.* To test this hypothesis, low doses of doxycycline could be administered to mice infected with the inducible *brlA* overexpression strain of *A. fumigatus* in the presence and absence of HBO and fungal growth and virulence assessed using similar methods described in chapter two of this thesis.

In *brlA* overexpressing pre-competent hyphae, the most highly dysregulated gene identified by RNA deep-sequencing was that encoding a putative cell wall protein (AFUA_5G08180) with 130-fold upregulation relative to wild type *A. fumigatus.* Previously, this gene has been identified as significantly downregulated in a 5-azacytidine-induced developmental variant strain of *A. fumigatus* (111) which has impaired *brlA* expression and conidiation. These findings are consistent with direct regulation of this putative cell wall protein gene by BrlA, and further support that high-level *brlA* overexpression results in dysregulation of *A. fumigatus* cell wall biosynthesis. Determining the effects of *brlA* overexpression on cell wall composition will be an obvious next step in confirming this hypothesis.

The results of our transcriptome analysis provide several clues as to a possible mechanism of *brlA* overexpression-induced growth inhibition of pre-competent hyphae. These include alterations in plasma membrane composition through dysregulated expression of genes encoding a putative phosphatidyl synthase and a GPI-anchored serine-threonine rich protein gene with

predicted lysophospholipase activity, or alternatively, dysregulation of the cell cycle through *nimT* and *artA*, as discussed in detail in chapter two of this thesis. However, due to a lack of biological replicates in our RNA deep-sequencing assay, these findings must be replicated and further validated *in vitro*. Future studies will therefore include repeating the RNA deep-sequencing with at least two more biological replicates and validating differentially expressed genes *in vitro* using RT-qPCR. Differentially expressed genes that have an already well-defined phenotype can also be used to increase confidence in the findings of our RNA deep-sequencing analysis. For example, we observed strong downregulation of the UDP-glucose 4-epimerase (*uge3*) which is essential for GAG biosynthesis (97). To validate these findings, we will quantify the amount of cell wall-bound GAG on the inducible *brlA* overexpression strain of *A. fumigatus* after exposure to doxycycline using a fluorescent lectin stain known to bind GAG (97) and compare the mean fluorescence intensity with that of doxycycline untreated and wild type *A. fumigatus* with or without doxycycline.

Further studies will also be required to validate *brlA* induction as a viable new target for antifungal therapeutics. We have shown, as proof of concept, that targeted upregulation of *brlA* reduces *A. fumigatus* virulence in a mouse model of IA. The identification of small molecules that induce overexpression of native *brlA*, and elucidating their effects on the growth and virulence of wild type *A. fumigatus* isolates are important next steps. We are therefore in the process of cloning a fluorescent *brlA* reporter strain of *A. fumigatus*, in which the native *brlA* locus is replaced with the gene encoding green fluorescent protein (GFP). This strain will be tested for GFP expression in a high-throughput screening (HTS) assay with a library of small molecules. By replacing the native *brlA* ORF with GFP, any molecule that interacts with the native *brlA* promotor to induce gene expression will activate GFP expression, thus providing a fluorescent signal that can be

quantified. Molecules that induce GFP will then be validated using wild type *A. fumigatus*, assessing their effects on *brlA* expression, conidiation, growth, and virulence using methods previously described. Ultimately, any small molecule capable of inducing *brlA* overexpression in wild type *A. fumigatus* will also be tested in combination with conventional antifungals to assess their synergistic potential both *in vitro* and *in vivo*.

The natural polyphenol curcumin inhibits the growth and filamentation of C*andida albicans* as well as several other non-*albicans Candida spp. in vitro,* and treating infected mice with curcumin significantly reduces the fungal load within their kidneys (112). The antifungal effects of curcumin are attributed to the upregulation of *tup1*, which encodes a global suppressor of genes involved in the filamentation pathway of *Candida spp.* (112, 113). Another naturally occurring polyphenol eugenol inhibits the growth of *A. fumigatus* (114) and *Aspergillus flavus* (115) *in vitro*. In *A. flavus,* eugenol treatment leads to a marked increase in the expression of *brlA,* as determined through RNA deep-sequencing (115). These findings suggest that naturally occurring polyphenols may serve as a logical starting point for the identification of natural inducers of conidiation.

