

# Characterization of a 5-azacytidine-induced developmental *Aspergillus fumigatus* variant

Ronen Ben-Ami,<sup>1</sup> John Varga,<sup>3</sup> Russell E. Lewis,<sup>4</sup> Gregory S. May,<sup>2</sup> William C. Nierman<sup>3,5</sup> and Dimitrios P. Kontoyiannis<sup>1,4,\*</sup>

<sup>1</sup>Department of Infectious Diseases and <sup>2</sup>Department of Pathology; Infection Control and Employee Health; The University of Texas MD Anderson Cancer Center; Houston, TX USA; <sup>3</sup>J. Craig Venter Institute; Rockville, MD USA; <sup>4</sup>College of Pharmacy; University of Houston; Houston, TX USA; <sup>5</sup>Department of Biochemistry and Molecular Biology; George Washington University School of Medicine; Washington, DC USA

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The hypomethylating agent 5-azacytidine (5AC) is widely used in patients at risk of invasive mycoses. We sought to determine whether 5AC affects the developmental competence and virulence of *Aspergillus fumigatus*. Incubation of *A. fumigatus* strain 293 with 5AC induced high-frequency conversion to a fluffy-variant (Af293<sup>FL</sup>). The conidiation defect was bypassed by exposing Af293<sup>FL</sup> to light during the initial 18 hours of growth on solid media. Transcriptional profiling revealed differential expression of multiple genes involved in G-protein signaling, including a putative G-protein coupled photoreceptor (opsin), suggesting that impaired signaling through a light-responsive pathway upstream of *brlA* is responsible for this phenotype. Af293<sup>FL</sup> was fully virulent in fruit fly and murine models of invasive aspergillosis. Moreover, Af293<sup>FL</sup> overexpressed aspergillopepsin F, had increased elastase activity and was more angioinvasive than the parental wild-type strain. The 5AC-induced *A. fumigatus* fluffy variant illustrates the potential effects of chemotherapeutic agents on the developmental and pathobiologic characteristics of opportunistic fungi.

## Introduction

Asexual reproduction in *Aspergillus* species requires a transition from multicellular, highly polarized structures (vegetative hyphae) to unicellular budding spores (conidia). This phenotypic switch is coordinated by a limited set of regulatory genes, which in turn activate hundreds of genes involved in morphogenesis. The principal regulatory genes were first characterized in the model fungus *A. nidulans*. *Bristle* (*brlA*) encodes a transcription factor that induces expression of the developmental regulatory genes *Abacus* (*abaA*) and *wet-white conidia* (*wetA*). Loss-of-function of *brlA* results in the formation of defective conidiophore-like structures (bristles) that fail to produce the specialized cell types required for conidiation.

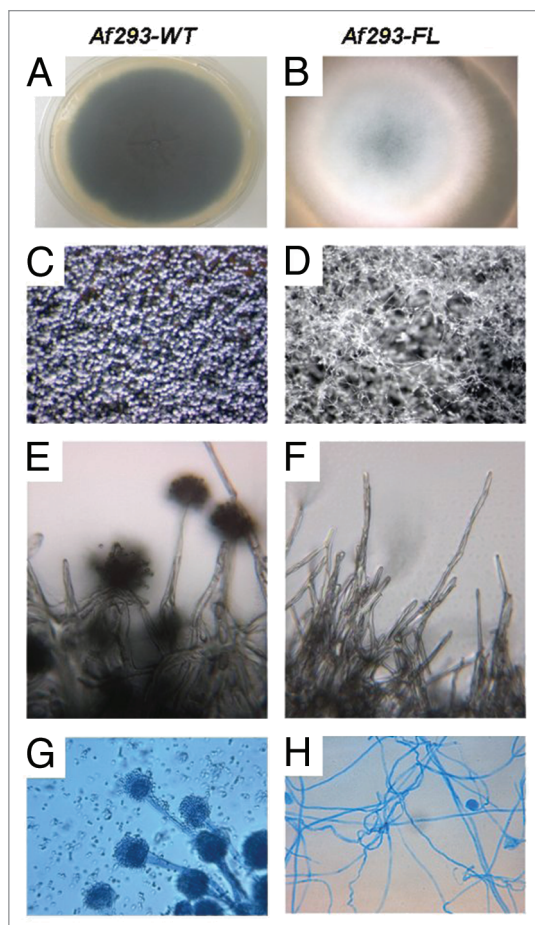
Twenty-five years ago, Tamame et al.<sup>1,2</sup> demonstrated that 5-azacytidine (5AC), a cytidine analog with DNA hypomethylating and mutagenic properties, can induce high-frequency conversion of *A. nidulans* and *A. niger* to a stable “fluffy” phenotypic variant that is characterized by severely impaired asexual sporulation and the formation of abundant vegetative hyphae. The authors further noted that fluffy variant colonies lacked contact inhibition and grew uncontrollably over other colonies. Complementation studies revealed that all 5AC-induced variants were defective in the same gene, leading the authors to postulate that a developmental gene is specifically targeted by 5AC at an early time point in development.<sup>1</sup>

Unlike *A. nidulans*, *A. fumigatus* is a common opportunistic pathogen that is associated with morbidity and mortality in immunocompromised patients, such as those with hematologic malignancies.<sup>3</sup> Given the increasing use of hypomethylating agents in these patients,<sup>4</sup> we sought to determine whether 5AC induces a developmental variant of *A. fumigatus* and whether this phenotype is associated with altered tissue invasiveness and pathogenicity. 5AC induced high-frequency conversion of *A. fumigatus* to a developmental mutant with impaired light-dependent conidiation. Whole-genome gene expression studies revealed early differential expression of an opsin-encoding gene and other genes with heterotrimeric G-protein regulatory functions [*flbA* (fluffy low *brlA*) and arrestin], followed by under-expression of *brlA*, suggesting that 5AC targets a light-sensing developmental pathway upstream of *brlA*. 5AC-induced *A. fumigatus* variants exhibited increased elastase activity and were fully pathogenic in fruit fly and murine model systems.

