H3K9 methylation regulates growth and development in *Aspergillus fumigatus*

Jonathan M. Palmer\(^1\)\(^†\), Robyn M. Perrin\(^1\)\(^†\), Taylor R.T. Dagenais\(^2\), and Nancy P. Keller\(^1,2,3\)*

\(^†\)Authors contributed equally to this work

\(^1\) Department of Plant Pathology, University of Wisconsin-Madison, Madison, WI

\(^2\) Department of Medical Microbiology and Immunology, University of Wisconsin-Madison, Madison, WI

\(^3\) Department of Bacteriology, University of Wisconsin-Madison, Madison, WI

Running title: epigenetic regulation in *Aspergillus*

Keywords: histone methylation, Clr4, BrlA, conidia, chromatin

* Corresponding Author:

Nancy P. Keller

3467 Microbial Sciences Building

1550 Linden Drive

Madison, WI 53706

Phone: (608) 262-9795

Fax: (608) 263-2626

npkeller@wisc.edu
Abstract

In most species, chromatin remodeling mediates critical biological processes ranging from development to disease states. In fungi within the genus *Aspergillus*, chromatin remodeling may regulate expression of metabolic gene clusters, but other processes regulated by chromatin structure remain to be elucidated. In many eukaryotic species, methylation of lysine 9 of histone 3 (H3K9) is a hallmark of heterochromatin formation and subsequent gene silencing. The sole H3K9 methyltransferase in *Schizosaccharomyces pombe* is Clr4. We report that disruption of the Clr4 homolog in the pathogenic mold *A. fumigatus* (ClrD) – involved in both mono and tri-methylation of H3K9 - results in several growth abnormalities. Developmental defects in ∆Af-clrD include reduction in radial growth, reduction in conidial production, and delayed conidiation after developmental competence mediated by delayed expression of *brlA*, the master regulator of conidiophore development. Sensitivity of ∆Af-clrD to 6-azauracil suggests that ClrD influences transcriptional processing in *A. fumigatus*. Despite growth abnormalities, macrophage assays suggest ClrD may be dispensable for host interactions.
Introduction

In eukaryotes, gene expression is greatly influenced by chromatin structure. Heterochromatin exists in either a constitutively transcriptionally silent state as at centromeres and telomeres, or as facultative heterochromatin capable of switching between transcriptionally active and silent states. The expression of developmentally critical genetic loci present in facultative heterochromatin can be regulated by this shift. Classical examples include X-chromosome inactivation in females (9), derepression of mating type loci in fission yeast (15), and position effect variegation (PEV) in *Drosophila* (13).

The “histone code” influences nucleosome positioning and chromatin compaction in facultative heterochromatin (Fig. 1). This code consists of specific patterns of post-translational modifications of key amino acids on histone tails (reviewed in 18, 20, 22, 44). Modifications may include acetylation, ubiquitination, phosphorylation, and/or methylation. Such modifications recruit regulatory binding proteins that direct processes such as chromatin compaction (which dictates steric accessibility of DNA to transcriptional activators and repressors) or stabilization of transcriptional machinery.

There is limited information available about chromatin remodeling proteins and chromatin-level regulation of gene expression in filamentous fungi of the genus *Aspergillus*. Three histone arginine methyltransferases (RmtA-RmtC) have been analyzed biochemically in the model species *A. nidulans*; however, detailed analysis of deletion strain phenotypes was not reported (50). An *A. nidulans* histone deacetylase, HdaA, is required for normal growth under oxidative stress (49) and is involved in regulating telomere-proximal secondary metabolite gene clusters (42). However, there is
a near-absence of studies on chromatin regulation in the opportunistic pathogen *A. fumigatus*. The degree to which such processes impact growth, development, and pathogenesis in this species is largely unknown.

A key histone modification influencing heterochromatin formation and transcriptional activation state is mono-, di-, or tri-methylation at lysine 9 of histone H3 (H3K9). Tri-methylation at H3K9 recruits heterochromatin protein 1 (encoded by *HP1* in mammals, *swi6* in fission yeast, and *su(var)205* in *Drosophila*), leading to heterochromatin spread (18) (Fig. 1). H3K9 methylation frequently demarks a transcriptionally silent state; however, a recent study found that H3K9 tri-methylation was associated with active expression in a variety of human cancer cell lines (53). H3K9 methylation regulates developmental processes in many species. Examples include the regulation of stem cell pluripotency via expression of the *HOXA* cluster (5), regulation of *KIR* gene expression in natural killer cells (37), vulval development in *Caenorhabditis elegans* (4), and pleiotropic regulation of *Drosophila* development (52).