Overall, the results of this study provide strong evidence that conidiation pathway dysregulation, through the activation of *brlA,* reduces *A. fumigatus* growth *in vitro* and attenuates the virulence of this opportunistic pathogen in two distinct *in vivo* models of IA. Further work will be required to overcome the limitations of our strategy, notably adapting this approach to wild type strains of *A. fumigatus.* The results of our proof-of-concept study support the induction of *brlA* as a novel target for antifungal therapy and thus lay the groundwork for the development of an entirely new class of antifungals against IA. More broadly, our study illustrates the diversity of functions

regulated by a single transcriptional regulator – *brlA*, thus highlighting the possibility of targeted dysregulation of other transcriptional regulators as an antifungal strategy against *A. fumigatus*.

References

1. Bennett JW. An overview of the genus Aspergillus. Aspergillus: molecular biology and genomics. 2010:1-17.
2. Krijgsheld P, Bleichrodt R, van Veluw GJ, Wang F, Müller WH, Dijksterhuis J, et al. Development in Aspergillus. Studies in Mycology. 2013;74:1-29.
3. Geiser DM, Klich MA, Frisvad JC, Peterson SW, Varga J, Samson RA. The current status of species recognition and identification in Aspergillus. Studies in Mycology. 2007;59:1-10.
4. Hawksworth DL. Naming Aspergillus species: progress towards one name for each species. Medical Mycology. 2011;49(Supplement_1):S70-S6.
5. Abe K, Gomi K, Hasegawa F, Machida M. Impact of Aspergillus oryzae genomics on industrial production of metabolites. Mycopathologia. 2006;162(3):143.
6. Pel HJ, de Winde JH, Archer DB, Dyer PS, Hofmann G, Schaap PJ, et al. Genome sequencing and analysis of the versatile cell factory Aspergillus niger CBS 513.88. Nature Biotechnology. 2007;25:221.
7. Soccol CR, Vandenberghe LP, Rodrigues C, Pandey A. New perspectives for citric acid production and application. Food Technology & Biotechnology. 2006;44(2).
8. Kwon-Chung KJ, Sugui JA. Aspergillus fumigatus—What Makes the Species a Ubiquitous Human Fungal Pathogen? PLoS Pathogens. 2013;9(12):e1003743.
9. Latgé J-P, Steinbach WJ. *Aspergillus fumigatus* and aspergillosis. Clinical Microbiology Reviews. 1999;12(2):310-50.
10. Tao L, Yu J-H. AbaA and WetA govern distinct stages of Aspergillus fumigatus development. Microbiology. 2011;157(2):313-26.
11. Yu J-H. Regulation of Development in Aspergillus nidulans and Aspergillus fumigatus. Mycobiology. 2010;38(4):229-37.

12. Lamarre C, Sokol S, Debeaupuis J-P, Henry C, Lacroix C, Glaser P, et al. Transcriptomic analysis of the exit from dormancy of Aspergillus fumigatus conidia. BMC Genomics. 2008;9(1):417.

13. Paris S, Debeaupuis J-P, Crameri R, Carey M, Charlès F, Prévost MC, et al. Conidial Hydrophobins of Aspergillus fumigatus. Applied and Environmental Microbiology. 2003;69(3):1581-8.

14. Dague E, Alsteens D, Latgé J-P, Dufrêne YF. High-Resolution Cell Surface Dynamics of Germinating Aspergillus fumigatus Conidia. Biophysical Journal. 2008;94(2):656-60.

15. Konzack S, Rischitor PE, Enke C, Fischer R. The Role of the Kinesin Motor KipA in Microtubule Organization and Polarized Growth of Aspergillus nidulans. Molecular Biology of the Cell. 2005;16(2):497-506.

16. Momany M, Taylor I. Landmarks in the early duplication cycles of Aspergillus fumigatus and Aspergillus nidulans: polarity, germ tube emergence and septation. Microbiology. 2000;146(12):3279-84.

17. Prosser JI, Tough AJ. Growth Mechanisms and Growth Kinetics of Filamentous Microorganisms. Critical Reviews in Biotechnology. 1991;10(4):253-74.

18. Park H-S, Yu J-H. Developmental regulators in Aspergillus fumigatus. Journal of Microbiology. 2016;54(3):223-31.

19. Sheppard DC, Doedt T, Chiang LY, Kim HS, Chen D, Nierman WC, et al. The Aspergillus fumigatus StuA Protein Governs the Up-Regulation of a Discrete Transcriptional Program during the Acquisition of Developmental Competence. Molecular Biology of the Cell. 2005;16(12):5866-79.