## Results

5AC induces high-frequency conversion of *A. fumigatus* to the fluffy phenotype (Af293<sup>FL</sup>). Exposure of submerged *A. fumigatus* cultures to 250 mM or 500 mM 5AC induced the fluffy phenotype in ~10% of colonies. Af293<sup>FL</sup> was characterized by the complete absence of conidiation when incubated in the dark (Fig. 1). As previously described for 5AC-induced fluffy

\*Correspondence to: Dimitrios P. Kontoyiannis; Email: dkontoyi@mdanderson.org  
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**Figure 1.** Morphologic characteristics of Af293<sup>FL</sup>. Af293<sup>FL</sup> (B, D, F and H) was compared with its parental wild-type strain (A, C, E and G). It demonstrated a lack of conidiophore formation and extensive proliferation of undifferentiated hyphae after 72 hours of incubation in the dark. Macroscopic colony appearance is shown in (A and B); appearance on dissecting microscopy (x40) is shown in (C and D); colony surface by light microscopy (x250) is shown in (E and F); and light microscopy (x250) of lactophenol cotton blue-stained tease slides is shown in (G and H).

*A. niger* and *A. nidulans* variants,<sup>2</sup> Af293<sup>FL</sup> formed extensive aerial hyphae that rose high above the surface of the solid medium. On microscopic examination, these aerial hyphae appeared as conidiophore-like structures that failed to differentiate into conidia-forming vesicles and grew indeterminately, a phenotype consistent with *brlA* silencing.<sup>16</sup> Growth in proximity to a wild-type Af293 colony did not induce conversion of Af293<sup>FL</sup> to the wild-type phenotype, indicating that the fluffy variant does not result from deficiency of a diffusible factor such as *FluG*.<sup>17</sup> Af293<sup>FL</sup> was similar to its parental wild-type strain with respect to its radial growth rate and susceptibility to antifungal drugs and the resistance of its conidia and hyphae to oxidative damage (data not shown).

Af293<sup>FL</sup> is a stable phenotype characterized by impaired light-dependent conidiation. Repeated passages (>20) of Af293<sup>FL</sup> on solid medium did not abrogate the conidiation defect, indicating that this is a stable phenotype. Exposure of Af293<sup>FL</sup> cultures

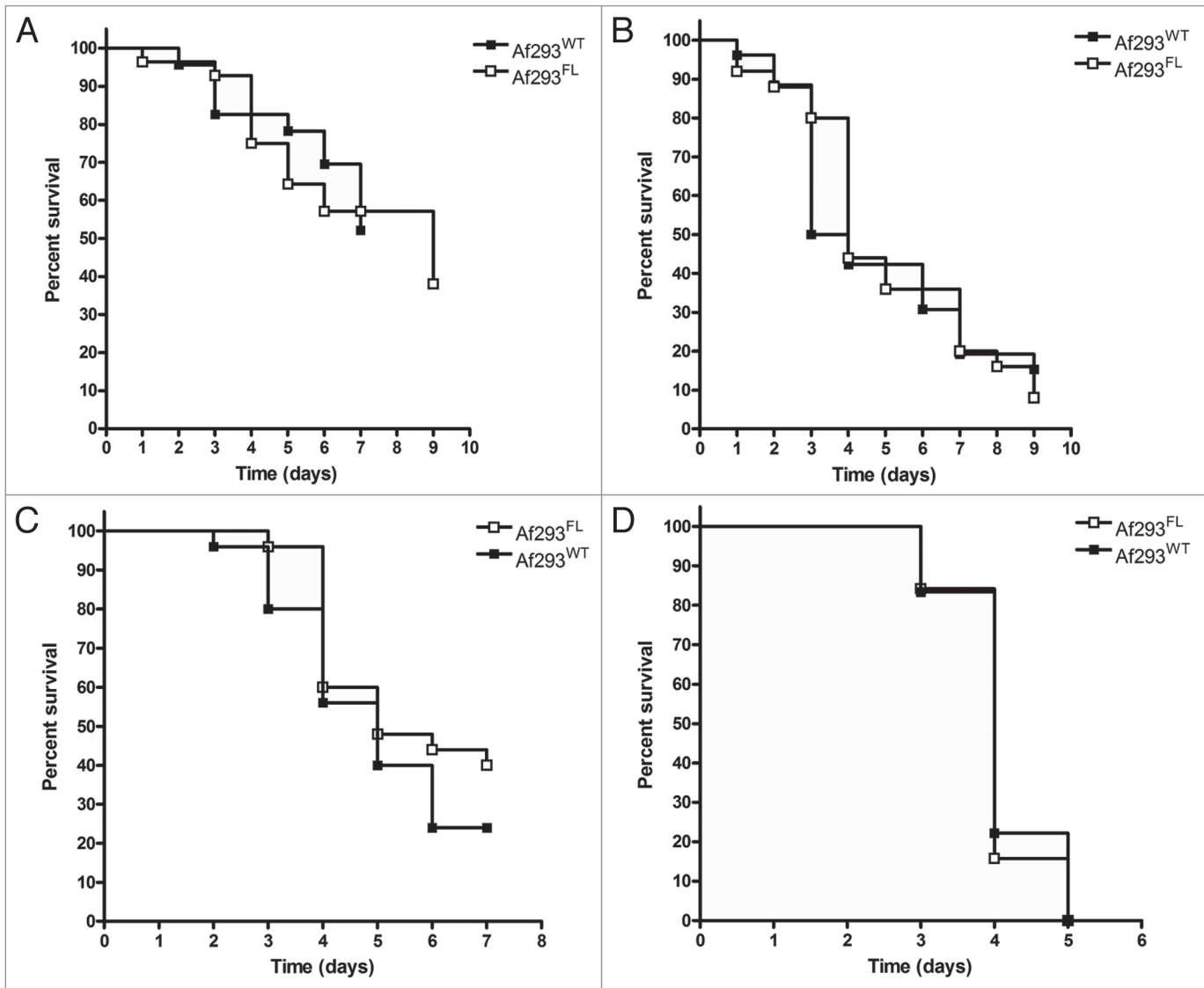
to light during the initial 18 hours of incubation induced conidiation equivalent to that of the wild-type strain. However, Af293<sup>FL</sup> cultures incubated in the dark for >18 hours failed to conidiate when transferred to light-exposed conditions, suggesting that light exposure is required during a critical timeframe before the acquisition of developmental competence. Light dependent conidiation was observed in all fluffy colonies tested (>10). Conidia collected from these strains invariably produced colonies exhibiting the fluffy phenotype.

