

## H3K9 Methylation Regulates Growth and Development in *Aspergillus fumigatus*<sup>∇</sup>

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**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 *Aspergillus fumigatus* (ClrD), which is involved in both mono- and trimethylation of H3K9, results in several growth abnormalities. Developmental defects in  $\Delta$ AfclrD 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  $\Delta$ AfclrD 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.**

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 in *Drosophila melanogaster* (13).

The “histone code” influences nucleosome positioning and chromatin compaction in facultative heterochromatin (Fig. 1). This code consists of specific patterns of posttranslational modifications of key amino acids on histone tails (reviewed in references 18, 20, 22, and 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 to 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 trimethylation at lysine 9 of histone H3 (H3K9). Trimethylation at H3K9 recruits heterochromatin protein 1 [encoded by *HPI* 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 trimethylation 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 trimethylation and results in decreased DNA methylation and growth abnormalities (47).

We investigated the role of the Clr4 homolog in the patho-

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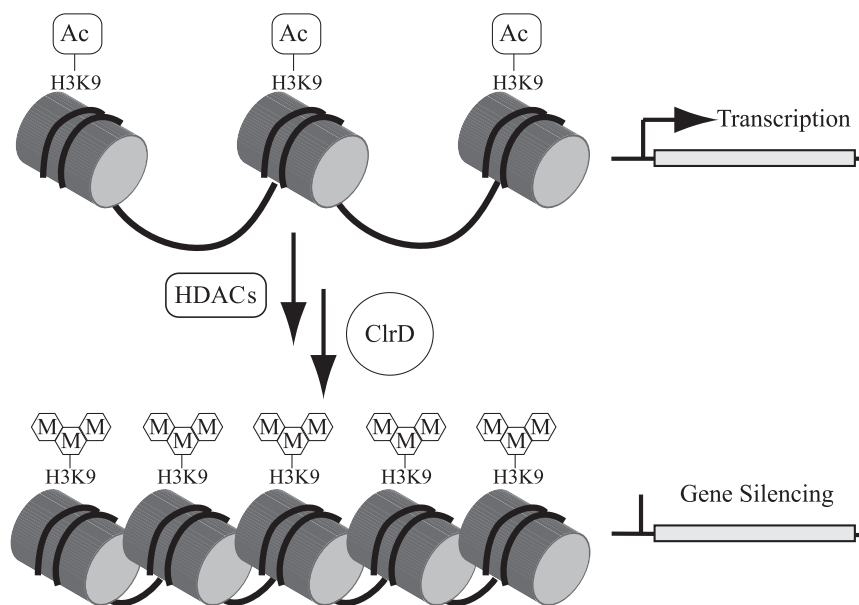


FIG. 1. Diagram illustrating the putative role of ClrD in chromatin remodeling. Histone tail modifications have been proposed to alter chromatin structure, thereby regulating access of transcriptional machinery to DNA. H3K9 has consistently been identified as a residue that can be either acetylated or 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 the 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.

genic mold *Aspergillus fumigatus* (*AfclrD*). We report that *AfClrD* methylates H3K9, mediates 6-azauracil (6AU) 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 medium (GMM) as described in reference 41 unless noted otherwise. When appropriate, medium 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 measurement of colony diameters every 24 h, and data are presented as the means of four replicates. Conidial production was quantified from two inoculation methods; point-inoculated cultures were inocu-

lated as per the radial growth experiment, and overlay-inoculated cultures were set up by pipetting  $1 \times 10^6$  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 spore suspensions were counted with a hemocytometer. All conidial quantification experiments were performed in triplicate. Graphs and statistical analysis were done using Prism 5.0 software. Genomic DNA was isolated from fungi and *Escherichia 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 the manufacturers' 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 the *A. fumigatus clrD* construct (AfuIg11090) were amplified from *A. fumigatus* AF293 genomic DNA. Primers RMP10 and RMP15 were used to amplify an 1.1-kb fragment upstream of the *AfclrD* open reading frame. 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 *AfclrD* disruption cassette pRMP7.