The major enzyme responsible for histone H3K9 methylation is SU(VAR)3-9 in *Drosophila* (38), Clr4 in *Schizosaccharomyces pombe* (32), and DIM-5 in *Neurospora crassa* (47). Mammals have a homologous H3K9 methyltransferase, *SUV39H1*; however, there are several other mammalian enzymes capable of methylating H3K9 (34).

A Clr4 null mutant in *S. pombe* shows derepression of silent mating-type loci (15), increased chromosome loss (2), improper chromosomal segregation, diminished growth, and increased susceptibility to microtubule destabilizing agents (14). Disruption of DIM-5 in *N. crassa* causes loss of H3K9 tri-methylation, results in decreased DNA methylation and growth abnormalities (47).
We investigated the role of the Clr4 homolog in the pathogenic mold *Aspergillus fumigatus* (Af-clrD). We report that Af-ClrD methylates H3K9, mediates 6-azauracil sensitivity and is required for normal growth and development, but does not affect *in vitro* conidial uptake by macrophages.
Materials and Methods

Culturing Conditions and DNA Isolations

Fungal strains used in this study are listed in Table 1. All growth assays were conducted with prototrophic strains at 37°C on glucose minimal media (GMM) as in (41) unless noted otherwise. When appropriate, media was supplemented with 5 mM of uridine and uracil for pyrG auxotrophs and 5 mM arginine for argB auxotrophs. Radial growth was assayed by inoculation of approximately 500 spores in the center of appropriate media and colony diameter was measured every 24 hours and represented as the mean of four replicates. Conidial production was quantified from two inoculation methods; point-inoculated cultures were inoculated as per the radial growth experiment and overlay inoculated cultures were set up by pipetting 1 x 10^6 conidia/mL into 0.75% GMM molten agar that was subsequently poured over 1.5% GMM solid agar petri dishes. One agar plug was removed from the center of point-inoculated plates and three agar plugs were removed from the overlay plates; subsequent spores suspensions were counted with a hemocytometer. All conidial quantification experiments were completed in triplicate. Graphs and statistical analysis was done using Prism 5.0 software.

Genomic DNA was isolated from fungi and E. coli using standard procedures (36, 41) and all primers used are listed in Table 2.

Generation of transformation cassettes and genetic manipulations

A plasmid (pRMPargB/Topo2) bearing the A. nidulans argB cassette (AN4409.3) was generated using primers RMP17 and RMP18 to amplify a 1.9-kb fragment beginning 0.4 kb upstream of the argB translational start codon and ending 0.35 kb downstream of
the translational stop codon. Following manufacturer’s protocols, the product was amplified from genomic DNA of *A. nidulans* FGSC4 using TripleMaster polymerase (Eppendorf), blunt ended with *PfuUltra* (Stratagene) and cloned into pCR-Blunt-II Topo (Invitrogen). Flanking sequences of *A. fumigatus clrD* construct (Afu1g11090) were amplified from *A. fumigatus* AF293 genomic DNA. Primers RMP10 and RMP15 were used to amplify a 1.1 kb fragment upstream of the *Af-clrD* ORF. The resulting PCR product was blunt ended with *PfuUltra* and the fragment was cloned into pCR-Blunt-II. A downstream flanking fragment 0.9 kb in length was generated using primers RMP14 and RMP13 and was cloned into this pCR-Blunt-II/5’ *clrD* intermediate construct. Finally, a 1.9 kb *XhoI* fragment from pRMPargB/Topo2 containing the *A. nidulans argB* gene was cloned in between the flanking fragments of the intermediate construct to create the final *Af-clrD* disruption cassette pRMP7.

The *Af-clrD* complementation cassette was amplified from AF293 genomic DNA using primers RMP21 and RMP22, yielding a 3.1 kb fragment composed of approximately 1 kb upstream and 0.5 kb downstream of the putative open reading frame. Product was generated using TripleMaster polymerase, blunt ended with *PfuUltra* polymerase, and ligated with pCR-Blunt-II vector to create pRMPclrD/comp#1. The *EcoRI* fragment of pRMPclrD/comp#1 was ligated with pJW24 (10) to create the *A. fumigatus clrD* complementation construct pRMP10.

