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April 2002

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Peter R. Kraus
University of Connecticut Health Center

Amy F. Hoffman
University of Connecticut Health Center, Farmington, CT

Steven D. Harris
University of Nebraska-Lincoln, Steven.Harris@umanitoba.ca

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Published in *FEMS Microbiology Letters* **210**:1 (April 2002), pp. 61-66; doi:10.1111/j.1574-6968.2002.tb11160.x
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http://www.sciencedirect.com/science/journal/03781097

Submitted January 2, 2002; revised and accepted February 25, 2002; published online March 20, 2002.

Characterization of the *Aspergillus nidulans* 14-3-3 homologue, ArtA

Peter R. Kraus[1]*, Amy F. Hofmann[1], and Steven D. Harris[1,2,†]

[1] Department of Microbiology, University of Connecticut Health Center, Farmington, CT 06030-3205, USA
[2] Plant Science Initiative, University of Nebraska–Lincoln, N234 Beadle Center, Lincoln, NE 68588-0660, USA

* Current address: Department of Genetics, Duke University Medical School, Durham, NC 27710, USA.
[†] Corresponding author, N234 Beadle Center, University of Nebraska–Lincoln, Lincoln, NE 68588-0660; Tel. (402) 472-2938; Fax (402) 472-3139; Email: sharri1@unlnotes.unl.edu

Abstract: The 14-3-3 family of proteins function as small adaptors that facilitate a diverse array of cellular processes by mediating specific protein interactions. One such process is the DNA damage checkpoint, where these proteins prevent inappropriate activation of cyclin-dependent kinases. The filamentous fungus *Aspergillus nidulans* possesses a highly conserved 14-3-3 homologue (*artA*) that may function in an analogous manner to prevent septum formation. However, instead of blocking septation, over-expression of *artA* causes a severe delay in the polarization of conidiospores. This observation suggests that these proteins play an important role in hyphal morphogenesis.

Keywords: Fungal, Cell cycle checkpoint, Polarity, 14-3-3, Septation, *Aspergillus*

1 Introduction

14-3-3 proteins are a group of small, highly conserved, acidic proteins that have been implicated in a wide variety of cellular processes in eukaryotes. Although these proteins function in apoptosis, signal transduction, cell cycle regulation and transcriptional regulation, their exact role in these processes remains unclear [1]. Recently, general themes have begun to emerge which have led to a better understanding of 14-3-3 function [2]. First, 14-3-3 proteins may function as scaffold proteins in various processes. Second, they may act as specific determinants that alter the cellular localization of binding partners. Third, they may be involved in direct regulation of enzymatic activity.

The two yeast model organisms, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, provide excellent systems for the study of 14-3-3 proteins due to the availability of powerful genetic and biochemical techniques. *S. cerevisiae* possesses two 14-3-3 isoforms, encoded by the *BMH1* and *BMH2* genes [3]. Several interacting partners of Bmh1p and Bmh2p have been identified, implicating these proteins in numerous cellular processes such as vesicle transport [3,4], TOR-mediated signaling [5], regulation of transcription [6] and Ras/MAPK signaling during pseudohyphal development [7]. In the latter process, Bmh1p and Bmh2p associate with the Ste20p kinase, which is an upstream activator of a MAP kinase pathway responsible for triggering the cell elongation that is characteristic of pseudohyphal development.

In *S. pombe*, the *rad24* and *rad25* genes encode 14-3-3 homologues. These genes play a role in the timing of mitosis and the regulation of the DNA damage checkpoint [8]. Rad24 functions as an inhibitor of the Tyr-15 phosphatase Cdc25 when the DNA damage checkpoint is activated, thus preventing activation of the cyclin-dependent kinase Cdc2. The mechanism of Rad24 action is unclear at present. Early models suggested that Rad24 functioned by sequestering Cdc25 in the cytoplasm away from nuclear Cdc2, thus preventing the activating dephosphorylation catalyzed by Cdc25 [9]. Recent studies have shown this model to be inadequate, and current possibilities for the function of Rad24 include facilitating a direct inhibition of Cdc25 catalytic activity by Chk1 [10,11].

