CHARACTERIZATION OF SEPTINS ASPA AND ASPC IN *ASPERGILLUS NIDULANS*

by

REBECCA LINDSEY

(Under the Direction of Michelle Momany)

ABSTRACT

To divide each cell must determine a division site and form an organizational scaffold to recruit the proper cellular machinery. This machinery works to accurately replicate and distribute cell contents. Septin proteins are important members of this division scaffold, they affect cell growth and morphology in fungi and animals.

Fungi grow in one of two forms: hyphae or yeasts. *Saccharomyces cerevisiae* grows as yeast under most conditions. Here we show: introduction of the septin AspC from the filamentous fungus *A. nidulans* caused *S. cerevisiae* to make elongated cells similar to those of hyphal fungi. AspC localizes to bud and filament necks, while its *S. cerevisiae* ortholog, Cdc12, localizes only to bud necks. Our results suggest that AspC competes with Cdc12 for incorporation into the yeast septin scaffold and once there alters cell shape.

In *A. nidulans* there are five septins AspA, AspB, AspC, AspD, and AspE. We characterized *A. nidulans* septins AspA and AspC in an attempt to learn more. In *S. cerevisiea*, Cdc11 and Cdc12, orthologs of AspA and AspC are essential so results from our studies shed new light on septin function. Deletion mutants show early germination, increased germ tube and branch emergence, abnormal septation and disorganized conidiophores. The unexpected increased growth phenotypes in deletion mutants suggest septins may function as negative regulators or interact with proteins that are negative regulators. AspA-GFP and AspC-GFP show novel fungal septin localization; it is dynamic and found at all stages of growth examined so far. Both septins show different types of animal-like septin localization which is punctate or as micro-filaments. In mammalian cells this type of localization is associated with membrane trafficking or the cytoskeleton. In other organisms septins form multi-septin scaffolds. In *S. cerevisiae* when Cdc11 or Cdc12 is mutated the other septins do not localize. AspA-GFP localizes in *ΔaspC* and therefore septin structure may still be intact. However, AspC-GFP does not show localization in *ΔaspA* suggesting that discrete septin structures are not formed. Our results suggest that in *A. nidulans* AspA and AspC may have different roles in septin complex formation, membrane trafficking and cytoskeletal interactions.

INDEX WORDS: Septin, AspA, AspC, cell division, germ tube emergence, septation

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May 2007

DEDICATION

I would like to dedicate this thesis to my husband Christian Lindsey for his support during this quest. Also to my grandparents Adolf and Virginia Feren who are unable it to see me finish but always supported me.

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INTRODUCTION

To divide, all cells must go through a regulated process. They must determine a division site and form an organizational scaffold to recruit the proper cellular machinery to complete cytokinesis. This machinery works to accurately replicate and then distribute cell contents. Septin proteins are important members of this division scaffold. Septins have been shown to play roles as cytoskeletal elements, in coordinating nuclear division, and in membrane trafficking.

Septins are GTPases that are not found in plants, but they form filaments in all other eukaryotes from fungi to humans (Neufeld and Rubin, 1994; Longtine *et al.*, 1996; Nguyen *et al.*, 2000; Gale *et al.*, 2001; Kartmann and Roth, 2001). Within and between species, septins usually have at least 30% amino acid sequence identity. Their structure is highly conserved, with an amino terminal basic region, a central GTP-binding domain, and in most septins a coiled-coil domain at the carboxy terminus (Figure 1,Longtine *et al.*, 1996).

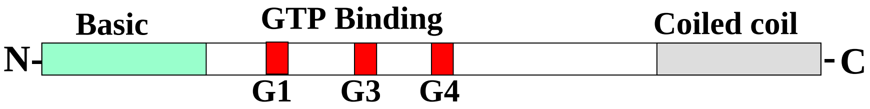

Figure 1.1. General septin protein structure. Septins typically contain a N-terminal basic region; conserved GTP binding domains G1 (GXXXGKT), G3 (DTPG) and G4 (XKXD) a C-terminal coiled coil.

Septin function and localization in *Saccharomyces cerevisiae*

Septins are a family of proteins that were first discovered in *Saccharomyces cerevisiae* (Hartwell, 1971). The original septin mutants, *cdc3, cdc10, cdc11* and *cdc12,* identified in a screen for temperature sensitive cell cycle mutants, make elongated buds at restrictive temperature and are unable to complete cytokinesis. Septins were first visualized by transmission electron microscopy as a set of highly ordered 10 nm filaments forming rings around the mother-bud neck of *S. cerevisiae* (Byers and Goetsch, 1976).

In *S. cerevisiae* there are seven septins, five of which (Cdc3p, Cdc10p, Cdc11p, Cdc12p and Sep7p) co-localize to the neck where they interact with each other to form a scaffold that recruits other proteins to the bud neck. There are over 35 proteins known to interact directly or indirectly with septins at the bud neck (Gladfelter *et al.*, 2001). Cdc3p, Cdc10p, Cdc11p, Cdc12p and Sep7p initially localize as a ring with actin, in late G1 phase, about 15 minutes before bud emergence. Chitin is deposited in the cell wall where the septins are present. New cell wall material is deposited in this area to form the wall of the growing bud. As the bud emerges the septins broaden at the neck and remain there for the rest of the cell cycle. Once the bud is mature Chs3p and Chs4p are localized to the septum and synthesize chitin to form the primary septum (DeMarini *et al.*, 1997). Eventually, some of the primary septum degrades so the mother and daughter cells can separate (Gladfelter *et al.*, 2001; Faty *et al.*, 2002; Kinoshita, 2003; Longtine and Bi, 2003). The remaining two septins, Spr3p and Spr28p, are involved in formation of the double membrane or prospore that encapsulates the haploid nucleus during ascospore formation. These septins along with Cdc3p and Cdc11p localize to the leading edge of the prospore wall (Ozsarac *et al.*, 1995; De Virgilio *et al.*, 1996; Fares *et al.*, 1996).

***S. cerevisiae* septin interaction with actin cytoskeleton**

Septins and actin localize to the bud site at about the same time, but further details are unclear. A number of studies found that septins are able to localize as rings at bud necks when actin is depolymerized and actin is able to localize to bud necks when the septins are mutated (Adams and Pringle, 1984; Ford and Pringle, 1991; Ayscough *et al.*, 1997). Studies in which actin was depolymerized and deletions or mutants of downstream effectors were used suggest that actin may be required to maintain a mature septin ring (Kadota *et al.*, 2004; Kozubowski *et al.*, 2005). The formins, Bni1 and Bnr1, which nucleate or polymerize actin filaments, do interact with the septins at the bud neck (Evangelista *et al.*, 2002; Sagot *et al.*, 2002). Septins and actin are two of many proteins found at the bud neck and it is probable that other proteins are involved.

Septin function and localization in other eukaryotes

Chapter 2 is a review of septin localization across kingdoms (Lindsey and Momany, 2006). We reviewed septin localization and function in *A. nidulans, Candida albicans, Ashbya gossypii, S. cerevisiae, Schizosaccharomyces pombe, Caenorhabditis elegans, Drosophila melanogaster* and mammals. We found that septins with similar localization patterns have been implicated in similar processes: septins at the base of projections (as at the base of the conidium) shape emerging growth and serve as barriers; septins at partitions (as in fly cellularization) compartmentalize previously existing cytoplasm without affecting its overall shape; septins at the base of projections and at partitions frequently coordinate nuclear division with cell division; septins at the cell periphery or throughout the cytoplasm in a punctate pattern are often involved in membrane trafficking; septins that form long filaments co-localize with actin and/or tubulin. These septins either organize the other elements of the cytoskeleton or are organized by them.

Other Eukaryotes and septin interaction with actin cytoskeleton

In mammalian cells, septins and actin co-localize and have been shown to be structurally dependent on each other. In mouse NIH3T3 fibroblasts Sept2, Sept6 and Sept7 localize with long bundles of actin fibers and at cortical arcs and when actin was depolymerized with cytochalasin D septin bundle localization changed to small rings, while the arcs remained (Kinoshita *et al.*, 2002). Similar results were seen in mammalian fibroblast cells with Sept2 and Sept4 (Xie *et al.*, 1999). Kinoshita et. al. suggest septin rings are possibly a default septin form, as pure septin complexes of Sept2, Sept6 and Sept7 form rings in *in vivo* reconstitution studies (Kinoshita *et al.*, 2002). When anillin, a protein that co-localizes with septins and binds F-actin, was added to the reconstitution mix, septin filaments formed (Kinoshita *et al.*, 2002). When RNAi was used to deplete Sept2 and Sept7 in NIH3T3 cells there was a loss of actin bundles (Kinoshita *et al.*, 2002). The authors conclude that septins either stabilize or promote assembly of actin bundles.

Septin complex formation in S. cerevisiae

To date, septins are always found in complexes. They form multi-septin heteropolymers that act as scaffolds to organize other proteins. In *S. cerevisiae* mutants in which *CDC3*, *CDC10*, *CDC11*, or *CDC12* is mutated, the remaining three septins are unable to localize normally to the bud neck (Haarer and Pringle, 1987; Ford and Pringle, 1991; Kim *et al.*, 1991). Versele *et al.* used electron microscopic analysis and reconstitution studies of multi-septin complexes to predict a model of heteropentamer septin complex and sequential filament formation in mitotic cells in which Cdc12 is at the center of the septin complex. They propose each individual septin forms dimers then Cdc3 and Cdc12 associate with each other through their C-terminal ends, Cdc10 then associates with the N-terminal domains of Cdc3 and Cdc12. Cdc11 and Cdc12 are predicted to associate with each other through their N-terminal domains while Shs1 (which is dispensable to complex formation) and Cdc11 associate through C-terminal domains. Paired heteropentamer septin complexes then assemble into filaments that are held together by Cdc10 bridges (Versele *et al.*, 2004).

In *S. cerevisiae* and *Drosophila* septin complexes show very low rates of GTP hydrolysis and guanine-nucleotide exchange (Field and Kellogg, 1999; Vrabioiu *et al.*, 2004). So what role do GTP hydrolysis and guanine-nucleotide exchange have in septin complex or filament formation? Using mutants with defective nucleotides in conserved locations Versele *et. al.* were able to block GTP hydrolysis and/or binding *in vitro.*   They found that *in vivo* septin filaments formation at the bud neck was dependant on GTP binding, while GTP hydrolysis did not have an effect of filament formation (Versele and Thorner, 2004).

Septin complex formation in Other Eukaryotes

Septin-septin complexes are found in higher eukaryotes. In *Drosophila* septin proteins Pnut and Sep1 co-immunoprecipitate and co-sediment suggesting formation of a complex (Fares *et al.*, 1995). In *pnut* null *Drosophila* embryos there was reduced or no staining with Sep1 antibodies (Fares *et al.*, 1995). In mouse studies, Sept5 co-immunoprecipitated with Sept2 and Sept7 and in Sept5 null mice reduced expression levels of Sept7 were found (Peng *et al.*, 2002). In NIH3T3 cells RNAi knockdown of

Sept2 resulted in a slight reduction of Sept7 levels and visa versa (Kinoshita *et al.*, 2002). In mammalian cells the minimal septin formation is three septin dimers forming a heterotrimeric complex (Joberty *et al.*, 2001; Kinoshita *et al.*, 2002; Sheffield *et al.*, 2003). SEPT7 is always present and binds proteins from the SEPT2 (SEPT1, SEPT4, SEPT5) and SEPT6 (SEPT8, SEPT10, SEPT11) groups to form the multiseptin complex (Kinoshita, 2003).

Aspergillus nidulans vegetative growth

A. nidulans is a simple, well-characterized filamentous fungus well suited to the study of septins especially since there are major developmental differences between budding yeast and filamentous fungi (Figure 1.2.). In yeast after mitosis cell division takes place. In *A. nidulans*, asexual conidia break dormancy and grow isotropically and soon after the first round of mitosis they switch to polar growth. The germ tube grows by tip extension while several rounds of mitosis take place and fill the germ tube with new nuclei. The third mitotic division triggers formation of the first septum near the basal end of the germ tube (Figure 2,Momany and Taylor, 2000). After further mitotic divisions septa divide the hypha into regular multinucleate sections. Basal compartments are arrested in interphase until branch formation, at which time mitosis begins in that compartment again. In *A. nidulans,* only the tip cell, with 8-20 nuclei, remains

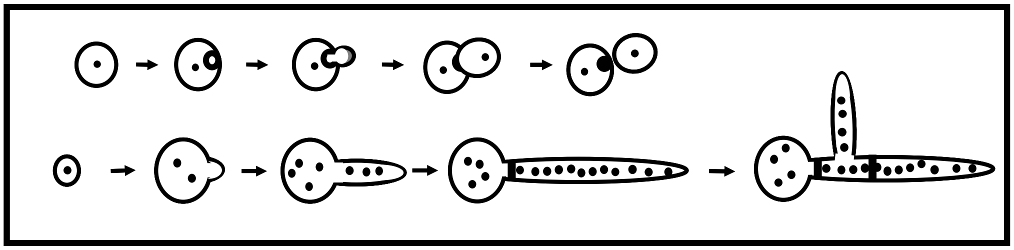

Figure 1.2. *S. cerevisiae* vs. *A. nidulans* development.

mitotically active (Fiddy and Trinci, 1976; Kaminskyj and Hamer, 1998). In *A. nidulans* cytokinesis does not always follow mitosis and does not result in a separate daughter cell allowing separation of septin roles in partitioning vs. organization of new growth.

Septins in A. nidulans

Four of the five *A. nidulans* septins are related by homology to the four main septins in *S. cerevisiae* (Table 1,Momany and Hamer, 1997; Momany *et al.*, 2001; Westfall and Momany, 2002). The fifth, *aspE,* is not closely related to any of the *S. cerevisiae* septins. Previous mutant and immunofluorescence localization studies show that AspB plays a role in cytokinesis, branch initiation and asexual development (Westfall and Momany, 2002).

Table 1.1. *A. nidulans* septin orthologs

***A. nidulans* gene**	***S. cerevisiae* ortholog**
aspA	Cdc11*
aspB	Cdc3*
aspC	Cdc12*
aspD	Cdc10
aspE	n/a
* essential	

A. nidulans septin structure including predicted introns, exons, coiled coils and GTPase domains are shown below (Figure 1.3, Momany *et. al.*, 2001). AspD does not contain a coiled coil which is expected since its *S. cerevisiae* ortholog Cdc10, does not contain one either (Momany *et al.*, 2001). Based on previous work in *A.*

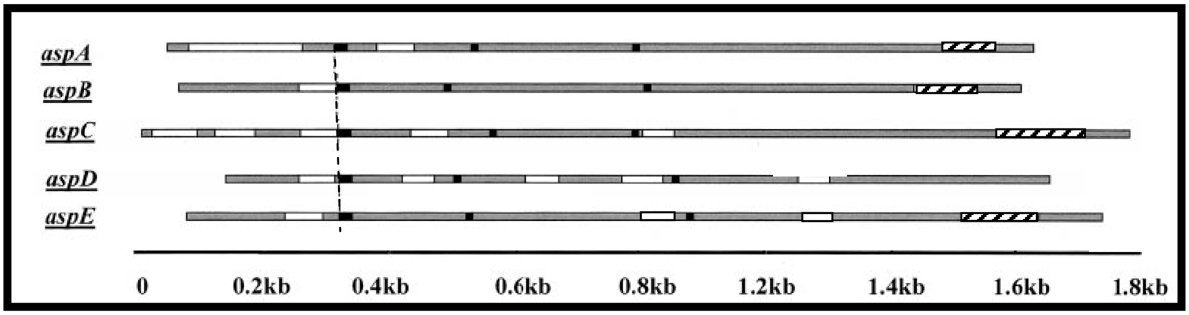

Figure 1.3. Comparison of septin structure *A. nidulans*. Shaded boxes represent exons and open boxes represent introns (shown relative to predicted amino acid sequence of exons). Coiled coil domains are indicated by striped boxes. Solid boxes represent the predicted positions of GTPase domains. A vertical dashed line indicates the first G1 GTPase domain. Redrawn from Momany *et. al.* 2001.

nidulans and other systems, I predict septins AspA and AspC could have roles in the formation of septa, branches, and conidiophores. Potential deletion mutant phenotypes would show abnormalities in septation, branches and conidiophore development. Potential localization would be at septa. AspA and AspC will probably interact with many proteins including other septins, actin and the formin, SepA. Using the model of yeast septin complex and filament formation from Versele *et. al.* as a template (Versele *et al.*, 2004), and incorporating results described in other chapters of this dissertation. I predict that *A. nidulans* septin complex formation is similar except AspA would be at the center of the complex (Figure 1.4., Versele *et al.*, 2004).

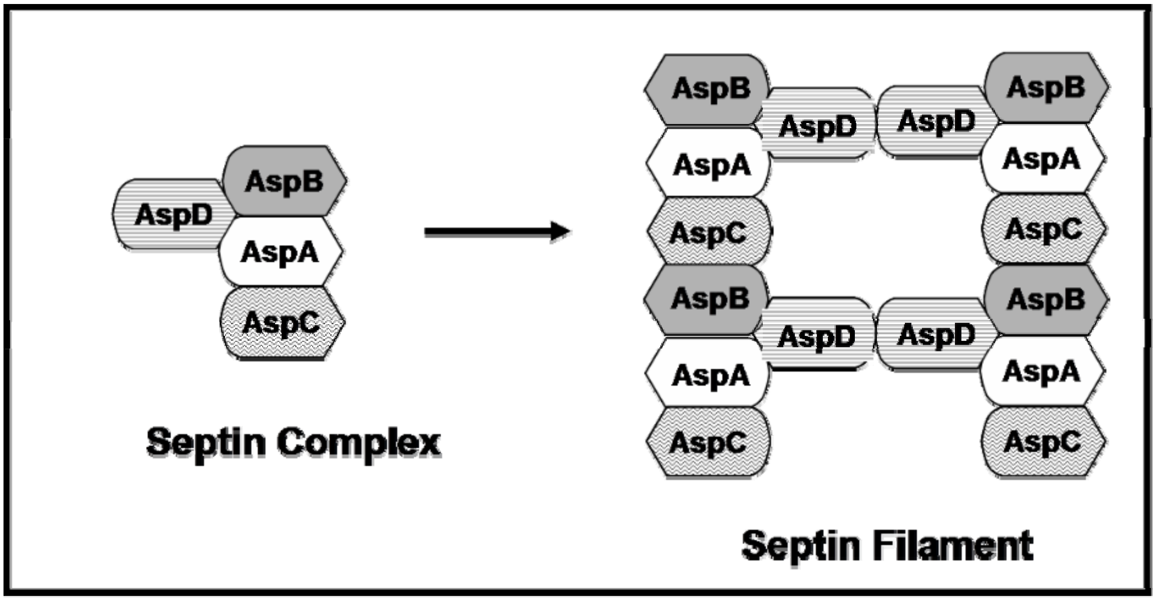

Figure 1.4. Septin complex and filament formation at *A. nidulans* septa. Each septin protein forms a dimer (shown only as single septins) which interacts through N or C-terminal domains to form a complex or filament. AspE is absent from the model above because its role is thought to be elsewhere. Septin interactions and model based on studies conducted in yeast (Versele *et. al.* 2004).

In chapter 2 of this dissertation “Septin localization across kingdoms: three themes with variations” is a literature review that focuses on localization of septins and their corresponding functions across kingdoms. Chapter 3 “Expression of a single *A. nidulans* septin gene is sufficient to induce filamentous growth in the budding yeast *S.*

cerevisiae" examines how the addition of an *A. nidulans* septin changes the morphology of *S. cerevisiae* cells from round to filamentous. Chapter 4 "Characterization of septins AspA/Cdc11 and AspC/Cdc12 in Aspergillus nidulans" examines two *A. nidulans* septins. Chapter 5 is a conclusion chapter with possible future studies.

REFERENCES

Adams, A.E., and Pringle, J.R. (1984). Relationship of actin and tubulin distribution to bud growth in wild-type and morphogenetic-mutant Saccharomyces cerevisiae. The Journal of cell biology *98,* 934-945.

Ayscough, K.R., Stryker, J., Pokala, N., Sanders, M., Crews, P., and Drubin, D.G. (1997). High rates of actin filament turnover in budding yeast and roles for actin in establishment and maintenance of cell polarity revealed using the actin inhibitor latrunculin-A. The Journal of cell biology *137,* 399-416.

Byers, B., and Goetsch, L. (1976). A highly ordered ring of membrane-associated filaments in budding yeast. The Journal of cell biology *69,* 717-721.

De Virgilio, C., DeMarini, D.J., and Pringle, J.R. (1996). SPR28, a sixth member of the septin gene family in Saccharomyces cerevisiae that is expressed specifically in sporulating cells. Microbiology (Reading, England) *142 (Pt 10),* 2897-2905.

DeMarini, D.J., Adams, A.E., Fares, H., De Virgilio, C., Valle, G., Chuang, J.S., and Pringle, J.R. (1997). A septin-based hierarchy of proteins required for localized deposition of chitin in the Saccharomyces cerevisiae cell wall. The Journal of cell biology *139*, 75-93.

Evangelista, M., Pruyne, D., Amberg, D.C., Boone, C., and Bretscher, A. (2002). Formins direct Arp2/3-independent actin filament assembly to polarize cell growth in yeast. Nature cell biology *4*, 32-41.

Fares, H., Goetsch, L., and Pringle, J.R. (1996). Identification of a developmentally regulated septin and involvement of the septins in spore formation in Saccharomyces cerevisiae. The Journal of cell biology *132*, 399-411.

Fares, H., Peifer, M., and Pringle, J.R. (1995). Localization and possible functions of Drosophila septins. Molecular biology of the cell *6*, 1843-1859.

Faty, M., Fink, M., and Barral, Y. (2002). Septins: a ring to part mother and daughter. Curr Genet *41*, 123-131.

Fiddy, C., and Trinci, A.P. (1976). Mitosis, septation, branching and the duplication cycle in Aspergillus nidulans. Journal of general microbiology *97*, 169-184.

Field, C.M., and Kellogg, D. (1999). Septins: cytoskeletal polymers or signalling GTPases? Trends in cell biology *9*, 387-394.

Ford, S.K., and Pringle, J.R. (1991). Cellular morphogenesis in the Saccharomyces cerevisiae cell cycle: localization of the CDC11 gene product and the timing of events at the budding site. Developmental genetics *12*, 281-292.

Gale, C., Gerami-Nejad, M., McClellan, M., Vandoninck, S., Longtine, M.S., and Berman, J. (2001). Candida albicans Int1p interacts with the septin ring in yeast and hyphal cells. Molecular biology of the cell *12*, 3538-3549.

Gladfelter, A.S., Pringle, J.R., and Lew, D.J. (2001). The septin cortex at the yeast mother-bud neck. Current opinion in microbiology *4*, 681-689.

Haarer, B.K., and Pringle, J.R. (1987). Immunofluorescence localization of the Saccharomyces cerevisiae CDC12 gene product to the vicinity of the 10-nm filaments in the mother-bud neck. Molecular and cellular biology *7*, 3678-3687.

Hartwell, L.H. (1971). Genetic control of the cell division cycle in yeast. IV. Genes controlling bud emergence and cytokinesis. Exp Cell Res *69*, 265-276.

Joberty, G., Perlungher, R.R., Sheffield, P.J., Kinoshita, M., Noda, M., Haystead, T., and Macara, I.G. (2001). Borg proteins control septin organization and are negatively regulated by Cdc42. Nature cell biology *3*, 861-866.

Kadota, J., Yamamoto, T., Yoshiuchi, S., Bi, E., and Tanaka, K. (2004). Septin ring assembly requires concerted action of polarisome components, a PAK kinase Cla4p, and the actin cytoskeleton in Saccharomyces cerevisiae. Molecular biology of the cell *15*, 5329-5345.

Kaminskyj, S.G., and Hamer, J.E. (1998). hyp loci control cell pattern formation in the vegetative mycelium of Aspergillus nidulans. Genetics *148*, 669-680.

Kartmann, B., and Roth, D. (2001). Novel roles for mammalian septins: from vesicle trafficking to oncogenesis. J Cell Sci *114*, 839-844.