The *AfclrD* 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 *AfclrD* disruption fragment by amplification of pRMP7 with primers RMP10 and RMP13 to generate TRMP1.60 ( $\Delta AfclrD pyrG1$ ). Fungal transformation was done essentially as described previously (29), with the modification of embedding the protoplasts in top agar

TABLE 1. Fungal strains used in this study

Strain	Genotype	Source
AF293	<i>Aspergillus fumigatus</i> wild type	54
AF293.6	<i>argB1 pyrG1</i>	54
TJW55.1	<i>pyrG1; A. parasiticus pyrG</i>	8
TRMP1.60	$\Delta AfclrD::A. nidulans argB argB1 pyrG1$	This study
TJMP1.11	$\Delta AfclrD::A. nidulans argB A. parasiticus pyrG argB1 pyrG1$	This study
TRMP4.18	$\Delta AfclrD::A. nidulans argB A. fumigatus clrD::A. parasiticus pyrG argB1 pyrG1$	This study
TRMP4.25	$\Delta AfclrD::A. nidulans argB A. fumigatus clrD::A. parasiticus pyrG argB1 pyrG1$	This study
TRMP4.40	$\Delta AfclrD::A. nidulans argB A. fumigatus clrD::A. parasiticus pyrG argB1 pyrG1$	This study

TABLE 2. Primers used in this study

Primer	Sequence (5' to 3') <sup>a</sup>	Purpose
RMP10	<u>TTAATCTAGAGCCTGATTATGACTGGGTTTATTC</u>	Forward for <i>AfclrD</i> 5' flank
RMP15	<u>AATTAGCGGCCGCTATTTGGGTTAGCCGTGTCT</u>	Reverse for <i>AfclrD</i> 5' flank
RMP14	<u>TTAAGGGCCCCATGGATTTCAGGGCATGT</u>	Forward for <i>AfclrD</i> 3' flank
RMP13	<u>AATTGGGCCCCGACCTTACTTCATCGTCA</u>	Reverse for <i>AfclrD</i> 3' flank
RMP17	<u>TTAACTCGAGAACGCTGTGTAAGCGGAGT</u>	Forward for <i>A. nidulans</i> ArgB
RMP18	<u>AATTCTCGAGCCATTGCGAAACCTCAGAAG</u>	Reverse for <i>A. nidulans</i> ArgB
RMP23	<u>TTCCCTTTAGCATTGGAACG</u>	Forward for <i>AfclrD</i> internal probe
RMP24	<u>CTTCTCCCCACAGAGACAGC</u>	Reverse for <i>AfclrD</i> internal probe
RMP21	<u>TTAAGAACTAGTCAGGAGGCTTAATCTCAGG</u>	Forward for <i>AfclrD</i> complementation
RMP22	<u>TTAAGAGAGCTCCGAGTTACAATGTTGCTGC</u>	Reverse for <i>AfclrD</i> complementation
JP Act F	<u>TGACAATGTTACCGTAGAGATCC</u>	Forward for actin probe
JP Act R	<u>GGAGAAGATCTGGCATCACA</u>	Reverse for actin probe
JP SSM1 F	<u>CGACTACGGAATGTC</u>	Forward for SSM1 probe
JP SSM1 R	<u>GCTCCTCAAGGCTGCGAATC</u>	Reverse for SSM1 probe
JP IMD2 F	<u>GTCACATCCAGAGATATCCAG</u>	Forward for IMD2 probe
JP IMD2 R	<u>TTGACGCCGTCGTTGGAGCTC</u>	Reverse for IMD2 probe
JP PPR2 F	<u>GATGCGAAGGAAATCGAGCTC</u>	Forward for DST1 probe
JP PPR2 R	<u>CCTCCACGATTTACCACAGTTC</u>	Reverse for DST1 probe
JP RTF1 F	<u>TGATGCTGGGTCTCACCATT</u>	Forward for RTF1 probe
JP RTF1 R	<u>GGCGACATACTCTTGATCAA</u>	Reverse for RTF1 probe
JP SPT3 F	<u>AGCTTGACGAAGCAGCATG</u>	Forward for SPT3 probe
JP SPT3 R	<u>CTATCGTAGTATAGCTCCGC</u>	Reverse for SPT3 probe
JP POP2 F	<u>CATAGGATACGGAGTTCCC</u>	Forward for POP2 probe
JP POP2 R	<u>CACCAGGAGTTAGTGCACC</u>	Reverse for POP2 probe

<sup>a</sup> Restriction sites are underlined.

(0.75%) as described above. The transformant TRMP1.60 was analyzed by PCR and Southern analysis, confirming the disruption of *AfclrD*. This strain was then transformed with *A. parasiticus pyrG*(pJW24) to generate TJMP1.11 and with 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.