Af293<sup>FL</sup> has preserved virulence in fly and murine models of aspergillosis. Inoculation with Af293<sup>FL</sup> conidia produced rapidly fatal infection in *Tl<sup>-/-</sup>* flies and neutropenic and non-neutropenic BALB/c mice. The survival rates of Af293<sup>FL</sup>-infected animals did not significantly differ from those of Af293<sup>WT</sup>-infected animals in any of the models evaluated (Fig. 2). In addition, pulmonary fungal burden was similar for Af293<sup>WT</sup> and Af293<sup>FL</sup>-infected neutropenic and non-neutropenic mice (median burden, 2.46 x 10<sup>6</sup> versus 1.64 x 10<sup>6</sup> conidial equivalents respectively; *p* = 0.72).

Af293<sup>FL</sup> exhibits increased elastase activity. We measured the elastase activity of five independent Af293<sup>FL</sup> strains and compared it with that of Af293<sup>WT</sup>. Af293<sup>FL</sup> strains exhibited significantly higher elastase activity on elastin-Congo-red medium than did the parental wild-type strain. The mean ( $\pm$ standard error of the mean) elastase activity indices were 0.72  $\pm$  0.04 for Af293<sup>FL</sup> and 0.53  $\pm$  0.02 for Af293<sup>WT</sup> (*p* = 0.002; Fig. 3A). Moreover, an examination of EVG-stained lung sections from BALB/c mice infected with Af293<sup>FL</sup> revealed extensive angiogenesis and disruption of elastin fibers in the walls of invaded blood vessels (Fig. 3B). Angiogenesis was observed in 19 of 104 blood vessels (18.2%) in Af293<sup>FL</sup>-infected lungs versus 5 of 84 blood vessels (5.9%) in Af293<sup>WT</sup>-infected lungs (odds ratio, 3.5; *p* = 0.01 by Fisher's exact test).

Af293<sup>FL</sup> differentially expresses opsin and G-protein regulatory genes. Forty-five genes were differentially expressed by a factor of 1.5 or more during submerged growth in Af293<sup>FL</sup> compared with the parental wild-type strain (Fig. 4A, Table 1). Hierarchical clustering revealed four distinct clusters among these 45 genes. The largest of these four groups included 24 genes that were underexpressed in Af293<sup>FL</sup> during precompetent growth. This cluster contained developmentally regulated genes such as heat-shock protein (HSP) Awh11,<sup>18</sup> HSP 9/12,<sup>18</sup> and NAD-dependent formate dehydrogenase AciA/Fdh.<sup>19</sup> At the 30-hour time point, Af293<sup>FL</sup> underexpressed the developmentally regulated genes *esdC*, *rodA* and *rodB* but overexpressed genes encoding for cell wall-associated proteins AspF13, IgE-binding protein, and galactomannoprotein Mp2 as well as aspergillopepsin F, a secreted elastinolytic aspartic protease.<sup>20</sup>

Interestingly, two genes that are homologous to members of the rhodopsin light-responsive complex were differentially expressed in Af293<sup>FL</sup> in the precompetent phase. AFUA\_7G01430, which encodes a predicted seven-helix transmembrane opsin-related protein,<sup>21</sup> was underexpressed in Af293<sup>FL</sup>. The arrestin gene homolog AFUA\_6G13380, which encodes an opsin regulator in vertebrates that blocks signal transduction by preventing the interaction between cytoplasmic domains and heterotrimeric G-proteins, was upregulated in Af293<sup>FL</sup>. In addition, the



**Figure 2.** Assessment of Af293<sup>FL</sup> virulence in fly and mouse models of invasive aspergillosis. The virulence of Af293<sup>FL</sup> relative to that of its parental wild-type strain was assessed in Toll-deficient flies using an inoculum suspension of 10<sup>6</sup> conidia/ml (A) or 10<sup>7</sup> conidia/ml (B), and in BALB/c mice immunosuppressed with a neutropenia-inducing drug regimen (cyclophosphamide plus cortisone acetate) (C) or a non-neutropenic regimen (cortisone acetate) (D). In each model system, survival rates were similar between animals infected with Af293<sup>FL</sup> and those infected with Af293<sup>WT</sup> (p = NS by the log-rank test).