20. Alkhayyat F, Chang Kim S, Yu JH. Genetic control of asexual development in aspergillus fumigatus. Advances in applied microbiology. 2015;90:93-107.

21. O'Gorman CM, Fuller HT, Dyer PS. Discovery of a sexual cycle in the opportunistic fungal pathogen Aspergillus fumigatus. Nature. 2008;457:471.

22. Adams TH, Boylan MT, Timberlake WE. brlA is necessary and sufficient to direct conidiophore development in Aspergillus nidulans. Cell. 1988;54(3):353-62.

23. Aguirre J. Spatial and temporal controls of the Aspergillus brlA developmental regulatory gene. Molecular Microbiology. 1993;8(2):211-8.

24. Mah J-H, Yu J-H. Upstream and Downstream Regulation of Asexual Development in Aspergillus fumigatus. Eukaryotic Cell. 2006;5(10):1585-95.

25. Upadhyay S, Torres G, Lin X. Laccases Involved in 1,8-Dihydroxynaphthalene Melanin Biosynthesis in Aspergillus fumigatus Are Regulated by Developmental Factors and Copper Homeostasis. Eukaryotic Cell. 2013;12(12):1641-52.

26. Twumasi-Boateng K, Yu Y, Chen D, Gravelat FN, Nierman WC, Sheppard DC. Transcriptional Profiling Identifies a Role for BrlA in the Response to Nitrogen Depletion and for StuA in the Regulation of Secondary Metabolite Clusters in Aspergillus fumigatus. Eukaryotic Cell. 2009;8(1):104-15.

27. Chang YC, Timberlake WE. Identification of Aspergillus brlA response elements (BREs) by genetic selection in yeast. Genetics. 1993;133(1):29-38.

28. Ni M, Yu J-H. A Novel Regulator Couples Sporogenesis and Trehalose Biogenesis in Aspergillus nidulans. PLOS ONE. 2007;2(10):e970.

29. Fillinger S, Chaveroche M-K, van Dijck P, de Vries R, Ruijter G, Thevelein J, et al. Trehalose is required for the acquisition of tolerance to a variety of stresses in the filamentous fungus Aspergillus nidulans. Microbiology. 2001;147(7):1851-62.

30. Ahmed YL, Gerke J, Park H-S, Bayram Ö, Neumann P, Ni M, et al. The Velvet Family of Fungal Regulators Contains a DNA-Binding Domain Structurally Similar to NF-κB. PLOS Biology. 2014;11(12):e1001750.

31. Beyhan S, Gutierrez M, Voorhies M, Sil A. A Temperature-Responsive Network Links Cell Shape and Virulence Traits in a Primary Fungal Pathogen. PLOS Biology. 2013;11(7):e1001614.

32. Park H-S, Bayram Ö, Braus GH, Kim SC, Yu J-H. Characterization of the velvet regulators in Aspergillus fumigatus. Molecular Microbiology. 2012;86(4):937-53.

33. Dhingra S, Andes D, Calvo AM. VeA Regulates Conidiation, Gliotoxin Production, and Protease Activity in the Opportunistic Human Pathogen Aspergillus fumigatus. Eukaryotic Cell. 2012;11(12):1531-43.

34. Kwon N-J, Shin K-S, Yu J-H. Characterization of the developmental regulator FlbE in Aspergillus fumigatus and Aspergillus nidulans. Fungal Genetics and Biology. 2010;47(12):981-93.

35. Xiao P, Shin K-S, Wang T, Yu J-H. Aspergillus fumigatus flbB Encodes Two Basic Leucine Zipper Domain (bZIP) Proteins Required for Proper Asexual Development and Gliotoxin Production. Eukaryotic Cell. 2010;9(11):1711-23.

36. Garzia A, Etxebeste O, Herrero-Garcia E, Fischer R, Espeso EA, Ugalde U. Aspergillus nidulans FlbE is an upstream developmental activator of conidiation functionally associated with the putative transcription factor FlbB. Molecular Microbiology. 2009;71(1):172-84.