**Western blot assays 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 h. 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 (1 M sorbitol, 10 mM Tris-HCl, pH 7.5, 0.15 mM spermine, 0.5 mM spermidine, 10 mM EDTA, 2.5 mM phenylmethylsulfonyl fluoride) on ice. Samples were centrifuged in a GSA rotor (1,000  $\times$  g) at 4°C for 10 min, and supernatant was filtered through two layers of Miracloth. The filtrate was centrifuged at 10,000  $\times$  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 an SS-35 rotor (9,700  $\times$  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 (1,500  $\times$  g) in a microcentrifuge for 30 s. The protein concentration was determined in a Bradford assay in triplicate. Approximately 50  $\mu$ g protein per sample was subjected to electrophoresis on a 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred to a 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 h in TBS-T. Antibodies were anti-histone H3 (catalog no. 07-690; Upstate Biotechnology) used at 1:5,000, anti-H3K9 monomethyl (catalog no. 07-450; Upstate) used at 1:100, anti-H3K9 di-methyl (catalog no. ab7312; AbCam) used at 1:20, and anti-H3K9 tri-methyl (catalog no. 07-442; Upstate) used at 1:2,000. Blots were washed three times for 10 min each in TBS-T. Secondary antibodies (goat anti-rabbit-horse radish peroxidase conjugate; 1:20,000; Pierce) were applied in TBS-T for 1 h at room temperature. Blots were washed and used for chemiluminescence detection.

**Developmental competence.** Developmental competence (synchronous asexual development) was assayed as described elsewhere (27). A follow-up experiment was performed identically, with the exception that liquid shake cultures were incubated for 24 h before switching to solid medium. Briefly, 100 ml of liquid medium (GMM plus 0.1% yeast extract) was inoculated with  $1 \times 10^6$  conidia/ml and incubated in a shaking culture at 250 rpm at 37°C for 18 or 24 h.

Mycelia were collected on sterile filter paper, transferred to solid medium, 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 h. Northern analysis was conducted using standard techniques (36), and probes were generated with the following PCR primers: *AfclrD* with RMP23 and RMP24, *AfbrlA* with OJH81 and OJH82 (27), *AfwetA* with OJH25 and OJH29 (27), *AfosA* with oMN164 and oMN165 (33), *AffluG* with OJH91 and OJH92 (27), and 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 h at 250 rpm and 37°C. 6AU (100  $\mu$ g/ml) was added to the liquid shake cultures, and total RNA was extracted at 0, 3, 6, 12, and 24 h. A 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: *Afdst1* with JP PPR2 F and JP PPR2 R, *Afpop2* with JP POP2 F and JP POP2 R, *Aftrf1* with JP RTF1 F and JP RTF1 R, *Afspt3* with JP SPT3 F and JP SPT3 R, *Afimd2* with JP IMD2 F and JP IMD2 R, and *Afsdt1* 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 gentamicin (Sigma, St. Louis, MO). RAW cells ( $5 \times 10^4$ /well) were plated onto 16-well chamber slides (Nunc, Rochester, NY) and incubated at 37°C, 5% CO<sub>2</sub>. After overnight incubation (18 h), freshly harvested conidia ( $2.5 \times 10^5$  conidia/well) were added to the macrophages for 1 hour, wells were washed twice with RPMI medium, and macrophages were allowed to incubate for an additional 2 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 per total number of macrophages  $\times$  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 *AfclrD* null mutant, and subsequent complementation.** We identified *AfclrD* by a BLASTp search (3) of the *A. fumigatus* genomic