Kim, H.B., Haarer, B.K., and Pringle, J.R. (1991). Cellular morphogenesis in the Saccharomyces cerevisiae cell cycle: localization of the CDC3 gene product and the timing of events at the budding site. The Journal of cell biology *112*, 535-544.

Kinoshita, M. (2003). Assembly of mammalian septins. J Biochem (Tokyo) *134*, 491-496.

Kinoshita, M., Field, C.M., Coughlin, M.L., Straight, A.F., and Mitchison, T.J. (2002). Self- and actin-templated assembly of Mammalian septins. Dev Cell *3*, 791-802.

Kozubowski, L., Larson, J.R., and Tatchell, K. (2005). Role of the septin ring in the asymmetric localization of proteins at the mother-bud neck in Saccharomyces cerevisiae. Molecular biology of the cell *16*, 3455-3466.

Lindsey, R., and Momany, M. (2006). Septin localization across kingdoms: three themes with variations. Current opinion in microbiology *9*, 559-565.

Longtine, M.S., and Bi, E. (2003). Regulation of septin organization and function in yeast. Trends in cell biology *13*, 403-409.

Longtine, M.S., DeMarini, D.J., Valencik, M.L., Al-Awar, O.S., Fares, H., De Virgilio, C., and Pringle, J.R. (1996). The septins: roles in cytokinesis and other processes. Current opinion in cell biology *8*, 106-119.

Momany, M., and Hamer, J.E. (1997). The Aspergillus nidulans Septin Encoding Gene, aspB, Is Essential for Growth. Fungal Genet Biol *21*, 92-100.

Momany, M., and Taylor, I. (2000). Landmarks in the early duplication cycles of Aspergillus fumigatus and Aspergillus nidulans: polarity, germ tube emergence and septation. Microbiology (Reading, England) *146 Pt 12*, 3279-3284.

Momany, M., Zhao, J., Lindsey, R., and Westfall, P.J. (2001). Characterization of the Aspergillus nidulans septin (asp) gene family. Genetics *157*, 969-977.

Neufeld, T.P., and Rubin, G.M. (1994). The Drosophila peanut gene is required for cytokinesis and encodes a protein similar to yeast putative bud neck filament proteins. Cell *77*, 371-379.

Nguyen, T.Q., Sawa, H., Okano, H., and White, J.G. (2000). The C. elegans septin genes, unc-59 and unc-61, are required for normal postembryonic cytokineses and

morphogenesis but have no essential function in embryogenesis. J Cell Sci *113 Pt 21*, 3825-3837.

Ozsarac, N., Bhattacharyya, M., Dawes, I.W., and Clancy, M.J. (1995). The SPR3 gene encodes a sporulation-specific homologue of the yeast CDC3/10/11/12 family of bud neck microfilaments and is regulated by ABFI. Gene *164*, 157-162.

Peng, X.R., Jia, Z., Zhang, Y., Ware, J., and Trimble, W.S. (2002). The septin CDCrel-1 is dispensable for normal development and neurotransmitter release. Molecular and cellular biology *22*, 378-387.

Sagot, I., Klee, S.K., and Pellman, D. (2002). Yeast formins regulate cell polarity by controlling the assembly of actin cables. Nature cell biology *4*, 42-50.

Sheffield, P.J., Oliver, C.J., Kremer, B.E., Sheng, S., Shao, Z., and Macara, I.G. (2003). Borg/septin interactions and the assembly of mammalian septin heterodimers, trimers, and filaments. The Journal of biological chemistry *278*, 3483-3488.

Versele, M., Gullbrand, B., Shulewitz, M.J., Cid, V.J., Bahmanyar, S., Chen, R.E., Barth, P., Alber, T., and Thorner, J. (2004). Protein-Protein Interactions Governing Septin Heteropentamer Assembly and Septin Filament Organization in Saccharomyces cerevisiae. Molecular biology of the cell.

Versele, M., and Thorner, J. (2004). Septin collar formation in budding yeast requires GTP binding and direct phosphorylation by the PAK, Cla4. The Journal of cell biology *164*, 701-715.

Vrabioiu, A.M., Gerber, S.A., Gygi, S.P., Field, C.M., and Mitchison, T.J. (2004). The majority of the Saccharomyces cerevisiae septin complexes do not exchange guanine nucleotides. The Journal of biological chemistry *279*, 3111-3118.

Westfall, P.J., and Momany, M. (2002). Aspergillus nidulans septin AspB plays pre- and postmitotic roles in septum, branch, and conidiophore development. Molecular biology of the cell *13*, 110-118.

Xie, H., Surka, M., Howard, J., and Trimble, W.S. (1999). Characterization of the mammalian septin H5: distinct patterns of cytoskeletal and membrane association from other septin proteins. Cell Motil Cytoskeleton *43*, 52-62.

CHAPTER 2

SEPTIN LOCALIZATION ACROSS KINGDOMS: THREE THEMES WITH VARIATIONS[1]

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[1] Lindsey, R. and M. Momany (2006). Septins across kingdoms: three themes with variations. Current Opinion in Microbiology *9*:1-7.

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SUMMARY

Septins are GTPases that form filaments in fungi and animals. In addition to their original role in cell division, septins have been shown to play roles in coordinating nuclear division, membrane trafficking and organizing the cytoskeleton. Many recent studies have examined subcellular localization of septins in a wide range of fungi and animals. Septin localization shows three patterns which generally correspond to function across kingdoms. Septins that localize to projections shape and partition emerging growth. Septins that localize to partitions compartmentalize pre-existing cellular material. Septins that localize to the whole cell are involved in membrane trafficking and organizing the cytoskeleton.

Septins were first observed thirty years ago associated with filamentous rings at the mother-bud neck in *Saccharomyes cerevisiae* (Longtine *et al.*, 1996). During the next two decades septins were found in other fungi and some animals. With the increased availability of genome sequences it is now clear that septins are found in fungi, animals and microsporidia and that they are absent from plants. The number of septin proteins in a single organism ranges from two in *Caenorhabditis elegans* to thirteen in humans (Neufeld and Rubin, 1994; Fares *et al.*, 1995; Longtine *et al.*, 1996; Nguyen *et al.*, 2000; Gale *et al.*, 2001; Momany *et al.*, 2001; Spiliotis and Nelson, 2006) (M Momany *et al.*, unpublished). Septins are members of the P loop GTPase family and septin heteropolymers form filaments. The combination of GTPase activity and filament formation has led to comparisons between the septins and the cytoskeletal elements actin and tubulin (Field and Kellogg, 1999; Kinoshita, 2006).

Recent reviews have focused on septins in fungi (Douglas *et al.*, 2005; Gladfelter, 2006) and mammals (Spiliotis and Nelson, 2006), on the scaffolding role of septins (Kinoshita, 2006) and on septin filament formation (Versele and Thorner, 2005). Here we will focus on the subcellular localization of septins. All fungi and animals have multiple septin genes, ranging from two in *C. elegans* to thirteen in humans (M Momany *et al*, unpublished) and splice variants have also been reported (Spiliotis and Nelson, 2006). Further complicating matters, septin localization is dynamic, changing during development and the cell cycle (Gladfelter, 2006; Spiliotis and Nelson, 2006). In the past few years, many studies have examined septins in fungi and animals yielding a bewildering array of subcellular localization patterns. However, upon closer examination subcellular septin localization falls into variations of three distinct patterns: localization to projections, to partitions or to whole cells (Figure 2.1).

Projections

Septins localize to projections, outgrowths from a larger cell, in fungi and animals (Figure 2.1, pattern 1; Table 2.1). Projections can be round (*e.g.*, yeast buds and fungal spores) or tubular (*e.g.*, fungal hyphae and mammalian neurites) and arise from cells of any shape.

Septins at the projection base:

Septin localization to the base of a projection is common in fungi where round projections include buds, reproductive structures and spores, and tubular projections include germ tubes, hyphae and branches. Indeed the first septin structure observed was the prominent ring formed by Cdc3p, Cdc10p, Cdc11p and Cdc12p at the base of the projecting daughter bud in *S. cerevisiae* (Longtine *et al.*, 1996). Later work showed that the septin ring forms on the mother cell along with an actin ring late in G1 phase, circumscribing the site where the bud will emerge in S phase (Gladfelter *et al.*, 2001). As the bud emerges through the ring, the septins broaden at the neck and remain there for the rest of the cell cycle. Septins at the neck form a barrier to diffusion of products between mother and daughter cells (Dobbelaere and Barral, 2004), are part of the morphogenesis checkpoint that monitors the progression of budding and the spindle checkpoint that monitors chromosome segregation (Gladfelter, 2006).

In the filamentous fungi *Aspergillus nidulans* and *Ashbya gossypii*, a similar pattern is seen in branching (Westfall and Momany, 2002) (AS Gladfelter, personal communication). The septin ring appears, the branch emerges within the circumference of the ring and nuclear division occurs. Though details are not yet clear, it appears that septins play a role in coordinating branch emergence and mitosis. In *Candida albicans* hyphae a broad, diffuse septin band appears at the base of the emerging germ tube before nuclear division (Sudbery, 2001; Warenda and Konopka, 2002). In the *A. nidulans* reproductive structure the conidiophore (Westfall and Momany, 2002), septins localize to the base of round projections, though in this case many projections emerge in synchrony forming multiple layers of cells before spores are made.

In animals, sperm cells furnish the only example of septin localization to the base of a projection. In mice Sept4, along with other septins, forms the annulus, a cortical ring separating the sperm into two segments, the midpiece and the primary piece. Sperm from mice lacking Sept4 also lack the annulus and are sterile (Ihara *et al.*, 2005; Kissel *et al.*, 2005). Though the septins have not yet been localized during early sperm development, EM studies have followed the annulus, now thought to be composed of septins (Clermont

Y, 1993). Early in sperm development, before the tail is made, an electron dense rudimentary annulus appears near the centrosome. The flagellum develops within the annulus, much as a bud or branch emerges from the septin ring in fungi. During flagellum development the sperm nucleus is reorganized, though it is not clear if the septins have anything to do with this process. After the flagellum is made the annulus moves down the tail to serve as a barrier between the midpiece and primary piece. Interestingly septins remain at the mature annulus making this the only clear case of a permanent septin ring (Kissel *et al.*, 2005).

Septins at the base of a projection define its circumference before it emerges from the main cell. These septins appear to couple growth with nuclear division. Septins at the base of a projection serve as diffusion barriers, though in some cases the barrier function occurs long after the projection is made.

Septins at the projection tip and punctate throughout the projection:

Septins are at the tips of *C. albicans* and *A. gossypii* hyphae. The tip is where all growth occurs in fungal hyphae (Harris *et al.*, 2005), thus tip-localized septins are well-positioned to play a role in the addition of new membrane and cell wall. Mammalian Sept 5 is localized to plasma membranes and concentrated in tips of neurites where it associates with vesicles and is thought to regulate vesicle targeting and fusion (Beites *et al.*, 2005). The single example of punctuate localization to a projection is also consistent with a role in secretion and membrane fusion. Sept3, a septin expressed in brain, localizes to synaptic vesicles in mammalian neurons (Xue *et al.*, 2004).

Septins localized at projection tips and throughout the cytoplasm in a punctate pattern appear to direct new growth through membrane trafficking.

Partitions

Septins localize to partitions, barriers that divide existing cytoplasm, in animals and fungi (Figure 2.1, pattern 2; Table 2.1). Septins at the base or tip of projections also form barriers; however we consider this a different pattern because in projections septins shape the new growth they circumscribe, while in partitions septins do not alter the overall shape of the cell, only its organization. When fungal septa and animal cleavage furrows separate cytoplasm into two compartments that will occupy the same plane septins localize only to the partition between the cells, not to the periphery of the cells. During cellularization of embryos partitions separate cytoplasm into multiple compartments that form a network extending in all directions and septin localization is peripheral, forming a cage around the new compartment.

Septins at partitions:

In the fission yeast *Schizosaccharomyces pombe* septins form a ring late in mitosis at the center of the elongated, tubular cell. As the contractile ring constricts, the septin ring splits (Berlin *et al.*, 2003; Tasto *et al.*, 2003). In the filamentous fungus *A. nidulans*, septins form rings that partition the tubular hypha into compartments after mitosis is complete (Westfall and Momany, 2002). Septins partition hyphae of *C. albicans*, though in this case mitosis occurs after the septin ring appears (Warenda and Konopka, 2002). Septins also partition the hyphae of *A. gossypii*, where septation and mitosis appear to be much less coordinated (AS Gladfelter, personal communication). Similarly, *C. elegans* and *Drosophila melanogaster* septins localize to cleavage furrows during partitioning of cells. After cleavage, septins are often found at the midbody, an intercellular bridge of inter-digitated spindle microtubules seen in animal cell cytokinesis

(Fares *et al.*, 1995; Adam *et al.*, 2000; Nguyen *et al.*, 2000; Finger *et al.*, 2003). This same pattern of septin localization to constricting cleavage furrows often followed by localization to the midbody has been reported for a variety of mammalian cell types (Hsu *et al.*, 1998; Xie *et al.*, 1999; Surka *et al.*, 2002; Qi *et al.*, 2005; Spiliotis *et al.*, 2005; Ahuja *et al.*, 2006). Interestingly, in addition to its localization at the cleavage furrow and midbody, Sept1 in HeLa cells localizes to the spindle poles throughout mitosis (Qi *et al.*, 2005). This association of septins with both spindle and cleavage plane led to the suggestion that mammalian septins form a scaffold that coordinates chromosome segregation and division plane specification (Qi *et al.*, 2005; Spiliotis *et al.*, 2005). In *S. cerevisiae* ascospore formation and *D. melanogaster* embryo cellularization, partitions divide compartments in more than one plane and septins localize to the periphery of compartments (Fares *et al.*, 1995; Fares *et al.*, 1996; Adam *et al.*, 2000; Field *et al.*, 2005).

Septins localized to partitions do not alter the cell's overall shape, only its organization into compartments. Though the mechanisms are not clear, septins in partitions appear to relay information between sites of nuclear division and sites of cell division.

Whole cells

Septins localize throughout the cell most often in animals, though examples from a single fungus have been reported (Figure 2.1, pattern 3, Table 2.1).

Septins at the whole cell periphery and punctate throughout:

The single published example of septin localization to the periphery of a fungal cell is found in *C. albicans* chalmydospores where short septin filaments localize to the cortex (Martin *et al.*, 2005). Since no divisions occur in the spore, it is thought that these septins direct vesicles carrying cell wall biosynthetic enzymes to the plasma membrane. Peripheral and punctate septin localization has been seen in a number of mammalian cell types (Hsu *et al.*, 1998; Beites *et al.*, 1999; Walikonis *et al.*, 2000; Takahashi *et al.*, 2003; Kinoshita *et al.*, 2004; Martinez *et al.*, 2004; Xue *et al.*, 2004; Martinez *et al.*, 2006; Spiliotis and Nelson, 2006) where it is also thought to be involved in membrane trafficking. In platelets punctate Sept5 and Sept6 have been associated with secretion (Martinez *et al.*, 2004; Martinez *et al.*, 2006) and rat brain septins co-precipitate with proteins needed for vesicle delivery (Hsu *et al.*, 1998). One interesting exception is the punctate localization of a Sept3 splice variant to mitochondria in neurons where it is thought to be involved in neural development (Takahashi *et al.*, 2003).

Septins filaments throughout the whole cell:

The only published report of septin filaments in fungi comes from *C. albicans* chalmydospores (Martin *et al.*, 2005). Though the filament pattern is reminiscent of actin or tubulin, no co-localization experiments were reported. Septin filaments have been reported in a variety of mammalian cell types (Xie *et al.*, 1999; Kinoshita *et al.*, 2002; Surka *et al.*, 2002; Hanai *et al.*, 2004; Nagata *et al.*, 2004; Spiliotis *et al.*, 2005; Ahuja *et al.*, 2006) and in all cases they co-localize with actin stress filaments and/or cytoplasmic microtubules throughout the cytoplasm. One interesting variation on filament localization is seen in platelets. Platelets are formed by the extrusion of cytoplasm from larger proplatelets. This process is driven by microtubules which remain coiled around the platelet forming a circumferential band (Italiano *et al.*, 1999). Septins co-localize with this cortical microtubule band where they help maintain the discoid shape of resting platelets (Martinez *et al.*, 2006).

The co-localization of septin filaments with actin and/or microtubules led to the question of whether actin or microtubules were serving as templates for septin, or visa versa. Disruption of mammalian actin caused septins to shift from filaments to small circles similar to septins reconstituted *in vitro* suggesting that actin directs the formation of septin filaments and that circles might be the default septin organization when actin is absent (Kinoshita *et al.*, 2002). However, other work has shown that disruption of actin or microtubules causes septins to lose their filamentous form in some cell types and not others (Hanai *et al.*, 2004). There is only a single report of septin localization to the cytoplasm and its physiological significance is not yet clear (Sui *et al.*, 2003).

Septins that localize to the cell periphery or cytoplasm in a punctate pattern are mostly involved in membrane trafficking. Septins that localize as filaments co-localize with actin or microtubules where they are thought to organize the cytoskeleton via interactions with cytoskeletal binding proteins (Spiliotis et al., 2005).

CONCLUSION

Septin form and function across kingdoms

Examples of septin localization to partitions (Figure 2.1, pattern 2) are common in both fungi and animals; however, most examples of localization to projections (Figure 2.1, pattern 1) are in fungi and most examples of localization to whole cells (Figure 2.1, pattern 3) are in animals. This has led to the assumption that, except for cytokinesis, fungi and animals use septins in very different ways. However, the apparent differences in septin localization between fungi and animals probably have more to do with experimental systems than with septin function.

Fungi have between five and seven septins (M Momany *et al.*, unpublished). The full complement of fungal septins has been examined only in the budding yeast *S. cerevisiae* and its close relative *C. albicans*. *S. cerevisiae* is unicellular and microtubules have little or no role in its growth. Thus septin localization specific to coordination of multiple cells or transport over long distances would not be expected in budding yeast. In this context, it is significant that the only published examples of whole cell septin localization (pattern 3) in fungi are from *C. albicans* - a multicellular fungus that undergoes a number of developmental programs. Further in our unpublished work, we have seen punctuate and filamentous whole cell septin localization in *A. nidulans*. As more septins are studied in multicellular fungi, we'll undoubtedly find more examples of whole cell localization.

Septin localization data in animals are similarly skewed, with mammalian cell culture furnishing most examples. In humans there are thirteen septin genes, many with alternate splice forms (Spiliotis *et al.*, 2005). Given the likely number of septin isoforms and complexity of tissue types, it is not surprising that no one has localized the full complement of septins in any mammal. Studies of septins in early development of cells that form projections would be especially informative. In fungi the septin ring circumscribes the region from which the bud or germ tube will emerge. It seems likely that studies of early sperm and neuron development will reveal similar septin localization.

Though the classification of septin localization into three patterns is undoubtedly an oversimplification, it does point to trends across kingdoms. Septins that show similar localization patterns have been implicated in similar processes. Based on what we know so far, we predict that the following will hold true across kingdoms:

1. Septins at the base of projections (Figure 2.1, pattern 1b) shape emerging growth and serve as barriers, though shape determination and barrier functions might be temporally separated.
2. Septins at partitions (Figure 2.1, pattern 2) compartmentalize previously existing cytoplasm without affecting its overall shape.
3. Septins at the base of projections (pattern 1b) and at partitions (pattern 2) coordinate nuclear division with cell division, though mechanism is not always clear.
4. Septins at the cell periphery (pattern 3per) or throughout the cytoplasm in a punctate pattern (pattern 3p) are involved in membrane trafficking or, less commonly, development of mitochondria or other organelles.
5. Septins that form filaments (pattern 3f) co-localize with actin and/or tubulin. These septins either organize the other elements of the cytoskeleton or are organized by them.

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Figure 2.1. Septins localize in three basic patterns in fungal and animal cells. Septins localize to: (a) projections at the base, tip or throughout; (b) partitions between cells; (c) whole cells at the periphery or throughout in a punctate, filamentous or cytoplasmic pattern. Green shading represents septin labeling.

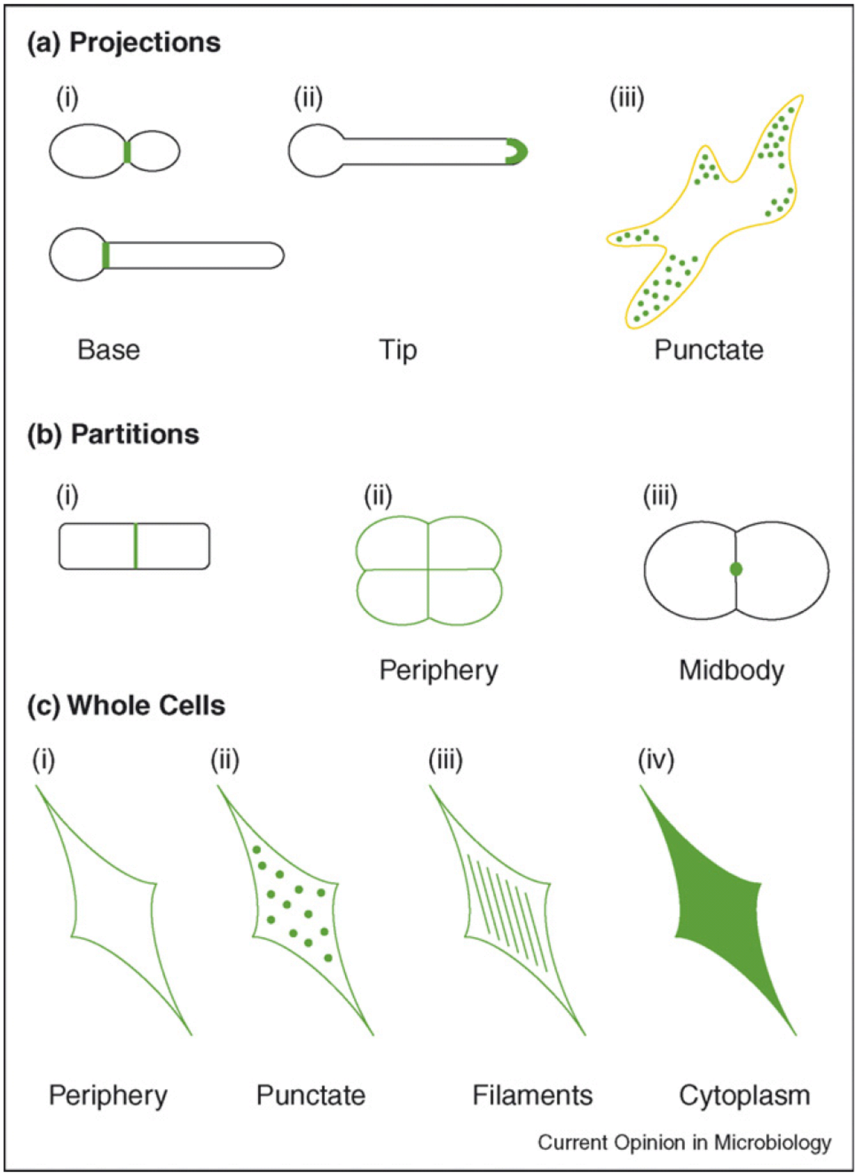
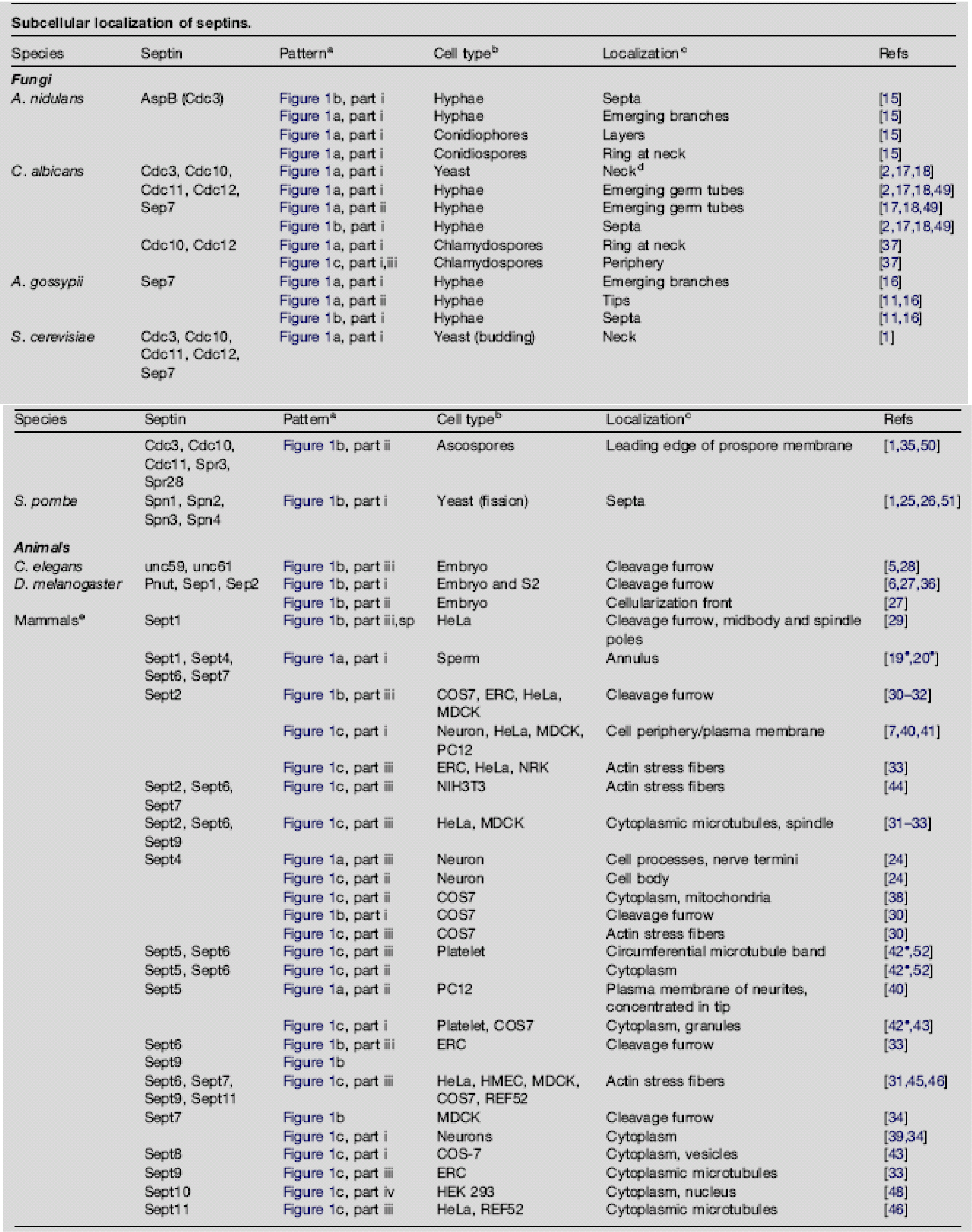

(a) Projections
(i)
(ii)
(iii)
Base
Tip
Punctate
(b) Partitions
(i)
(ii)
(iii)
Periphery
Midbody
(c) Whole Cells
(i)
(ii)
(iii)
(iv)
Periphery
Punctate
Filaments
Cytoplasm
Current Opinion in Microbiology

Table 2.1. Subcellular localization of septins.