37. Neves SR, Ram PT, Iyengar R. G Protein Pathways. Science. 2002;296(5573):1636-9.

38. Liebmann B, Gattung S, Jahn B, Brakhage AA. cAMP signaling in Aspergillus fumigatus is involved in the regulation of the virulence gene pksP and in defense against killing by macrophages. Molecular Genetics and Genomics. 2003;269(3):420-35.

39. Tsai H-F, Chang YC, Washburn RG, Wheeler MH, Kwon-Chung KJ. The Developmentally Regulated *alb1* Gene of *Aspergillus fumigatus*: Its Role in Modulation of Conidial Morphology and Virulence. Journal of Bacteriology. 1998;180(12):3031-8.

40. Shin K-S, Kwon N-J, Yu J-H. Gβγ-mediated growth and developmental control in Aspergillus fumigatus. Current Genetics. 2009;55(6):631.

41. Lin C-J, Sasse C, Gerke J, Valerius O, Irmer H, Frauendorf H, et al. Transcription Factor SomA Is Required for Adhesion, Development and Virulence of the Human Pathogen Aspergillus fumigatus. PLOS Pathogens. 2015;11(11):e1005205.

42. Gravelat FN, Ejzykowicz DE, Chiang LY, Chabot JC, Urb M, Macdonald KD, et al. Aspergillus fumigatus MedA governs adherence, host cell interactions and virulence. Cellular Microbiology. 2010;12(4):473-88.

43. Balloy V, Chignard M. The innate immune response to Aspergillus fumigatus. Microbes and Infection. 2009;11(12):919-27.

44. Camargo JF, Husain S. Immune Correlates of Protection in Human Invasive Aspergillosis. Clinical Infectious Diseases. 2014;59(4):569-77.

45. Kousha M, Tadi R, Soubani AO. Pulmonary aspergillosis: a clinical review. European Respiratory Review. 2011;20(121):156-74.

46. Lee HJ, Lee JH, Hwang BY, Kim HS, Lee JJ. Anti-angiogenic activities of gliotoxin and its methylthio-derivative, fungal metabolites. Archives of Pharmacal Research. 2001;24(5):397.

47. Ben-Ami R. Angiogenesis at the mold-host interface: a potential key to understanding and treating invasive aspergillosis. Future Microbiology. 2013 2013/11//:1453+.

48. Sin N, Meng L, Wang MQW, Wen JJ, Bornmann WG, Crews CM. The anti-angiogenic agent fumagillin covalently binds and inhibits the methionine aminopeptidase, MetAP-2. Proceedings of the National Academy of Sciences. 1997;94(12):6099-103.

49. Bok JW, Balajee SA, Marr KA, Andes D, Nielsen KF, Frisvad JC, et al. LaeA, a Regulator of Morphogenetic Fungal Virulence Factors. Eukaryotic Cell. 2005;4(9):1574-82.

50. Spikes S, Xu R, Nguyen CK, Chamilos G, Kontoyiannis DP, Jacobson RH, et al. Gliotoxin Production in Aspergillus fumigatus Contributes to Host-Specific Differences in Virulence. The Journal of Infectious Diseases. 2008;197(3):479-86.

51. Prystowsky SD, Vogelstein B, Ettinger DS, Merz WG, Kaizer H, Sulica VI, et al. Invasive aspergillosis. New England Journal of Medicine. 1976;295(12):655-8.

52. Young RC, Bennett JE, Vogel CL, Carbone PP, Devita VT. The spectrum of the disease in 98 patients. Medicine. 1970;49(2):147-73.

53. David WD. Invasive Aspergillosis. Clinical Infectious Diseases. 1998;26(4):781-803.

54. Brown GD, Denning DW, Gow NAR, Levitz SM, Netea MG, White TC. Hidden Killers: Human Fungal Infections. Science Translational Medicine. 2012;4(165):165rv13-rv13.

55. Feldmesser M. Role of Neutrophils in Invasive Aspergillosis. Infection and Immunity. 2006;74(12):6514-6.

56. Pagano L, Valentini CG, Fianchi L, Caira M. The role of neutrophils in the development and outcome of zygomycosis in haematological patients. Clinical Microbiology and Infection. 2009;15:33-6.

57. Dagenais TRT, Keller NP. Pathogenesis of *Aspergillus fumigatus* in Invasive Aspergillosis. Clinical Microbiology Reviews. 2009;22(3):447-65.