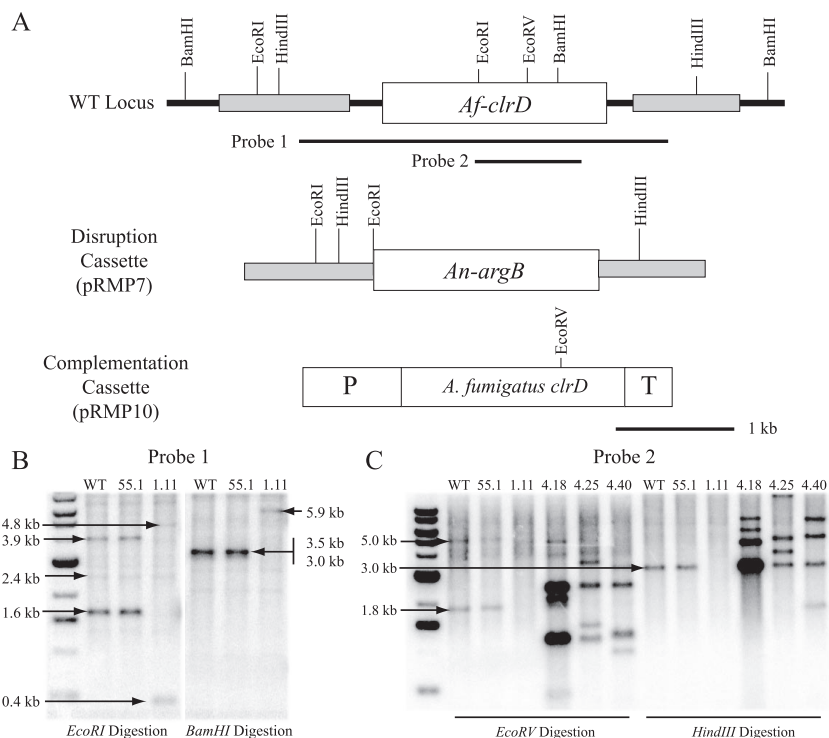


FIG. 2. Schematic overview and confirmation of the constructs used to disrupt and complement *AfclrD*. (A) *AfclrD* was replaced with the *A. nidulans argB* gene as a selectable marker in *A. fumigatus* AF293.6 (*argB1 pyrG1*) and complemented with pRMP10 containing a 3.1-kb fragment composed of 1 kb upstream of the putative start codon (P) and 0.5 kb downstream of the putative stop codon (T) of the *AfclrD* 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 *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 the wild type and 4.8-kb, 2.4-kb, and 0.4-kb bands for the  $\Delta AfclrD$  strain. Additionally, *BamHI* digestion was expected to show 3.5-kb and 3.0-kb bands for the wild type and a single 5.9-kb band for  $\Delta AfclrD$ . (C) Ectopic integration of at least two 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.

sequence (40) at the *Aspergillus* Comparative Database ([www.broad.harvard.edu/annotation/genome/aspergillus\\_group/](http://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 the presence of the conserved methyltransferase PRE-SET and SET domains (28).

The *AfclrD* 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  $\Delta AfclrD$  transformants (Fig. 2B). To confirm that the  $\Delta AfclrD$  phenotype was due solely to loss of *AfclrD*, one  $\Delta AfclrD$  transformant (TRMP1.60) was transformed with the *AfclrD* 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 prototrophic  $\Delta AfclrD$  strain TJMP1.11 (data not shown).

**AfClrD methylates lysine 9 of histone 3 in *A. fumigatus*.** Functionality of AfClrD was examined by Western analysis of nuclear protein extracts from the wild type with *pyrG*

(TJW55.1),  $\Delta AfclrD$  (TJMP1.11), and complemented  $\Delta AfclrD$  (TRMP4.40) with antibodies to H3K9-me<sub>1</sub>, H3K9-me<sub>2</sub>, and H3K9-me<sub>3</sub>. Figure 3 shows that *AfclrD* is primarily involved in trimethylation and monomethylation of H3K9. No substantial difference in dimethylation between the strains was observed under the conditions tested.

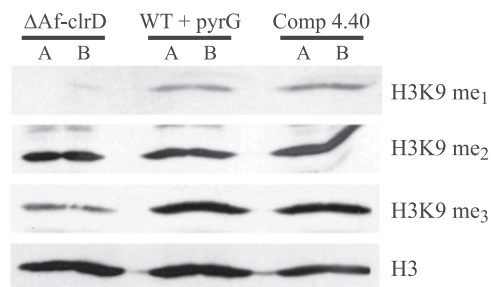


FIG. 3. Immunoblot detection of H3K9 methylation patterns in nuclear protein extracts of selected strains. Nuclear protein extracts of  $\Delta AfclrD$  (TJMP1.11), the wild type with *pyrG* (TJW55.1), and a complemented control strain (TRMP4.40) were assayed via Western blotting with antibodies to monomethylated (me<sub>1</sub>), dimethylated (me<sub>2</sub>), and trimethylated (me<sub>3</sub>) H3K9. Immunoblot analysis was conducted in duplicate for each strain, corresponding to lanes A and B. The faint upper band seen in the dimethylation panel is nonspecific background.