Subcellular localization of septins.

Species	Septin	Pattern[a]	Cell type[b]	Localization[c]	Refs
Fungi					
A. nidulans	AspB (Cdc3)	Figure 1b, part i	Hyphae	Septa	[15]
		Figure 1a, part i	Hyphae	Emerging branches	[15]
		Figure 1a, part i	Conidiophores	Layers	[15]
		Figure 1a, part i	Conidiospores	Ring at neck	[15]
C. albicans	Cdc3, Cdc10, Cdc11, Cdc12, Sep7	Figure 1a, part i	Yeast	Neck[d]	[2,17,18]
		Figure 1a, part i	Hyphae	Emerging germ tubes	[2,17,18,49]
		Figure 1a, part ii	Hyphae	Emerging germ tubes	[17,18,49]
		Figure 1b, part i	Hyphae	Septa	[2,17,18,49]
	Cdc10, Cdc12	Figure 1a, part i	Chlamydospores	Ring at neck	[37]
		Figure 1c, part i,iii	Chlamydospores	Periphery	[37]
A. gossypii	Sep7	Figure 1a, part i	Hyphae	Emerging branches	[16]
		Figure 1a, part ii	Hyphae	Tips	[11,16]
		Figure 1b, part i	Hyphae	Septa	[11,16]
S. cerevisiae	Cdc3, Cdc10, Cdc11, Cdc12, Sep7	Figure 1a, part i	Yeast (budding)	Neck	[1]
	Cdc3, Cdc10, Cdc11, Spr3, Spr28	Figure 1b, part ii	Ascospores	Leading edge of prospore membrane	[1,35,50]
S. pombe	Spn1, Spn2, Spn3, Spn4	Figure 1b, part i	Yeast (fission)	Septa	[1,25,26,51]
Animals					
C. elegans	unc59, unc61	Figure 1b, part iii	Embryo	Cleavage furrow	[5,28]
D. melanogaster	Pnut, Sep1, Sep2	Figure 1b, part i	Embryo and S2	Cleavage furrow	[6,27,36]
		Figure 1b, part ii	Embryo	Cellularization front	[27]
Mammals[e]	Sept1	Figure 1b, part iii,sp	HeLa	Cleavage furrow, midbody and spindle poles	[29]
	Sept1, Sept4, Sept6, Sept7	Figure 1a, part i	Sperm	Annulus	[19*,20*]
	Sept2	Figure 1b, part iii	COS7, ERC, HeLa, MDCK	Cleavage furrow	[30–32]
		Figure 1c, part i	Neuron, HeLa, MDCK, PC12	Cell periphery/plasma membrane	[7,40,41]
		Figure 1c, part iii	ERC, HeLa, NRK	Actin stress fibers	[33]
	Sept2, Sept6, Sept7	Figure 1c, part iii	NIH3T3	Actin stress fibers	[44]
	Sept2, Sept6, Sept9	Figure 1c, part iii	HeLa, MDCK	Cytoplasmic microtubules, spindle	[31–33]
	Sept4	Figure 1a, part iii	Neuron	Cell processes, nerve termini	[24]
		Figure 1c, part ii	Neuron	Cell body	[24]
		Figure 1c, part ii	COS7	Cytoplasm, mitochondria	[38]
		Figure 1b, part i	COS7	Cleavage furrow	[30]
		Figure 1c, part iii	COS7	Actin stress fibers	[30]
	Sept5, Sept6	Figure 1c, part iii	Platelet	Circumferential microtubule band	[42*,52]
	Sept5, Sept6	Figure 1c, part ii		Cytoplasm	[42*,52]
	Sept5	Figure 1a, part ii	PC12	Plasma membrane of neurites, concentrated in tip	[40]
		Figure 1c, part i	Platelet, COS7	Cytoplasm, granules	[42*,43]
	Sept6	Figure 1b, part iii	ERC	Cleavage furrow	[33]
	Sept9	Figure 1b			
	Sept6, Sept7, Sept9, Sept11	Figure 1c, part iii	HeLa, HMEC, MDCK, COS7, REF52	Actin stress fibers	[31,45,46]
	Sept7	Figure 1b	MDCK	Cleavage furrow	[34]
		Figure 1c, part i	Neurons	Cytoplasm	[39,34]
	Sept8	Figure 1c, part i	COS-7	Cytoplasm, vesicles	[43]
	Sept9	Figure 1c, part iii	ERC	Cytoplasmic microtubules	[33]
	Sept10	Figure 1c, part iv	HEK 293	Cytoplasm, nucleus	[48]
	Sept11	Figure 1c, part iii	HeLa, REF52	Cytoplasmic microtubules	[46]

[a] Numbers denote patterns described in Figure 2.1: a, projection, part i, base; part ii, tip; part iii, punctuate. b, part i, cleavage furrow or septum; part ii, periphery of dividing cells; partition: part iii, midbody after completion of cleavage; sp, spindle pole. c, whole cell: part i, periphery; part ii,

punctate; part iii, filaments; part iv, cytoplasm.

[b] Fungal or animal cell type. Cell culture sources: COS-7, African green monkey kidney fibroblast; ERC, embryonic rat cardiomyocytes; HeLa, human epithelial; HEK 293, human embryonic kidney; HIT T15, hamster insulinoma; HMEC, human mammary epithelial; MDCK, Madin–Darby canine kidney epithelial; NIH 3T3, mouse embryonic fibroblast; N18, mouse neuroblastoma; NRK, normal rat kidney; PC12-rat pheochromocytoma; REF52, rat embryo fibroblasts; S2, fly.

[c] Only localization resolved at the individual cell level is included. Localization at the tissue level is not included. Most septin localization is transient.

[d] Septin was overexpressed.

[e] Most mammalian septin localizations used antibodies raised against heterologous protein and are listed with the cell type examined.

The mammalian septin-naming convention is described by Macara et al. [53] and Martinez and Ware [54].

REFERENCES

1. Longtine MS, DeMarini DJ, Valencik ML, Al-Awar OS, Fares H, De Virgilio C, Pringle JR: **The septins: roles in cytokinesis and other processes**. *Curr Opin Cell Biol* 1996, **8**:106-119.

2. Gale C, Gerami-Nejad M, McClellan M, Vandoninck S, Longtine MS, Berman J: **Candida albicans Int1p interacts with the septin ring in yeast and hyphal cells**. *Mol Biol Cell* 2001, **12**:3538-3549.

3. Momany M, Zhao J, Lindsey R, Westfall PJ: **Characterization of the Aspergillus nidulans septin (asp) gene family**. *Genetics* 2001, **157**:969-977.

4. Neufeld TP, Rubin GM: **The Drosophila peanut gene is required for cytokinesis and encodes a protein similar to yeast putative bud neck filament proteins**. *Cell* 1994, **77**:371-379.

5. Nguyen TQ, Sawa H, Okano H, White JG: **The C. elegans septin genes, unc-59 and unc-61, are required for normal postembryonic cytokineses and morphogenesis but have no essential function in embryogenesis**. *J Cell Sci* 2000, **113 Pt 21**:3825-3837.

6. Fares H, Peifer M, Pringle JR: **Localization and possible functions of Drosophila septins**. *Mol Biol Cell* 1995, **6**:1843-1859.

7. Spiliotis ET, Nelson WJ: **Here come the septins: novel polymers that coordinate intracellular functions and organization**. *J Cell Sci* 2006, **119**:4-10.

8. Field CM, Kellogg D: **Septins: cytoskeletal polymers or signalling GTPases**. *Trends in Cell Biology* 1999, **9**:387-394.

9. Kinoshita M: **Diversity of septin scaffolds**. *Curr Opin Cell Biol* 2006, **18**:54-60.

●10. Douglas LM, Alvarez FJ, McCreary C, Konopka JB: **Septin function in yeast model systems and pathogenic fungi**. *Eukaryot Cell* 2005, **4**:1503-1512.

An excellent review of septins in fungal systems.

11. Gladfelter AS: **Control of filamentous fungal cell shape by septins and formins**. *Nat Rev Microbiol* 2006, **4**:223-229.

12. Versele M, Thorner J: **Some assembly required: yeast septins provide the instruction manual**. *Trends Cell Biol* 2005, **15**:414-424.

13. Gladfelter AS, Pringle JR, Lew DJ: **The septin cortex at the yeast mother-bud neck**. *Curr Opin Microbiol* 2001, **4**:681-689.

●●14. Dobbelaere J, Barral Y: **Spatial coordination of cytokinetic events by compartmentalization of the cell cortex**. *Science* 2004, **305**:393-396.

Elegant study showing a septin-based diffusion barrier is formed at the bud neck by septins.

15. Westfall PJ, Momany M: **Aspergillus nidulans septin AspB plays pre- and postmitotic roles in septum, branch, and conidiophore development**. *Mol Biol Cell* 2002, **13**:110-118.

16. Helfer H, Gladfelter AS: **AgSwe1p regulates mitosis in response to morphogenesis and nutrients in multinucleated A. gossyppii**. *Mol Bio Cell* 2006, **17:**4494-4512.

17. Warenda AJ, Konopka JB: **Septin function in Candida albicans morphogenesis**. *Mol Biol Cell* 2002, **13**:2732-2746.

18. Sudbery PE: **The germ tubes of Candida albicans hyphae and pseudohyphae show different patterns of septin ring localization**. *Mol Microbiol* 2001, **41**:19-31.

●19. Ihara M, Kinoshita A, Yamada S, Tanaka H, Tanigaki A, Kitano A, Goto M, Okubo K, Nishiyama H, Ogawa O, et al.: **Cortical organization by the septin**

cytoskeleton is essential for structural and mechanical integrity of mammalian spermatozoa. *Dev Cell* 2005, **8**:343-352.

●20. Kissel H, Georgescu MM, Larisch S, Manova K, Hunnicutt GR, Steller H: **The Sept4 septin locus is required for sperm terminal differentiation in mice**. *Dev Cell* 2005, **8**:353-364.

Along with previous reference describes the septin annulus in sperm.

21. Clermont Y, Oko R, Hermo L: **Cell biology of mammalian spermiogenesis.** In *Cell and Molecular Biology of the Testis*. Edited by Desjardins C, Ewing LL: Oxford University Press; 1993:332–376.

22. Harris SD, Read ND, Roberson RW, Shaw B, Seiler S, Plamann M, Momany M: **Polarisome meets spitzenkorper: microscopy, genetics, and genomics converge**. *Eukaryot Cell* 2005, **4**:225-229.

23. Beites CL, Campbell KA, Trimble WS: **The septin Sept5/CDCrel-1 competes with alpha-SNAP for binding to the SNARE complex**. *Biochem J* 2005, **385**:347-353.

24. Xue J, Tsang CW, Gai WP, Malladi CS, Trimble WS, Rostas JA, Robinson PJ: **Septin 3 (G-septin) is a developmentally regulated phosphoprotein enriched in presynaptic nerve terminals**. *J Neurochem* 2004, **91**:579-590.

25. Berlin A, Paoletti A, Chang F: **Mid2p stabilizes septin rings during cytokinesis in fission yeast**. *J Cell Biol* 2003, **160**:1083-1092.

26. Tasto JJ, Morrell JL, Gould KL: **An anillin homologue, Mid2p, acts during fission yeast cytokinesis to organize the septin ring and promote cell separation**. *J Cell Biol* 2003, **160**:1093-1103.

27. Adam JC, Pringle JR, Peifer M: **Evidence for functional differentiation among Drosophila septins in cytokinesis and cellularization**. *Mol Biol Cell* 2000, **11**:3123-3135.

28. Finger FP, Kopish KR, White JG: **A role for septins in cellular and axonal migration in C. elegans**. *Dev Biol* 2003, **261**:220-234.

29. Qi M, Yu W, Liu S, Jia H, Tang L, Shen M, Yan X, Saiyin H, Lang Q, Wan B, et al.: **Septin1, a new interaction partner for human serine/threonine kinase aurora-B**. *Biochem Biophys Res Commun* 2005, **336**:994-1000.

30. Xie H, Surka M, Howard J, Trimble WS: **Characterization of the mammalian septin H5: distinct patterns of cytoskeletal and membrane association from other septin proteins**. *Cell Motil Cytoskeleton* 1999, **43**:52-62.

31. Surka MC, Tsang CW, Trimble WS: **The mammalian septin MSF localizes with microtubules and is required for completion of cytokinesis**. *Mol Biol Cell* 2002, **13**:3532-3545.

32. Spiliotis ET, Kinoshita M, Nelson WJ: **A mitotic septin scaffold required for Mammalian chromosome congression and segregation**. *Science* 2005, **307**:1781-1785.

33. Ahuja P, Perriard E, Trimble W, Perriard JC, Ehler E: **Probing the role of septins in cardiomyocytes**. *Exp Cell Res* 2006, **312**:1598-1609.

34. Hsu SC, Hazuka CD, Roth R, Foletti DL, Heuser J, Scheller RH: **Subunit composition, protein interactions, and structures of the mammalian brain sec6/8 complex and septin filaments**. *Neuron* 1998, **20**:1111-1122.

35. Fares H, Goetsch L, Pringle JR: **Identification of a developmentally regulated septin and involvement of the septins in spore formation in Saccharomyces cerevisiae**. *J Cell Biol* 1996, **132**:399-411.

36. Field CM, Coughlin M, Doberstein S, Marty T, Sullivan W: **Characterization of anillin mutants reveals essential roles in septin localization and plasma membrane integrity**. *Development* 2005, **132**:2849-2860.

37. Martin SW, Douglas LM, Konopka JB: **Cell cycle dynamics and quorum sensing in Candida albicans chlamydospores are distinct from budding and hyphal growth**. *Eukaryot Cell* 2005, **4**:1191-1202.

38. Takahashi S, Inatome R, Yamamura H, Yanagi S: **Isolation and expression of a novel mitochondrial septin that interacts with CRMP/CRAM in the developing neurones**. *Genes Cells* 2003, **8**:81-93.

39. Walikonis RS, Jensen ON, Mann M, Provance DW, Jr., Mercer JA, Kennedy MB: **Identification of proteins in the postsynaptic density fraction by mass spectrometry**. *J Neurosci* 2000, **20**:4069-4080.

40. Beites CL, Xie H, Bowser R, Trimble WS: **The septin CDCrel-1 binds syntaxin and inhibits exocytosis**. *Nat Neurosci* 1999, **2**:434-439.

41. Kinoshita N, Kimura K, Matsumoto N, Watanabe M, Fukaya M, Ide C: **Mammalian septin Sept2 modulates the activity of GLAST, a glutamate transporter in astrocytes**. *Genes Cells* 2004, **9**:1-14.

●42. Martinez C, Corral J, Dent JA, Sesma L, Vicente V, Ware J: **Platelet septin complexes form rings and associate with the microtubular network**. *J Thromb Haemost* 2006, **4**:1388-1395.

This study examines Sept5 and septin localization at the periphery and as a ring in human platelets.

43. Martinez C, Sanjuan MA, Dent JA, Karlsson L, Ware J: **Human septin-septin interactions as a prerequisite for targeting septin complexes in the cytosol**. *Biochem J* 2004, **382**:783-791.

44. Kinoshita M, Field CM, Coughlin ML, Straight AF, Mitchison TJ: **Self- and actin-templated assembly of Mammalian septins**. *Dev Cell* 2002, **3**:791-802.

45. Nagata K, Asano T, Nozawa Y, Inagaki M: **Biochemical and cell biological analyses of a mammalian septin complex, Sept7/9b/11**. *J Biol Chem* 2004, **279**:55895-55904.

46. Hanai N, Nagata K, Kawajiri A, Shiromizu T, Saitoh N, Hasegawa Y, Murakami S, Inagaki M: **Biochemical and cell biological characterization of a mammalian septin, Sept11**. *FEBS Lett* 2004, **568**:83-88.

47. Italiano JE, Jr., Lecine P, Shivdasani RA, Hartwig JH: **Blood platelets are assembled principally at the ends of proplatelet processes produced by differentiated megakaryocytes**. *J Cell Biol* 1999, **147**:1299-1312.

48. Sui L, Zhang W, Liu Q, Chen T, Li N, Wan T, Yu M, Cao X: **Cloning and functional characterization of human septin 10, a novel member of septin family cloned from dendritic cells**. *Biochem Biophys Res Commun* 2003, **304**:393-398.

49. Martin SW, Konopka JB: **SUMO modification of septin-interacting proteins in Candida albicans**. *J Biol Chem* 2004, **279**:40861-40867.

50. De Virgilio C, DeMarini DJ, Pringle JR: **SPR28, a sixth member of the septin gene family in Saccharomyces cerevisiae that is expressed specifically in sporulating cells**. *Microbiology* 1996, **142 (Pt 10)**:2897-2905.

51. An H, Morrell JL, Jennings JL, Link AJ, Gould KL: **Requirements of fission yeast septins for complex formation, localization, and function**. *Mol Biol Cell* 2004, **15**:5551-5564.

52. Blaser S, Horn J, Wurmell P, Bauer H, Strumpell S, Nurden P, Pagenstecher A, Busse A, Wunderle D, Hainmann I, et al.: **The novel human platelet septin SEPT8 is an interaction partner of SEPT4**. *Thromb Haemost* 2004, **91**:959-966.

53. Macara IG, Baldarelli R, Field CM, Glotzer M, Hayashi Y, Hsu SC, Kennedy MB, Kinoshita M, Longtine M, Low C, et al.: **Mammalian septins nomenclature**. *Mol Biol Cell* 2002, **13**:4111-4113.

54. Martinez C, Ware J: **Mammalian septin function in hemostasis and beyond**. *Exp Biol Med (Maywood)* 2004, **229**:1111-1119.

CHAPTER 3

EXPRESSION OF A SINGLE *ASPERGILLUS NIDULANS* SEPTIN GENE IS SUFFICIENT TO INDUCE FILAMENTOUS GROWTH IN THE BUDDING YEAST *SACCHAROMYCES CEREVISIAE*[1]

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[1] Rebecca Lindsey, Youngsil Ha and Michelle Momany. Submitted to *Eukaryotic Cell*.

ABSTRACT

Fungi grow in one of two forms: hyphae (elongated tubular compartments joined end to end) or yeasts (round unicells). Septin proteins form scaffolds that organize other proteins at division planes and affect cell morphology in animals and fungi. *Saccharomyces cerevisiae* grows in the yeast form under most conditions. Introduction of the septin AspC from the filamentous fungus *Aspergillus nidulans* caused *S. cerevisiae* to make elongated cells and spore-producing structures similar to those of hyphal fungi. AspC induced filaments when *S. cerevisiae* pseudohyphal or haploid invasive genes were deleted, but not when the *CDC10* septin was deleted. AspC localized to bud and filament necks, while its *S. cerevisiae* ortholog, Cdc12, localized only to bud necks. AspC did not induce filaments in strains deleted for the Cdc12-interacting formin Bni1. Our results suggest that AspC competes with Cdc12 for incorporation into the yeast septin scaffold and once there alters cell shape by altering interactions with the formin Bni1. Our results further suggest that morphological transitions in evolution, like that from filamentous fungi to yeast, could have been accomplished through changes in key scaffold proteins.

Most fungi grow either as multicellular, tubular hyphae or unicellular, spherical yeasts. Some fungi switch between hyphae and yeasts and the ability to change growth form has been implicated in fungal pathogenicity. Since hyphae are found in all fungal lineages and yeasts are in the most derived lineages, the common ancestor of all fungi is thought to have been hyphal.

Laboratory strains of *Saccharomyces cerevisiae* generally grow as budding yeasts; however when incubated on low nitrogen solid medium, diploids of certain strains, such as Σ1278b, form pseudohyphae, filamentous chains of elongated cells that invade the agar (Gimeno *et al.*, 1992). Similarly, haploids of certain strains form filaments that invade solid rich medium (Liu *et al.*, 1996). Both diploid pseudohyphal and haploid invasive growth require Flo11, a GPI-anchored cell surface glycoprotein that is transcriptionally regulated by the cAMP pathway (via Flo8) and the MAPK pathway (via Ste12 and Tec1) (Lo and Dranginis, 1998).

Septins are members of the P-loop GTPase family and have been shown to bind and hydrolyze guanine-nucleotide (Versele *et al.*, 2004). The septin GTPase motif combined with the ability of septin heteropolymers to form filaments has led to the suggestion that septins are cytoskeletal elements (Field and Kellogg, 1999). Animal septins often co-localize with actin filaments or microtubules and, like microtubules, some septins localize to the spindle and play a role in chromosome segregation (Lindsey and Momany, 2006). Like other cytoskeletal elements, septins have been implicated in cell morphogenesis and motility.