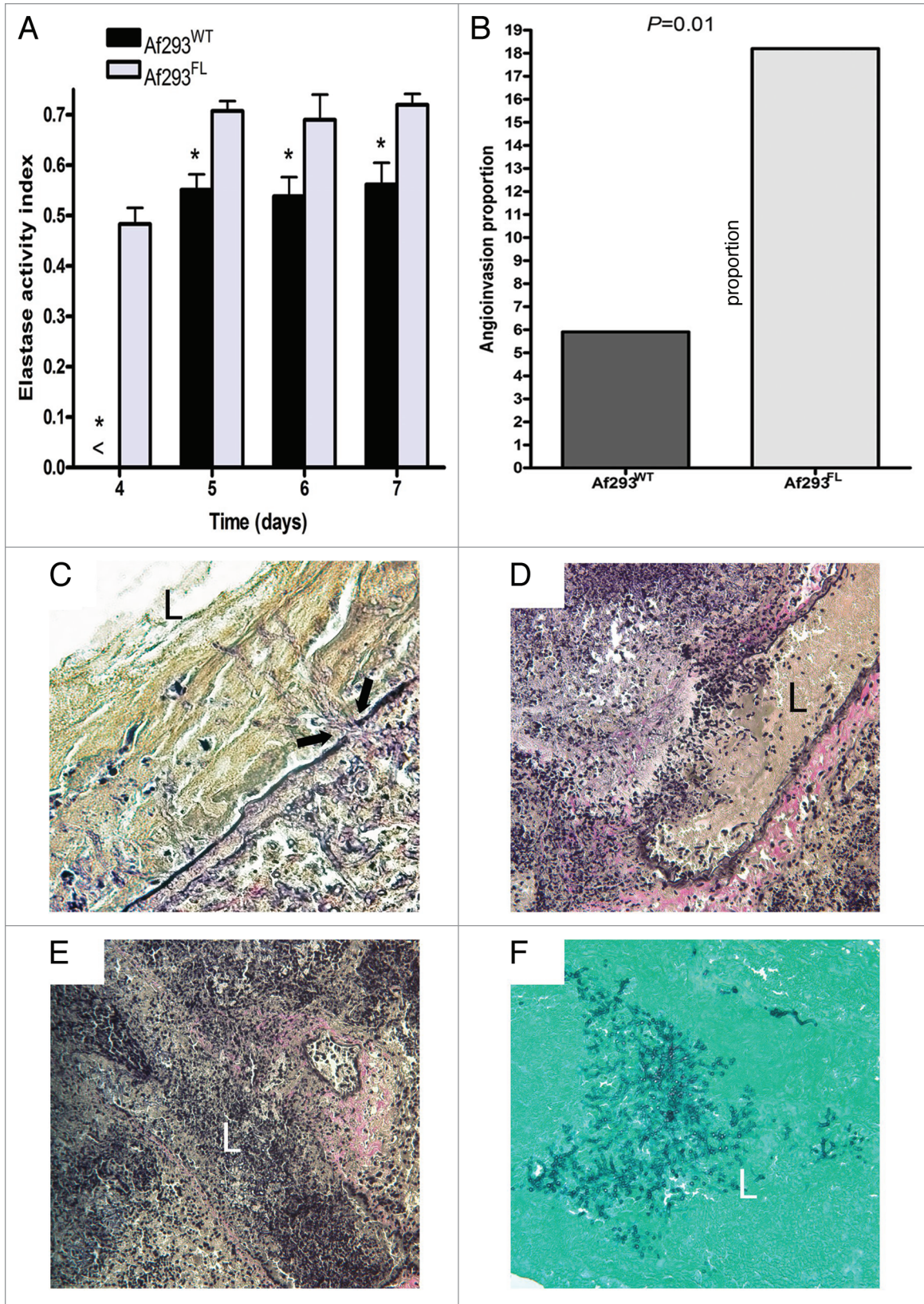
*flbA* gene (AFUA\_2G11180), which encodes a regulator of G-protein signaling (RGS) protein that activates conidiation, was concurrently upregulated in Af293<sup>FL</sup>. *brlA*, whose expression is positively regulated by *flbA*,<sup>22</sup> was underexpressed by Af293<sup>FL</sup> at the 30-hour time point. The significant differential expression of *opsin*, *arrestin*, *brlA*, aspergillopepsin F, *rodA* and *rodB* genes in Af293<sup>FL</sup> was confirmed with the use of RT-qPCR analysis (Fig. 4B).

### Discussion

In this study, we determined the phenotypic characteristics and differential gene expression patterns of 5AC-induced *A. fumigatus* developmental mutants (Af293<sup>FL</sup>). A striking feature of Af293<sup>FL</sup>

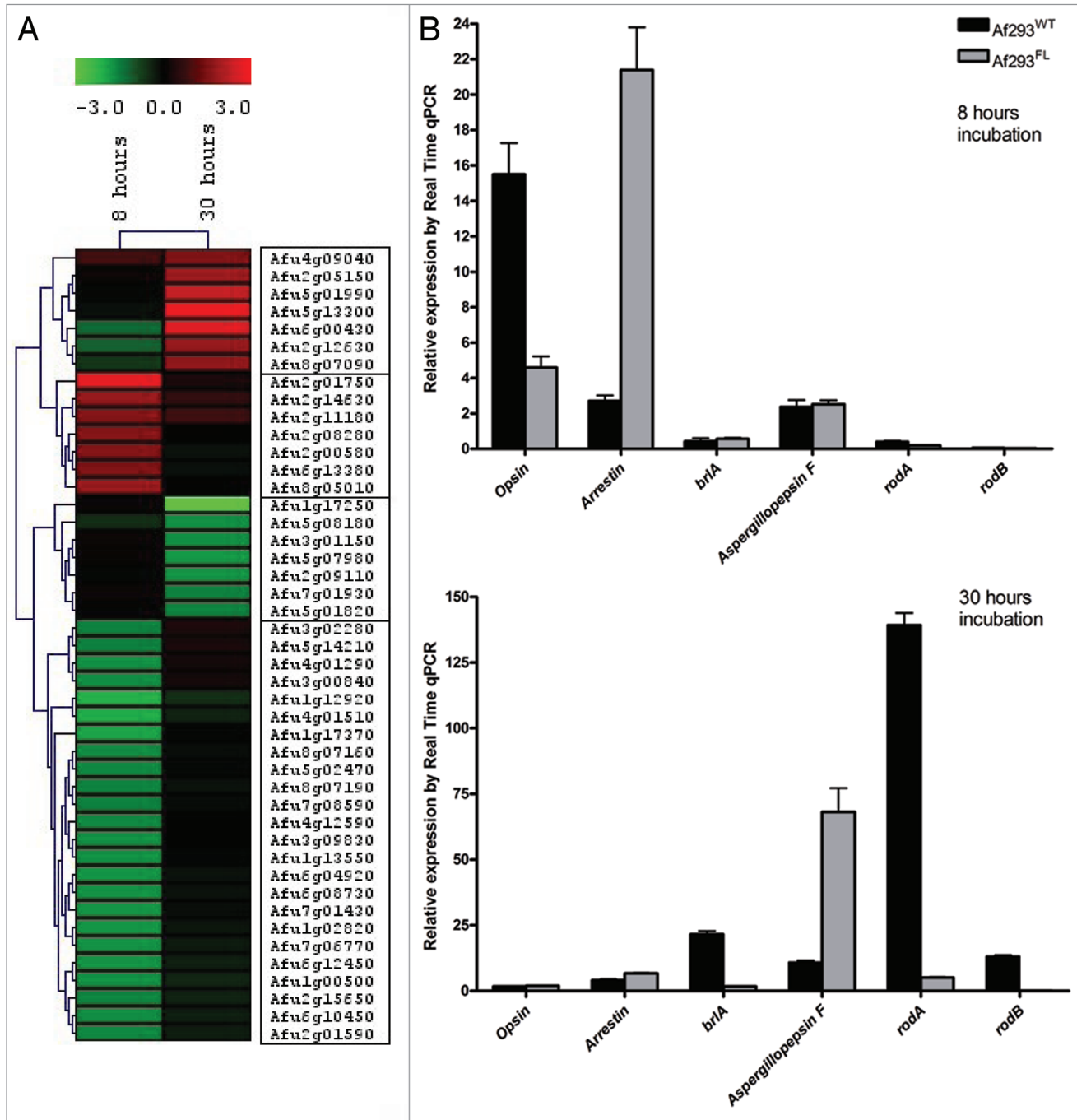
is its requirement for light exposure to achieve developmental competence, indicating a defect in a light-responsive signaling pathway. Af293<sup>FL</sup> was fully virulent in two disparate model host systems; moreover, this strain overexpressed the elastolytic protease aspergillopepsin F, a putative virulence factor,<sup>20</sup> and displayed enhanced elastase activity and angioinvasiveness.