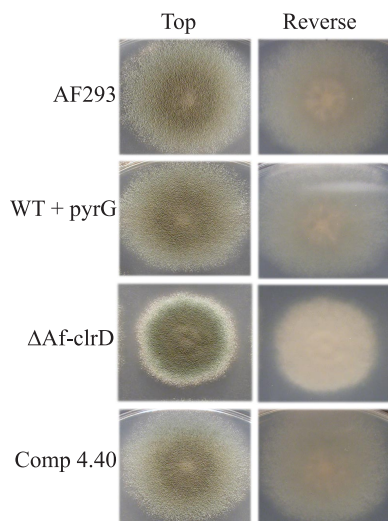


FIG. 4. Phenotype of  $\Delta Afc1rD$ , control, and wild-type strains on glucose minimal medium plates. Gross phenotypic differences observed for the  $\Delta Afc1rD$  mutant include reduced radial growth, reduced conidiation, presence of a white colony margin, and colony crenulation after growth for 3 days at 37°C on GMM. Strain numbers correspond to the following genotypes: WT, AF293; WT with *pyrG*, TJW55.1;  $\Delta Afc1rD$ , TJMP1.11; Comp 4.40, TRMP4.40.

**AfClrD is required for normal growth and BrIA-mediated conidiophore development.** The  $\Delta Afc1rD$  mutant had reduced pigmentation on the reverse side of the colony, an increased white colony margin, and increased colony crenulation on GMM at 37°C (Fig. 4). The deletion strain showed a significant reduction in radial growth when cultures were grown on GMM at 37°C (Fig. 5A). Furthermore, conidial production was comparatively decreased in the  $\Delta Afc1rD$  mutant after 5 days of growth on GMM using both an overlay inoculation method and a point-inoculation method (Fig. 5B and C). 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 the methods of Mah and Yu (27). 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 h and then shifted to solid medium. Conidiophore formation was temporally identical in both regimens after the shift, indicating that  $\Delta Afc1rD$  is delayed in conidiogenesis after reaching competence.  $\Delta Afc1rD$  was delayed in conidiophore formation by 3 to 4 h (Fig. 6C).

Conidiophore development is under strict control of several genes in *Aspergillus* spp. (1). Northern analysis of four conidiogenesis genes (*AfbrlA*, *AfwetA*, *AffluG*, and *AfvosA* [27, 33]) indicated a clear delay in expression of the transcriptional activator *AfbrlA* and possibly a slight delay in *AfwetA*, but not *AffluG* or *AfvosA* (Fig. 6B).

**$\Delta Afc1rD$  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

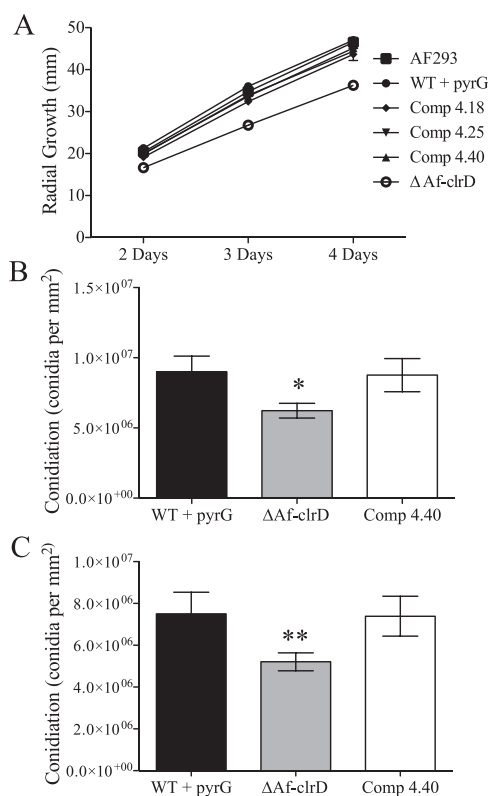


FIG. 5. Quantification of colony diameter and conidial production in  $\Delta Afc1rD$ , complemented control, and wild-type strains. Standard physiological experiments demonstrated growth abnormalities in the  $\Delta Afc1rD$  strain. (A) Radial growth of  $\Delta Afc1rD$  on GMM at 37°C was significantly reduced compared to the 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 37°C in  $\Delta Afc1rD$  (TJMP1.11) compared to wild type with *pyrG* (TJW55.1) and an *Afc1rD*-complemented control strain (TRMP4.40) (\*,  $P = 0.026$ ; \*\*,  $P = 0.028$ ). Error bars indicate 1 standard deviation.