Strain AF293.6 was transformed with the *Af-clrD* disruption fragment by amplification of pRMP7 with primers RMP10 and RMP13 to generate TRMP1.60 (Δ*Af-clrD; pyrG1*). Fungal transformation was done essentially as described by (29), with the modification of embedding the protoplasts in top agar (0.75%) as described above. The
transformant TRMP1.60 was analyzed by PCR and Southern analysis confirming the
disruption of Af-clrD. This strain was then transformed with A. parasiticus pyrG
(pJW24) to generate TJMP1.11 and the A. fumigatus clrD complementation cassette
pRMP10 to generate TRMP4.18, TRMP4.25, and TRMP4.40. All strains were
confirmed by preliminary PCR screens and secondarily by Southern analysis.

Westerns to detect global histone H3K9 modifications

Nuclei were prepared from TJW55.1, TJMP1.11, and TRMP4.40 strains.
Cultures (500 mL) of GMM were inoculated with $1 \times 10^6$ conidia/mL and incubated at
37°C for 48 hours. Mycelia were collected by vacuum filtration, frozen in liquid
nitrogen, and ground to powder under liquid nitrogen. Ground mycelial powder was
suspended in 200 mL nuclei isolation buffer (1M sorbitol, 10 mM Tris-HCl pH 7.5, 0.15
mM spermine, 0.5 mM spermidine, 10 mM EDTA, 2.5 mM PMSF) on ice. Samples
were centrifuged in a GSA rotor (1000 x g) at 4°C for 10 min, and supernatant was
filtered through two layers of Miracloth. The filtrate was centrifuged at 10,000 x g for 15
min at 4°C. The pellet was then resuspended in 15 mL resuspension buffer (identical to
nuclei isolation buffer but containing 1 mM EDTA) and then centrifuged in a SS-35 rotor
(9700 x g) for 15 min at 4°C. Supernatant was removed and crude nuclei were
resuspended in 0.6 mL of ST buffer (1 M sorbitol, 10 mM Tris-HCl pH 7.5, protease
inhibitor cocktail for fungi (Sigma)). Debris was pelleted by centrifugation (1500 x g) in
a microfuge for 30 sec. Protein concentration was quantified by Bradford assay in
triPLICATE. Approximately 50 µg protein per sample was subjected to electrophoresis on a
15% SDS-PAGE gel and transferred to nitrocellulose membrane. Membranes were
blocked for 30 min at room temperature on a rocking platform using 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T). Samples were incubated with primary antibodies for 1 hour in TBS-T. Antibodies were anti-histone H3 (Upstate, Cat. No. 07-690) used at 1:5,000; anti-H3K9 mono-methyl (Upstate, Cat. No. 07-450) used at 1:100; anti-H3K9 di-methyl (AbCam, Cat. No. ab7312) used at 1:20; or anti-H3K9 tri-methyl (Upstate, Cat. No. 07-442) used at 1:2,000. Blots were washed three times for 10 min each in TBS-T. Secondary antibodies (goat anti-rabbit-HRP, Pierce, 1:20,000) were applied in TBS-T for 1 hour at room temperature. Blots were washed and used for chemiluminescent detection.

Developmental Competence

Developmental competence (synchronous asexual development) was assayed as in (27). A follow-up experiment was performed identically, with the exception that liquid shake cultures were incubated for 24 hours before switching to solid media. Briefly, 100 mL of liquid media (GMM + 0.1% yeast extract) was inoculated with 1 x 10^6 conidia/mL and incubated in shaking culture at 250 rpm at 37°C for 18 or 24 hours. Mycelia were collected on sterile filter paper, transferred to solid media, and further incubated at 37°C. Cultures were examined microscopically and total RNA was extracted using Trizol (Invitrogen) at 0, 3, 6, 9, 12, and 24 hours. Northern analysis was conducted using standard techniques (36) and probes were generated with the following PCR primers: Af-clrD with RMP23 and RMP24, Af-brlA with OJH81 and OJH82 (27), Af-wetA with OJH25 and OJH29 (27), Af-vosA with oMN164 and oMN165 (33), Af-fluG with OJH91 and OJH92 (27), and Af-actin with JP Act F and JP Act R.
Expression analysis upon challenge with 6-azauracil

Liquid GMM was inoculated with $1 \times 10^6$ spores/mL and grown for 24 hours at 250 rpm and 37°C. 6AU (100 µg/mL) was added to the liquid shake cultures and total RNA was extracted at 0, 3, 6, 12, and 24 hours. Blastp search of the A. fumigatus genome identified putative homologs of the yeast genes DST1 (Afu3g07660), POP2 (Afu5g07370), RTF1 (Afu2g01900), SPT3 (Afu1g14030), IMD2 (Afu2g03610), and SDT1 (Afu2g13470). Probes for northern analysis were constructed with the following PCR primer sets: Af-dst1 with JP PPR2 F and JP PPR2 R, Af-pop2 with JP POP2 F and JP POP2 R, Af-rtf1 with JP RTF1 F and JP RTF1 R, Af-spt3 with JP SPT3 F and JP SPT3 R, Af-imd2 with JP IMD2 F and JP IMD2 R, and Af-sdt1 with JP SSM1 F and JP SSM1 R.

