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Location and functional analysis of the *Aspergillus nidulans* Aurora kinase confirm mitotic functions and suggest non-mitotic roles

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ABSTRACT

Filamentous fungi have devastating negative impacts as pathogens and agents of food spoilage but also have critical ecological importance and are utilized for industrial applications. The characteristic multinucleate nature of filamentous fungi is facilitated by limiting if, when and where septation, the fungal equivalent of cytokinesis, occurs. In the model filamentous fungus *Aspergillus nidulans* septation does not occur immediately after mitosis and is an incomplete process resulting in the formation of a septal pore whose permeability is cell cycle regulated. How mitotic regulators, such as the Aurora kinase, contribute to the often unique biology of filamentous fungi is not well understood. The Aurora B kinase has not previously been investigated in any detail during hyphal growth. Here we demonstrate for the first time that Aurora displays cell cycle dependent locations to the region of forming septa, the septal pore and mature septa as well as the mitotic apparatus. To functionally analyze Aurora, we generated a temperature sensitive allele revealing essential mitotic and spindle assembly checkpoint functions consistent with its location to the kinetochore region and spindle midzone. Our analysis also reveals that cellular and kinetochore Aurora levels increase during a mitotic spindle assembly checkpoint arrest and we propose that this could be important for checkpoint inactivation when spindle formation is prevented. We demonstrate that Aurora accumulation at mature septa following mitotic entry does not require mitotic progression but is dependent upon a timing mechanism. Surprisingly we also find that Aurora inactivation leads to cellular swelling and lysis indicating an unexpected function for Aurora in fungal cell growth. Thus in addition to its conserved mitotic functions our data suggest that Aurora has the capacity to be an important regulator of septal biology and cell growth in filamentous fungi.

1. Materials and methods

1.1. A. nidulans manipulation and strain generation

Standard media and techniques were used to culture and genetically manipulate *A. nidulans* as previously described (Oakley and Osmani, 1993; Todd et al., 2007). Endogenous C-terminal GFP tagging of Aurora (AN5815) was carried out using fusion PCR to generate a gene replacement construct incorporating a GFP::*pyrG*^{Af} cassette amplified from plasmid pFN03 (Yang et al., 2004). The full length *aurora*-GFP::*pyrG*^{Af} gene replacement construct was transformed into the SO451 recipient strain containing the *pyrG*89 nutritional marker and the Δ*nkuA*^{ku70} mutation to facilitate a high frequency of homologous recombination (Nayak et al., 2006). *pyrG*$^{+}$ transformants were selected on media lacking uridine and uracil and streaked to single colony. Site specific integration was confirmed by diagnostic PCR using primers flanking the site of gene replacement. Endogenously tagged versions of Aurora are functional as, unlike the null allele (De Souza et al., 2013), they are viable and also display no growth defects. Tagged versions of Ndc80, Nup49 and the TubA α-tubulin have been used previously and are functional as strains grow normally and do not display the growth defects of the null alleles (De Souza et al., 2009; Osmani et al., 2006; Ovechkina et al., 2003). Strains and primers used in this study are listed in Supplementary Tables S1 and S2 respectively.

1.2. Generation of the aurA3 allele

An *aurA*3::*pyroA*^{Af} construct to replace the C-terminus of Aurora substituting histidine 365 with tyrosine was generated by fusion PCR (Supplementary Fig. S1). Initially 2070 bp, 262 bp and 965 bp fragments were amplified from genomic DNA using primer pairs

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CDS228/CDS376, CDS372/CDS369 and CDS373/Nat4 respectively of which primers CDS372 and CDS376 introduced the point mutation. Extensions on primers CDS369, CDS372 and CDS373 facilitated fusion of the 3 fragments together with the *pyroA*^{Af} nutritional marker to generate the full length 3267 bp *aurA*3:: *pyroA*^{Af} construct using the nested primers Nat3 and Nat8 (Supplementary Fig. S1) (Osmani et al., 2006; Yang et al., 2004). This construct was transformed into strain SO451 selecting for complementation of the *pyroA*4 nutritional marker. Transformants were tested for colony formation at 20°, 25°, 32°, 37° and 42° and temperature sensitive transformants streaked to single colony. Introduction of the *aurA*3 mutation in temperature sensitive transformants was confirmed by sequencing the *aurora* locus. Propagation and genetic crossing of *aurA*3 strains was carried out below 25° due to the high degree of temperature sensitivity of the mutant.

1.3. Microscopic imaging and analysis

Imaging was carried out at room temperature in liquid cultures of germlings grown in 35 mm glass-bottom microwell dishes (MatTech) using minimal media with 55 mM glucose as the carbon source and 10 mM urea as the nitrogen source. Time lapse images were collected using an Ultraview ERS spinning disk confocal system (Perkin-Elmer) fitted with an Orca-AG camera (Hamamatsu) on a TE2000-U inverted microscope (Nikon) using a 60× 1.40 NA Plan Apochromatic objective (Nikon). For experiments collecting DIC images, imaging was with an Ultraview Vox spinning disk confocal system (Perkin-Elmer) fitted with dual C9100-13 cameras (Hamamatsu) run by Volocity software (Perkin-Elmer). To depolymerize microtubules, cultures were exchanged to media containing 2.4 μg/ml Benomyl (Sigma) 15 min before imaging (Horio and Oakley, 2005). Image analysis, pixel line intensity profiles, kymograph generation, and digital straightening of germlings were carried out using ImageJ freeware (Rasband, WS, ImageJ, US National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/, 1997–2008). Data are displayed as maximal intensity profiles. Statistical analysis and graph generation was carried out using Excel (Microsoft).