replication efficiency), and 6AU (an inhibitor of guanine nucleotide synthesis and indicator of transcriptional defects). The  $\Delta Afc1rD$  strain did not show differential sensitivity to thiabendazole, and there was only a slight trend toward higher susceptibility to hydroxyurea than the wild type (data not shown). However, the mutant did show increased sensitivity to 6AU at all concentrations tested (25  $\mu\text{g/ml}$ , 50  $\mu\text{g/ml}$ , 100  $\mu\text{g/ml}$ , and 300  $\mu\text{g/ml}$ ) in a radial growth assay (data not shown). Additionally,  $\Delta Afc1rD$  remained sensitive to 6AU (100  $\mu\text{g/ml}$ ) over time (Fig. 7A).

To explore in more detail the mechanism of the 6AU response, we analyzed expression of IMP dehydrogenase (*Afimd2*) and pyrimidine nucleotidase (*Afsdt1*). Neither *Afimd2* nor *Afsdt1* was differentially induced in  $\Delta Afc1rD$  compared to control strains upon challenge with 100  $\mu\text{g/ml}$  6AU (Fig. 7B). Additionally, genes involved in 6AU sensitivity that are independent of IMP dehydrogenase activity in yeast (*Afdst1*, *Afspt3*, *Afpop2*, and *Atrf1*) were examined but were not differentially expressed in the  $\Delta Afc1rD$  mutant compared to control strains (Fig. 7B).

**Conidial uptake by macrophages is unaltered in  $\Delta Afc1rD$ .** Alveolar macrophages represent the first line of host defense to inhaled *Aspergillus* conidia. To determine whether *Afc1rD*