In the 5AC-induced *A. fumigatus* strain Af293<sup>FL</sup>, we observed early differential expression of multiple genes encoding for proteins with known or predicted roles in G-protein signaling, including a putative G-protein-coupled photoreceptor (*opsin*), *arrestin*, *flbA*, and at a later time point, *brlA* and *esdC*, both developmentally important genes regulated by G-protein signaling. This pattern of gene expression suggests that altered G-protein signaling is responsible for the Af293<sup>FL</sup> phenotype.



**Figure 3.** For figure legend, see page 168.

**Figure 3. (See previous page).** Elastase activity and angioinvasiveness. (A) The elastase activity indices (EAI) of Af293<sup>FL</sup> and its parental wild-type strain were evaluated on elastin-Congo red agar according to the method described by Blanco et al.<sup>11</sup> Values represent the means of experiments with five different Af293<sup>FL</sup> strains. No elastase activity was detected prior to day 4. \*p < 0.01. (B) The proportion of blood vessels invaded by fungal hyphae was evaluated in tissue sections from mouse lungs stained with the EVG stain. Proportions were compared with Fisher's exact test. (C–E) Representative tissue sections from Af293<sup>FL</sup>-infected lungs: (C) penetration of the vascular lumen by hyphae is associated with thinning and disruption of elastin fibers (arrows mark site of penetration) (EVG x400 magnification), (D) complete disruption of vessel wall by a penetrating fungal mass (EVG x200), (E) vessel lumen packed with proliferating hyphae (EVG, x200), (F) same vessel as (E) observed in a GMS-stained specimen (x200). L, vascular lumen.



**Figure 4.** Whole-genome expression studies in Af293<sup>FL</sup>. Transcriptional analysis of Af293<sup>FL</sup> using cDNA microarrays revealed differential expression of four gene clusters (A); the expression of selected genes was confirmed by RT-PCR (B). After 8 hours of growth in submerged culture, Af293<sup>FL</sup> underexpressed the gene encoding opsin-related-protein as well as several developmentally regulated genes (HSP 9/12, HSP *Awh11*, and NAD-dependent formate dehydrogenase *AciA/Fdh*). The genes encoding arrestin and the RGS protein *flbA* were concurrently overexpressed. At the 30-hour time point, Af293<sup>FL</sup> underexpressed the key developmental regulator *brlA* and conidia-specific genes *rodA* and *rodB*, whereas aspergillopepsin F was overexpressed.

**Table 1.** Af293FL differentially expresses opsin and G-protein regulatory genes

Cluster	Gene name	ORF	Relative expression	
			8 hours	30 hours
1	high affinity methionine permease	Afu4g09040	0.848774	1.513
	cell wall galactomannoprotein Mp2/allergen F17-like	Afu2g05150	0.143108	1.86916
	BYS1 domain protein, putative	Afu5g01990	-0.04796	2.342465
	aspartic endopeptidase <i>Pep1</i> /aspergillopepsin F	Afu5g13300	-0.13327	2.861234
	IgE-binding protein	Afu6g00430	-1.23993	2.657625
	allergenic cerato-platanin Asp F13	Afu2g12630	-1.13578	1.781088
	extracellular proline-serine rich protein	Afu8g07090	-0.65047	1.7238
	methionine aminopeptidase, type II, putative	Afu2g01750	2.730707	0.315456
2	cell wall glycosyl hydrolase <i>YteR</i> , putative	Afu2g14630	1.760417	0.661531
	developmental regulator <i>FbA</i>	Afu2g11180	1.571861	0.837913
	NADP-dependent malic enzyme <i>MaeA</i>	Afu2g08280	1.581181	0.007476
	conserved hypothetical protein	Afu2g00580	1.584199	-0.15622
	arrestin (or S-antigen), N-terminal domain protein	Afu6g13380	1.538449	-0.13847
	C2H2 finger domain protein, putative	Afu8g05010	1.800728	0.011303
	conidial hydrophobin <i>RodB</i>	Afu1g17250	0.112375	-3.19989
	cell wall protein, putative	Afu5g08180	-0.53011	-1.66332
3	GPI anchored cell wall protein, putative	Afu3g01150	0.144695	-1.66902
	hypothetical protein	Afu5g07980	0.095115	-1.77793
	phenylalanine ammonia-lyase	Afu2g09110	-0.05489	-1.73881
	GTP-binding protein <i>EsdC</i>	Afu7g01930	0.143474	-1.5393
	DUF221 domain protein, putative	Afu5g01820	0.042208	-1.53861
	alpha, alpha-trehalose glucohydrolase <i>TreA/Ath1</i>	Afu3g02280	-1.52994	0.386307
	glucose repressible protein <i>Grg1</i> , putative	Afu5g14210	-1.51353	0.32186
	endo-chitosanase, pseudogene	Afu4g01290	-1.67239	0.274825
4	FAD-dependent oxygenase, putative	Afu3g00840	-1.65143	0.259612
	glycogen phosphorylase <i>GlpV/Gph1</i> , putative	Afu1g12920	-2.10915	-0.55088
	C6 transcription factor, putative	Afu4g01510	-2.02165	-0.39924
	HSP9/12 family heat shock protein	Afu1g17370	-1.92717	-0.02969
	conserved hypothetical protein	Afu8g07160	-1.6317	-0.11533
	thiamine biosynthesis protein ( <i>Nmt1</i> ), putative	Afu5g02470	-1.60505	-0.06664
	conserved hypothetical protein	Afu8g07190	-1.55362	-0.15525
	hypothetical protein	Afu7g08590	-1.51399	-0.09198
	conserved hypothetical protein	Afu4g12590	-1.61718	0.025716
	conserved hypothetical protein	Afu3g09830	-1.71358	0.020823
	conserved hypothetical protein	Afu1g13550	-1.70911	-0.05334
	NAD-dependent formate dehydrogenase <i>AciA/Fdh</i>	Afu6g04920	-1.74368	-0.15843
	6-phosphogluconate dehydrogenase, decarboxylating	Afu6g08730	-1.70504	-0.15849
	opsin, putative	Afu7g01430	-1.75658	-0.11454
	NADH-quinone oxidoreductase <i>Pst2</i> , putative	Afu1g02820	-1.76404	-0.22796
	conserved hypothetical protein	Afu7g06770	-1.72165	-0.27497
	chaperone/heat shock protein <i>Awh11</i>	Afu6g12450	-1.7315	-0.3533
	FMN dependent dehydrogenase, putative	Afu1g00500	-1.70418	-0.43737
	N-methyltransferase, putative	Afu2g15650	-1.57065	-0.37734
	conserved hypothetical protein	Afu6g10450	-1.6392	-0.36357
non-classical export protein <i>Nce102</i> , putative	Afu2g01590	-1.59545	-0.31464	