Macrophage phagocytosis assay

The RAW 264.7 and MH-S cell lines were purchased from ATCC (Manassas, VA) and maintained in RPMI-1640 supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and gentamycin (Sigma, St. Louis, MO). RAW cells (5x10^4/well) were plated onto 16-well chamber slides (Nunc, Rochester, NY) and incubated at 37°C, 5% CO₂. After overnight incubation (18 hours), freshly harvested conidia (2.5x10^5 conidia/well) were added to the macrophages for one hour, wells were washed twice with RPMI media and macrophages were allowed to incubate for an additional two hours to allow complete uptake of conidia. Macrophages were fixed in 2% formaldehyde, washed three times with phosphate-buffered saline and stained with
calcofluor white (Sigma) to label extracellular conidia, which were excluded from analysis. The percent uptake (number of macrophages with conidia/total number of macrophages x 100) and the average number of conidia per macrophage (of macrophages containing conidia) were determined. These experiments were conducted in triplicate.
**Results**

**Identification of *Aspergillus fumigatus clrD*, creation of Af-ClrD null mutant and subsequent complementation**

We identified *Af-clrD* by Blastp (3) search of the *A. fumigatus* genomic sequence (40) at the *Aspergillus* Comparative Database (www.broad.harvard.edu/annotation/genome/aspergillus_group/) with the amino acid sequence of Clr4 from *S. pombe* (CAA07709). Locus Afu1g11090 was identified with an e value of 0.0 despite only 39% identity overall. Further *in silico* analysis using the Conserved Domain Database indicated presence of the conserved methyltransferase PRE-SET and SET domains (28).

The *Af-clrD* locus was replaced by double homologous recombination with the *A. nidulans* argB marker gene by transforming *A. fumigatus* strain AF293.6 (54) with the gene replacement construct pRMP7. Gene replacement was confirmed by PCR of over 100 transformants and Southern blot analysis of a subset of transformants. Several strains showing obvious growth defects proved to be Δ*Af-clrD* transformants (Fig 2B). To confirm that the Δ*Af-clrD* phenotype was due solely to loss of *Af-clrD*, one Δ*Af-clrD* transformant (TRMP1.60) was transformed with the *Af-clrD* complementation construct pRMP10. Resulting transformants were screened by PCR and Southern blot analysis (Fig. 2C) and TRMP4.18, TRMP4.25, and TRMP4.40 were chosen for further analysis. TRMP1.60 was transformed with pJW24 harboring the *A. parasiticus* pyrG gene to generate a prototrophic Δ*Af-clrD* strain TJMP1.11 (data not shown).

**Af-ClrD methylates lysine 9 of histone 3 in *A. fumigatus***
Functionality of Af-ClrD was examined by western analysis of nuclear protein extracts from wild type + pyrG (TJW55.1), ΔAf-clrD (TJMP1.11) and complemented ΔAf-clrD (TRMP4.40) with antibodies to H3K9-me₁, H3K9-me₂ and H3K9-me₃. Figure 3 shows that Af-clrD is primarily involved in tri-methylation and mono-methylation of H3K9. No substantial difference in di-methylation between the strains was observed under the conditions tested.

Af-ClrD is required for normal growth and BrlA-mediated conidiophore development

The ΔAf-clrD mutant had reduced pigmentation on the reverse side of the colony, an increased white colony margin, and increased colony crenulation on glucose minimal medium (GMM) at 37°C (Fig. 4). The deletion strain showed significant reduction in radial growth when cultures were grown on glucose minimal medium (GMM) at 37°C (Fig. 5A). Furthermore, conidial production was comparatively decreased in the ΔAf-clrD mutant after 5 days of growth on GMM using both an overlay inoculation method and a point inoculation method (Fig. 5B and 5C). No significant differences in germination rates from liquid stationary culture were observed (data not shown).

To further examine the mechanism underlying the delayed conidiogenesis, developmental competence assays were performed as per Mah et al. 2006. These assays distinguish between the ability to initiate conidiophore formation (e.g. competence) versus a delay in conidiophore formation once development has started (Fig. 6A). Liquid shake cultures were grown for either 18 or 24 hours and then shifted to solid medium. Conidiophore formation was temporally identical in both regimes after the shift,
indicating that ΔAf-clrD is delayed in conidiogenesis after reaching competence. ΔAf-clrD was delayed in conidiophore formation by 3 – 4 hours (Fig. 6C).