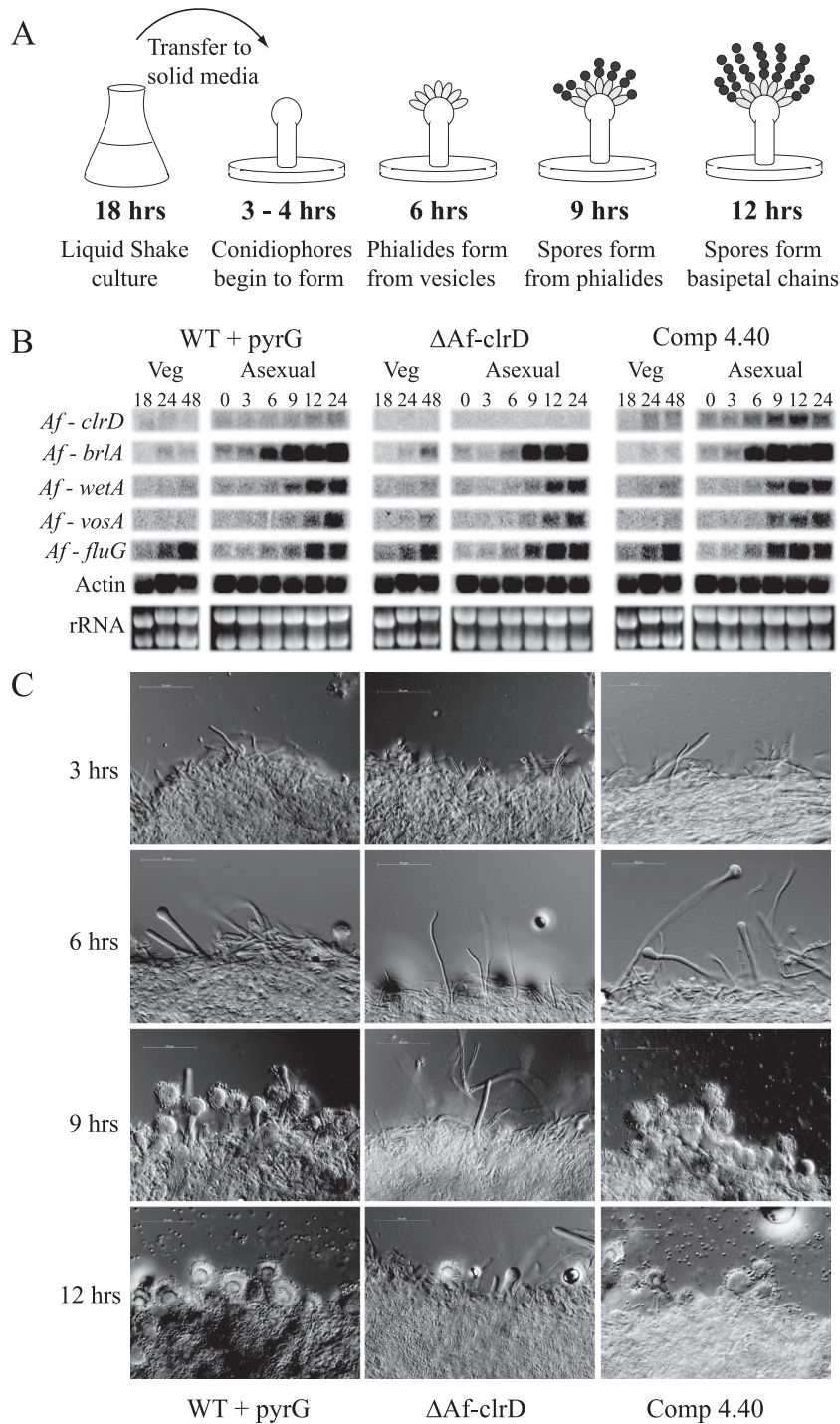


FIG. 6. Developmental competence assays to compare  $\Delta$ AfclrD, control, and wild-type strains. (A) Diagram of the developmental competence assay and relative timing of conidiophore development in wild-type *A. fumigatus*. (B) Northern analysis to determine expression of key genes in the conidiogenesis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

contributes 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 similarly to primary murine alveolar mac-

rophages (unpublished results). Neither the number of macrophages containing conidia nor the average number of conidia per macrophage was significantly different among strains (Fig. 8 and data not shown). Additionally,  $\Delta$ AfclrD

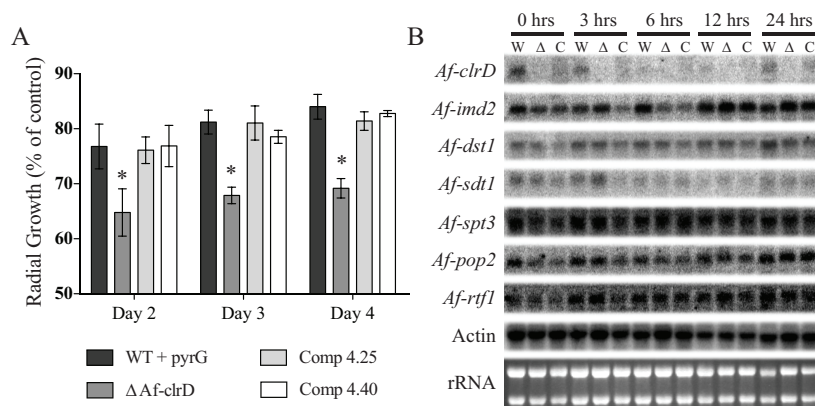


FIG. 7.  $\Delta$ *AfclrD* is more sensitive to 6AU than wild-type or complemented control strains. (A) The  $\Delta$ *AfclrD* mutant (TJMP1.11) remained sensitive over 3 days of exposure to 100  $\mu$ g/ml 6AU compared to the wild type with *pyrG* (TJW55.1) and two complemented control strains (TRMP4.25 and TRMP4.40). Error bars indicate 1 standard deviation (\*,  $P < 0.001$ ). (B) Northern analysis of mycelia challenged with 6AU resulted in induction of *Afimd2* and *Afsdt1* 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 (*Afdst1*, *Afspt3*, *Afpop2*, and *Aftrf1*). Abbreviations: W, TJW55.1;  $\Delta$ , TJMP1.11; C, TRMP4.40.

conidia displayed similar susceptibility as wild-type conidia to hydrogen peroxide, an indicator of reaction to host reactive oxygen species (data not shown). These experiments suggest that *AfclrD* may be dispensable for defense against host alveolar macrophages.