ORF, open reading frame.

G-protein signaling plays a crucial role in regulating development and growth in *Aspergillus* spp.<sup>22-24</sup> and is required for vegetative growth.<sup>23,24</sup> Inhibition of G-protein signaling, such as is mediated by the RGS protein *flbA*, results in upregulation of *brlA*, which leads to asexual sporulation, and *esdC*, which initiates sexual sporulation.<sup>23</sup> Indeed, both inactivating mutations of *flbA* and activating mutations of *fadA* (encoding the G $\alpha$  subunit) result in fluffy strains with uncontrolled vegetative growth and absent sexual and asexual sporulation.<sup>23,24</sup>

Opsins are photoreceptor proteins that use retinal as a chromophore; they are found in all major taxa and are involved in various light-driven processes.<sup>25</sup> Arrestin proteins are a family of ubiquitous eukaryotic regulators of G-protein-coupled receptors, including photoreceptors.<sup>26</sup> Thus, Af293<sup>FL</sup> differentially expresses genes that are homologous for both a seven-helix transmembrane receptor and arrestin, suggesting that the fluffy phenotype arises from impaired light-sensing through an opsin-like G-protein-coupled photoreceptor. Although intriguing, these results are preliminary and further work is required to characterize the role of opsin/G-protein signaling and its contribution to conidiation in *A. fumigatus*.

The failure of Af293<sup>FL</sup> to form conidiophores was coupled with exuberant proliferation of vegetative hyphae, similar to that reported by Tamame et al.<sup>1,2</sup> for fluffy phenotypic variants of *A. nidulans*. Not surprisingly, the dysregulated proliferation of vegetative hyphae was accompanied by upregulation of genes that encode for cell-wall constituents, such as galactomannoprotein Mp2 and Asp F13, whereas conidia-specific genes such as the hydrophobins *rodA* and *rodB* were concurrently downregulated. Interestingly, aspergillopepsin F expression was increased in Af293<sup>FL</sup> mycelia, suggesting that the production and secretion of proteolytic enzymes is required to accommodate the expanding mycelial biomass. This notion was further supported by increased *in vitro* elastase activity of Af293<sup>FL</sup> compared with the parental wild-type strain. Elastase production by *A. fumigatus* was previously shown to be correlated with pulmonary invasion by hyphae and virulence, both in experimental models and the clinical setting.<sup>11,12</sup> These observations are consistent with Af293<sup>FL</sup>'s pathogenicity in fruit flies and immunosuppressed mice and increased angioinvasiveness in mouse lungs.

In spite of the increased proteolytic activity of Af293<sup>FL</sup>, this strain was not associated with reduced survival rates in model hosts. This paradox is consistent with the results of recent studies that failed to find an association between secreted protease production and *A. fumigatus* virulence.<sup>27,28</sup> It is possible that the increased proteolytic activity seen *in vitro* does not reflect protease secretion and activity during infection. Protease inhibitors present within host lungs might attenuate the proteolytic activity of secreted *A. fumigatus* enzymes.<sup>29</sup> Furthermore, given the multitude of proteolytic enzymes produced by *A. fumigatus*, the relative importance of aspergillopepsin F may be negligible. Alternatively, the animal models employed in the present study might have been insensitive for the detection of increments in virulence. Use of smaller *A. fumigatus* inoculums, resulting in a more subacute infection, might have uncovered subtle differences in virulence associated with the fluffy phenotype.

In summary, we demonstrated an interaction between a chemotherapeutic agent (5AC) and the pathogenic mold *A. fumigatus* that resulted in a distinct developmental variant. Whether such interactions have clinical relevance is unclear as the concentrations of 5AC required to induce the fluffy phenotype far exceed those achieved in patient's tissues during treatment.<sup>30</sup> Of note, in addition to its use as an antineoplastic drug, 5AC has been used in recent years in epigenetic manipulations of crop plants.<sup>31</sup> Moreover, an oral formulation of 5AC is currently being explored in preliminary clinical trials.<sup>32</sup> Such applications could unintentionally expose saprophytic or mucosal colonizing molds to high concentrations of 5AC. Af293<sup>FL</sup> characteristics, such as increased protease secretion and upregulation of allergenic cell-wall components, indicate that 5AC-*A. fumigatus* interactions could have potentially important consequences for human health. Additionally, although Af293<sup>FL</sup> is a fully virulent *A. fumigatus* strain, its failure to conidiate at standard incubation conditions may delay its identification using standard microbiologic methods and may lead to it being considered a harmless saprophyte. The pleiotropic effects of chemotherapeutic agents on fungal biology add another level of complexity to the pathogenesis of invasive aspergillosis in immunocompromised hosts.