Septins were first discovered in *S. cerevisiae* where they form a filamentous scaffold that organizes the bud site and are a component of the morphogenesis checkpoint that coordinates budding with mitosis (Longtine *et al.*, 1996; Longtine and Bi, 2003). Five of the seven *S. cerevisiae* septins (Cdc3, Cdc10, Cdc11, Cdc12 and Shs1) co-localize to the neck region between the mother cell and daughter bud, the eventual site of septation (cytokinesis). At least 35 other proteins, including cell cycle regulators, localize to the neck region in a septin-dependent manner (Gladfelter *et al.*, 2001; Cid *et al.*, 2002). Mutation of *CDC3, CDC10, CDC11,* or *CDC12* prevents formation of the septin scaffold, resulting in elongated buds and mitotic delay (Hartwell, 1971; Longtine *et al.*, 2000). During sexual reproduction, the Spr3 and Spr28 septins localize with Cdc3, Cdc11 and Cdc12 at the leading edge of the prospore membrane as it encapsulates the nucleus (Ozsarac *et al.*, 1995; De Virgilio *et al.*, 1996; Fares *et al.*, 1996).

The filamentous fungus *Aspergillus nidulans* grows exclusively in the hyphal form and contains five septins (AspA, AspB, AspC, AspD, and AspE) (Momany *et al.*, 2001). We previously showed that one of the *A. nidulans* septins, AspB, localizes to sites of septation, branching and spore formation (Westfall and Momany, 2002).

RESULTS

To investigate the role of septins in fungal morphogenesis, we expressed septins from the filamentous fungus *A. nidulans* in the budding yeast *S. cerevisiae*. When the *A. nidulans* septin *aspC* was expressed in *S. cerevisiae*, we observed elongated, filamentous cells on solid and in liquid medium (Figure 3.1.). The *A. nidulans* septin *aspC* caused elongated growth in 27-54% of cells in haploid and diploid *S. cerevisiae* driven by the inducible *GAL1* promoter or the constitutive *ADH1* promoter. GFP-AspC also induced filaments in haploid and diploid derivatives of the *S. cerevisiae* pseudohyphal strain Σ1278b in liquid medium without nitrogen limitation (data not shown). On solid medium, *S. cerevisiae* AspC-induced filaments did not invade agar compared to control haploid invasive strains (data not shown).

To determine whether AspC induces filaments via the previously described cAMP or MAPK Flo11-dependent pseudohyphal or haploid invasive pathways (Rupp *et al.*, 1999), we introduced *A. nidulans aspC* into nine *S. cerevisiae* haploid strains with deletions in genes important for these pathways. AspC induced filaments in all cAMP, MAPK and invasive growth deletion strains tested, including *flo11*Δ (Table 3.1.). Additionally, the *S. cerevisiae* wildtype strains used in this study are derived from S288C, which has previously been shown to be unable to undergo haploid invasive or pseudohyphal growth because of a mutation in *FLO8* (Liu *et al.*, 1996). AspC induction of filaments in strains deleted for genes known to be required for pseudohyphal and haploid invasive growth and in S288C derivates is consistent with filament formation via an alternate pathway.

A. nidulans AspC is most closely related to the *S. cerevisiae* septin Cdc12 based on phylogenetic analysis (Momany *et al.*, 2001). To determine whether overexpression of *CDC12* might induce filaments, we introduced an extra copy of *CDC12* driven by its own promoter into haploid and diploid *S. cerevisiae*. The extra copy of *CDC12* did not alter the budding phenotype (data not shown). Further, previous work has shown that *CDC12* driven by the *GAL1* promoter does not induce filamentation, nor does it perturb the bud-neck localization of septins Cdc3, Cdc10 and Cdc11 (Versele *et al.*, 2004).

To determine whether *A. nidulans* AspC could substitute for any of the *S. cerevisiae* septins, we introduced *aspC* into *S. cerevisiae* septin deletion mutants. Because *CDC3, CDC11* and *CDC12* are essential genes we used heterozygous diploids (strains in which one copy of the gene has been deleted while the other copy remains intact) for some experiments. Expression of *aspC* induced filaments in haploid *shs1*Δ*, spr3*Δ*, spr28*Δ*,* and heterozygous diploid *CDC3/cdc3*Δ*, CDC11/cdc11*Δ and *CDC12/cdc12*Δ. However, *aspC* did not induce filaments in *cdc10*Δ (Table 3.1.). The failure of *aspC* to induce filaments in *cdc10*Δ suggested that AspC might require a functional *S. cerevisiae* septin neck scaffold to induce filaments.

To determine if AspC localizes to the neck scaffold, we introduced GFP-AspC into *S. cerevisiae*. The GFP-AspC fusion protein induced filaments just as AspC had. GFP-AspC label was seen with similar frequency at the necks of yeasts and of filaments. Label was also seen at the tips of filaments (Figure 3.2. a, b, e, f). To determine whether the *A. nidulans* septin AspC affected the localization of the orthologous yeast septin, we introduced Cdc12-GFP into *S. cerevisiae* carrying *aspC* under the control of the constitutive *ADH1* promoter. Surprisingly, Cdc12-GFP localized to the necks of buds, but not of filaments. Virtually all Cdc12-GFP label was at yeast necks (99.5%, n=300) and almost none was at filament necks (0.5%, n=300) (Figure 3.2 c, d, g, h).

To determine whether the *A. nidulans* septin AspC affected the localization of other yeast septins, we used an antibody to localize Cdc11 in *S. cerevisiae* carrying GFP-AspC. We found examples of co-localization of GFP-AspC and Cdc11 at the necks of both yeasts and filaments (Figure.3.3.). We also observed GFP-AspC, but not Cdc11, at the tips of some filaments and large buds. The presence of Cdc12-GFP exclusively at bud necks and of GFP-AspC and Cdc11 at both bud and filament necks suggests that AspC competes with Cdc12 for incorporation into the septin scaffold at the neck and that the substitution of AspC for Cdc12 induces filaments.

The *A. nidulans aspC* septin also induced abnormal morphology during sexual reproduction in *S. cerevisiae*. When diploid *S. cerevisiae* is placed on sporulation medium it undergoes meiosis and packages four spores into an ascus (a round protective sac). When *S. cerevisiae CDC12/cdc12Δ* carrying GFP-AspC was induced to sporulate asci were enlarged with spores arranged in long chains or clusters reminiscent of the asci of filamentous fungi (Figure 3.4.). Over half (65%) of the resulting asci contained more than four spores, with as many as twelve seen in some cases. Five apparently normal tetrad asci and five abnormal enlarged asci were dissected. Each of the normal tetrads had two viable spores while no spores from abnormal asci were viable (data not shown).

Given the scaffolding role of septins, a likely mechanism for AspC-induced morphological changes is inappropriate recruitment or regulation of proteins that localize in a septin-dependent manner. To investigate this possibility, we introduced *aspC* into seven *S. cerevisiae* strains with deletions in genes encoding Cdc12-interacting proteins or a morphogenesis checkpoint component (*AFR1*, *BEM4*, *BNI1*, *CLA4*, *GIC1*, *GIC2* and *SWE1*) (Gladfelter *et al.*, 2001). Six of seven deletion strains formed filaments upon introduction of GFP-AspC (Table 3.1.). The one exception, *bni1*Δ*,* made triads or short chains of yeast cells with dramatically broader necks and GFP-AspC bars or dots in neck regions and at bud tips (data not shown). Introduction of extra *CDC12* into *bni1*Δ carrying GFP-AspC restored a normal budding phenotype to virtually all cells, consistent with the notion that AspC competes with Cdc12 for incorporation into the septin scaffold and that AspC inappropriately recruits Bni1. In control experiments, *bni1*Δ transformed with Cdc12-GFP made normal budding cells with necks slightly broader than those of wildtype and Cdc12-GFP localizing to neck rings.

The formins Bnr1 and Bni1 nucleate actin filament assembly in *S. cerevisiae* (Pruyne *et al.*, 2004). Though the functions of yeast formins overlap, Bnr1 assembles actin cables that form a stable axis between mother and daughter while Bni1 assembles dynamic actin cables that target vesicles to multiple locations in the bud and are required for polar growth (Pruyne *et al.*, 2004). To determine whether both formins might be required for AspC-induced filamentation, we introduced *aspC* into *bnr1*Δ (Table 3.1.).

AspC induced filaments in *bnr1*Δ showing that a stable mother/bud axis is not required for filament formation. The failure of AspC to induce filaments in *bni1*Δ suggests that the dynamic actin cables used in polar growth of buds are also used in polar growth of filaments.

DISCUSSION

The *S. cerevisiae* septins Cdc3, Cdc10, Cdc11 and Cdc12 form a filamentous collar at the mother neck. Disruption of any one of these septins leads to mislocalization the others. The introduction of the *A. nidulans* septin AspC induces filaments in wild-type *S. cerevisiae* likely via incorporation of AspC into the *S. cerevisiae* septin scaffold. The failure of *aspC* to induce filaments in *cdc10*Δ suggests that AspC requires a functional *S. cerevisiae* septin neck scaffold to induce filaments. The co-localization of the *S. cerevisiae* Cdc11 septin with GFP-AspC at the necks of yeasts and filaments argues that the septin scaffold is largely intact.

Our data further suggest that AspC competes with its ortholog Cdc12 for incorporation into the neck and that the replacement of Cdc12 by AspC results in filamentous growth. Indeed we observed that the percentage of filaments relative to yeast cells correlated with the strength of the promoter driving *aspC* expression (data not shown) and Cdc12-GFP was virtually never seen at filament necks while GFP-AspC was seen at yeast and filament necks. Our results further suggest that once it is incorporated into the septin scaffold AspC exerts its effects via the formin Bni1. Consistent with a role for the Bni1 formin in the polar growth of filaments, SepA, the *A. nidulans* Bni1 ortholog, localizes to hyphal tips along with actin and is required for polar growth (Sharpless and Harris, 2002).

There is increasing evidence from a variety of systems that septins play critical roles in directing cell shape and movement (Lindsey and Momany, 2006). Because they form scaffolds that recruit and organize many other proteins, including those that regulate morphogenesis and cell cycle (Gladfelter *et al.*, 2001), it seems reasonable that changes in septins would have large effects on cell shape by virtue of their binding partners.

That the *A. nidulans* septin AspC induces filaments in *S. cerevisiae* through incorporation into the septin scaffold and inappropriate recruitment and regulation of the actin-nucleator Bni1 raises the intriguing possibility that morphological transitions during evolution, such as that from hyphae to yeasts, could have proceeded by changes in key scaffold proteins.

MATERIALS AND METHODS

Yeast strains and plasmids used in this study are listed in Table 3.2. Yeast strains were incubated in YPD (rich medium), SC (synthetic complete medium with amino acids omitted as necessary for plasmid selection), sporulation medium or SLAD (sigma strains) (Ausubel, 1987) at 30ºC unless otherwise stated. Plasmids were constructed using standard methods (Ausubel, 1987; Sikorski and Hieter, 1989). *A. nidulans* AspC was amplified from cDNA using the Advantage High Fidelity 2 PCR kit (Clontech Laboratories, Inc, Mountain View, CA), cloned into PCR 2.1 TOPO (Invitrogen Corp., Carlsbad, CA) and sequenced. For expression in *S. cerevisiae, aspC* was cloned behind the inducible *GAL1* promoter or the constitutive *ADH1* promoter (Ruohonen *et al.*, 1991;

Ruohonen *et al.*, 1995) to yield plasmids pRL16 (*GAL1*-AspC) and pRL19 (*ADH1*-AspC). Plasmids were introduced into *S. cerevisiae* by transformation using LiAc (Elble, 1992).

Induction of filamentous growth and microscopy

Haploid Sc295 was transformed with pRL16 (*GAL1*-AspC) and pRS316 (empty vector). Single transformant colonies were transferred to liquid selective medium, grown to an OD_{600} of 0.4 and split into two tubes. One tube was induced by the addition of fresh medium with galactose (0.5% final concentration) while the second tube received fresh medium without galactose. Strains 4741 and 4743 were transformed with pRL19 (*ADH1*-AspC) and plated on selective medium without galactose. Cells were incubated for 24 or 48 hours and fixed using standard techniques (Pringle *et al.*, 1989). MLY40 and MLY61, derivatives of Σ1278b, were transformed with pJT2044 (cdc12-GFP), pYH35 (*ADH1*-AspC-GFP), and pRS316 (empty vector) and grown in SLAD liquid and solid medium. Samples were incubated overnight at 30°C in appropriate medium. Overnight cultures were used to inoculate fresh medium, incubated at 30°C for 2hrs and observed using a Zeiss Axioplan microscope. Digital images were acquired using an Optronics digital imaging system and were prepared using Adobe Photoshop cs version 8.0.

Counting

All morphology counts were performed at least twice by two or more investigators counting a minimum of 300 cells each. The average count is presented.

Invasive growth assay

ARL15 (pRS316GU in Sc295), AYH3 (pRL16 in Sc295), ARL21 (pRS316 in 4741), ARL22 (pRL16 in 4741), Sc295 and SY3721 (haploid invasive strain) were inoculated to SC –ura solid medium with and without 0.5% galactose. The above strains plus haploid strain 4741 were inoculated to YPD with and without 2% galactose. All strains were incubated for three days at 30ºC. On the third day plates were washed with gently running water and rubbed with a gloved finger (Liu *et al.*, 1996). Pictures were taken before and after washing with a Kodak CX6330 digital camera. Experiments were repeated three times with identical results.

Pathway mutant studies

We obtained seventeen haploid deletion strains (*afr1*Δ*, bem4*Δ*, bni1*Δ*, bnr1*Δ*, bud8*Δ*, cla4*Δ*, dfg16*Δ*, flo8*Δ*, flo11*Δ*, gic1*Δ*, gic2*Δ*, kss1*Δ*, ras2*Δ*, ste7*Δ*, ste11*Δ*, ste20*Δ*, swe1*Δ*, tec1*Δ) (Winzeler *et al.*, 1999) through Open Biosystems (AL). We confirmed the identity of all deletion strains by PCR amplification of unique tags using sequences provided by the *Saccharomyces* Genome Deletion Project web page (www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html). Five colonies from a streaked plate of each deletion strain were tested using colony PCR with modifications to protocols from the Stanford Yeast Deletion website. Modifications included making a master mix of diluted primer A and KanB for 5 individual Easy Start Micro 20 PCR tubes (Molecular Bioproducts, San Diego, CA). The final primer concentration in each tube was 0.37µM. A pipette tip was used transfer 0.25µL of cells (or less) from a yeast colony to the PCR tube. PCR was carried out with a RoboCycler Gradient 96 thermocycler (Stratagene, La Jolla, CA) under the following conditions: 1 cycle at 94C for 4 min, 35 cycles at 94C for 30 seconds, 64C for 1 min, and 72C for 2 min, and 1cycle at 72C for 10 min. The total contents of each tube were electrophoresed on a 1% TBE gel with a 100bp DNA ladder (Promega, Madison, WI). PCR products amplified from all five colonies were the expected size in all strains. These haploid deletion strains were transformed with pRS316 (empty vector control) and pRL16 (pRS316 with AspC) and plated on selective medium. For induction, single colonies were grown in selective liquid medium to an optical density at A_{600} of 0.4; the sample was split and centrifuged. Half of the sample was resuspended in two volumes of fresh medium; the other half was resuspended in two volumes of fresh medium with 0.5% galactose and no glucose. Samples were incubated overnight (16hrs) and examined microscopically. Most deletion strains were also transformed with pRL19 (*ADH1*-AspC) and plated on selective medium. We obtained this vector by cloning the constitutively active *ADH1* promoter into *Sal*I and *Kpn*I restriction sites in pRL16 (*GAL1*-AspC). Transformation and induction experiments for each strain were repeated at least twice.

GFP Localization

A triple GFP mutant pQBI25 (Quantum, Inc.) was fused inframe to the N-terminus of AspC in pRS316 using the *Sal*I-*Eco*RI restriction sites to give pYH33. The constitutively active *ADH1* promoter from pRL19 was cloned into the *Kpn*I and *Sal*I restriction sites to drive fusion protein expression to yield plasmid pYH35 (*ADH1*-GFP-AspC). For co-transformations the *LEU2* marker was cloned into a *Nco*I site in the middle of the *URA3* marker of pRL19 (*ADH1*-AspC, *URA3*) to get pRL20 (*ADH1*-AspC, *LEU2*). We transformed both pJT2044 (cdc12-GFP, *URA3*) and pRL20 (*ADH1*-AspC, *LEU2*) into the diploid yeast strain *CDC12/cdc12Δ.* In separate experiments

CDC12/cdc12Δ was transformed with pYH35 (*ADH1*-GFP-AspC). All transformants were incubated overnight at 30°C. Overnight cultures were transferred to fresh medium, incubated at 30°C for 2hrs and observed using a Zeiss Axioplan microscope. Digital images were acquired using an Optronics digital imaging system and were prepared using Adobe Photoshop cs version 8.0.

Septin deletion mutants

We obtained haploid (*cdc10*Δ*,sep7*Δ*, spr3*Δ*, spr28*Δ) and heterozygous diploid (*CDC3/cdc3*Δ*, CDC11/cdc11*Δ and *CDC12/cdc12*Δ) *S. cerevisiae* septin deletion strains (Winzeler *et al.*, 1999) through Open Biosystems (AL). Haploid deletion strains were transformed with pRS316 (control), pRL16 (*GAL1*-AspC) and pRL19 (*ADH1*-AspC) vectors and plated on selective medium. Strains with *GAL1*-AspC were induced as noted above in pathway mutant studies with fresh medium with or without 0.5% galactose. The diploid deletion strain *CDC12/cdc12*Δ was transformed with pYH35 (*ADH1*-GFP-AspC) or with both pJT2044 (Cdc12-GFP) and pRL20 (*ADH1*-AspC) and grown on selective media. Transformation and induction experiments for each strain were repeated at least twice. Counts of filaments vs. buds were made for each strain.

Sporulation

Diploid heterozygous septin deletion strains, with and without AspC were inoculated to sporulation medium (Ausubel, 1987) and incubated at room temperature for 4-7 days. Colonies were transferred to a slide with mounting solution and examined microscopically for ascospores. Septin deletion strain *CDC12/cdc12*Δ was transformed with *ADH1*-GFP-AspC and incubated on selective medium. Single colonies were streaked and transferred to sporulation medium (Ausubel, 1987) and incubated at room temperature for 4-7 days. For tetrad analysis, a micromanipulator was used to transfer ascospores to YPD plus sorbitol medium, then to selective medium and colonies were scored.

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Table 3.1. AspC-induced phenotype in *S. cerevisiae* deletion mutants

Deletion[a]	**Phenotype**[b]	**Function**[c]
*afr1*Δ[de]	F	Cdc12i, Shmoo
*bem4*Δ[d]	F	Cdc12i
*bni1*Δ[de]	Y, N	Cdc12i, Formin
*bnr1*Δ[de]	F	Formin
*CDC3/cdc3*Δ[d]	F	Septin
*cdc10*Δ[d]	Y	Septin
*CDC11/cdc11*Δ[d]	F	Septin
*CDC12/cdc12*Δ[de]	F	Septin
*cla4*Δ[d]	F	Cdc12i, Cell Pol
*dfg16*Δ[d]	F	Hap Inv
*flo8*Δ[d]	F	cAMP
*flo11*Δ[d]	F	cAMP
*gic1*Δ[de]	F	Cdc12i, Cell Pol
*gic2*Δ[de]	F	Cdc12i, Cell Pol
*kss1*Δ[d]	F	MAPK
*ras2*Δ[d]	F	Pseudohyphal
*shs1*Δ[d]	F	Septin
*spr3*Δ[d]	F	Septin
*spr28*Δ[d]	F	Septin
*ste7*Δ[d]	F	MAPK

*ste11*Δ[d]	F	MAPK
*ste20*Δ[d]	F	MAPK
*swe1*Δ[de]	F	Morph Ckpt
*tec1*Δ[d]	F	MAPK

[a] deletion mutants from Open Biosource.

[b] *S. cerevisiae* deletion mutants were transformed with *A. nidulans aspC* behind the *GAL1* inducible or the *ADH1* constitutive promoter as indicated. Phenotype after galactose induction (*GAL1* promoter) or with no induction (*ADH1* promoter) is indicated. F= filaments in >28%; Y= yeast; N = novel phenotype.

[c] cAMP, cAMP pathway component; Cdc12i, Cdc12-interacting (genetic or biochemical); Cell Pol, establishment of cell polarity; Hap Inv, Haploid invasive growth; MAPK, MAPK pathway component; Morph Ckpt, morphogenesis checkpoint component; Pseudohyphal, diploid pseudohyphal growth; shmoo, mating projection formation.

[d] *GAL1* promoter.

[e] *ADH1* promoter.

Table 3.2. *S. cerevisiae* strains and plasmids used in this study

Strain	Relevant genotype	Source
ARL7	Sc295 with pRL16 (AspC-*GAL1-URA3*)	this study
ARL15	Sc295 with pRS316GU (empty vector)	this study
ARL21	BY4741 with pRS316 (empty vector)	this study
ARL22	BY4741 with pRL16 (AspC-*GAL1-URA3*)	this study
ARL68	BY4741 with pRL19 (AspC-*ADH1-URA3*)	this study
AYH3	Sc295 with pRL16 (AspC-*GAL1-URA3*)	this study
BY4741	***a*** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
BY4742	***α*** *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Open Biosystems
BY4743	***a/α*** *4741/4742*	Open Biosystems
CDC12/cdc12Δ	BY4743 background	Open Biosystems
DDY1453	**a** *ura3-52 his7 ade2 trp1-289 cdc3-1ts*	J. Thorner (Hartwell, 1971; Roemer *et al.*, 1996)
DDY1462	**a** *ura3-52 cdc12-6ts*	J. Thorner
DDY1455	**a** *ura3-52 his7 tyr1 ade2 lys2 cdc11-1ts*	J. Thorner (Hartwell, 1971; Roemer *et al.*, 1996)
DDY1476	**a** *ura3-52 trp1-289 ade2 lys2 tyr1 cdc10-1ts*	J. Thorner
MLY40	**α** *ura3-52*	J. Heitman (Lorenz and Heitman, 1997)
MLY61	**a/α** *ura3-52/ura3-52*	J. Heitman (Lorenz and Heitman, 1997)
pJT2044/MVB89	pRS316 with CDC12-GFP	J. Thorner
pQBI25	triple GFP mutant −	Quantum Inc.
pRL16	pRS316 with *GAL1*-AspC, *URA3*	this study

pRL19	pRS316 with AspC-*ADH1*, *URA3*	this study
pRL20	pRS316 with AspC-*ADH1*, *LEU2*	this study
pRS315	pRS305 with *LEU2*	W. Schmidt (Sikorski and Hieter, 1989)
pRS316	pRS306 with *URA3*	W. Schmidt (Sikorski and Hieter, 1989)
pRS316GU	pRS316 with *GAL1*,*URA3*	W. Schmidt (Hovland *et al.*, 1989; Sikorski and Hieter, 1989; Joshua-Tor *et al.*, 1995)
pYH33	pRS316 with GFP-AspC	this study
pYH35	pRS316 with *ADH1*-GFP-AspC	this study
Sc295*	**a** *ura3-52 leu2-3 112 reg1-501 gal1 pep4-3*	W. Schmidt (Hovland *et al.*, 1989; Sikorski and Hieter, 1989; Joshua-Tor *et al.*, 1995)
SY3721	**α** *ura3 axl1::HIS3*	W. Schmidt (Cullen and Sprague, 2002)
Y187	**α** *ura3-52 his3-200 ade2-101 trp1-901*	Clontech (Harper *et al.*, 1993)

* Sc295 has a *reg1-501* mutation that prevents glucose-mediated repression of *GAL*-dependent gene expression (Hovland *et al.*, 1989).

Figure 3.1. ***A. nidulans*** **septin AspC induces filaments in *S. cerevisiae.*** *S. cerevisiae* transformed with the *A. nidulans* septin gene *aspC* under the control of the inducible *GAL1* promoter and incubated for 24 (a-c) or 48 hr (d-f). a, d) uninduced cultures grow only as budding yeasts. b, c, e, f) induced cultures grow as filaments and yeasts. Arrows indicate examples of filaments. Scale bar = 10µ.