We have identified a gene encoding a 14-3-3 protein in the filamentous fungus *Aspergillus nidulans*, which we named *artA*. Cytokinesis in *A. nidulans* germlings occurs by the formation of crosswalls called septa [12]. Septum formation is uncoupled from nuclear division in early *A. nidulans* germlings, and genetic evidence suggests that the ability to uncouple septum formation from nuclear division

depends upon regulation of the cyclin-dependent kinase NimX [13]. The phosphorylation state of NimX is a critical factor in the decision to undergo septation [13,14], and we hypothesized that ArtA regulates the timing of septum formation by preventing dephosphorylation of NimX in a manner similar to the regulation of *S. pombe* Cdc2. To test this hypothesis, we over-expressed the ArtA and assessed its ability to act as a dose-dependent inhibitor of septum formation. Our results suggest that ArtA is not involved in the regulation of septum formation; however, it may be involved in a pathway regulating morphogenesis during conidiospore germination.

2 Materials and methods

2.1 Cloning and identification of artA

A sequence found in the *A. nidulans* EST database compiled by the *Aspergillus* Sequencing Project at the University of Oklahoma showed significant homology to other fungal 14-3-3 proteins using the BLAST algorithm [15]. Oligonucleotide primers were constructed based on the EST sequence, and they were used in a PCR-based screen of an ordered, chromosome-specific cosmid library containing *A. nidulans* genomic DNA organized in 96-well microtiter plates (Fungal Genetics Stock Center, Kansas City, KS, USA). Sequential PCR reactions using whole-chromosome cosmid pools, and various sub-pools, revealed the presence of the 14-3-3 gene on cosmids W06C11 and W25G09 (based on the amplification of a band of the predicted size of 300 bp). The presence of the 14-3-3 homologue on these cosmids was confirmed by Southern analysis of cosmid DNA. The gene was named *artA*, for *Aspergillus*rad twenty-four, because the predicted amino acid sequence initially showed the greatest degree of similarity to *S. pombe* Rad24. To determine the cDNA sequence, oligonucleotide primers were constructed that corresponded to *artA* genomic sequences found upstream and downstream from the coding region. These primers were used in PCR reactions using a λZAP-based cDNA library (gift from G. May, Baylor College of Medicine, Houston, TX, USA). The largest fragment amplified was subcloned and sequenced, and the sequence was deposited in GenBank under accession No. AF348497. Sequence comparisons were obtained using the PSI-BLAST algorithm at NCBI [16]. Isolation of RNA and Northern analysis were performed as previously described [17].

2.2 Construction of an artA over-expression plasmid

A 1.6-kb *Xho* I genomic fragment which contains the entire *artA* coding sequence was subcloned into the plasmid pBluescript (Stratagene Corporation, La Jolla, CA, USA) to make pXH1.6. A 1.6-kb *Kpn* I-*Bam* HI fragment from pXH1.6 was subcloned into the *A. nidulans* expression plasmid pSDW194 (gift from S. James, Gettysburg College, Gettysburg, PA, USA), which contains the *argB* selectable marker and the alcohol-inducible *alcA* promoter [18]. The resulting plasmid, pSDWartA, was transformed into the arginine-requiring strain AML13 to produce APK99. Southern analysis of genomic DNA was used to confirm that a single copy of pSDWartA had integrated at the *artA* locus.

2.3 Strains and growth conditions

Media used were MNV (1% glucose, nitrate salts, trace elements and 0.01% vitamins) and MNV-EtOH (MNV with 20 ml l^{-1} ethanol as the only carbon source). Nitrate salts, trace elements, and vitamins were prepared and used as previously described [19]. Strains carrying the *argB2* mutation were supplemented with 0.02% arginine. For solid media, 1.8% agar was added. Strains used were: MO73 (*nimT23*; *pabaA6*, gift from Stephen Osmani, Ohio State University, Columbus, OH, USA), AML13 (*argB2*; *pabaA6*; *yA2*), AML30 (*argB2*; *pabaA6*; *yA2* [arg^+, pSDW194]), APK99 (*argB2*; *pabaA6*; *yA2* [arg^+, pSDWartA]), and APK118 (*argB2*; *yA2*; *nimT23* [arg^+ pSDWartA]).

2.4 Staining, microscopy, and measurements

Growth of strains on coverslips, fixation of samples, staining of coverslips, mounting of coverslips on glass slides, and measurements of cell length were performed as previously described [12,13].