## Materials and Methods

**5-Azacytidine.** 5-Azacytidine (Vidaza, Pharmion Corp., Boulder, CO) was reconstituted from 100-mg vials in 4 mL of sterile water and mixed by gentle rolling to yield a 25 mg/mL stock solution.

**Induction of *A. fumigatus* developmental mutants.** *A. fumigatus* (strain Af293) was grown on yeast-extract agar glucose (YAG) plates for 72 hours. Conidia were collected in sterile 0.9% saline with 0.08% Tween-20 and resuspended at 10<sup>5</sup> cells/mL in liquid minimal medium (2% [v/v] 50x salts, 2% [w/v] glucose, 1.2% 1 M KPO<sub>4</sub> [pH 6.8], and 0.1% trace elements) containing different 5AC concentrations (0  $\mu$ M, 250  $\mu$ M and 500  $\mu$ M). Conidial suspensions were incubated at 37°C with shaking for 72 hours. Next, the mycelia were transferred onto glass microfiber filters (Whatman, Piscataway, NJ). Filters were placed on a monolayer of sterile glass beads immersed in liquid YAG and maintained in a humidified 37°C incubator until conidiation (48 to 72 hours). Conidia from several regions of each filter disc were collected with a swab, diluted in 100- $\mu$ L saline, and spread on YAG plates. Plates were incubated at 37°C for an additional 48 hours. Colonies that exhibited fluffy morphologic characteristics (i.e., markedly reduced or absent conidiation and abundant formation of aerial hyphae) were counted and collected. In subsequent experiments, conidiation of Af293<sup>FL</sup> was induced when necessary by light exposure during the initial 18 hours of growth on YAG. To determine the stability of the fluffy phenotype, Af293<sup>FL</sup> was passaged repeatedly on solid medium. In each passage, conidia were collected by flooding the surface of the mycelium with 5 ml of sterile saline and lightly scraping with a Drigalsky spreader. The conidial suspension was aspirated, and conidia were washed three times in sterile normal saline by centrifugation. The resulting suspension was again plated on YAG.

**Growth rate.** The radial growth rate was determined by spotting ~1,000 conidia in 5  $\mu$ L of saline in the center of a minimal medium agar plate. Plates were incubated at 37°C, and the colony diameter was measured daily. The radial growth rate, expressed as the slope of the colony diameter over time, was compared between the parental wild-type Af293 (Af293<sup>WT</sup>) and fluffy strains (Af293<sup>FL</sup>).

**Analysis of fungal damage by exogenous H<sub>2</sub>O<sub>2</sub>.** Because resistance to oxidative stress is a virulence determinant of *A. fumigatus*,<sup>5</sup> we compared the resistance of Af293<sup>FL</sup> and Af293<sup>WT</sup> to H<sub>2</sub>O<sub>2</sub>. Conidial susceptibility to oxidative damage was measured using the method described by Eisendle et al.<sup>6</sup> Conidia (10<sup>5</sup>/mL) were incubated with H<sub>2</sub>O<sub>2</sub> at concentrations of 50 mM to 1.0 mM at 30°C for 30 minutes, with shaking. Conidial viability was determined by spreading 100  $\mu$ L of the 50-fold diluted suspension on YAG and counting colonies after 18 hours of incubation at 37°C. Hyphal susceptibility to H<sub>2</sub>O<sub>2</sub> was measured by spotting ~1,000 conidia onto YAG with 4 mM H<sub>2</sub>O<sub>2</sub> and peroxide-free YAG plates and measuring the growth rate at 37°C, as described above.

**Antifungal drug susceptibility testing.** We determined the susceptibility of *A. fumigatus* strains to the antifungal drugs amphotericin B, voriconazole, caspofungin and nikkomycin Z using the broth dilution method, as outlined in document M38-A2 of the Clinical and Laboratory Standards Institute.<sup>7</sup>

**Fruit fly model of invasive aspergillosis.** Toll-deficient (*Tl*<sup>-/-</sup>) *Drosophila melanogaster* has previously been shown to be a useful model host for assessing *A. fumigatus* virulence.<sup>8</sup> To compare the virulence of Af293<sup>FL</sup> and Af293<sup>WT</sup> in this model, we infected *Tl*<sup>-/-</sup> flies with conidial suspensions of each strain and compared their survival rates. Two–four day-old female *Tl*<sup>-/-</sup> flies (25 flies per group) were inoculated with *A. fumigatus* by insertion of a needle (0.25 mm diameter) dipped in a suspension of freshly collected conidia. Experiments were performed in triplicate for each of two conidial inoculums, 10<sup>6</sup> and 10<sup>7</sup> cells/mL. After inoculation, the flies were housed at 29°C to maximize expression of the *Tl*<sup>F632</sup> phenotype<sup>9</sup> and transferred into fresh vials every three days. Fly survival was assessed daily over seven days. Flies that died within 3 hours after inoculation were excluded from the survival analysis.

**Mouse model of invasive pulmonary aspergillosis.** To confirm our observations in fruit flies in a mammalian model host, we further assessed the virulence of Af293<sup>FL</sup> relative to Af293<sup>WT</sup> in immunosuppressed mice. Eight-week-old female BALB/c mice (weight, 18 to 20 gr, National Cancer Institute) were immunosuppressed with cortisone acetate alone (non-neutropenic model) or cortisone acetate in combination with cyclophosphamide (neutropenic model). In the non-neutropenic model, cortisone acetate (250 mg/kg, subcutaneously) was given 4 days and 1 day prior to inoculation and 2 days and 5 days after inoculation. In the neutropenic model, cyclophosphamide (100 mg/kg) was injected intraperitoneally 4 days and 1 day prior to inoculation, and a single cortisone acetate dose (250 mg/kg) was given 1 day prior to inoculation. Additional cyclophosphamide (100 mg/kg, intraperitoneally) was injected 2 days after inoculation to maintain neutropenia. Inoculation was performed under isoflurane-induced

anesthesia by intranasal instillation of a 35- $\mu$ L droplet containing 1.75 x 10<sup>6</sup> conidia. After inoculation, the animals were monitored for 7 days; any animal determined to be in a morbid state was euthanized by CO<sub>2</sub>-induced asphyxiation, and death was recorded as occurring 12 hours later. All procedures were performed according to the highest standards for humane handling, care, and treatment of research animals and were approved by the MD Anderson Cancer Center Institutional Animal Care and Use Committee.