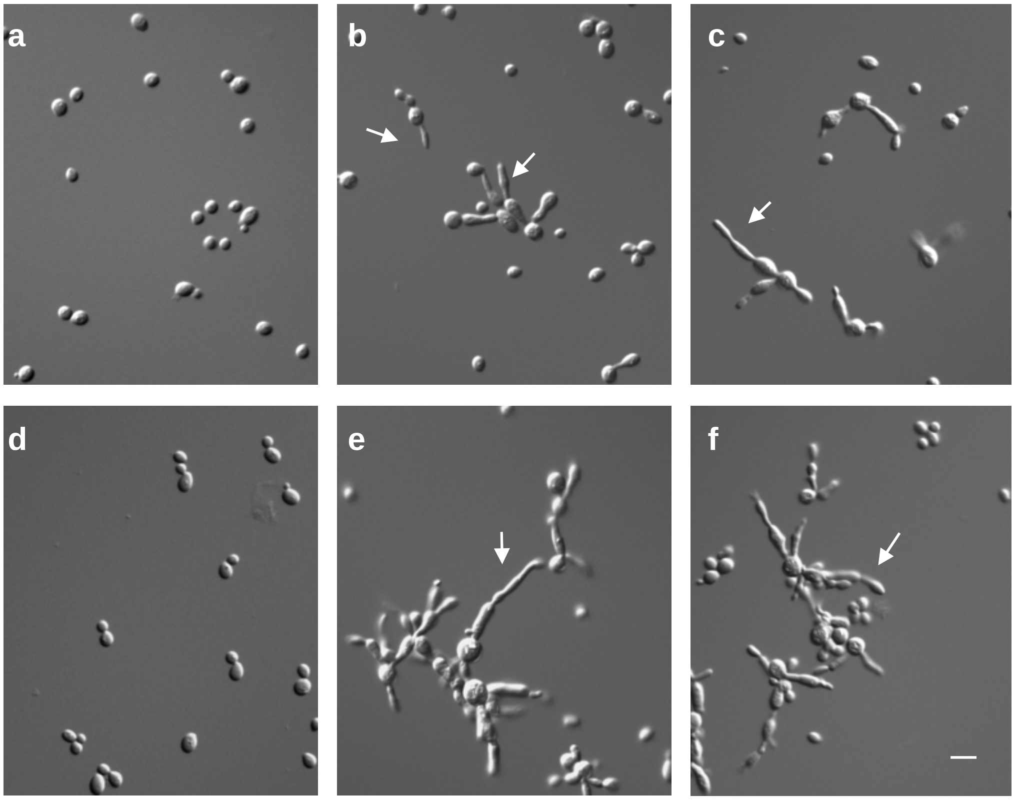
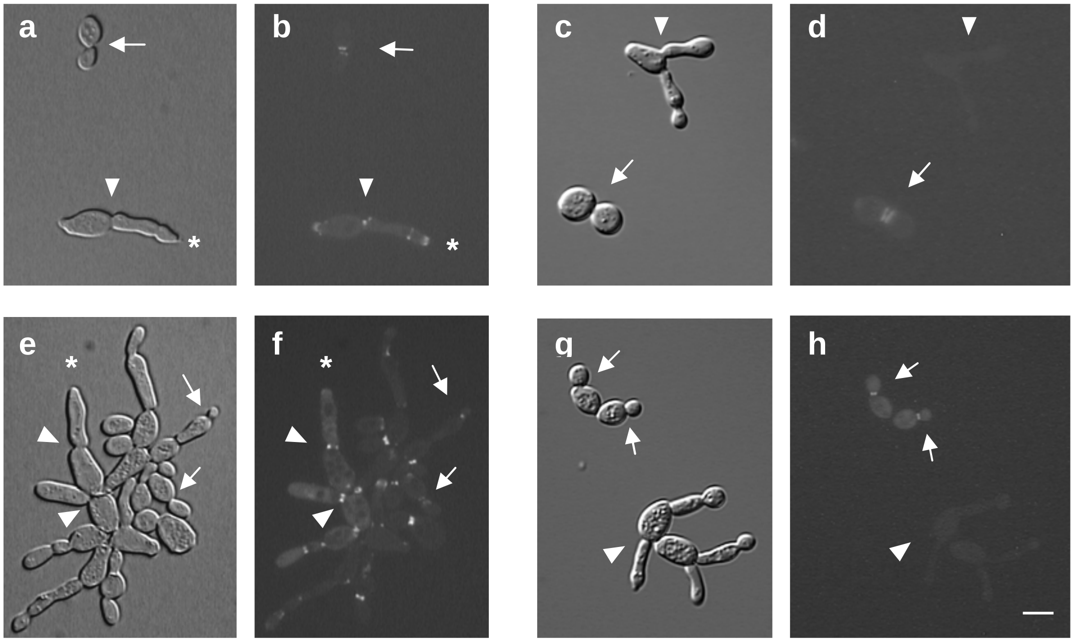

a
b
c
d
e
f

Figure 3.2. ***A. nidulans*** **GFP-AspC localizes to bud and filament necks and tips while** ***S. cerevisiae*** **Cdc12-GFP localizes exclusively to bud necks.** a,b,e,f) *S. cerevisiae* was transformed with the *A. nidulans* septin *aspC* fused to GFP under the control of the constitutive *ADH1* promoter. c,d,g,h) *S. cerevisiae* was transformed with the *A. nidulans* septin *aspC* under the control of the constitutive *ADH1* promoter and the *S. cerevisiae* septin *CDC12* fused to GFP. The *A. nidulans* septin is visible in both yeasts and filaments while the *S. cerevisiae* septin is visible only in yeasts. Arrows indicate examples of bud necks. Arrowheads indicate examples of filament necks. Asterisks indicate examples of filament tips. Scale bar = 10µ.


a
b
c
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e
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g
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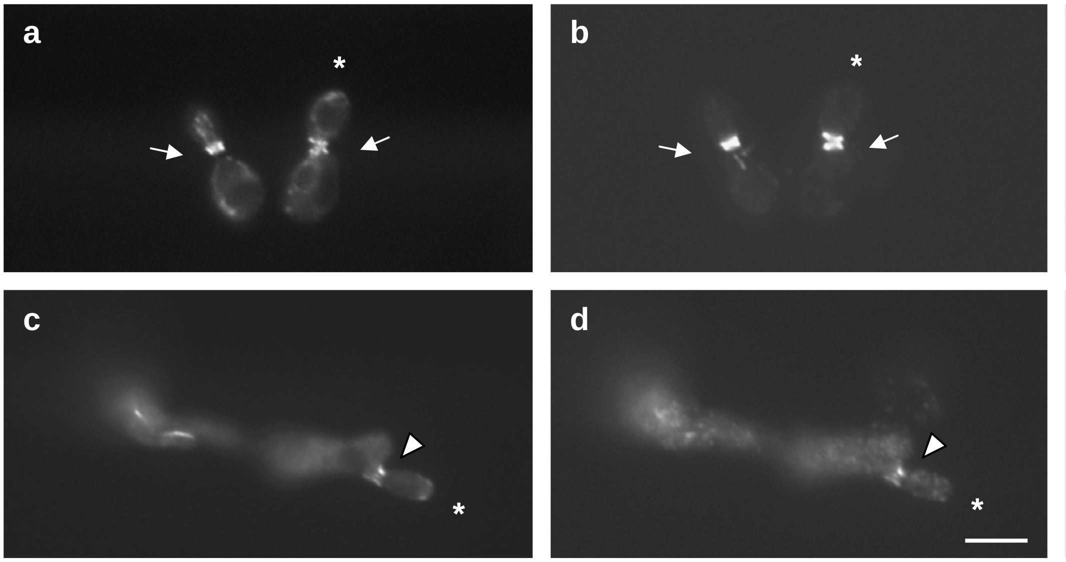
Figure 3.3. ***A. nidulans*** **GFP-AspC co-localizes with** ***S. cerevisiae*** **Cdc11 to bud and filament necks.** *S. cerevisiae* was transformed with the *A. nidulans* septin *aspC* fused to GFP under the control of the constitutive *ADH1* promoter and incubated for 48 hrs. a, c) GFP-AspC. b, d) Cdc11 detected by a commercially available primary antibody and rhodamine-coupled secondary antibody. Arrows indicate examples of bud necks. Arrowheads indicate examples of filament necks. Asterisks indicate examples of filament tips. Scale bar = 10µ.


a
*
b
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c
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d
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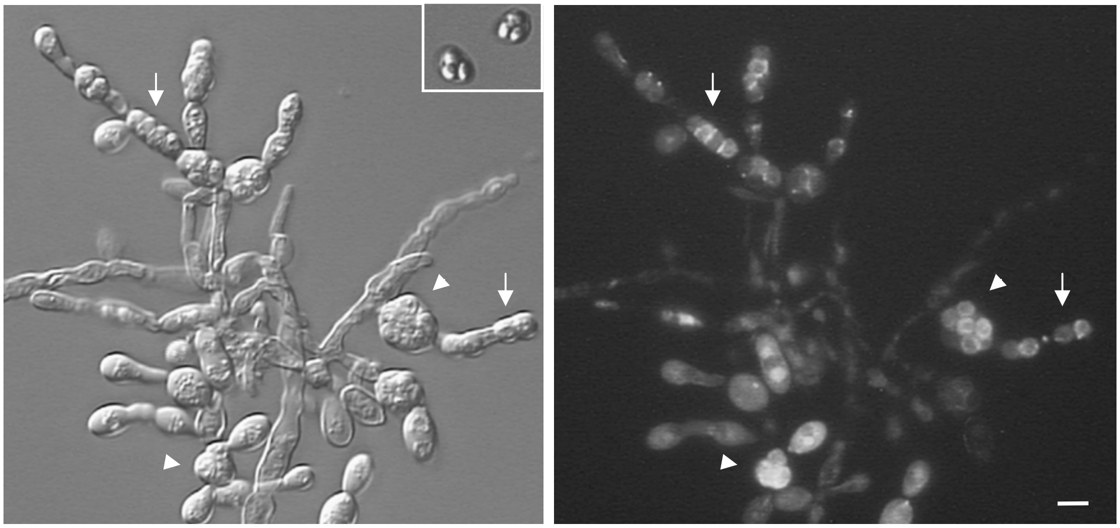
Figure 3.4. *A. nidulans* AspC induces abnormal asci in *S. cerevisiae*. After *S. cerevisiae* mates, it forms a tetrad ascus containing four spores. The *A. nidulans* septin *aspC* causes *S. cerevisiae* to make enlarged asci with up to twelve spores similar to asci made by filamentous fungi. *S. cerevisiae* was transformed with the *A. nidulans* septin *aspC* fused to GFP under the control of the constitutive *ADH1* promoter and induced to sporulate. Arrows indicate examples of linear asci. Arrowheads indicate examples of round asci. Inset shows normal yeast tetrad ascus for comparison. Scale bar = 10µ.



References

Ausubel, F.M. (1987). Current protocols in molecular biology. J. Wiley: New York.

Cid, V.J., Jimenez, J., Molina, M., Sanchez, M., Nombela, C., and Thorner, J.W. (2002). Orchestrating the cell cycle in yeast: sequential localization of key mitotic regulators at the spindle pole and the bud neck. Microbiology (Reading, England) *148*, 2647-2659.

Cullen, P.J., and Sprague, G.F., Jr. (2002). The roles of bud-site-selection proteins during haploid invasive growth in yeast. Molecular biology of the cell *13*, 2990-3004.

De Virgilio, C., DeMarini, D.J., and Pringle, J.R. (1996). SPR28, a sixth member of the septin gene family in Saccharomyces cerevisiae that is expressed specifically in sporulating cells. Microbiology (Reading, England) *142 (Pt 10)*, 2897-2905.

Elble, R. (1992). A simple and efficient procedure for transformation of yeasts. Biotechniques *13*, 18-20.

Fares, H., Goetsch, L., and Pringle, J.R. (1996). Identification of a developmentally regulated septin and involvement of the septins in spore formation in Saccharomyces cerevisiae. The Journal of cell biology *132*, 399-411.

Field, C.M., and Kellogg, D. (1999). Septins: cytoskeletal polymers or signalling GTPases? Trends in cell biology *9*, 387-394.

Gimeno, C.J., Ljungdahl, P.O., Styles, C.A., and Fink, G.R. (1992). Unipolar cell divisions in the yeast S. cerevisiae lead to filamentous growth: regulation by starvation and RAS. Cell *68*, 1077-1090.

Gladfelter, A.S., Pringle, J.R., and Lew, D.J. (2001). The septin cortex at the yeast mother-bud neck. Current opinion in microbiology *4*, 681-689.

Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K., and Elledge, S.J. (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell *75*, 805-816.

Hartwell, L.H. (1971). Genetic control of the cell division cycle in yeast. IV. Genes controlling bud emergence and cytokinesis. Exp Cell Res *69*, 265-276.

Hovland, P., Flick, J., Johnston, M., and Sclafani, R.A. (1989). Galactose as a gratuitous inducer of GAL gene expression in yeasts growing on glucose. Gene *83*, 57-64.

Joshua-Tor, L., Xu, H.E., Johnston, S.A., and Rees, D.C. (1995). Crystal structure of a conserved protease that binds DNA: the bleomycin hydrolase, Gal6. Science *269*, 945-950.

Lindsey, R., and Momany, M. (2006). Septin localization across kingdoms: three themes with variations. Current opinion in microbiology *9*, 559-565.

Liu, H., Styles, C.A., and Fink, G.R. (1996). Saccharomyces cerevisiae S288C has a mutation in FLO8, a gene required for filamentous growth. Genetics *144*, 967-978.

Lo, W.S., and Dranginis, A.M. (1998). The cell surface flocculin Flo11 is required for pseudohyphae formation and invasion by Saccharomyces cerevisiae. Molecular biology of the cell *9*, 161-171.

Longtine, M.S., and Bi, E. (2003). Regulation of septin organization and function in yeast. Trends in cell biology *13*, 403-409.

Longtine, M.S., DeMarini, D.J., Valencik, M.L., Al-Awar, O.S., Fares, H., De Virgilio, C., and Pringle, J.R. (1996). The septins: roles in cytokinesis and other processes. Current opinion in cell biology *8*, 106-119.

Longtine, M.S., Theesfeld, C.L., McMillan, J.N., Weaver, E., Pringle, J.R., and Lew, D.J. (2000). Septin-dependent assembly of a cell cycle-regulatory module in Saccharomyces cerevisiae. Molecular and cellular biology *20*, 4049-4061.

Lorenz, M.C., and Heitman, J. (1997). Yeast pseudohyphal growth is regulated by GPA2, a G protein alpha homolog. Embo J *16*, 7008-7018.

Momany, M., Zhao, J., Lindsey, R., and Westfall, P.J. (2001). Characterization of the Aspergillus nidulans septin (asp) gene family. Genetics *157*, 969-977.

Ozsarac, N., Bhattacharyya, M., Dawes, I.W., and Clancy, M.J. (1995). The SPR3 gene encodes a sporulation-specific homologue of the yeast CDC3/10/11/12 family of bud neck microfilaments and is regulated by ABFI. Gene *164*, 157-162.

Pringle, J.R., Preston, R.A., Adams, A.E., Stearns, T., Drubin, D.G., Haarer, B.K., and Jones, E.W. (1989). Fluorescence microscopy methods for yeast. Methods Cell Biol *31*, 357-435.

Pruyne, D., Gao, L., Bi, E., and Bretscher, A. (2004). Stable and dynamic axes of polarity use distinct formin isoforms in budding yeast. Molecular biology of the cell *15*, 4971-4989.

Roemer, T., Madden, K., Chang, J., and Snyder, M. (1996). Selection of axial growth sites in yeast requires Axl2p, a novel plasma membrane glycoprotein. Genes Dev *10*, 777-793.

Ruohonen, L., Aalto, M.K., and Keranen, S. (1995). Modifications to the ADH1 promoter of Saccharomyces cerevisiae for efficient production of heterologous proteins. J Biotechnol *39*, 193-203.

Ruohonen, L., Penttila, M., and Keranen, S. (1991). Optimization of Bacillus alpha-amylase production by Saccharomyces cerevisiae. Yeast *7*, 337-346.

Rupp, S., Summers, E., Lo, H.J., Madhani, H., and Fink, G. (1999). MAP kinase and cAMP filamentation signaling pathways converge on the unusually large promoter of the yeast FLO11 gene. Embo J *18*, 1257-1269.

Sharpless, K.E., and Harris, S.D. (2002). Functional characterization and localization of the Aspergillus nidulans formin SEPA. Molecular biology of the cell *13*, 469-479.

Sikorski, R.S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics *122*, 19-27.

Versele, M., Gullbrand, B., Shulewitz, M.J., Cid, V.J., Bahmanyar, S., Chen, R.E., Barth, P., Alber, T., and Thorner, J. (2004). Protein-Protein Interactions Governing Septin Heteropentamer Assembly and Septin Filament Organization in Saccharomyces cerevisiae. Molecular biology of the cell.

Westfall, P.J., and Momany, M. (2002). Aspergillus nidulans septin AspB plays pre- and postmitotic roles in septum, branch, and conidiophore development. Molecular biology of the cell *13*, 110-118.

Winzeler, E.A., Shoemaker, D.D., Astromoff, A., Liang, H., Anderson, K., Andre, B., Bangham, R., Benito, R., Boeke, J.D., Bussey, H., Chu, A.M., Connelly, C., Davis, K., Dietrich, F., Dow, S.W., El Bakkoury, M., Foury, F., Friend, S.H., Gentalen, E., Giaever, G., Hegemann, J.H., Jones, T., Laub, M., Liao, H., Liebundguth, N., Lockhart, D.J., Lucau-Danila, A., Lussier, M., M'Rabet, N., Menard, P., Mittmann, M., Pai, C., Rebischung, C., Revuelta, J.L., Riles, L., Roberts, C.J., Ross-MacDonald, P., Scherens, B., Snyder, M., Sookhai-Mahadeo, S., Storms, R.K., Veronneau, S., Voet, M., Volckaert, G., Ward, T.R., Wysocki, R., Yen, G.S., Yu, K., Zimmermann, K., Philippsen, P., Johnston, M., and Davis, R.W. (1999). Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis. Science *285*, 901-906.

CHAPTER 4

CHARACTERIZATION OF SEPTINS ASPA AND ASPC IN *ASPERGILLUS NIDULANS*[1]

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[1] Lindsey, R., Cowden, S., Momany, M. To be submitted to *Eukaryotic Cell.*

ABSTRACT

The septin family of proteins was first discovered in *Saccharomyces cerevisiae* in a screen for temperature sensitive cell cycle mutants. Septin mutants make elongated buds at restrictive temperature and are unable to complete cytokinesis. The septin proteins form filamentous rings at the mother-bud neck in yeast and are dependent on each other for localization. In addition to their original role in cell division, septins have been shown to have roles in cytoskeletal organization, coordination of nuclear division and trafficking across membranes. In *Aspergillus nidulans* there are five septins AspA, AspB, AspC, AspD and aspE. Here we characterize the *A. nidulans* septins AspA and AspC. Deletion mutants show varying degrees of early germination, increased germ tube and branch emergence, abnormal septation and disorganized conidiophores. Localization of AspA-GFP and AspC-GFP are dynamic, to all stages of growth examined and unique. AspA-GFP forms discrete structures in *ΔaspC* while AspC-GFP does not show localization in *ΔaspA*. Our results suggest that AspA and AspC have different roles in *A. nidulans*.

INTRODUCTION

Septins are a family of proteins that were discovered in *S. cerevisiae* (Hartwell, 1971). The original septin mutants, *cdc3, cdc10, cdc11* and *cdc12* are temperature sensitive cell cycle mutants that make elongated buds at restrictive temperature and are unable to complete cytokinesis (Hartwell, 1971). It is now known that five of *S. cerevisea's* seven septins (Cdc3p, Cdc10p, Cdc11p, Cdc12p and Sep7p) co-localize to the neck where they interact with each other to form a scaffold that recruits other proteins (Caviston *et al.*, 2003; Versele *et al.*, 2004; Iwase *et al.*, 2006). There are over 35 proteins known to interact directly or indirectly with septins at the bud neck (Gladfelter *et al.*, 2001).

Septins are found in fungi, animals and microsporidia but they are absent from plants. The number of septins in higher eukaryotes varies from two in *Caenorhabditis elegans* to 13 in humans (Neufeld and Rubin, 1994; Fares *et al.*, 1995; Nguyen *et al.*, 2000; Spiliotis and Nelson, 2006). Septins are always found in complexes. They form multiseptin heteropolymers that act as scaffolds which organize other proteins (Frazier *et al.*, 1998; Versele *et al.*, 2004). In *S. cerevisiae* mutants in which *CDC3*, *CDC10*, *CDC11* or *CDC12* is mutated the remaining three septins are unable to localize normally to the bud neck (Haarer and Pringle, 1987). Dependant co-localization of septins is also seen in Drosophila and mammalian systems (Fares *et al.*, 1995; Kinoshita *et al.*, 2002; Peng *et al.*, 2002).

A. nidulans has five septins AspA, AspB, AspC, AspD and aspE that were previously cloned and sequenced (Momany *et al.*, 2001). All five are expressed during vegetative and asexual growth, with AspB and then AspC having the highest expression levels respectively (Momany *et al.*, 2001). Immunofluorescence studies show AspB localizes to septa and conidiophore layers and was found as a marker for branch emergence (Westfall and Momany, 2002).

MATERIALS AND METHODS

Strains and media

Strains used in this study are listed in Table 4.1. Media used were previously described (Harris *et al.*, 1994). Strain growth and construction was by standard *A. nidulans* techniques (Kafer, 1977; Harris *et al.*, 1994). All incubations were at 30°C and in complete medium except where noted (1% glucose, 0.2% peptone, 0.1% casamino acids, 0.1% yeast extract, trace elements, nitrate salts and 0.01% vitamins, pH 6.5) with amino acid supplements, trace elements, nitrate salts, and vitamins (Kafer, 1977).

Growth conditions and microscopic observation

Preparation and growth of cells were as previously reported (Momany *et al.*, 1999). Briefly, conidia were inoculated on sterile coverslips in liquid medium and incubated at 30°C in a Petri dish. Cells were fixed, septa were stained with calcofluor (American Cyanamid, Wayne, NJ) and nuclei were stained with Hoechst 33258 (Sigma, St. Louis). Microscopic observations were made using a Zeiss (Thornwood, NY) Axioplan microscope and digital images were acquired using an Axion (Axion Technologies) digital imaging system. Images were prepared using Photoshop cs version 8.0 (Adobe, Mountain View, CA).

Asexual structures

Preparation of conidiophores were as previously reported (Lin and Momany, 2003). Briefly, conidia were inoculated on the edges of a square of complete agar medium and a coverslip was placed on top of the agar square. Plates were incubated inverted at 30°C for 2 days. To observe conidiophore structures, coverslips with aerial hyphae and conidiophores attached were fixed, mounted on slides, and observed microscopically.

Fluorescent tags and gene knockouts

We used a fusion PCR-based method to generate C-terminal green fluorescent protein (GFP) tagged septins expressed from their own promoters as previously reported (Yang *et al.*, 2004). Cassettes for GFP fusions were created in the Osmani lab and obtained through the Fungal Genetics Stock Center (http://www.fgsc.net/) (Yang *et al.*, 2004). Briefly, the tags with an additional 2 kb of flanking targeting sequence plus the fluorescent tag were transformed into an *A. nidulans* Δ*nkuA* strain in which a gene for non-homologous end-joining has been removed (Yang *et al.*, 2004). Homologous recombination generates a tagged version which replaces the wild-type allele. All strains were then crossed out of the Δ*nkuA* strain into an A850, A28 or A773 wild type strain. We also used this methodology to create gene deletions in which the target gene was replaced with the *pyrG* marker (Kuwayama *et al.*, 2002). For each strain PCR was used to confirm site-specific gene replacements with one primer anchored in the genome upstream of the flanking target sequence and the other primer anchored in the fluorescent tag or *pyrG* marker for gene replacement. At least four independent transformants were characterized.

