

INTERPLAY BETWEEN SEPTIN ASPD, NUCLEAR DIVISION AND THE NUCLEOLUS

by

ILKAY DÖRTER

(Under the Direction of Michelle Momany)

ABSTRACT

Cell division is one of the mysterious wonders of life. A great number of cells divide in our body. Thereby a new daughter cell always arises from a mother cell, never from scratch. The actual cell division involves two processes: mitosis (nuclear division) and cytokinesis (cytoplasmic division). DNA is the master instruction set for all that makes cells work. It is in the cell's best interest to duplicate the DNA properly and distribute the new copies to the daughter cells in a highly choreographed and controlled fashion. In *Saccharomyces cerevisiae*, septin proteins play a key role in coupling mitosis and cytokinesis. The filamentous fungus *Aspergillus nidulans* does not undergo complete cytokinesis, but needs to coordinate nuclear division with cellular partitioning nevertheless. It has four core septin genes, *aspA* (an ortholog of *S. cerevisiae* *CDC11*), *aspB* (*CDC3*), *aspC* (*CDC12*), and *aspD* (*CDC10*) and one septin gene with no *S. cerevisiae* ortholog, *aspE*. We have found that, unlike the other three core septin deletion mutants, the deletion of *aspD* does not alter the vegetative morphology of *A. nidulans*, but results in misshaped nuclei, asynchronous nuclear division and mislocalization of nucleolar proteins.

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CHAPTER 1

INTRODUCTION

Septins take on equally important roles as actin, microtubules and intermediate filaments [2, 3]. They are considered as components of the cytoskeleton and form nonpolar, heteropolymeric higher order (HO) structures within the cell. The formation of such structures is crucial for their function as barriers and scaffolds. Septin structures have no catalytic function. They subdivide the cell and support its architecture. The deletion of one or more septins is often lethal, reflecting their function in maintaining normal cell functions [4-7]. Septins are highly conserved between different eukaryotic species and all of them have a P-loop nucleotide binding motif [8]. Septins form heteropolymers, which then assemble into higher-order structures (HOs). These usually localize at the cortex, where they serve as a scaffold for membrane proteins or as a diffusion barrier retaining proteins in certain subcellular domains. HOs are considered the biologically active form of septins [9-11], which play an essential role in developmental processes, including the establishment of cell polarity, the support of cell architecture, and cytokinesis [2, 3]. In mammals, septins ensure proper growth, cell migration, and cell division [12-15]. Deficiencies in septin structure and organization are associated with growth defects in fungi and a variety of human diseases such as cancer and Alzheimer's disease [16].

Septins are divided into five phylogenetic groups. *S. cerevisiae* has seven septin proteins. Four of the seven *S. cerevisiae* septins belong to the core complex, the other two, SPR3 and SPR28, are only expressed during sporulation [17, 18]. Septins Cdc3, Cdc10, Cdc11 and Cdc12, locate at the budding zone where they form 10 nm thick filaments between the mother and the bud and

restrict and organize cell division proteins [5, 8, 19]. Studies on the septins of the core complex (Cdc3, Cdc10, Cdc11 and Cdc12 or their orthologues) in fungi and animals showed that septin monomers interact with one another at two interfaces: a nucleotide binding site, called the G-interface, and N- and C-terminal helices, called the NC-interface. Septin monomers assemble linearly into nonpolar oligomers using the G- and NC-dimerization interfaces in tandem [9-11]. Only certain septins can interact with each other. Within a heteropolymer a septin either interacts with itself or with a septin from another group [20, 21]. In *S. cerevisiae*, the predominant heterooctameric chain is derived from the interaction of core septins in the order Cdc11-Cdc12-Cdc3-Cdc10-Cdc10-Cdc3-Cdc12-Cdc11 [9]. The central Cdc10 monomers dimerize via the NC-interfaces and interact with neighboring Cdc3 monomers via their G-interface. The terminal Cdc11 interacts with itself via the NC-interface, which links the septin chains to filaments. If Cdc11 is replaced by Shs1, a septin which is not part of the core complex, the heterooctamers laterally associate with each other, forming a ring instead of linear filaments [22]. When certain septins are deleted, new combinations arise, which maintain the formation of heteropolymers and higher order structures [10].