Conidiophore development is under strict control of several genes in *Aspergillus* spp. (1). Northern analysis of four conidiogenesis genes (*Af-brlA, Af-wetA, Af-fluG,* and *Af-vosA*, (27, 33)) indicated a clear delay in expression of the transcriptional activator *Af-brlA* and possibly a slight delay in *Af-wetA*, but not *Af-fluG* or *Af-vosA* (Fig. 6B).

**ΔAf-clrD is sensitive to 6-Azauracil**

Histone methyltransferase mutants are frequently sensitive to chromatin-targeting inhibitors. Mutants generated in this study were screened for sensitivity to thiabendazole (a microtubule destabilizing agent), hydroxyurea (an inhibitor of ribonucleotide reductase and indicator of DNA replication efficiency), and 6-azauracil (6AU; an inhibitor of guanine nucleotide synthesis and indicator of transcriptional defects). The ΔAf-clrD strain did not show differential sensitivity to thiabendazole, and there was only a slight trend towards more susceptibility to hydroxyurea than wild type (data not shown).

However, it did show increased sensitivity to 6AU at all concentrations tested (25 µg/mL, 50 µg/mL, 100 µg/mL, and 300 µg/mL) in a radial growth assay (data not shown).

Additionally, ΔAf-clrD remained sensitive to 6AU (100 µg/mL) over time (Fig. 7A).

To explore in more detail the mechanism of the 6AU response, we analyzed expression of IMP dehydrogenase (*Af-imd2*) and pyrimidine nucleotidase (*Af-sdt1*). Neither *Af-imd2* nor *Af-sdt1* were differentially induced in ΔAf-clrD compared to control strains upon challenge with 100 µg/mL 6AU (Fig. 7B). Additionally, genes involved in 6AU sensitivity that are independent of IMP dehydrogenase activity in yeast (*Af-dst1, Af-...
spt3, Af-pop2, and Af-rtf1) were examined but were not differentially expressed in the 
ΔAf-clrD mutant compared to control strains (Fig. 7B).

Conidial uptake by macrophages is unaltered in ΔAf-clrD

Alveolar macrophages represent the first line of host defense to inhaled 
Aspergillus conidia. To determine whether Af-clrD may contribute to A. fumigatus 
pathogenesis, we conducted macrophage uptake experiments using the RAW 264.7 and 
MH-S cell lines. We have observed that both cell lines phagocytose Aspergillus conidia 
similar to primary murine alveolar macrophages (unpublished results). Neither the 
number of macrophages containing conidia nor the average number of conidia per 
macrophage was significantly different among strains (Figure 8 and data not shown). 
Additionally, ΔAf-clrD conidia displayed similar susceptibility as wild type conidia to 
hydrogen peroxide, an indicator of reaction to host ROS (data not shown). These 
experiments suggest that Af-clrD may be dispensable for defense against host alveolar 
macrophages.
Discussion

Chromatin remodeling affects a range of developmental processes in a variety of organisms, from mitotic defects in fission yeast (2, 14) to clinical diseases in humans (reviewed in refs 7, 19). These studies highlight the impact of H3K9 methylation on normal growth and development. Here, we provide evidence that Af-ClrD is a lysine-9 histone-3 methyltransferase required for normal growth and asexual development in the opportunistic pathogen A. fumigatus. Our data suggest that the growth abnormalities associated with loss of methylation at H3K9 could be due to transcriptional impairment, possibly associated with defects in transcriptional elongation machinery as evidenced by 6AU sensitivity of the ΔAf-clrD strain. Furthermore, Af-ClrD does not appear to be critical for host interactions with A. fumigatus, as sensitivity to hydrogen peroxide and phagocytosis of conidia by macrophages were unaffected in the ΔAf-clrD strain as compared to wild type and complemented control strains. Other A. fumigatus mutants harboring growth defects have similarly shown little to no effect on pathogenicity (12, 39).