Quantification of deletion mutants

After incubating 4 or 5hrs at 30°C the proportions of cells that had germinated and released germ tubes were noted (n=200). At 4 and 5hrs cells that were round and swollen but without germ tubes (0), cells that had one (1) or two or more germ tubes (2) were counted. Counts at six hours in each population all of the swollen conidia had one (1) or more germ tubes (2). Overall the deletion mutants growth was about 2 hours ahead of the wild-type due to the early germination, so all further counts were conducted with wild-type strains that grew two hours longer than the deletion mutants, resulting in individuals of about the same length. To assay reduced septation we examined cells that were fixed and labeled with calcofluor to visualize cell wall and septa and Hoechst to visualize nuclei. We did not count the total number of septa present in the cell. We only noted if septa were present (1) or not (0) and we only counted one hypha per cell. The first count was based on a time course, counts were made of A850 at 12 hrs and of the deletion mutants at 10hrs (n=200). The second, more stringent count we examined septation in cells with a germ tube holding at least 16 nuclei (n=30), If no septum was present the cells were scored as (0) if there was a septum present these cells were scored as (1). Cells were also counted for the presence (1) or absence (0) of septa after 15hr incubation. All the hyphae in these counts had at least 16 nuclei (n=30). Branch emergence was assayed by counting the number of branch initials that formed off of the main germ tube (n=200).

RESULTS

ΔaspA, ΔaspC **and the double deletion mutant show early germination, increased germ tube and branch emergence and abnormal septation.**

In wild-type *A. nidulans*, spores break dormancy and grow isotropically; next they switch to polar growth and send out a germ tube which grows by tip extension. Germ tube emergence is sequential and occurs in a bipolar pattern (Harris, 1999; Momany and Taylor, 2000). After incubation for 8hrs at 30°C, 8-16 nuclei are present in the hyphae, at which time septation occurs near the conidium, next a branch will form off of the main germ tube (Momany and Taylor, 2000).

To determine if the septins AspA and AspC might play roles in early *A. nidulans* development, we examined the phenotypes of strains in which individual septin genes had been replaced by markers (*ΔaspA*, ASH 5 and *ΔaspC,* ARL 161) or in which both septins were replaced by markers (*ΔaspAΔaspC*, ARL162). We observed the same phenotypes for the single and double deletion mutants we examined, though they varied in severity (Figure 4.1, Table 4.2). Germ tube emergence in the deletion mutants started 2-3 hours earlier than in wild-type. After 4 hrs at 30°C, germ tubes were visible in 0% of wild-type, 19% of *ΔaspA*, 8% *ΔaspC* and 24% of *ΔaspA ΔaspC* (n=200). At 4 hours the double deletion mutant was the only one that showed individuals with two or more germ tubes emerging (4%). By 5hrs at 30°C, germ tubes were not visible in the wild-type, but 52-58% of deletion strains had sent out germ tubes (Figure 4.1, Table 4.2). Overall the deletion mutants growth was about 2 hours ahead of the wild-type, so all subsequent observations were conducted with wild-type strains that grew two hours longer than the deletion mutants, resulting in individuals of about the same length and developmental stage. When incubated for 8hrs, 8% of wild-type cells had two or more germ tubes. At the developmentally equivalent 6 hr time point 59-62% of the single deletion mutants and 99% of the double deletion mutants had two or more germ tubes (Figure 4.1, Table 4.2). If two germ tubes formed in the wild-type strain they were usually separated by 180°. In the deletion mutants multiple germ tubes were adjacent to each other or separated by 45°, 90° or 180°.

We assayed septation using two different methods which gave very similar results. When incubated for 12 hr, at least one septum was seen in 97% of wild-type cells (n=200). At the developmentally equivalent 10hr time point, a septum was seen in 13-15% of single deletion mutants and 0% of the double deletion mutants. When at least 16 nuclei were present in a germ tube, 100% of wild-type cells, 12% of *ΔaspA*, 3% *ΔaspC* and 0% of the double deletion mutants had a septum (n=30, Figure 4.1, Table 4.2). At 15hrs, 95% *ΔaspA* cells had at least one septum suggesting septation did occur though it was delayed (Figure 4.1, Table 4.2). In contrast at 15 hrs, only 28% of *ΔaspC* and 38% of *ΔaspA ΔaspC* cells had at least one septum. Further, the intensity of staining with Calcofluor was reduced and difficult to see in *ΔaspC* and *ΔaspA ΔaspC* as compared to wild-type and *ΔaspA*.

Branch emergence was assayed by counting the number of branch initials that formed from the main germ tube (n=200). When incubated for 12 hr, a single branch was seen in 16% of wild-type hyphae and two branches were seen in 6% of wild-type. At the developmentally equivalent 10hr time point, a single branch was seen in 17-26% of single deletion mutant hyphae and two branches were seen in 32-39% of the single deletion mutants. At the10hr time point, a single branch was seen in 23% of double deletion mutant hyphae and two branches were seen in 59% of the double deletion mutants (Figure 4.1, Table 4.2).

ΔaspA, ΔaspC **and** ***ΔaspA ΔaspC*** **show reduced and fused layers in asexual structures.**

In asexual reproduction (Figure 4.2a) a single hyphal compartment sends up an aerial hypha and the tip of the aerial hypha swells to form a vesicle. From the vesicle a layer of metulae form, from the metulae layer the philades form. From the philade layer, chains of conidiospores form (Timberlake and Marshall, 1988; Adams *et al.*, 1998). Wild-type conidiospores are pigmented resulting in green colonies when examined. Deletion mutants had irregular and fused layers in the conidiophore which resulted in a reduced number of spores (Figure 4.2 b-d) making the colonies appear brown instead of green on agar plates. The *ΔaspA ΔaspC* conidiophores were slightly more abnormal than the single deletion mutants, but still produced a small number of conidiospores. The sexual reproduction structures huelle cells, cleistothecia and ascospores all formed and deletion mutants were able to cross. *ΔaspA* was successfully crossed with *ΔaspC* to obtain a double deletion strain. *ΔaspA ΔaspC* was successfully crossed with wild-type.

AspA localizes to conidia, germ tubes, septa, branches and conidiophore layers.

In order to localize the septins, we constructed C-terminal GFP fusions at the endogenous septin locus and behind the endogenous septin promoter. Initially AspA localization was a single spot, circle or short bar on the conidium (Figure 4.3a, 4.4a). As the conidium swelled localization became more punctuate and cortical (Figure 4.3a, b). As polarization occurred AspA-GFP was found at the base and growing tip of the germ tube (Figure 4.3b). In hyphae fluorescence was punctate at the cortex with bright caps at the tips (Figure 4.3c, 4.4a) or as filaments at the tips (Figure 4.4b). AspA-GFP formed a bright band transiently at septa (Figure 4.3c). In branch initials localization was diffuse in forming nubs (Figure 4.3d). As branches emerged AspA-GFP was filamentous (Figure 4.4c). In the asexual conidiophores AspA localization was to tips of emerging layers, transiently at metula/ phialide interfaces (not shown), and persistently to the phialide/conidiospore interface and as a dot, circle or short bar on the conidiospores (Figure 4.5).

AspC localizes to conidia, hyphae, presumptive septation site, branches and conidiophore layers.

Initially AspC was seen as a single spot, a single long bar or pair of short bars on the conidium (Figures 4.6a, b unpublished and data not shown). As the conidium swelled AspC-GFP became more punctate and cortical (Figures 4.6a, b). Localization to hyphae was as dots, short bars or long bars that frequently looped down along the edge of the growing tip (Figure 4.6b-f). AspC-GFP bright filaments were frequently seen throughout hyphae (Figures 4.6d, f) except at times these filaments were absent in compartments (Figures 4.6e). Faint localization was seen as a series of thin lines across the hypha at presumptive septation sites (Figures 4.6f). As branches emerged a long bar sometimes associated with dots extended into branch initials and continued to loop in as branch grows (Figure 4.7).

In conidiophores localization appears as a broad bar below the aerial hypha initial and then moved up into aerial hypha as it grew, this broad or elongated bar is sometimes seen at vesicles in mature conidiophores (Figure 4.8a, data not shown). Localization was to layers of conidiophores as dots, rings, filaments, and short or long bars (Figure 4.8).

AspC-GFP does not show localization in *ΔaspA*. AspA-GFP localizes abnormally in *ΔaspC*.

To investigate whether AspA and AspC require each other for localization we made strains in which one septin was fused to GFP and the other septin was deleted. We examined three different PCR positive progeny (ARL183-185) resulting from a cross of *ΔaspA* (ASH26) with AspC-GFP (ARL159). In all cases we no longer observed specific localization of AspC in a *ΔaspA* background in vegetative or asexual growth. At most there was a faint overall green fluorescence (Figure 4.9).

We examined progeny from two different AspA-GFP and *ΔaspC* crosses; ARL141 crossed with ARL157 (ARL198) and ARL141 crossed with ARL161 (ARL201) for localization. With the AspA-GFP in a *ΔaspC* background localization collapsed to a tiny bright dot in conidia (Figure 4.10a). Localization continued to be one very bright dot or a short bar per individual at different locations of the cells during the remaining stages of growth except in the conidiophore where there were multiple dots or bars (Figure 4.10b-d). This localization is reminiscent of, but not as extensive as, AspC in the wild-type background.

DISCUSSION

***ΔaspA, ΔaspC* and the double deletion mutant show early germination, increased germ tube and branch emergence and abnormal septation.**

We found that *ΔaspA, ΔaspC* and *ΔaspA ΔaspC* are viable. This is in sharp contrast with *S. cerevisiae,* where *CDC11* and *CDC12* are essential (Frazier *et al.*, 1998). *ΔaspA, ΔaspC* and *ΔaspA ΔaspC* showed increased germ tube and branch emergence (Figure 4.1, Table 4.2). Once more this is very different from the case in *S. cerevisiae*, where temperature sensitive *cdc11* and *cdc12* form elongated buds and are unable to complete cell division at restrictive temperature (Hartwell, 1971). However in *S. cerevisiae* a multiple bud phenotype is seen in *kcc4Δ*, and in *sec14* and *cog4* when their promoters were shut off (Okuzaki *et al.*, 2003; Mnaimneh *et al.*, 2004). Kcc4 is a protein kinase that localizes in a septin dependent manner to the bud neck and it is a negative regulator of Swe1 (Longtine *et al.*, 1998; Okuzaki and Nojima, 2001). The phosphatidylinositol/phosphatidylcholine transfer protein Sec14p is essential for transport of proteins from the Golgi complex in yeast (Bankaitis *et al.*, 1990). COG4 is an essential component of the conserved oligomeric Golgi (COG) complex in yeast, this complex is involved in vesicle transport to and from the Golgi (Kim *et al.*, 2001; Loh and Hong, 2004). In *C. albicans* CaCdc42p with the hyperactive G12V substitution cells arrested with multiple buds (Ushinsky *et al.*, 2002). CaCdc42 is a Ras-like small GTPase important for cell growth and morphology (Ushinsky *et al.*, 2002). In yeast Cdc42 interacts with the septins and many downstream protein effectors including kinases and proteins that regulate or nucleate the actin cytoskeleton (Caviston *et al.*, 2003). CaCdc42 and Kcc4 are in septin pathways (Caviston *et al.*, 2003).

In wild-type *A. nidulans*, germ tubes emerge over a period of hours and almost always opposite each other (Harris, 1999; Momany and Taylor, 2000). In the deletion strains we saw multiple germ tubes formed at the same time and in abnormal positions of 45°, 90°, 180° as well as axial to the other germ tube. This is somewhat analogous to the effect of perturbing septins on polarity in *S. cerevisiae* where haploid α and a cells typically undergo axial budding with both mother and daughter cells forming new buds adjacent to the preceding division site. Mutations in the septins *CDC3, CDC10, CDC11* or *CDC12* result in a switch to bipolar budding in haploids (Chant *et al.*, 1995; Chant and Pringle, 1995; Sanders and Herskowitz, 1996).

In *A. nidulans* polarity genes are proposed to form two groups (Momany *et al.*, 1999; Momany, 2002): those that establish a location for germ tube emergence and those that maintain polar growth. Branch and germ tube emergence are conceptually similar and some of the same genes could be involved in both. The deletion phenotype of increased germ tube and branch emergence suggests that AspA and AspC may have a role in polarity establishment. The role of AspA and AspC could be to activate a single site for germ tube emergence or to suppress alternate sites thus allowing the germ tube to emerge from a single site. The single and double deletion mutants showed a similar proportion of individuals with one germ tube or branch emerging however the proportion of two or more germ tubes or branches was increased in *ΔaspA ΔaspC*.

It is not surprising that *ΔaspA, ΔaspC* and *ΔaspA ΔaspC* have defects in the formation of septa since septin mutants in *S. cerevisiae* are unable to complete cell division (Hartwell, 1971). The *A. nidulans* single and double deletion mutants show a reduction in septation and frequently the septation site is further from the conidium as compared to wild-type cells. In AspA by 15 hours the cells are septating almost at the wild-type level, so septation in these cells is delayed (Table 4.2). In contrast septation in *ΔaspC* and *ΔaspA ΔaspC* remains reduced and calcofluor label of the septa is very faint relative to wild type cells. This suggests that the septal cell wall is not forming properly and that AspA and AspC may have roles in recruiting or stabilizing cell wall materials to the septation site.

The *A. nidulans* the conidiophore is formed from an aerial hypha which swells at the tip to form a vesicle from which three layers emerge sequentially: metulae, philades and conidiospores (Adams *et al.*, 1998). Septin deletion mutants show reduced and fused layers of conidiophores (Figure 4.2b-d). During the formation of the complex conidiophore, with its many layers of emerging growth there are multiple requirements for polarity establishment. Improperly formed conidiophores suggest septin roles in polarity establishment and/or shaping emerging growth. In each phenotype examined: early germination, increased germ tubes and branches and abnormal septation, we saw an increase in the proportion of affected cells in the double deletion mutant as compared to the single deletion mutants suggesting that AspA/Cdc11 and AspC/Cdc12 may have somewhat different roles (Table 4.2).

AspA and AspC show unique localization patterns.

Consistent with the idea that AspA and AspC might not have identical roles, they do not localize identically. The septin-GFP fusions we observed are single copy behind the endogenous septin promoter at the endogenous septin chromosomal location and show wild-type phenotypes. AspA and AspC localization is dynamic throughout vegetative and asexual growth of *A. nidulans*. Initially in conidiospores AspA and AspC localization is almost identical as a dot, ring or bar, but AspC also is seen as a pair of short bars. AspA localizes to areas of new growth including the base and growing tips of germ tubes and branch initials as a diffuse cap (Figures 4.3b-d and 4.4a). AspC localization is more distinct in growing tips of germ tubes and branches where it is frequently seen as a long filament along the outer cell edge or as a pair of short bars, dots or a long bar closer to the conidium (Figure 4.6b-f). AspA transiently localizes as a bright band to forming septa (Figure 4.3c) while AspC is only seen before septation as a series of fine lines at the presumptive septation site (Figure 4.6f). AspA conidiophore localization is more ordered as diffuse bands to tips of metulae and philade layers (Figure 4.5) while AspC localizes throughout these layers as dots, rings, short and long bars (Figures 4.8). These localizations are consistent with the reduced and fused layers we saw in the deletion mutant phenotype (Figure 4.2, Table 4.2).

In addition to AspA and AspC localization being different from each other, they are different from that previously reported for AspB. Immunofluorescence localization studies showed AspB was not found at tips of polarizing cells or as filaments in hyphae or branches but it was found as a marker for branch emergence (Westfall and Momany, 2002). AspB, AspA and AspC (at presumptive bud site) all localize to septa and conidiophore layers (this paper, Westfall and Momany, 2002). It is somewhat surprising that the localization of individual septins differs in *A. nidulans* because all five *S. cerevisiae* vegetative septins localize identically to the ring at the bud neck (Caviston *et al.*, 2003; Versele *et al.*, 2004; Iwase *et al.*, 2006).

AspA and AspC require each other for proper localization.

In *S. cerevisiae* the septins Cdc3p, Cdc10p, Cdc11p, and Cdc12p have been shown to form multiseptin heteropolymers (Frazier *et al.*, 1998; Versele *et al.*, 2004). In *S. cerevisiae* mutants in which one septin: CDC3, CDC10, CDC11, or CDC12 gene is mutated the remaining three septin proteins are unable to localize normally to the bud neck (Haarer and Pringle, 1987; Ford and Pringle, 1991; Kim *et al.*, 1991). In *Drosophila,* septin proteins Pnut and Sep1 both co-immunoprecipitate and co-sediment suggesting formation of a complex (Fares *et al.*, 1995). In *pnut* null *Drosophila* embryos there was reduced or no staining with Sep1 antibodies (Fares *et al.*, 1995). In mouse studies, Sept5 co-immunoprecipitated with Sept2, Sept5 and Sept7 and in Sept5 null mice reduced expression levels of Sept7 were found (Peng *et al.*, 2002). In NIH3T3 cells RNAi knockdown of Sept2 resulted in a slight reduction of Sept7 levels and visa versa (Kinoshita *et al.*, 2002). In *A. nidulans,* AspC-GFP does not show localization in *ΔaspA* suggesting that AspC needs AspA to localize properly (Figure 4.9). AspA-GFP localizes in *ΔaspC* as a single dot or bar in vegetative growth and as multiple dots or bars in conidiophores (Figure 4.10). This localization is reminiscent but not as extensive as AspC-GFP in a wild type background suggesting that AspA may have some redundant function or can partially replace AspC-GFP. The removal of one septin affects the localization of the other suggesting that though localization is not identical, septins may depend on each other to form or stabilize complexes *A. nidulans.*

ACKNOWLEDGEMENTS

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Figure 4.1. A. nidulans deletion mutanta show early germination (at 5 hrs), increased germ tube (8 hrs) and branch emergence and abnormal septation (14hrs). Cells were incubated at 30C for 5 and 8 hour time points. The 14hr time point was incubated at room temperature. All micrographs are the same magnification. Scale bar = 10µ.

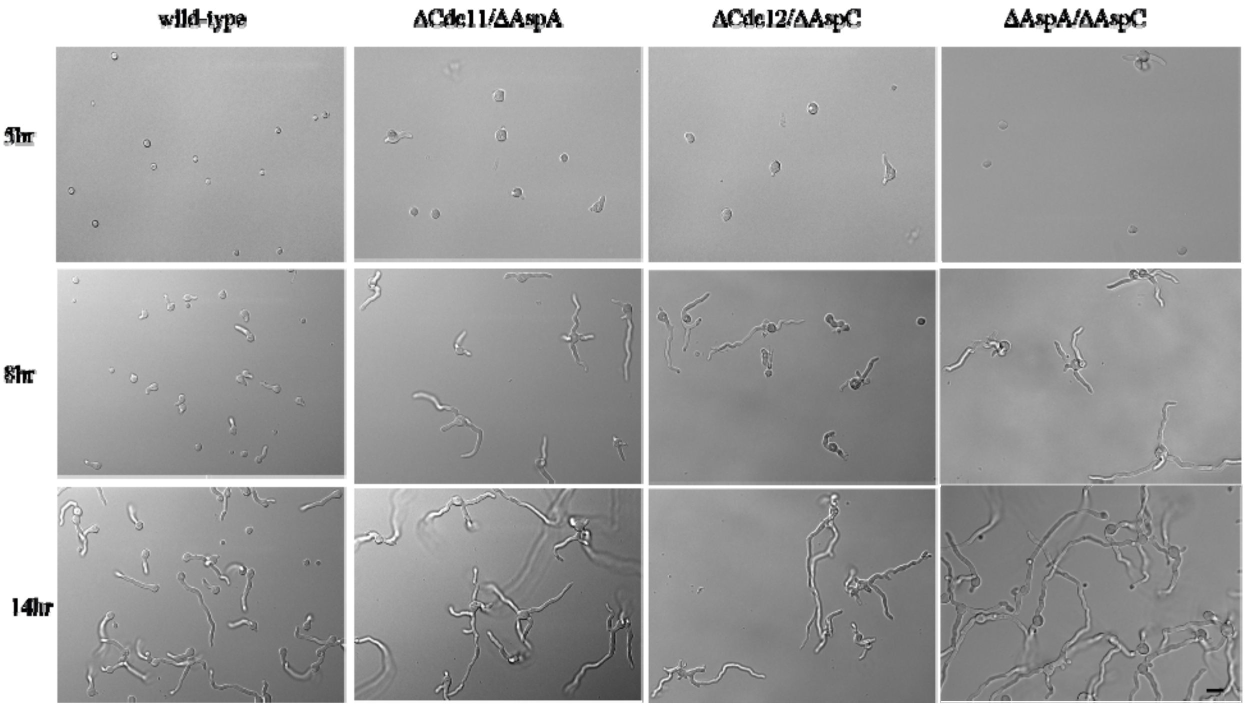

wild-type
ΔCdc11/ΔAspA
ΔCdc12/ΔAspC
ΔAspA/ΔAspC
5hr
8hr
14hr

Figure 4.2. *ΔaspC* (b-d) shows reduced and fused layers in asexual structures compared to wild type (a). Plates were incubated at 30°C for 2 days. All micrographs are the same magnification. Scale bar= 5μ


a
b
c
d

Table 4.1. *A. nidulans* strains used in this paper**.**

strain	**genotype**
A28[a]	*pabaA6; biA1*
A773 [a]	*pyrG89; wA3; pyroA4*
A850 [a]	*biA1; ΔargB::trpC ΔB; methG1; veA1;trpC801*
TNO2A5 [a]	*pabaB22;ΔnkuA::argB;riboB2*
TNO2A7 [a]	*pyroA4;ΔnkuA::argB;riboB2*
ASH5	*ΔaspA::pyrG89; biA1; ΔargB::trpC ΔB; methG1; veA1;trpC801*
ASH26	*ΔaspA::pyrG89; pyroA4; methG1*
ARL141	*aspA-GFP; biA1; ΔargB::trpC ΔB; methG1; veA1;trpC801*
ARL159	*aspC-GFP; pabaA6; biA1*
ARL161	*ΔaspC::pyrG89; biA1; ΔargB::trpC ΔB; methG1; veA1;trpC801*
ARL162	*ΔaspAΔaspC::pyrG89; pyroA4;biA1; ΔargB::trpC ΔB; veA1;trpC801*
ARL182	*ΔaspA::pyrG89; aspC-GFP; methG1;pabaA6*
ARL183	*ΔaspA::pyrG89; aspB-RFP; aspC-GFP; pabaA6*
ARL184	*ΔaspA::pyrG89; aspB-RFP; aspC-GFP; pabaA6*
ARL185	*ΔaspA::pyrG89; aspB-RFP; aspC-GFP*
ARL198	*ΔaspC::pyrG89;* AspA-GFP; *pyrG89; pyroA4*
ARL201	*ΔaspC::pyrG89;* AspA-GFP; *pyrG89; pyroA4*

[a] Available from Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical Center (Kansas City, KS).

Table 4.2. Deletion mutants show early germination, increased germ tube and branch emergence and abnormal septation as compared to wild type. All numbers as a percent.

Δ PHENOTYPE	**A850 Control**			**Δ *aspA***			**Δ *aspC***			**Δ *aspA* Δ *aspC***		
	0	**1**	**2**	**0**	**1**	**2**	**0**	**1**	**2**	**0**	**1**	**2**
Early germination – 5hr 30°C[a]	100	0	0	42	40	18	48	44	8	45	35	20
Multiple germ tubes - 30°C[ab]	--	92	8	--	41	59	--	38	62	--	1	99
Branch emergence[ab]	78	16	6	42	26	32	44	17	39	18	23	59
Nuclear number and septation (16 nuclei)[bc]	0	100	--	88	12	--	97	3	--	100	0	--
Septation - 15hr 30°C [bc]	0	100	--	5	95	--	72	28	--	62\	38	--

[a] At least 200 individuals counted

[b] Cells grown different times but equivalent development

[c] At least 30 individuals counted

Figure 4.3. AspA localizes to conidia (a), hyphae (b), septa (c), and branches (d). The upper row and lower row show DIC and fluorescent images of the same field respectively. All micrographs are the same magnification. Scale bar = 5μ.