58. Kosmidis C, Denning DW. The clinical spectrum of pulmonary aspergillosis. Thorax. 2015;70(3):270-7.

59. Gerson SL, Talbot GH, Hurwitz S, Strom BL, Lusk EJ, Cassileth PA. Prolonged granulocytopenia: The major risk factor for invasive pulmonary aspergillosis in patients with acute leukemia. Annals of Internal Medicine. 1984;100(3):345-51.

60. Segal BH, Barnhart LA, Anderson VL, Walsh TJ, Malech HL, Holland SM. Posaconazole as Salvage Therapy in Patients with Chronic Granulomatous Disease and Invasive Filamentous Fungal Infection. Clinical Infectious Diseases. 2005;40(11):1684-8.

61. Allison TL. Immunosuppressive Therapy in Transplantation. Nursing Clinics of North America. 2016;51(1):107-20.

62. Lionakis MS, Kontoyiannis DP. Glucocorticoids and invasive fungal infections. The Lancet. 2003;362(9398):1828-38.

63. Marr KA, Carter RA, Boeckh M, Martin P, Corey L. Invasive aspergillosis in allogeneic stem cell transplant recipients: changes in epidemiology and risk factors. Blood. 2002;100(13):4358-66.

64. Segal BH, Walsh TJ. Current approaches to diagnosis and treatment of invasive aspergillosis. American journal of respiratory and critical care medicine. 2006;173(7):707-17.

65. Baddley JW, Stephens JM, Ji X, Gao X, Schlamm HT, Tarallo M. Aspergillosis in Intensive Care Unit (ICU) patients: epidemiology and economic outcomes. BMC Infectious Diseases. 2013;13(1):29.

66. Parcell BJ, B C Raju PK, Johnson EM, Fardon TC, Olver WJ. Invasive pulmonary aspergillosis post extracorporeal membrane oxygenation support and literature review. Medical Mycology Case Reports. 2014;4:12-5.

67. Wauters J, Baar I, Meersseman P, Meersseman W, Dams K, De Paep R, et al. Invasive pulmonary aspergillosis is a frequent complication of critically ill H1N1 patients: a retrospective study. Intensive Care Medicine. 2012;38(11):1761-8.

68. Chiang LY, Sheppard DC, Gravelat FN, Patterson TF, Filler SG. Aspergillus fumigatus stimulates leukocyte adhesion molecules and cytokine production by endothelial cells in vitro and during invasive pulmonary disease. Infection and immunity. 2008;76(8):3429-38.

69. Stergiopoulou T, Meletiadis J, Roilides E, Kleiner DE, Schaufele R, Roden M, et al. Host-dependent patterns of tissue injury in invasive pulmonary aspergillosis. American journal of clinical pathology. 2007;127(3):349-55.

70. Denning DW. Echinocandin antifungal drugs. The Lancet. 2003;362(9390):1142-51.

71. Chowdhary A, Sharma C, Meis JF. Azole-Resistant Aspergillosis: Epidemiology, Molecular Mechanisms, and Treatment. The Journal of Infectious Diseases. 2017;216(suppl_3):S436-S44.

72. Denning DW, Tucker RM, Hansen LH, Stevens DA. Treatment of invasive aspergillosis with itraconazole. The American journal of medicine. 1989;86(6):791-800.

73. Hof H. A new, broad-spectrum azole antifungal: posaconazole–mechanisms of action and resistance, spectrum of activity. Mycoses. 2006;49:2-6.

74. Miceli MH, Kauffman CA. Isavuconazole: a new broad-spectrum triazole antifungal agent. Clinical Infectious Diseases. 2015;61(10):1558-65.

75. Da Silva Ferreira M, Colombo A, Paulsen I, Ren Q, Wortman J, Huang J, et al. The ergosterol biosynthesis pathway, transporter genes, and azole resistance in Aspergillus fumigatus. Medical Mycology. 2005;43(Supplement_1):S313-S9.

76. Odds FC, Brown AJ, Gow NA. Antifungal agents: mechanisms of action. Trends in microbiology. 2003;11(6):272-9.

77. Denning DW, Venkateswarlu K, Oakley KL, Anderson M, Manning N, Stevens DA, et al. Itraconazole resistance in Aspergillus fumigatus. Antimicrobial agents and chemotherapy. 1997;41(6):1364-8.