To quantify Aurora-GFP fluorescence levels the average pixel intensity less the background was determined for an identical sized region of the nucleus and cytoplasm. Aurora-GFP cytoplasmic, nuclear and bystander fluorescence was normalized relative to the maximal nuclear level during the time course which was set to 100%. Different time series were aligned based upon the time of mitotic entry and the average nuclear or cytoplasmic fluorescence intensity calculated. Due to differences in the length of mitotic arrest similar calculations were carried out for cells aligned based on the time of mitotic exit. Series aligned for mitotic entry and mitotic exit were plotted on the same graph as indicated.

1.4. Generation of mitotic samples for Aurora-DLAP affinity purification

Cultures of strain CDS1057 were inoculated with 2.5×10^6 conidia/ml (Liu et al., 2010) and grown overnight at room temperature to a packed cell volume of 0.2 ml/10 ml. Cultures were then shifted to the *nimT23*^{cdc25} restrictive temperature of 42° for 4 h to cause G2 arrest. After collecting a G2 sample, the remaining culture was shifted to 30° to release cells into mitosis and samples collected. Samples for Aurora-DLAP affinity purification and LC-MS/MS analysis were generated as described (De Souza et al., 2014; Liu et al., 2010). Samples for microscopic analysis were fixed in PBS containing 6% EM grade paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) which maintained the GFP fluorescence of the DLAP tag.

2. Introduction

The filamentous fungi are a fascinating group of organisms with important beneficial ecological, pharmaceutical and industrial impact, but which also have devastating effects as pathogens and agents of food spoilage (Borkovich and Ebbole, 2010). As such understanding filamentous fungal biology is both fundamentally and economically important. Mitosis and cytokinesis are complex processes whose regulation is critical to ensure genetic stability. In most cell types cytokinesis is linked to mitosis such that cell division follows nuclear division. One notable exception occurs during the formation of the syncytial hyphae of filamentous fungi. In the model filamentous fungus *Aspergillus nidulans* germinating uninucleate conidiospores generally undergo 2 rounds of synchronous mitotic nuclear division in which septation, the fungal equivalent of cytokinesis, is suppressed (Clutterbuck, 1970; Harris, 2001). Subsequent to this, septation becomes linked to the synchronous mitoses of tip cells although septa do not form between every post mitotic nucleus thereby maintaining the multinucleate nature of hyphae. Another feature of hyphal septation is that it is an incomplete process in which septal cell walls do not seal resulting in the formation of a central septal pore which connects the hyphal cells facilitating communication and nutrient exchange (Jedd and Pieuchot, 2012). Although many genes involved in regulating septation have been identified (Bruno et al., 2001; De Souza et al., 2013; Harris, 2001; Harris et al., 1994; Kim et al., 2006, 2009; Morris, 1976; Si et al., 2010; Takeshita et al., 2007; Westfall and Momany, 2002), little is known about how septation is initially suppressed following mitosis, spatially regulated along hyphae and halted to form septal pores.

Mitosis is in large part orchestrated by members of the mitotic Cdk, Polo, NIMA related kinase (NEK) and Aurora kinase families which are found in all eukaryotes (Ma and Poon, 2011; Nigg, 2001). A critical feature impacting the function of these kinases is the dynamic localization pattern each displays as cells progress from G2 and through mitosis. For example, as mammalian cells complete mitosis the Aurora B kinase sequentially locates to chromosomal arms, centromeres and the spindle midzone and these locations are important for its roles in regulating chromatin and mitotic spindle formation and function (Carmena et al., 2012; van der Waal et al., 2012). Aurora B is also present at the equatorial cortex during anaphase where it has been suggested to be involved in contractile ring assembly and to initiate cleavage furrow ingression (Carmena et al., 2012; Kitagawa et al., 2013). As cells exit mitosis Aurora B remains associated with microtubules as they rearrange to form the midbody present in the intercellular bridge connecting daughter cells where Aurora B helps coordinate cytokinesis (Agromayor and Martin-Serrano, 2013; Carmena et al., 2012; Murata-Hori et al., 2002).

To help ensure the fidelity of mitosis and cytokinesis cells monitor these processes and delay cell cycle progression in response to certain defects. The spindle assembly checkpoint (SAC) is a surveillance system which delays anaphase onset until a bipolar spindle has been correctly assembled (Lara-Gonzalez et al., 2012; Musacchio and Salmon, 2007). A hallmark of SAC activation is the recruitment of SAC regulators to kinetochores which assemble around centromeric DNA (Lara-Gonzalez et al., 2012; Musacchio and Salmon, 2007) and which provide the connection sites between chromosomes and the mitotic spindle. Much is known about how the SAC is first activated to inhibit anaphase and then, once the spindle is correctly attached to chromosomes, inactivated to allow anaphase and Aurora B kinases are involved in regulating these processes (Campbell and Desai, 2013; Jin and Wang, 2013; Liu et al., 2009; Nigg,