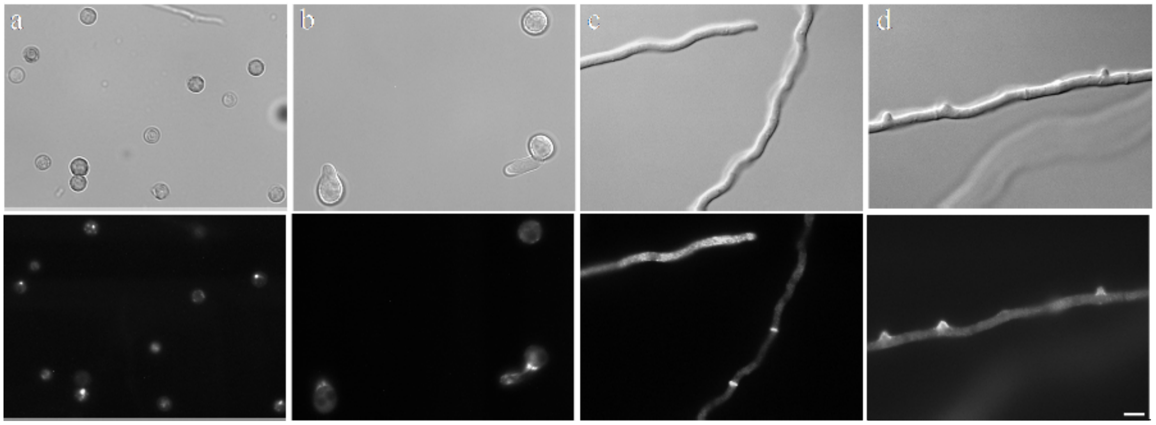

a
b
c
d

Figure 4.4. AspA localizes as a single spot in conidium (a), punctuate in hyphae (a-c), at septa (a), as filaments in tips and branches (b, c). All micrographs are the same magnification. Scale bar = 5µ.

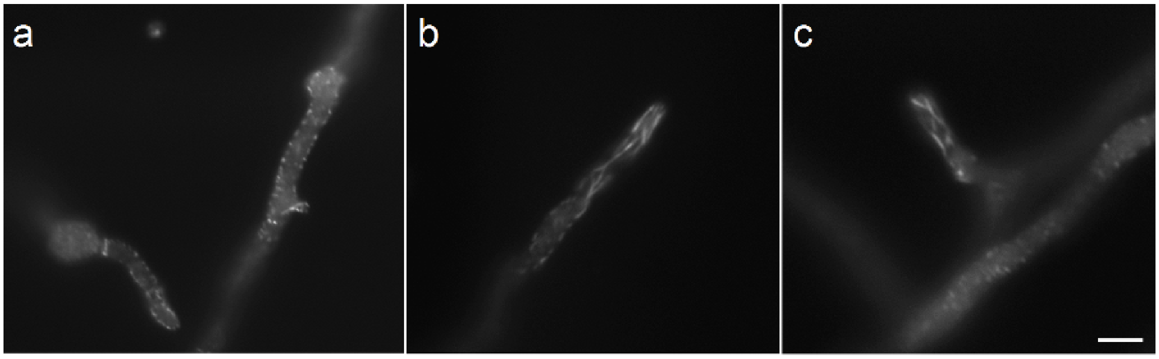

a
b
c

Figure 4.5. AspA localizes to metulae/conidiospore layer and spores. The left and right column show DIC and fluorescent images of the same field respectively. Scale bar = 5μ.

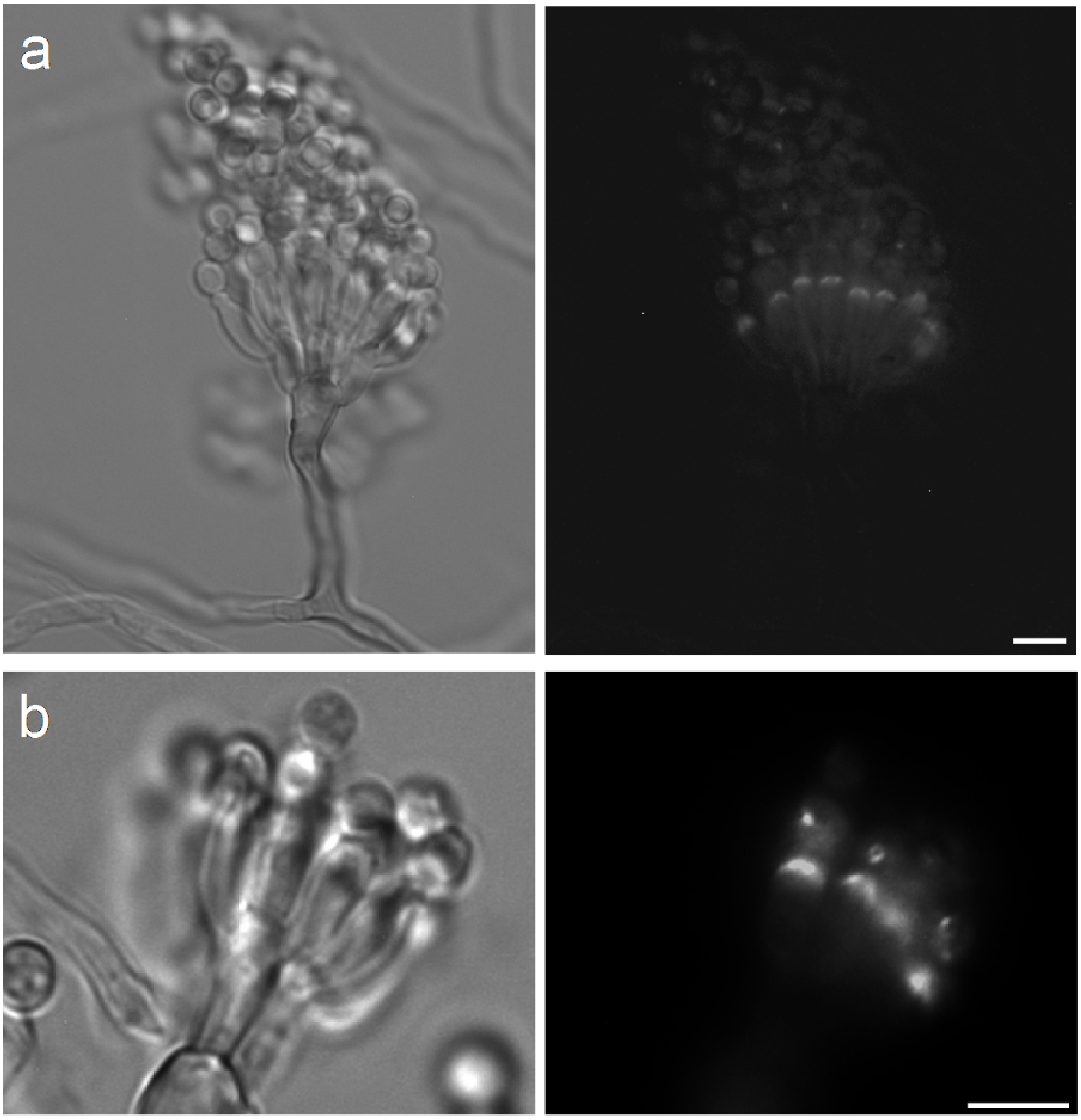

a
b

Figure 4.6. AspC localizes to conidia (a,b), hyphae (b-f) and presumptive septation site (f). Localization as filaments throughout hyphae except at times these filaments fade in whole compartments (e). The upper row and lower row show DIC and fluorescent images of the same field respectively. Scale bar = 5µ.

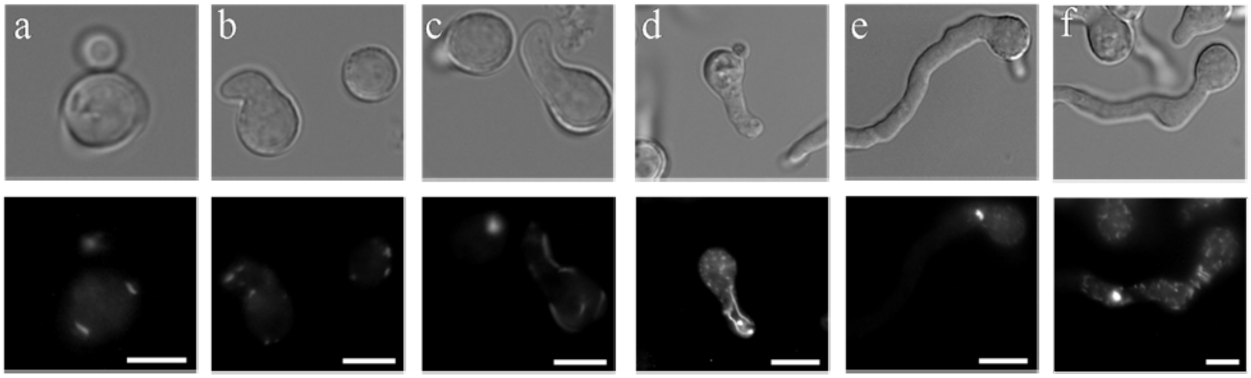

a
b
c
d
e
f

Figure 4.7. AspC localizes as long bar extended in branch initials and as loop in emerging branches. The left and right column show DIC and fluorescent images of the same field respectively. All micrographs are the same magnification. Scale bar = 5μ.

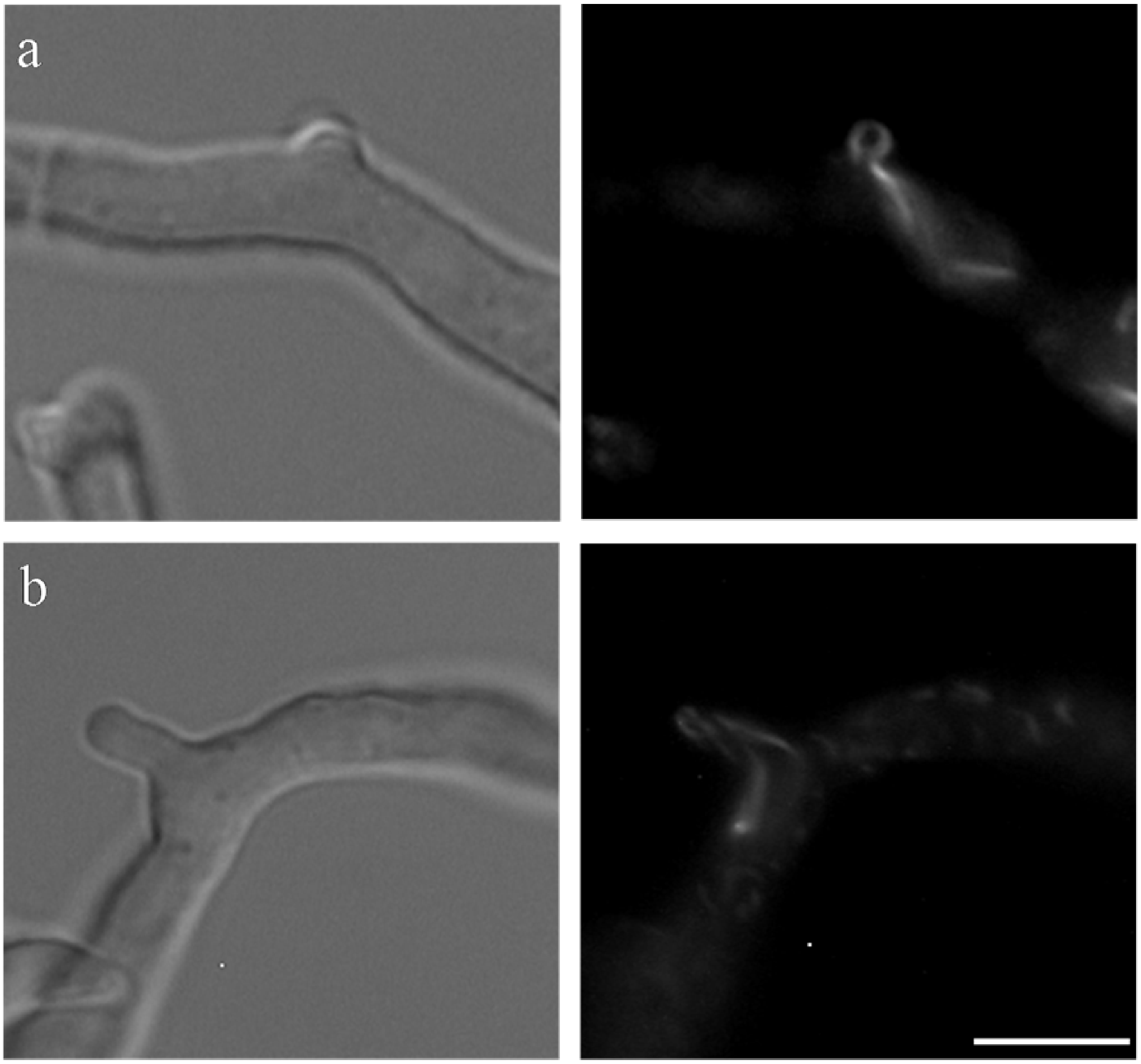

a
b

Figure 4.8. AspC localizes to conidiophores. The left column shows DIC and middle and right columns show fluorescent images at different planes of focus of the same field respectively. All micrographs are the same magnification. Scale bar = 5μ.

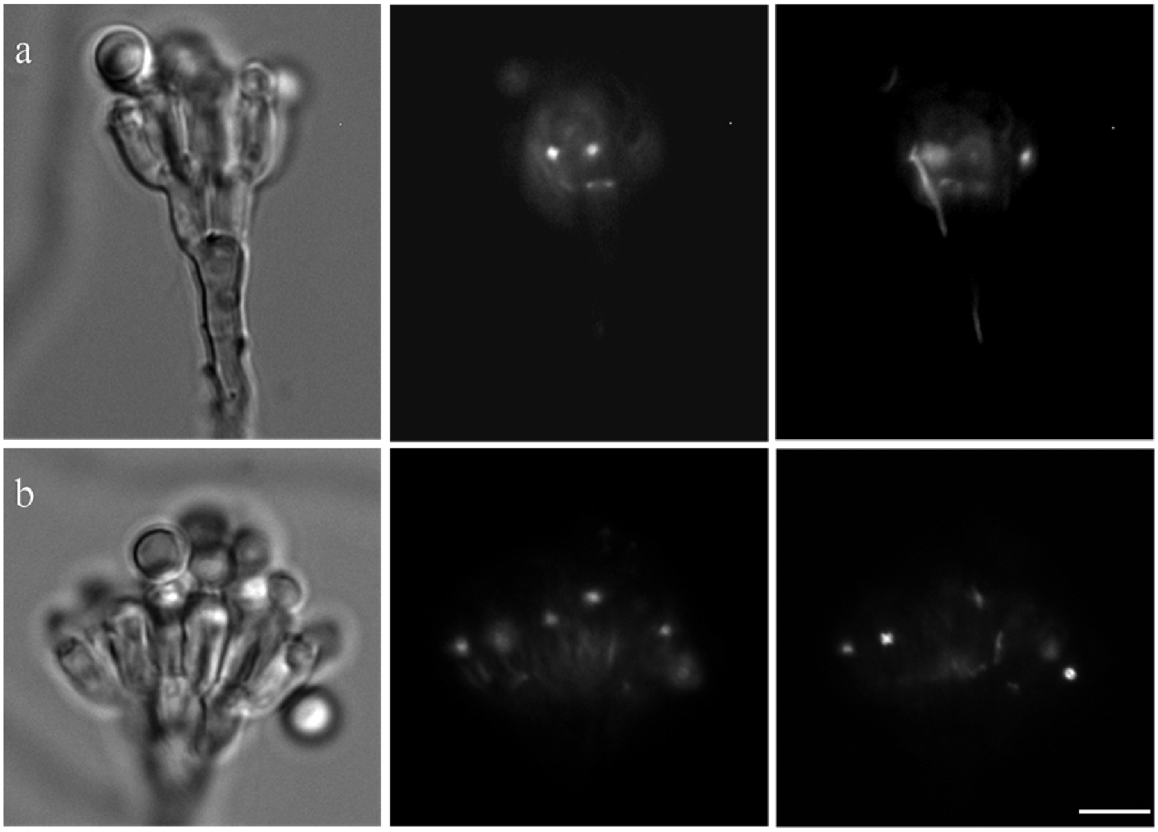

a
b

Figure 4.9. AspC-GFP does not show localization in *ΔaspA*. The upper row (a-c) and lower row show DIC and fluorescent images of the same field respectively. All micrographs (a-c) are the same magnification. The left (d) and right column show DIC and fluorescent images of the same field respectively. Scale bar = 5µ.

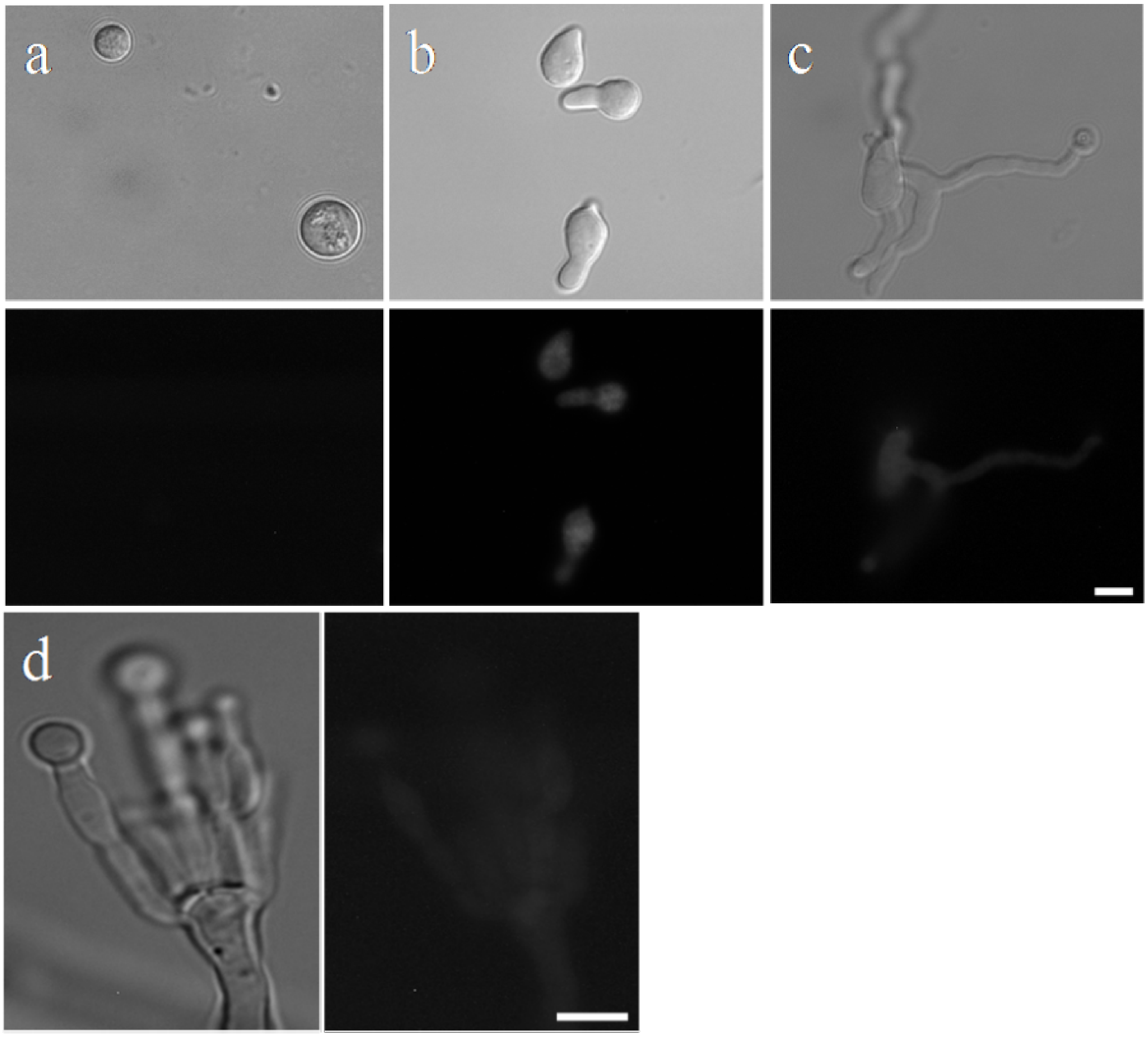
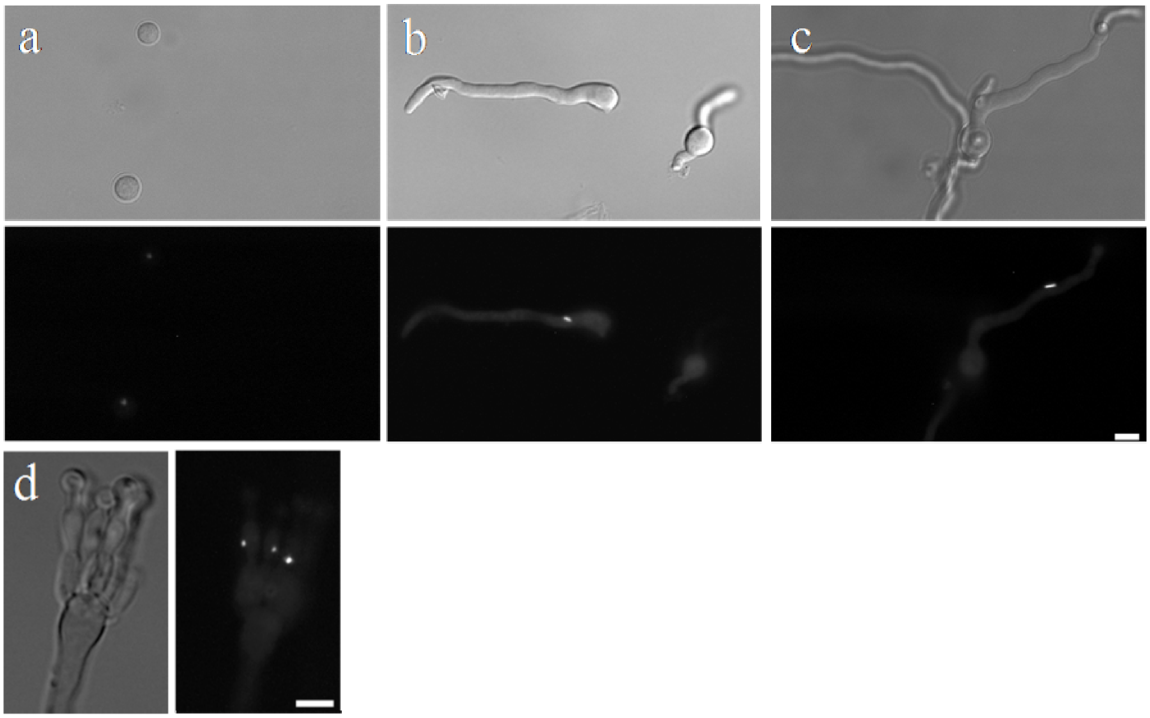

a
b
c
d

Figure 4.10. AspA-GFP forms discrete structures in *ΔaspC* vegetative growth and in conidiophores. The upper row (a-c) and lower row show DIC and fluorescent images of the same field respectively. All micrographs (a-c) are the same magnification. The left (d) and right column show DIC and fluorescent images of the same field respectively. Scale bar = 5μ.


a
b
c
d

REFERENCES

Adams, T.H., Wieser, J.K., and Yu, J.H. (1998). Asexual sporulation in Aspergillus nidulans. Microbiol Mol Biol Rev *62*, 35-54.

Bankaitis, V.A., Aitken, J.R., Cleves, A.E., and Dowhan, W. (1990). An essential role for a phospholipid transfer protein in yeast Golgi function. Nature *347*, 561-562.

Caviston, J.P., Longtine, M., Pringle, J.R., and Bi, E. (2003). The role of Cdc42p GTPase-activating proteins in assembly of the septin ring in yeast. Molecular biology of the cell *14*, 4051-4066.