Immunoblot analysis of nuclear extracts from ΔAf-clrD and complemented control strain indicates strong involvement of Af-ClrD in mono- and tri-methylation of H3K9, but not di-methylation. Several studies from model organisms suggest that different methyltransferases are involved in differential methylation of H3K9 at distinct chromosomal regions. For example, mammalian H3K9 tri-methylation by SUV39H1 is associated with pericentric heterochromatin, but centromeric regions contain di-methylated H3K9 independent of SUV39H1 activity (25). Additionally, the human G9a histone methyltransferase is associated with euchromatic gene activation by mono- and
di-methylation of H3K9 (24, 45). Our work implies the existence of at least one other H3K9 methyltransferase in A. fumigatus. Of 13 SET domain-containing proteins we have identified in the A. fumigatus genome, at least five have very low homology to other described methyltransferases (data not shown). A possible A. fumigatus homolog of Drosophila Ash1 is a plausible candidate because Ash1 has been shown to methylate H3K4, H3K9, and H4K20 (6).

Growth abnormalities have been reported for mutants lacking H3K9 tri-methylation. Tamaru and Selker (2001) reported that dim-5 null mutants of N. crassa had reduced apical growth and altered asexual development reminiscent of the decreased radial growth and delayed conidiation observed in ∆Af-clrD. The authors suggested that tri-methylation at H3K9 was necessary for DNA methylation by DIM-2 (47, 48). However, it is important to note that DNA methylation is minimal in Aspergillus spp. (23, 46) and therefore is not likely to play a key role in epigenetic regulation in this genus. Moreover, N. crassa DIM-2 null mutants do not show the same growth abnormalities as dim-5 mutants, suggesting that tri-methylation at H3K9 in N. crassa affects growth and development independently of DNA methylation (11). Contrary to the methylation patterns of H3K9 observed in A. fumigatus, mono- and di-methylation of H3K9 was not detected in wild-type N. crassa (11, 48). We propose that despite similarities in developmental phenotypes of the N. crassa dim-5 and A. fumigatus clrD mutants, the mechanisms leading to these aberrations may take place through alternative routes.

Significantly reduced radial growth, a decrease in conidial production, and a temporal shift in conidiophore maturation were observed in the ∆Af-clrD strain, but
competence (the ability to form conidiophores) was not affected. Decreased conidial production and delay in maturation may be a function of delayed Af-brlA expression. BrlA is an essential transcription factor coordinating asexual development, and null mutants of BrlA in both A. nidulans and A. fumigatus exhibit completely suppressed conidial production (27). Aberrations in brlA expression are commonly associated with delays or reduction in conidiophore formation (43, 51). BrlA is also a primary regulator of development-specific gene expression during conidiation, including wetA. Accordingly, the slight delay in Af-wetA expression observed in ∆Af-clrD may be the result of delayed Af-brlA expression.

Recently, a novel regulator of sporogenesis, VosA, has been described. VosA contributes to asexual development, specifically to spore viability (33). We did not detect a delay in Af-vosA expression pattern, suggesting that once formed, conidia were fully functional in ∆Af-clrD. Interestingly, Mah and Yu (2006) reported that Af-fluG was not required for conidiation in A. fumigatus, contrary to its role in A. nidulans. However, Af-brlA expression was delayed in the ∆Af-fluG mutant. Af-fluG expression was unaltered in the ∆Af-clrD mutant background compared to wild type, indicating that Af-ClrD affects conidiation through an Af-FluG independent pathway and functions upstream of Af-brlA. Since the ∆Af-clrD phenotype was pleiotropic, we investigated whether normal cellular metabolism was altered. Although fission yeast Clr4 null mutants were susceptible to the microtubule destabilizing agent thiabendazole (TBZ) (14), we did not observe a reduction in radial growth on TBZ compared to control plates for the ∆Af-clrD mutant (data not shown). ∆Af-clrD mutants were slightly more susceptible to HU treatment, but this trend was not statistically significant (data not
shown). From these experiments, we concluded that ∆Af-clrD does not have a serious mitotic defect.

In contrast, the ∆Af-clrD strain showed increased sensitivity to 6AU, an indicator of defects in transcriptional elongation via inhibition of guanine nucleotide biosynthesis (17). 6AU has been used to identify mutants, including histone methyltransferases of *Saccharomyces cerevisiae*, which are defective in transcriptional elongation (30, 40).

Our results are similar to those of other histone methyltransferase mutants (21, 26) and could imply a defect in GMP synthesis and/or impairment in transcriptional activity. 6AU inhibits IMP dehydrogenase (*IMD2/PUR5*), an enzyme catalyzing the first step of the GMP synthesis pathway (16). In *S. cerevisiae*, exposure to 6AU results in increased expression of *IMD2/PUR5* as well as *SDT1/SSM1*, a pyrimidine nucleotidase required for detoxification of 6AU (31). *S. cerevisiae* mutants defective in transcriptional elongation pathways fail to induce IMP dehydrogenase after being challenged with 6AU. However, differences in IMP dehydrogenase (*Af-imd2*) or pyrimidine nucleotidase (*Af-sdt1*) transcript levels were not observed in ∆Af-clrD, wild type, and complemented control strains.