Pulmonary fungal burden was determined in the lungs of mice using real-time-qPCR (RT-qPCR) as previously described.<sup>10</sup> In brief, DNA samples isolated from homogenized lungs were assayed in duplicate using an ABI PRISM 7000 sequence detection system (Applied Biosystems) with primers and a dually labeled fluorescent hybridization probe specific for the *Aspergillus* 18S rRNA gene (Table 1).<sup>10</sup> The cycle threshold of each sample was interpolated from a seven-point standard curve of cycle threshold values prepared by spiking uninfected mouse lungs with known amounts of conidia (10<sup>1</sup> to 10<sup>7</sup>) from *A. fumigatus* 293. The results are reported as conidial equivalents of *A. fumigatus* DNA. Representative lung specimens from each infection group were fixed in 10% formalin and embedded in paraffin wax. Matched sections were stained with Grocott-Gomori methenamine-silver to visualize fungal elements and the elastic Van Gieson (EVG) stain, which enhances elastin and collagen. Angioinvasion, defined as the proportion of vessel lumens containing hyphae, was assessed in a blinded fashion in randomly chosen fields of each lung specimen. At least 80 vessels were evaluated per experimental group.

**Elastase activity assay.** Elastase activity has been shown to correlate with the invasiveness of *A. fumigatus* isolates.<sup>11,12</sup> We measured elastase activity in a solid medium containing 0.1% elastin-Congo-red (Sigma, St. Louis, MO), 0.05% yeast carbon base, 1.5% agar, and 0.05 M borate buffer, pH 7.6, as described by Kothary et al.<sup>12</sup> Plates were inoculated centrally with ~1,000 conidia and incubated at 37°C for 7 days. The colony diameters and elastase lysis zones were measured daily, and the elastase activity index was calculated as [elastase lysis diameter]/[growth diameter].<sup>11</sup>

**Whole-genome gene expression analysis.** To analyze differential gene expression in the 5AC-induced *A. fumigatus* fluffy variant, we used *A. fumigatus* strain Af293 amplicon microarrays containing 9,516 genes.<sup>13</sup> *A. fumigatus* conidia (5 x 10<sup>7</sup> cells/mL) were incubated in liquid minimal medium in the dark at 37°C, with shaking. Mycelia were collected by filtration through four layers of miracloth (EMD Chemicals, Gibbstown, NJ) at two time points: predevelopmental competence (8 hours incubation) and postdevelopmental competence (30 hours incubation). Gene expression for Af293<sup>FL</sup> was determined at each time point with a corresponding parental wild-type strain as the reference. RNA was extracted from Af293<sup>FL</sup> mycelia in three replicate experiments using TRIzol (Invitrogen) and pooled and hybridized with RNA extracted in parallel from Af293<sup>WT</sup>. cDNA microarrays were performed as described by the J. Craig Venter Institute Standard Operating Procedures ([pfgc.jvci.org/index.php/microarray/protocols.html](http://pfgc.jvci.org/index.php/microarray/protocols.html)). In brief, cDNA was generated from 2  $\mu$ g



of total RNA by incubating for 18 hours at 42°C with Superscript II reverse transcriptase (Invitrogen), a dNTP mixture containing amino-allyl dTTP and primed with random hexamers. Unincorporated dNTPs and hexamers were removed by purification with a Qiagen polymerase chain reaction (PCR) purification column. Samples were then incubated for 18 hours at ambient temperature with Cy-3 or Cy-5 dye. After labeling, unincorporated dye was removed by purification with a Qiagen PCR purification column. Dye-coupled cDNA probes were evaporated and resuspended in hybridization buffer. Each sample was hybridized with a flip-dye replicate to account for labeling bias. Resuspended probes were mixed and spotted on the array for an 18-hour incubation. After postincubation washes, slides were scanned with a Genepix 6000B scanner and analyzed with Spotfinder.<sup>14</sup> Exported fluorescence intensities were normalized in MIDAS<sup>14</sup> using LOWESS, followed by flip-dye and in-slide replicate analyses. Heat maps and hierarchical expression clustering were performed using the MeV software program version 4.3.<sup>15</sup>

**Real-time quantitative PCR.** cDNA was generated from 1 µg of total RNA using the Taqman reverse transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions in a total volume of 100 µL. Twenty-microliter quantitative (qPCR) reactions were set up using 1 µL of cDNA, 10 µL of 2x SYBR green qPCR master mix (Applied Biosystems), 2 µL of 5 M Betaine (Sigma), and 4 pmol of each primer (Table 1). qPCR reactions were performed in 96-well plates in an ABI PRISM 7900HT Fast Real-Time thermocycler. The program used an initial 10-minute incubation at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 62°C. Primer specificity was verified using a denaturation step after the

last amplification cycle.  $C_T$  values were collected with a manual threshold of 0.2. Each target was tested in quadruplicate, and the mean of the four  $C_T$  values was used for analysis. The mean  $C_T$  was converted to an approximate transcript number by the equation  $n = 2^{(40-C_T)}$ . These values were then standardized to the determined value for *actA* and are reported as the number of target transcripts per *actA* transcript.

**Statistical analysis.** Survival rates in animal models were calculated using the Kaplan-Meier method; the survival curves of Af293<sup>FL</sup>- and Af293<sup>WT</sup>-infected animals were compared using the log-rank test. Pulmonary fungal burdens were compared using the Mann-Whitney test. The elastase activity indexes of different *A. fumigatus* strains were compared using the unpaired Student's t-test. The angioinvasion scores (the proportion of blood vessels with fungal hyphae in their lumens) of different specimens were compared with Fisher's exact test. Gene expression by RT-qPCR, expressed as folds of *actA* transcript, was compared between strains using the unpaired Student's t-test. A two-tailed p value <0.05 was considered statistically significant. Calculations were performed using InStat and Prism 4.0 (GraphPad, San Diego, CA) software.

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