Chant, J., Mischke, M., Mitchell, E., Herskowitz, I., and Pringle, J.R. (1995). Role of Bud3p in producing the axial budding pattern of yeast. The Journal of cell biology *129*, 767-778.

Chant, J., and Pringle, J.R. (1995). Patterns of bud-site selection in the yeast Saccharomyces cerevisiae. The Journal of cell biology *129*, 751-765.

Fares, H., Peifer, M., and Pringle, J.R. (1995). Localization and possible functions of Drosophila septins. Molecular biology of the cell *6*, 1843-1859.

Ford, S.K., and Pringle, J.R. (1991). Cellular morphogenesis in the Saccharomyces cerevisiae cell cycle: localization of the CDC11 gene product and the timing of events at the budding site. Developmental genetics *12*, 281-292.

Frazier, J.A., Wong, M.L., Longtine, M.S., Pringle, J.R., Mann, M., Mitchison, T.J., and Field, C. (1998). Polymerization of purified yeast septins: evidence that organized filament arrays may not be required for septin function. The Journal of cell biology *143*, 737-749.

Gladfelter, A.S., Pringle, J.R., and Lew, D.J. (2001). The septin cortex at the yeast mother-bud neck. Current opinion in microbiology *4*, 681-689.

Haarer, B.K., and Pringle, J.R. (1987). Immunofluorescence localization of the Saccharomyces cerevisiae CDC12 gene product to the vicinity of the 10-nm filaments in the mother-bud neck. Molecular and cellular biology *7*, 3678-3687.

Harris, S.D. (1999). Morphogenesis is coordinated with nuclear division in germinating Aspergillus nidulans conidiospores. Microbiology (Reading, England) *145 (Pt 10)*, 2747-2756.

Harris, S.D., Morrell, J.L., and Hamer, J.E. (1994). Identification and characterization of Aspergillus nidulans mutants defective in cytokinesis. Genetics *136*, 517-532.

Hartwell, L.H. (1971). Genetic control of the cell division cycle in yeast. IV. Genes controlling bud emergence and cytokinesis. Exp Cell Res *69*, 265-276.

Iwase, M., Luo, J., Nagaraj, S., Longtine, M., Kim, H.B., Haarer, B.K., Caruso, C., Tong, Z., Pringle, J.R., and Bi, E. (2006). Role of a Cdc42p effector pathway in recruitment of the yeast septins to the presumptive bud site. Molecular biology of the cell *17*, 1110-1125.

Kafer, E. (1977). Meiotic and mitotic recombination in Aspergillus and its chromosomal aberrations. Advances in genetics *19*, 33-131.

Kim, D.W., Massey, T., Sacher, M., Pypaert, M., and Ferro-Novick, S. (2001). Sgf1p, a new component of the Sec34p/Sec35p complex. Traffic (Copenhagen, Denmark) *2*, 820-830.

Kim, H.B., Haarer, B.K., and Pringle, J.R. (1991). Cellular morphogenesis in the Saccharomyces cerevisiae cell cycle: localization of the CDC3 gene product and the timing of events at the budding site. The Journal of cell biology *112*, 535-544.

Kinoshita, M., Field, C.M., Coughlin, M.L., Straight, A.F., and Mitchison, T.J. (2002). Self- and actin-templated assembly of Mammalian septins. Dev Cell *3*, 791-802.

Kuwayama, H., Obara, S., Morio, T., Katoh, M., Urushihara, H., and Tanaka, Y. (2002). PCR-mediated generation of a gene disruption construct without the use of DNA ligase and plasmid vectors. Nucleic acids research *30*, E2.

Lin, X., and Momany, M. (2003). The Aspergillus nidulans swoC1 mutant shows defects in growth and development. Genetics *165*, 543-554.

Loh, E., and Hong, W. (2004). The binary interacting network of the conserved oligomeric Golgi tethering complex. The Journal of biological chemistry *279*, 24640-24648.

Longtine, M.S., Fares, H., and Pringle, J.R. (1998). Role of the yeast Gin4p protein kinase in septin assembly and the relationship between septin assembly and septin function. The Journal of cell biology *143*, 719-736.

Mnaimneh, S., Davierwala, A.P., Haynes, J., Moffat, J., Peng, W.T., Zhang, W., Yang, X., Pootoolal, J., Chua, G., Lopez, A., Trochesset, M., Morse, D., Krogan, N.J., Hiley, S.L., Li, Z., Morris, Q., Grigull, J., Mitsakakis, N., Roberts, C.J., Greenblatt, J.F., Boone, C., Kaiser, C.A., Andrews, B.J., and Hughes, T.R. (2004). Exploration of essential gene functions via titratable promoter alleles. Cell *118*, 31-44.

Momany, M. (2002). Polarity in filamentous fungi: establishment, maintenance and new axes. Current opinion in microbiology *5*, 580-585.

Momany, M., and Taylor, I. (2000). Landmarks in the early duplication cycles of Aspergillus fumigatus and Aspergillus nidulans: polarity, germ tube emergence and septation. Microbiology (Reading, England) *146 Pt 12*, 3279-3284.

Momany, M., Westfall, P.J., and Abramowsky, G. (1999). Aspergillus nidulans swo mutants show defects in polarity establishment, polarity maintenance and hyphal morphogenesis. Genetics *151*, 557-567.

Momany, M., Zhao, J., Lindsey, R., and Westfall, P.J. (2001). Characterization of the Aspergillus nidulans septin (asp) gene family. Genetics *157*, 969-977.

Neufeld, T.P., and Rubin, G.M. (1994). The Drosophila peanut gene is required for cytokinesis and encodes a protein similar to yeast putative bud neck filament proteins. Cell *77*, 371-379.

Nguyen, T.Q., Sawa, H., Okano, H., and White, J.G. (2000). The C. elegans septin genes, unc-59 and unc-61, are required for normal postembryonic cytokineses and morphogenesis but have no essential function in embryogenesis. J Cell Sci *113 Pt 21*, 3825-3837.

Okuzaki, D., and Nojima, H. (2001). Kcc4 associates with septin proteins of Saccharomyces cerevisiae. FEBS letters *489*, 197-201.

Okuzaki, D., Watanabe, T., Tanaka, S., and Nojima, H. (2003). The Saccharomyces cerevisiae bud-neck proteins Kcc4 and Gin4 have distinct but partially-overlapping cellular functions. Genes & genetic systems *78*, 113-126.

Peng, X.R., Jia, Z., Zhang, Y., Ware, J., and Trimble, W.S. (2002). The septin CDCrel-1 is dispensable for normal development and neurotransmitter release. Molecular and cellular biology *22*, 378-387.

Sanders, S.L., and Herskowitz, I. (1996). The BUD4 protein of yeast, required for axial budding, is localized to the mother/BUD neck in a cell cycle-dependent manner. The Journal of cell biology *134*, 413-427.

Spiliotis, E.T., and Nelson, W.J. (2006). Here come the septins: novel polymers that coordinate intracellular functions and organization. J Cell Sci *119*, 4-10.

Timberlake, W.E., and Marshall, M.A. (1988). Genetic regulation of development in Aspergillus nidulans. Trends Genet *4*, 162-169.

Ushinsky, S.C., Harcus, D., Ash, J., Dignard, D., Marcil, A., Morchhauser, J., Thomas, D.Y., Whiteway, M., and Leberer, E. (2002). CDC42 is required for polarized growth in human pathogen Candida albicans. Eukaryotic cell *1*, 95-104.

Versele, M., Gullbrand, B., Shulewitz, M.J., Cid, V.J., Bahmanyar, S., Chen, R.E., Barth, P., Alber, T., and Thorner, J. (2004). Protein-Protein Interactions Governing Septin Heteropentamer Assembly and Septin Filament Organization in Saccharomyces cerevisiae. Molecular biology of the cell.

Westfall, P.J., and Momany, M. (2002). Aspergillus nidulans septin AspB plays pre- and postmitotic roles in septum, branch, and conidiophore development. Molecular biology of the cell *13*, 110-118.

Yang, L., Ukil, L., Osmani, A., Nahm, F., Davies, J., De Souza, C.P., Dou, X., Perez-Balaguer, A., and Osmani, S.A. (2004). Rapid production of gene replacement constructs and generation of a green fluorescent protein-tagged centromeric marker in Aspergillus nidulans. Eukaryotic cell *3*, 1359-1362.

CHAPTER 5

CONCLUSION

The conservation of septins in microsporidia, fungi, and animals, suggests they are evolutionarily significant. Mammalian septin defects are linked to infertility, leukemia, neurodegenerative disease and ovarian and breast cancer (Burrows *et al.*, 2003; Montagna *et al.*, 2003; Xue *et al.*, 2004; Ihara *et al.*, 2005; Kissel *et al.*, 2005; Russell and Hall, 2005). Characterization of the 13 septin genes in humans is difficult, there are multiple splice variants and localization is dynamic and frequently dependent on cell type and cell cycle stage (McIlhatton *et al.*, 2001; Hall and Russell, 2004). *A. nidulans* has well documented and easy to discern growth stages, straight forward genetics and well-characterized cell biology making it an ideal organism in which to study septins. Septin localization patterns in *A. nidulans* include both those seen in other fungi and those seen in mammalian cells (Lindsey and Momany, 2006).

Data presented in this dissertation show how septins affect morphology in fungi. Introduction of the septin AspC from the filamentous fungus *A. nidulans* causes the normally unicellular *S. cerevisiae* to make elongated cells and spore producing structures similar to those in hyphal fungi. In *A. nidulans* AspA and AspC deletion mutants show changes in morphology including early budding, increased germ tube and branch emergence and abnormal septation. These phenotypes of increased growth suggest that AspA and AspC either determine the sites for this new growth or function as or influence proteins that are negative regulators of germination, germ tube and/or branch emergence.

The deletion mutant phenotype of unorganized conidiophore layers suggests a function in determining the structure and development of conidiophores. AspA and AspC have unique localizations. Especially interesting is the localization of these septins as “animal like” micro-filaments (Figure 5.1.). This novel result of mammalian type localization and different localization patterns in *A. nidulans* is very exciting and suggests much can be learned about septin biology.

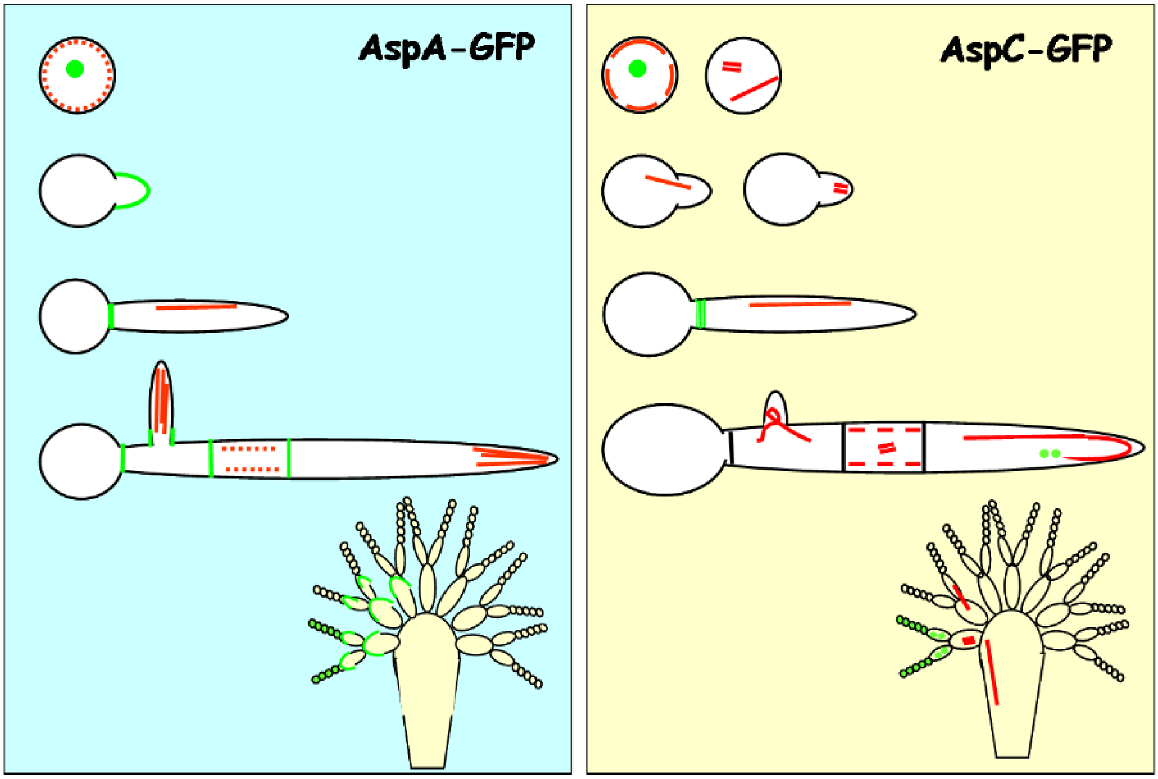


Figure 5.1. *A. nidulans* AspA and AspC septin localization in vegetative and asexual growth. Localizations labeled in green have been previously reported in fungi. Localizations in red are novel to fungi and are “animal like” localizations (Lindsey and Momany, 2006).

Septin scaffolds in polarity

Septins and the proteins they recruit are important in cell morphology and function. The addition of a single septin gene caused yeast to go from a budding shape to filamentous. AspA and AspC deletion mutants in *A. nidulans* showed changes in cell morphology. The deletion phenotypes and GFP fusion localizations suggest AspA and

AspC may have a role in polarity establishment. In *A. nidulans* polarity genes form two groups (Momany *et al.*, 1999; Momany, 2002): those that establish a location for germ tube emergence and those that maintain polar growth. Defects in germination and germ tube or branch emergence could be due to failure or improper recruitment of cortical markers, cell cycle regulators or proteins that interact with the actin cytoskeleton.

Septin scaffolds in cytokinesis

When AspC was introduced into *S. cerevisiae* it localized to the site of cell division, bud and filament necks while Cdc12 only localized to bud necks. Overall our studies suggest AspC competes with Cdc12 for incorporation into the septin scaffold. The deletion mutant phenotypes and GFP fusion localizations suggests AspA and AspC may have a role in where septation occurs and recruitment of components to build the septum. AspA and AspC deletion mutants show abnormal placement of septa and decreased chitin staining with a calcofluor label. These defects could be due to failure to recruit proteins involved in cell wall formation like chitin synthase. In *S. cerevisiae* septins have a role in directing Chs3p and Chs4p to the septum of the mature bud where they synthesize chitin to form the primary septum (DeMarini *et al.*, 1997). AspA and AspC may have roles in the recruitment and deposition of the cell wall machinery needed to synthesize the septum.

The deletion phenotypes and GFP fusion localization suggests AspA and AspC may have a role in asexual reproduction. AspA and AspC deletion mutants show defects in conidiophore formation. Improperly formed conidiophores suggest septin roles in polarity establishment, shaping emerging growth and in developmental growth. Defects in polarity establishment were covered above. Defects in shaping emerging growth could be due to failure to recruit proteins that couple growth and nuclear division. One such protein NimX, is thought to function in morphogenesis and regulation of the cell cycle (Ye *et al.*, 1999). Interestingly the conidiophore phenotype we saw in our septin deletion mutants and those seen in *nimX*^{cdc2AF} mutants are almost identical (Ye *et al.*, 1999). Developmental regulatory genes, *stuA* and *medA*, are necessary to form conidiophore layers (reviewed in Adams *et al.*, 1998). Abnormal conidiophores are found in *stuA* and *medA* mutant alleles (Clutterbuck, 1969; Busby *et al.*, 1996; Adams *et al.*, 1998). Defects in asexual development could be due to failure or improper recruitment of proteins involved in asexual development like StuA and MedA or in proteins that link cell cycle regulation with morphogenesis like NimX.

The *A. nidulans aspC* septin also induced abnormal morphology during sexual reproduction in *S. cerevisiae*. When the yeast *CDC12/cdc12*Δ with GFP-AspC was induced to sporulate, asci were enlarged and formed structures similar to those in hyphal fungi. We did not see any sexual reproduction defects in our *aspA* and *aspC* deletion mutants. The defects seen in yeast studies could be due improper formation of a septin scaffold involved in ascospore formation or the proper proteins were not recruited to the correct location. Further studies may find localization of AspC to sexual reproductive structures.

Conclusion

We have shown that AspC competes with Cdc12 for incorporation into the yeast septin scaffold and once there it may alter cell morphology by altering interactions with Bni1. Our results further suggest that morphological transitions in evolution, like those from hyphae to yeast could have been accomplished through changes in key scaffold proteins.

We have shown that *aspA* and *aspC* deletion mutant phenotypes include early germination, abnormal septation, increased germ tubes and branch emergence and reduced and fused layers in conidiophores. AspA and AspC show dynamic localization to conidia, hyphae, septa, branches and conidiophores. Localization as micro-filaments is also seen and could possibly be related to actin. AspA and AspC may depend on each other to form or stabilize complexes since the removal of one septin affects the localization of the other. In the model below, we hypothesize that AspA localizes first in most cases quickly recruiting AspC and then actin. We also hypothesize that septins and actin are required for septin stabilization and for complete wild-type function (Figure 5.2.).

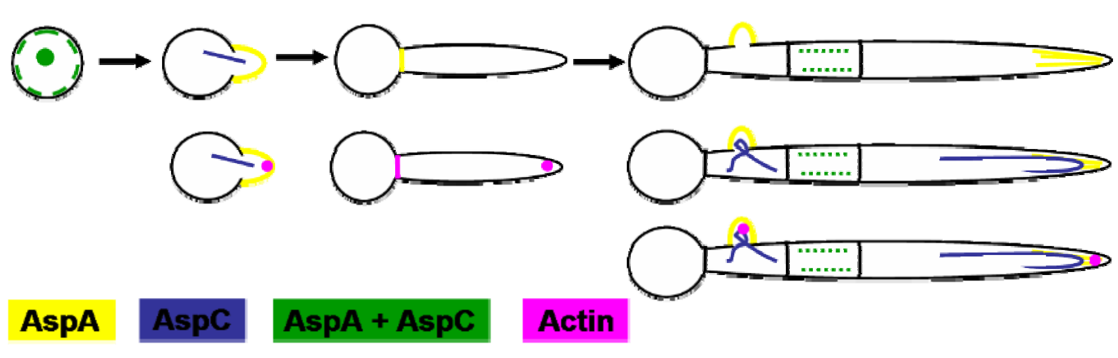


Figure 5.2. Postulated order of septin (AspA and AspC) and actin localization during the vegetative lifecycle of *A. nidulans*. Stages of the lifecycle go across the figure while timing of localization is drawn below each stage of the lifecycle. AspA is thought to localize first in most cases quickly recruiting AspC and then actin. The septins need each other and actin for stabilization and for complete wild-type function.

Future Septin Studies in *A. nidulans*

Results to date from the characterization of septins AspA, AspB and AspC in *A. nidulans* are especially exciting due to the differences in localization we have seen among the three. Due to the dynamic nature of septin localization confocal microscopy of GFP or RFP septin fusions throughout the lifecycle would be especially informative. We have the tools to make a strain carrying both an AspA-GFP and AspC-RFP fusion. A movie with a double labeled strain would add insight as to how septins AspA and AspC interact with each other.

The filamentous localization of AspA and AspC suggest that they may interact with the actin cytoskeleton. Studies localizing septin GFP fusions when actin is depolymerized and localizing actin GFP fusions in septin deletion mutants may shed light on cytoskeletal interactions. Localization of an actin GFP fusion with a septin RFP fusion would also give valuable interaction information. Similar co-localization studies conducted with septins and SepA, the only *A. nidulans* formin, which nucleates actin would be interesting.

Further studies where multiple septin deletion strains are generated would be informative. For instance a double *aspA* and *aspC* deletion mutant was able to complete sexual crosses with wild type. The a*spD* deletion mutant is able to cross with wild type. But when a cross to construct a triple deletion strain lacking *aspA*, *aspC* and *aspD* was attempted, no sexual structures formed. Would the same triple deletion mutant lack asexual structures? It is probable that if enough septins are deleted then *A. nidulans* is no longer viable. Which septins and how many of them would it take? Or what are the minimal septins required for asexual reproduction, sexual reproduction or viability in *A. nidulans*?

REFERENCES

Adams, T.H., Wieser, J.K., and Yu, J.H. (1998). Asexual sporulation in Aspergillus nidulans. Microbiol Mol Biol Rev *62*, 35-54.

Burrows, J.F., Chanduloy, S., McIlhatton, M.A., Nagar, H., Yeates, K., Donaghy, P., Price, J., Godwin, A.K., Johnston, P.G., and Russell, S.E. (2003). Altered expression of the septin gene, SEPT9, in ovarian neoplasia. J Pathol *201*, 581-588.

Busby, T.M., Miller, K.Y., and Miller, B.L. (1996). Suppression and enhancement of the Aspergillus nidulans medusa mutation by altered dosage of the bristle and stunted genes. Genetics *143*, 155-163.

Clutterbuck, A.J. (1969). A mutational analysis of conidial development in Aspergillus nidulans. Genetics *63*, 317-327.

DeMarini, D.J., Adams, A.E., Fares, H., De Virgilio, C., Valle, G., Chuang, J.S., and Pringle, J.R. (1997). A septin-based hierarchy of proteins required for localized deposition of chitin in the Saccharomyces cerevisiae cell wall. The Journal of cell biology *139*, 75-93.

Hall, P.A., and Russell, S.E. (2004). The pathobiology of the septin gene family. J Pathol *204*, 489-505.

Ihara, M., Kinoshita, A., Yamada, S., Tanaka, H., Tanigaki, A., Kitano, A., Goto, M., Okubo, K., Nishiyama, H., Ogawa, O., Takahashi, C., Itohara, S., Nishimune, Y., Noda, M., and Kinoshita, M. (2005). Cortical organization by the septin cytoskeleton is essential for structural and mechanical integrity of mammalian spermatozoa. Dev Cell *8,* 343-352.

Kissel, H., Georgescu, M.M., Larisch, S., Manova, K., Hunnicutt, G.R., and Steller, H. (2005). The Sept4 septin locus is required for sperm terminal differentiation in mice. Dev Cell *8,* 353-364.

Lindsey, R., and Momany, M. (2006). Septin localization across kingdoms: three themes with variations. Current opinion in microbiology *9,* 559-565.

McIlhatton, M.A., Burrows, J.F., Donaghy, P.G., Chanduloy, S., Johnston, P.G., and Russell, S.E. (2001). Genomic organization, complex splicing pattern and expression of a human septin gene on chromosome 17q25.3. Oncogene *20,* 5930-5939.

Momany, M. (2002). Polarity in filamentous fungi: establishment, maintenance and new axes. Current opinion in microbiology *5,* 580-585.

Momany, M., Westfall, P.J., and Abramowsky, G. (1999). Aspergillus nidulans swo mutants show defects in polarity establishment, polarity maintenance and hyphal morphogenesis. Genetics *151,* 557-567.

Montagna, C., Lyu, M.S., Hunter, K., Lukes, L., Lowther, W., Reppert, T., Hissong, B., Weaver, Z., and Ried, T. (2003). The Septin 9 (MSF) gene is amplified and overexpressed in mouse mammary gland adenocarcinomas and human breast cancer cell lines. Cancer research *63*, 2179-2187.

Russell, S.E., and Hall, P.A. (2005). Do septins have a role in cancer? British journal of cancer *93*, 499-503.

Xue, J., Tsang, C.W., Gai, W.P., Malladi, C.S., Trimble, W.S., Rostas, J.A., and Robinson, P.J. (2004). Septin 3 (G-septin) is a developmentally regulated phosphoprotein enriched in presynaptic nerve terminals. J Neurochem *91*, 579-590.

Ye, X.S., Lee, S.L., Wolkow, T.D., McGuire, S.L., Hamer, J.E., Wood, G.C., and Osmani, S.A. (1999). Interaction between developmental and cell cycle regulators is required for morphogenesis in Aspergillus nidulans. The EMBO journal *18*, 6994-7001.