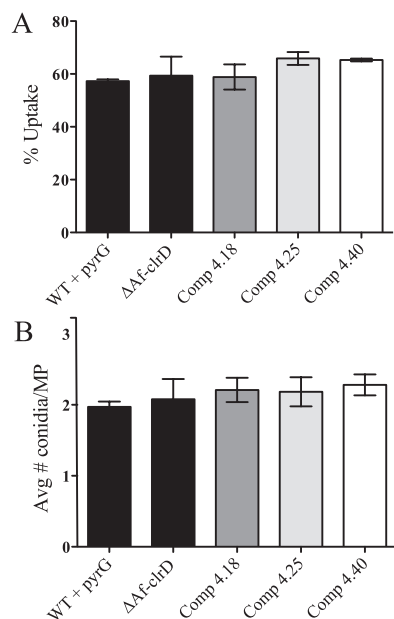


FIG. 8. Conidial uptake by macrophages is not altered in  $\Delta$ *AfclrD*. RAW 264.7 macrophages were coincubated with freshly harvested conidia of the wild-type,  $\Delta$ *AfclrD*, and three complemented controls for 1 hour at 37°C, washed, and incubated an additional 2 hours to permit conidial phagocytosis. Cultures were fixed and stained with calcofluor white to label extracellular conidia. The percent uptake (percentage of macrophages containing one or more conidia) (A) and the average number of conidia per macrophage (of those containing conidia) (B) were calculated from triplicate wells per strain. At least 100 macrophages were counted per well. Means are presented, and error bars indicate 1 standard deviation. Data represent at least three independent experiments.

## 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 references 7 and 19). These studies highlight the impact of H3K9 methylation on normal growth and development. Here, we provide evidence that AfClrD 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  $\Delta$ *AfclrD* strain. Furthermore, AfClrD 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  $\Delta$ *AfclrD* strain 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  $\Delta$ *AfclrD* and complemented control strains indicates strong involvement of AfClrD in mono- and trimethylation of H3K9, but not in dimethylation. Several studies from model organisms suggest that different methyltransferases are involved in differential methylation of H3K9 at distinct chromosomal regions. For example, mammalian H3K9 trimethylation by SUV39H1 is associated with pericentric heterochromatin, but centromeric regions contain dimethylated H3K9 independent of SUV39H1 activity (25). Additionally, the human G9a histone methyltransferase is associated with euchromatic gene activation by mono- and dimethylation 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 5 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 trimethylation. Tamaru and Selker (47) 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  $\Delta AfclrD$ . The authors suggested that trimethylation 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 trimethylation 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 dimethylation of H3K9 were 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  $\Delta AfclrD$  strain, but competence (the ability to form conidiophores) was not affected. Decreased conidial production and delay in maturation may be a function of delayed *AfbrlA* 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 *AfwetA* expression observed in  $\Delta AfclrD$  may be the result of delayed *AfbrlA* 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 *AfvosA* expression, suggesting that once formed, conidia were fully functional in  $\Delta AfclrD$ . Interestingly, Mah and Yu (27) reported that *AffluG* was not required for conidiation in *A. fumigatus*, contrary to its role in *A. nidulans*. However, *AfbrlA* expression was delayed in the  $\Delta AfclrD$  mutant. *AffluG* expression was unaltered in the  $\Delta AfclrD$  mutant background compared to wild type, indicating that *AfClrD* affects conidiation through an *AfFluG*-independent pathway and functions upstream of *AfBrlA*. Since the  $\Delta AfclrD$  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 (14), we did not observe a reduction in radial growth on thiabendazole compared to control plates for the  $\Delta AfclrD$  mutant (data not shown).  $\Delta AfclrD$  mutants were slightly more susceptible to hydroxyurea treatment, but this trend was not statistically significant (data not shown). From these experiments, we concluded that  $\Delta AfclrD$  does not have a serious mitotic defect.

In contrast, the  $\Delta AfclrD$  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 (*Afimd2*) or pyrimidine nucleotidase (*Afsdt1*) transcript levels were not observed in  $\Delta AfclrD$ , wild-type, or 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 the wild type and  $\Delta AfclrD$  for transcriptional elongation factor S-II (*Afsdt1*), a member of the PAF complex (*Aftrf1*), a member of the SAGA histone acetyltransferase complex (*Afspt3*), or a transcription factor associated with transcriptional processes (*Afpop2*). While a defect in transcriptional machinery could explain the pleiotropic phenotype of  $\Delta AfclrD$ , understanding the mechanism of *AfClrD* involvement in 6AU sensitivity requires further analysis.

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