3 Results

3.1 Isolation and characterization of artA

The DNA damage checkpoint pathway in *S. pombe* requires the function of the 14-3-3 proteins Rad24 and Rad25 [8]. Rad24 functions as an inhibitor of Cdc25 when the DNA damage checkpoint is activated, thus allowing the maintenance of Cdc2 Tyr-15 phosphorylation. Furthermore, *Δrad24* cells have a "semi-wee" phenotype, possibly due to a premature entry into mitosis [8]. This demonstrates that the Rad24-mediated inhibition of Cdc25 may be important for the timing of mitosis during normal cell cycles in *S. pombe*. Based on these results, we hypothesized that 14-3-3 protein-mediated inhibition of the Cdc25 ortholog NimT may contribute to the regulation of septum formation in *A. nidulans*. To test this, a gene encoding a 14-3-3 homologue (ArtA) was isolated and characterized based on sequences found in the *Aspergillus* EST database [15]. A comparison of the predicted amino acid sequence

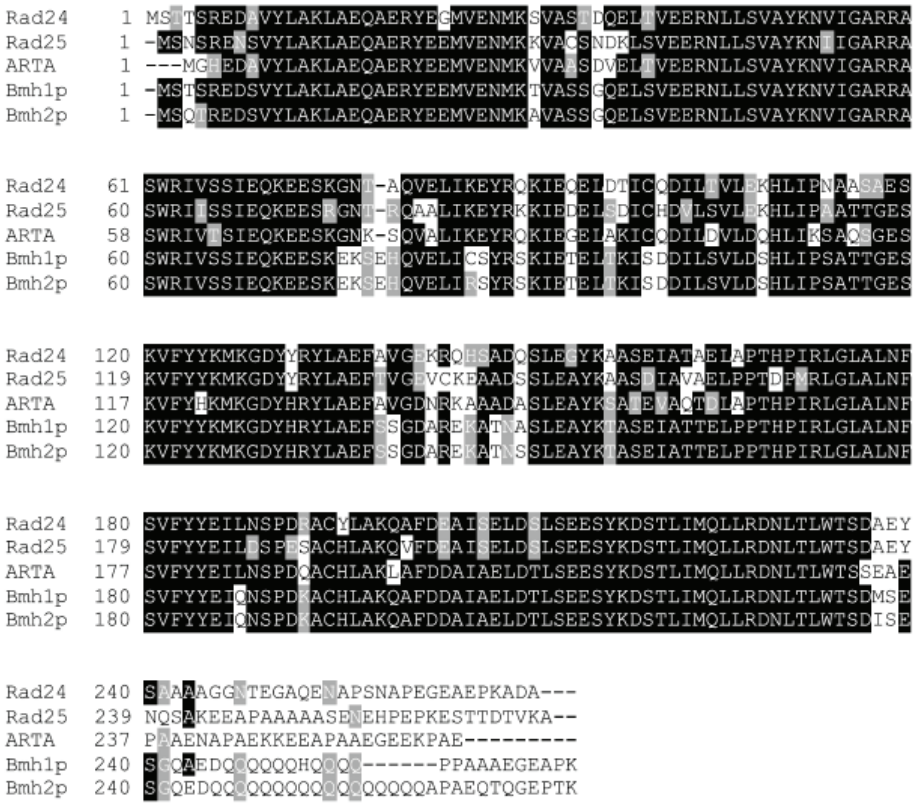

Figure 1. Alignment of 14-3-3 protein sequences from unicellular fungi and *A. nidulans*. Rad24 and Rad25 are from *S. pombe*, and Bmh1p and Bmh2p are from *S. cerevisiae.* Note the high degree of identity throughout the majority of the N-terminal portions of the proteins, and the divergent C-terminal regions.

of ArtA with the yeast 14-3-3 proteins is shown in Figure 1. The level of identity between ArtA and the yeast homologues ranges from 75 to 80%, and the level of similarity ranges from 84 to 89%. Moreover, ArtA displays greater than 80% identity to 14-3-3 homologues recently identified in several filamentous fungi, including *Schizophyllum commune*. Notably, like other 14-3-3 proteins, the fungal homologues share little sequence homology within the carboxy-terminal tail region (Figure 1).