A large-scale screen of single gene disruption mutants in *S. cerevisiae* identified several mutants susceptible to 6AU that did not differ in their ability to transcribe *IMD2*, suggesting alternative mechanisms of 6AU sensitivity (35). We examined expression patterns of genes known to be sensitive to 6AU in an *IMD*-independent manner in *S. cerevisiae* (35). However, no differences were observed in transcriptional patterns between wild-type and ∆Af-clrD for transcriptional elongation factor S-II (*Af-dst1*), a member of the PAF complex (*Af-rtf1*), a member of the SAGA histone acetyltransferase
complex (Af-spt3), or a transcription factor associated with transcriptional processes (Af-
pop2). While a defect in transcriptional machinery could explain the pleiotropic
phenotype of ΔAf-clrD, understanding the mechanism of Af-ClrD involvement in 6AU
sensitivity requires further analysis.

Acknowledgements

This material is based upon work supported in part by the U.S. Department of
Agriculture, under Agreement No. 59-0790-3-081. This is a cooperative project with the
U.S. Wheat & Barley Scab Initiative. Any opinions, findings, conclusions, or
recommendations expressed in this publication are those of the author(s) and do not
necessarily reflect the view of the U.S. Department of Agriculture. This research was
also funded in part by NSF MCB-0236393 and NIH R01 AI065728-01A1 to N.P.K.
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Figure Legends

Figure 1. Diagram illustrating the putative role of ClrD in chromatin remodeling. Histone tail modifications have been proposed to alter chromatin structure thereby regulating access of transcriptional machinery to DNA. H3K9 has consistently been identified as a residue that can be either acetylated or methylated and modifications of this residue are associated with different chromatin arrangements; in general acetylation is associated with transcriptionally activated regions and trimethylation with silenced regions. In an oversimplified model depicted here, histone deacetylases (HDACs) remove acetyl groups from ‘transcriptionally active’ regions followed by subsequent methylation by ClrD, which leads to inactivation of loci regulated by this mechanism. Under this model, heterochromatic silenced regions can be activated by removal of methyl groups from H3K9 followed by subsequent acetylation.

Figure 2. Schematic overview and confirmation of the constructs used to disrupt and complement Af-clrD. (A) Af-clrD was replaced with the A. nidulans argB gene as a selectable marker in A. fumigatus AF293.6 (argB1; pyrG1) and complemented with pRMP10 containing a 3.1 kb fragment; composed of 1 kb upstream of the putative start codon (P) and 0.5 kb downstream of the putative stop codon (T) of the Af-clrD open reading frame. This fragment was coupled with the A. parasiticus pyrG marker gene for selection. (B) Resulting transformants were confirmed by Southern digestion of genomic DNA. With an EcoRI digestion, a 3.1 kb probe (Probe 1) was expected to hybridize to
3.9 kb, 2.4 kb, and 1.6 kb bands for wild type and 4.8 kb, 2.4 kb, and 0.4 kb for a ΔAf-clrD strain. Additionally, a BamHI digestion was expected to show a 3.5 kb and 3.0 kb for wild type and a single 5.9 kb band for ΔAf-clrD. (C) Ectopic integration of at least 2 copies of the complementation cassette was confirmed by Southern analysis for all three complementation strains (TRMP4.18, TRMP4.25, and TRMP4.40) with an internal probe (Probe 2). All schematics are drawn to scale.

Figure 3. Immunoblot detection of H3K9 methylation patterns in nuclear protein extracts of selected strains. Nuclear protein extracts of ΔAf-clrD (TJMP1.11), wild type + pyrG (TJW55.1) and a complemented control strain (TRMP4.40) were assayed via western blot with antibodies to mono- (me$_1$), di- (me$_2$) and tri- (me$_3$) methylated H3K9. Immunoblot analysis was conducted in duplicate for each strain corresponding to lanes A and B. The faint upper band seen in the di-methylation panel is non-specific background.

Figure 4. Phenotype of ΔAf-clrD, control, and wild-type strains on glucose minimal media plates. Gross phenotypic differences observed for ΔAf-clrD mutant include reduced radial growth, reduced conidiation, presence of a white colony margin, and colony crenulation after growth for 3 days at 37°C on GMM. Strain numbers correspond to these genotypes: WT (AF293); WT + pyrG (TJW55.1); ΔAf-clrD (TJMP1.11); and Comp 4.40 (TRMP4.40).