78. Verweij PE, Mellado E, Melchers WJ. Multiple-triazole–resistant aspergillosis. New England Journal of Medicine. 2007;356(14):1481-3.

79. Verweij PE, Snelders E, Kema GHJ, Mellado E, Melchers WJG. Azole resistance in Aspergillus fumigatus: a side-effect of environmental fungicide use? The Lancet Infectious Diseases. 2009;9(12):789-95.

80. Kanafani ZA, Perfect JR. Resistance to Antifungal Agents: Mechanisms and Clinical Impact. Clinical Infectious Diseases. 2008;46(1):120-8.

81. Messer SA, Jones RN, Fritsche TR. International surveillance of Candida spp. and Aspergillus spp.: report from the SENTRY Antimicrobial Surveillance Program (2003). Journal of clinical microbiology. 2006;44(5):1782-7.

82. Sabatelli F, Patel R, Mann P, Mendrick C, Norris C, Hare R, et al. In vitro activities of posaconazole, fluconazole, itraconazole, voriconazole, and amphotericin B against a large collection of clinically important molds and yeasts. Antimicrobial Agents and Chemotherapy. 2006;50(6):2009-15.

83. Herbrecht R, Denning DW, Patterson TF, Bennett JE, Greene RE, Oestmann J-W, et al. Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. New England Journal of Medicine. 2002;347(6):408-15.

84. Arendrup MC, Cuenca-Estrella M, Lass-Flörl C, Hope WW. EUCAST Technical Note on Aspergillus and amphotericin B, itraconazole, and posaconazole. Clinical Microbiology and Infection. 2012;18(7):E248-E50.

85. Katiyar S, Edlind T. Echinocandins: Resistance mechanisms. Antifungals. 2015:55.

86. Verwer PEB, van Duijn ML, Tavakol M, Bakker-Woudenberg IAJM, van de Sande WWJ. Reshuffling of Aspergillus fumigatus cell wall components chitin and β-glucan under the influence of caspofungin or nikkomycin Z alone or in combination. Antimicrobial agents and chemotherapy. 2012;56(3):1595-8.

87. Arathoon EG. Clinical efficacy of echinocandin antifungals. Current Opinion in Infectious Diseases. 2001;14(6):685.

88. Perlin DS. Resistance to echinocandin-class antifungal drugs. Drug Resistance Updates. 2007;10(3):121-30.

89. Wiederhold NP, Lewis RE. The echinocandin antifungals: an overview of the pharmacology, spectrum and clinical efficacy. Expert opinion on investigational drugs. 2003;12(8):1313-33.

90. Denning DW. Therapeutic Outcome in Invasive Aspergillosis. Clinical Infectious Diseases. 1996;23(3):608-15.

91. Vallabhaneni S, Mody RK, Walker T, Chiller T. The Global Burden of Fungal Diseases. Infectious Disease Clinics of North America. 2016;30(1):1-11.

92. Warnock DW. Trends in the epidemiology of invasive fungal infections. Nippon Ishinkin Gakkai Zasshi. 2007;48(1):1-12.

93. Baddley JW. Clinical risk factors for invasive aspergillosis. Medical Mycology. 2011;49(Supplement_1):S7-S12.

94. Helmschrott C, Sasse A, Samantaray S, Krappmann S, Wagener J. Upgrading Fungal Gene Expression on Demand: Improved Systems for Doxycycline-Dependent Silencing in Aspergillus fumigatus. Applied and Environmental Microbiology. 2013;79(5):1751-4.

95. Vogt K, Bhabhra R, Rhodes JC, Askew DS. Doxycycline-regulated gene expression in the opportunistic fungal pathogen Aspergillus fumigatus. BMC microbiology. 2005;5.

96. Helmschrott C, Sasse A, Samantaray S, Krappmann S, Wagener J. Upgrading fungal gene expression on demand: improved systems for doxycycline-dependent silencing in Aspergillus fumigatus. Appl Environ Microbiol. 2013;79(5):1751-4.

97. Gravelat FN, Beauvais A, Liu H, Lee MJ, Snarr BD, Chen D, et al. Aspergillus galactosaminogalactan mediates adherence to host constituents and conceals hyphal β-glucan from the immune system. PLoS pathogens. 2013;9(8):e1003575.

98. Lee MJ, Geller AM, Bamford NC, Liu H, Gravelat FN, Snarr BD, et al. Deacetylation of fungal exopolysaccharide mediates adhesion and biofilm formation. MBio. 2016;7(2):e00252-16.