3.2 Over-expression of artA does not block septation

If ArtA functions as an inhibitor of NimT, its over-expression could conceivably inhibit septum formation in a dose-dependent fashion. To test this, we constructed a strain, APK99, possessing an additional copy of the *artA* gene behind the ethanol-inducible *alcA* promoter (see Section 2). Formation of conidiating colonies by the *alcA::artA* strain is severely impaired on plates containing ethanol (Figure 2), suggesting that over-expression of *artA* may be interfering with the initiation of asexual development. This was confirmed by demonstrating that conidiation is severely reduced in the *alcA::artA* strain compared to wild-type when grown on MNV-EtOH (Table 1), and that developmental structures were completely absent following microscopic examination. To test the effect of over-expressing *artA* on the regulation of septum formation, we germinated *alcA::artA* cells in MNV-EtOH media for 32 h and compared the septation index to a control strain transformed with pSDW194 vector alone. The septation index for the *alcA::artA* strain was 54.7±3.0, compared to 69.2±5.5 for the strain containing the vector alone (mean±S.E., n=3). Although the *alcA::artA* strain displayed a slight reduction in septation, it was not accompanied by an increase in the length or number of nuclei in hyphae containing a single septum, which would be indicative of a septation delay [13]. Rather, the slight difference is likely due to a defect in germ tube establishment, as described below.

Table 1: Reduced conidiation by the *alcA::artA* strain on ethanol media

	Conidia ml^{-1} ($\times 10^6$)	
	vector alone	*alcA::artA*
Dextrose		
72 h	330	430
96 h	460	590
EtOH		
96 h	240	0.16
120 h	460	0.3

Conidial suspensions containing 5×10^3 conidia from strains with the indicated genotype were spread on MNV or MNV-EtOH agar and allowed to incubate at 28 °C for the time indicated. The entire contents of the plates were harvested in 4 ml of water and the conidia were counted using a hemacytometer. Strains used were AML30 (vector alone) and APK99 (*alcA::artA*).

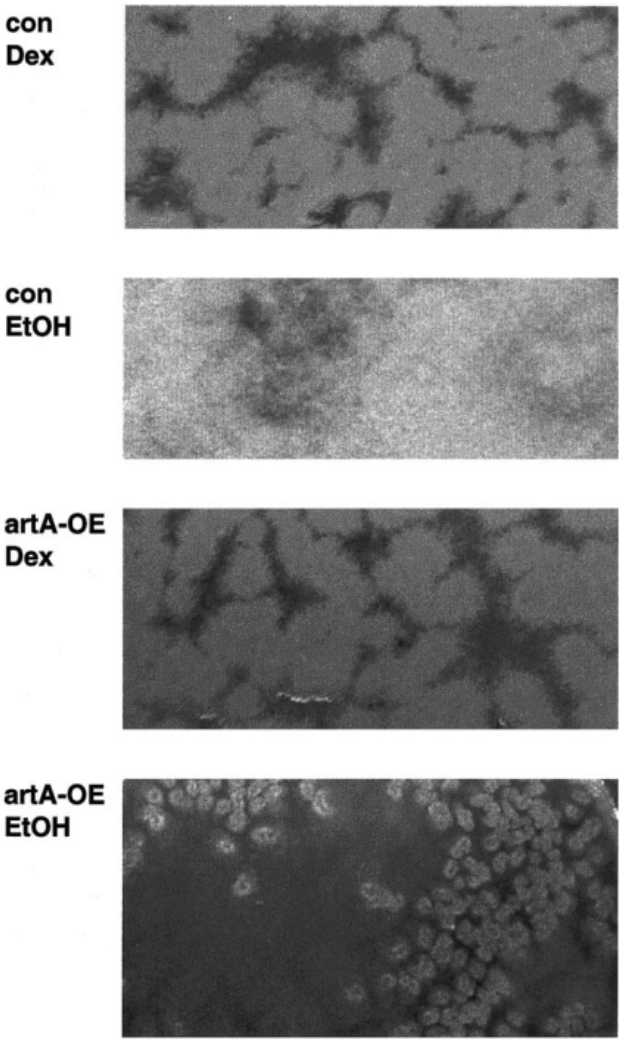

Figure 2. Over-expression of *artA* impairs colony formation. Conidiospores from strains AML30 (vector control; con) and APK99 (*alcA::artA*; artA-OE) were plated on MNV (Dex) or MNV-EtOH (EtOH) plates and incubated for 5 days at 28 °C. AML30 forms diffuse, conidiating colonies on MNV-EtOH. In contrast, APK99 forms compact, aconidial colonies.