Figure 5. Quantification of colony diameter and conidial production in ΔAf-clrD, complemented control, and wild type strains. Standard physiological experiments demonstrate growth abnormalities in the ΔAf-clrD strain. (A) Radial growth of ΔAf-clrD
on GMM at 37ºC was significantly reduced compared to wild type (AF293; p < 0.001) and complemented control strains were not significantly different than wild type (AF293). (B and C) Conidial production is reduced after 5 days incubation at 37ºC in ΔAf-clrD (TJMP1.11) compared to wild type + pyrG (TJW55.1) and an Af-clrD complemented control strain (TRMP4.40) (*p = 0.026; **p = 0.028). Error bars indicate one standard deviation.

**Figure 6. Developmental competence assays to compare ΔAf-clrD, control, and wild type strains.** (A) Diagram of the developmental competence assay and relative timing of conidiophore development in wild type A. fumigatus. (B) Northern analysis to determine expression of key genes in the conidiogenesis pathway. Expression of a critical activator, Af-brlA, is delayed in ΔAf-clrD. (C) Microscopic analysis of tissue from a developmental competence assay. The ΔAf-clrD mutant (TJMP1.11) does not start to produce phialides until ~ 9 hours after reaching competence, while wild type + pyrG (TJW55.1) and the complemented control (TRMP4.40) strains produced phialides by the 6-hour time point.

**Figure 7. ΔAf-clrD is more sensitive to 6AU than wild type or complemented control strains.** (A) The ΔAf-clrD mutant (TJMP1.11) remained sensitive over 3 days of exposure to 100 µg/mL 6AU when compared to wild type + pyrG (TJW55.1), and two complemented control strains (TRMP4.25 and TRMP4.40). Error bars indicate one standard deviation (* p < 0.001). (B) Northern analysis of mycelia challenged with 6AU resulted in induction of Af-imd2 and Af-sdt1 to similar levels in all three strains. In addition, no detectable difference in expression between strains was seen for genes
involved in IMP dehydrogenase independent 6AU sensitivity in yeast (Af-dstl, Af-spt3, Af-pop2, and Af-rtf1). Abbreviations as follows: W (TJW55.1), ∆ (TJMP1.11), and C (TRMP4.40).

Figure 8. Conidial uptake by macrophages is not altered in ∆Af-clrD.

RAW 264.7 macrophages were co-incubated with freshly harvested conidia of the wild type, ∆Af-clrD and three complemented controls for one hour at 37°C, washed, and incubated an additional two hours to permit conidial phagocytosis. Cultures were fixed and stained with calcofluor white to label extracellular conidia. (A) Percent uptake (percent of macrophages containing one or more conidia) and (B) the average number of conidia per macrophage (of those containing conidia) were calculated from triplicate wells per strain. At least 100 macrophages were counted per well. Means are presented and error bars indicate one standard deviation. Data represent at least three independent experiments.
## Tables

### TABLE 1. Fungal strains used in this study

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<td>argB1; pyrG1</td>
<td>(54)</td>
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A. fumigatus clrD Complementation Cassette (pRMP10)

WT Locus

Disruption Cassette (pRMP7)

Complementation Cassette (pRMP10)

B

Probe 1

WT  55.1  1.11

EcoRI Digestion

BamHI Digestion

Probes:

EcoRI

HindIII

EcoRV

Probe 2

An-argB

P

A. fumigatus clrD

T

Digestion:

EcoRI

HindIII

EcoRV

C

Probe 2

WT  55.1  1.11  4.18  4.25  4.40

EcoRV Digestion

HindIII Digestion

Results:

1.6 kb

3.9 kb

2.4 kb

1.6 kb

0.4 kb

EcoRI Digestion

BamHI Digestion

5.9 kb

3.5 kb

3.0 kb

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A

Radial Growth (mm)

0 10 20 30 40 50

2 Days 3 Days 4 Days

AF293

WT + pyrG

Comp 4.18

Comp 4.25

Comp 4.40

ΔAf-clrD

B

Conidiation (conidia per mm²)

WT + pyrG

ΔAf-clrD

Comp 4.40

C

Conidiation (conidia per mm²)

WT + pyrG

ΔAf-clrD

Comp 4.40

*

**
A

Transfer to solid media

18 hrs Liquid Shake culture
3 - 4 hrs Conidiophores begin to form
6 hrs Phialides form from vesicles
9 hrs Spores form from phialides
12 hrs Spores form basipetal chains

B

<table>
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C

3 hrs

6 hrs

9 hrs

12 hrs

WT + pyrG

ΔAf-clrD

Comp 4.40