99. Bossche HV, Koymans L. Review Article Cytochromes P450 in fungi: Cytochrome P450 bei Pilzen. Mycoses. 1998;41:32-8.

100. Hatefi Y. The mitochondrial electron transport and oxidative phosphorylation system. Annual review of biochemistry. 1985;54(1):1015-69.

101. Joseph-Horne T, Hollomon DW, Wood PM. Fungal respiration: a fusion of standard and alternative components. Biochimica et Biophysica Acta (BBA) - Bioenergetics. 2001;1504(2):179-95.

102. Kathiresan M, Martins D, English AM. Respiration triggers heme transfer from cytochrome c peroxidase to catalase in yeast mitochondria. Proceedings of the National Academy of Sciences of the United States of America. 2014;111(49):17468-73.

103. Chung D, Barker BM, Carey CC, Merriman B, Werner ER, Lechner BE, et al. ChIP-seq and in vivo transcriptome analyses of the Aspergillus fumigatus SREBP SrbA reveals a new regulator of the fungal hypoxia response and virulence. PLoS pathogens. 2014;10(11):e1004487.

104. Kroll K, Pähtz V, Hillmann F, Vaknin Y, Schmidt-Heck W, Roth M, et al. Identification of hypoxia-inducible target genes of Aspergillus fumigatus by transcriptome analysis reveals cellular respiration as important contributor to hypoxic survival. Eukaryotic cell. 2014:EC. 00084-14.

105. Shimizu M, Fujii T, Masuo S, Fujita K, Takaya N. Proteomic analysis of Aspergillus nidulans cultured under hypoxic conditions. Proteomics. 2009;9(1):7-19.

106. Grahl N, Puttikamonkul S, Macdonald JM, Gamcsik MP, Ngo LY, Hohl TM, et al. In vivo Hypoxia and a Fungal Alcohol Dehydrogenase Influence the Pathogenesis of Invasive Pulmonary Aspergillosis. PLOS Pathogens. 2011;7(7):e1002145.

107. Willger SD, Puttikamonkul S, Kim K-H, Burritt JB, Grahl N, Metzler LJ, et al. A sterol-regulatory element binding protein is required for cell polarity, hypoxia adaptation, azole drug resistance, and virulence in Aspergillus fumigatus. PLoS pathogens. 2008;4(11):e1000200.

108. Bonomo SR, Davidson JD, Tyrone JW, Lin X, Mustoe TA. Enhancement of wound healing by hyperbaric oxygen and transforming growth factor β3 in a new chronic wound model in aged rabbits. Archives of surgery. 2000;135(10):1148-53.

109. Tibbles PM, Edelsberg JS. Hyperbaric-oxygen therapy. New England Journal of Medicine. 1996;334(25):1642-8.

110. Dhingra S, Buckey JC, Cramer RA. Hyperbaric Oxygen Reduces *Aspergillus fumigatus* Proliferation *In Vitro* and Influences *In Vivo* Disease Outcomes. Antimicrobial Agents and Chemotherapy. 2018;62(3):e01953-17.

111. Ben-Ami R, Varga V, Lewis RE, May GS, Nierman WC, Kontoyiannis DP. Characterization of a 5-azacytidine-induced developmental Aspergillus fumigatus variant. Virulence. 2010;1(3):164-73.

112. Sharma M, Manoharlal R, Puri N, Prasad R. Antifungal curcumin induces reactive oxygen species and triggers an early apoptosis but prevents hyphae development by targeting the global repressor TUP1 in Candida albicans. Bioscience reports. 2010;30(6):391-404.

113. Braun BR, Johnson AD. Control of filament formation in Candida albicans by the transcriptional repressor TUP1. Science. 1997;277(5322):105-9.

114. Khan MSA, Ahmad I. In vitro antifungal, anti-elastase and anti-keratinase activity of essential oils of Cinnamomum-, Syzygium-and Cymbopogon-species against Aspergillus fumigatus and Trichophyton rubrum. Phytomedicine. 2011;19(1):48-55.

115. Lv C, Wang P, Ma L, Zheng M, Liu Y, Xing F. Large-scale comparative analysis of eugenol-induced/repressed genes expression in Aspergillus flavus using RNA-seq. Frontiers in microbiology. 2018;9.