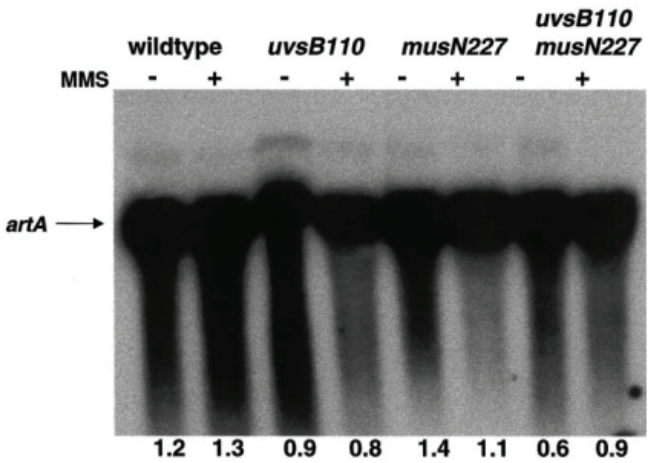

Figure 3. *artA* transcript levels are not affected by DNA damage. RNA was prepared from hyphae exposed to 0.025% MMS for 3 h (+) and from untreated controls (–). *artA* was detected on Northern blots using a non-radioactively labeled 300-bp PCR fragment. Transcript levels, normalized to a control probe from the *acnA* gene and determined using a phosphoimager, are shown below each lane.

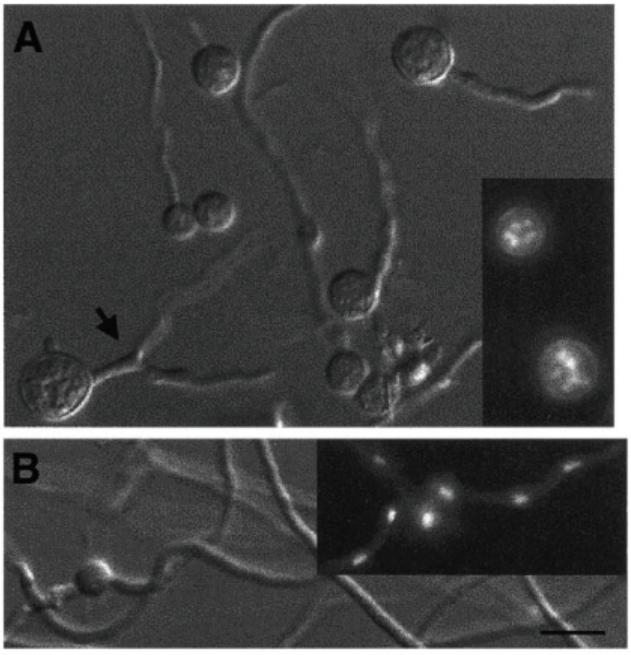

Figure 4. Over-expression of *artA* affects hyphal morphogenesis. Strains were incubated on coverslips in MNV-EtOH for 42 h at 28 °C. Coverslips were visualized by DIC optics. A: APK99 (*alcA::artA*). The arrow depicts an aberrant branch site. B: AML30 (vector control). Insets show swollen conidiospores (A) or hyphae (B) stained with Hoechst 33258 and Calcofluor to visualize nuclei and cell wall material, respectively. Bar, 10 μm.

In *A. nidulans*, activation of the DNA damage response triggers a block to septum formation [13]. Accordingly, if *artA* functions as an inhibitor of septation, its expression may be induced by DNA damage. However, *artA* transcript levels show little change in wild-type hyphae that have been treated with the genotoxin MMS, or in mutants defective in the induction (*uvsB110*) or termination (*musN227*) of the DNA damage response [17] (Figure 3).

3.3 Over-expression of artA impairs germ tube formation

Microscopic examination of *alcA::artA* hyphae grown on MNV-EtOH for 42 h revealed the presence of swollen conidiospores and aberrantly branched hyphae (Figure 4). To further characterize this phenotype, populations of *alcA::artA* cells containing two and four nuclei were scored for the presence of a germ tube and compared to vector-transformed controls. Whereas >95% of control spores had germinated prior to reaching the two nuclei stage, a significant proportion of *artA::alcA* spores possessing four nuclei (31%) had not formed polarized hyphae (Figure 5). These results suggest that ArtA has an important function in the regulation of germ tube formation and hyphal morphogenesis,

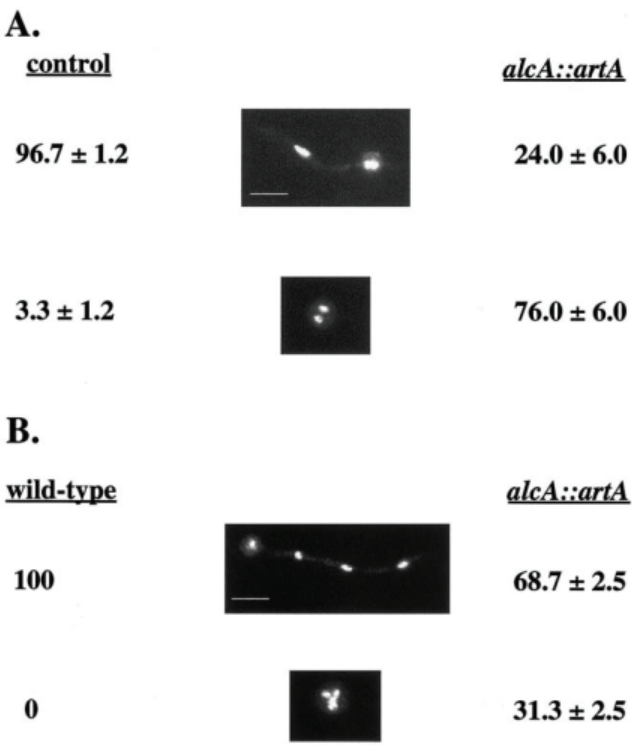

Figure 5. Over-expression of *artA* impairs germ tube formation. Strains with the indicated genotype were incubated on coverslips in MNVEtOH for 20 h (A) or 24 h (B) at 28 °C. Coverslips were stained with Hoechst 33258 to visualize nuclei. Populations of germlings (n = 200) with two nuclei (A) or four nuclei (B) were scored for the presence of a germ tube. Representative germlings or conidiospores containing two nuclei (A) or four nuclei (B) are shown. Bars, 10 μm.

which could account for the reduced size and colonial appearance of *alcA::artA* colonies formed on MNV-EtOH plates (Figure 2). Moreover, inactivation of the NimT phosphatase by the *nimT23* mutation had no effect on the kinetics of germ tube formation in *alcA::artA* spores, suggesting that the effects of *artA* over-expression are not mediated by inhibition of NimT.

4 Discussion

Negative regulation of the Cdc25 phosphatase by 14-3-3 proteins in the simple eukaryote *S. pombe* and the complex eukaryotes *Xenopus laevis* and *Homo sapiens* is involved in regulation of the DNA damage checkpoint as well as aspects of development [10,20,21]. Therefore, we hypothesized that the timing of septum formation in *A. nidulans* may be controlled by negative regulation of NimT by a 14-3-3 protein. However, we found that over-expression of the 14-3-3 protein ArtA causes defects in germ tube establishment and asexual development, and has no apparent effect on the timing of septum formation. Since exhaustive attempts to construct an *artA* gene replacement using conventional approaches [22] and a recently developed high-throughput strategy [23] have been unsuccessful, we have been unable to further characterize the potential role of ArtA in septum formation.

In *S. cerevisiae*, the 14-3-3 proteins Bmh1p and Bmh2p function in a signal transduction pathway that regulates pseudohyphal growth [7]. Notably, pseudohyphal cells share several morphological features with both elongating germ tubes and specific cell types (i.e. metulae and phialides) found in *A. nidulans* conidiophores [24,25]. Based on the similarities, we suggest that ArtA functions in a pathway regulating polarized growth in *A. nidulans*. Bmh1p and Bmh2p associate with the Ste20p kinase, which is an upstream activator of the MAP kinase pathway responsible for activating several aspects of pseudohyphal development [7]. In addition, they also appear to affect cellular morphogenesis by regulating the actin cytoskeleton and exocytosis [4]. Indeed, over-expression of the carboxy-terminal region of Bmh2p causes defects in vesicle targeting [4]. Accordingly, we propose that excess ArtA may titrate several proteins (i.e. such as the *A. nidulans* ortholog of Ste20p), and thus disrupt the pathways leading to germ tube establishment and conidiophore development.

The presence of multiple 14-3-3 proteins appears to be a typical feature of eukaryotic proteomes. For example, complex eukaryotes may contain up to seven 14-3-3 isoforms, each of which may be involved in different processes [26]. Like the yeasts *S. pombe* and *S. cerevisiae*, *A. nidulans* possesses two 14-3-3 proteins (S. Harris, unpublished observations). Although the yeast paralogues apparently function in related processes, it remains possible that the function of the different 14-3-3 homologues has diverged in *A. nidulans*. Accordingly, ArtA may regulate aspects of cellular morphogenesis, whereas its paralogue modulates cell cycle progression by affecting the Tyr-15 phosphorylation state of NimX.

Acknowledgements

This work was supported by awards from the University of Connecticut Health Center Research Advisory Council and the American Cancer Society (RPG-99-214-01-MBC).

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