The interaction of the septins with the plasma membrane (PM) determines many of their functions. During cytokinesis, the septin ring serves as a framework for the formation of the cytokinetic actin ring at the division site. Furthermore, it acts as a diffusion barrier for diffusible exocyst and polarisome proteins [4, 23, 24]. Figure 01.1 shows a schematic representation of the septin monomers, heteropolymers and HO structures in *S. cerevisiae* and *A. nidulans*. Septins of *A. nidulans* are homologous to the core complex septins Cdc3, Cdc10, Cdc11 and Cdc12 from *S. cerevisiae*, namely AspA^{Cdc11}, AspB^{Cdc3}, AspC^{Cdc12} and AspD^{Cdc10}. Another septin, which does not have a homologue in *S. cerevisiae*, is called AspE [6]. AspA^{Cdc11}, AspB^{Cdc3}, AspC^{Cdc12} and

AspD^{Cdc10} localize as septin rings on septa and as thick rods and thinner filaments in other areas of the cell [25, 26]. AspE localizes in cortical points and at septa throughout the development of *A. nidulans* and is, unlike the other four core septins, not found in conidiophores [27]. All five *A. nidulans* septins are expressed during vegetative and asexual development, whereas AspB^{Cdc3} shows the highest expression [6]. AspE needs the core complex septins, except AspD^{Cdc10}, for its normal localization and in return localization of these core complex septins requires AspE [27]. AspE is not part of the septin heteropolymer and does not substitute any of the core complex septins in an alternative heteropolymer [12].

These findings along with mutant phenotypes described below suggest a hypothesis for the formation of heteropolymers which states that at least two classes of septin heteropolymers co-exist during the vegetative development of *A. nidulans*: polymers with all four core septins form heterooctamers and polymers with AspA^{Cdc11}, AspB^{Cdc3}, AspC^{Cdc12}, but without AspD^{Cdc10} form heterohexamers [12]. The putative heterooctamer assembles into HO structures independent of AspE. These are observed as dots and rods in isotropic stages and as septal rings in multicellular stages. The putative hexamer seems to require AspE to form HO structures. These function as cortical rods, collars and caps in developing branches and at hyphal tips. The formation of hexamer HO structures could be initiated by a transient association of AspE and AspB^{Cdc3}. This short-term interaction could cause a change or stabilization of the conformation, which in turn facilitates the construction of HO structures [12]. The $\Delta aspA^{cdc11}$, $\Delta aspB^{cdc3}$ and $\Delta aspC^{cdc12}$ mutants form additional germ tubes and branches [15, 16], whereas the $\Delta aspD^{cdc10}$ mutant does not show any obvious changes in the early vegetative phenotype [12], but exhibits abnormally shaped nuclei.

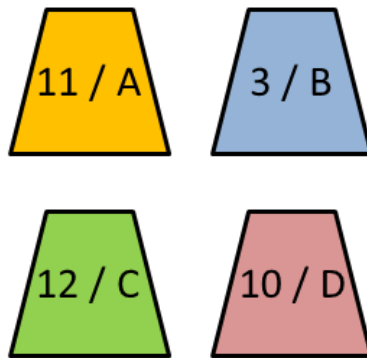
In contrast to the endosymbiotic origin of other organelles, such as mitochondria and chloroplasts, the phylogenetic origin of the nucleus is relatively unclear. Evidence suggests that the nucleus was not created by a bacterial endosymbiosis, but through symbiosis with a complex DNA virus [28]. The largest part of the cell's DNA is stored in the nucleus, highly compressed by histones. DNA replication, transcription, RNA processing, and regulation of gene expression, all take place in the nucleus. In some syncytial fungi it can also serve as storage for nitrogen and phosphate and behave autophagically [29-31]. The synthesis of ribosomes takes place in the nucleolus, a small, compact region within the nucleus. The nucleolus is generated around regions of the genome that encode repeated rRNA genes, referred to as nucleolar organizing regions (NORs) [32], and is embedded in the nucleoplasm, an amorphous substance consisting of different proteins. The nucleoplasm is surrounded by the nuclear envelope, a double membrane that is composed of the outer and inner nuclear membrane (ONM and INM). The outer membrane and the lumen between the membranes are identical in composition and are in direct continuity with the endoplasmic reticulum (ER). The nucleus has special channels, the nuclear pore complexes (NPCs), which regulate the exchange of molecules between the cytoplasm and the nucleoplasm. The structure of the NPCs is conserved from lower to higher eukaryotes and each pore consists of ~ 30 nucleoporins (Nups) forming an octagonal ring.

The nuclear membrane is either broken down in the "open" mitosis of mammals or remains intact in the "closed" mitosis of some yeast cells [33]. Some organisms, like *A. nidulans*, undergo an intermediate form of mitosis, a so called "semi-open" mitosis, in which peripheral Nups disassemble from core Nups that remain embedded in the NE [34, 35]. This allows the entry of cytoplasmic molecules, such as tubulin, into the nuclear space [34]. Since nuclei undergo synchronous mitoses in *A. nidulans*, the core Nups might protect the kinetochores from the

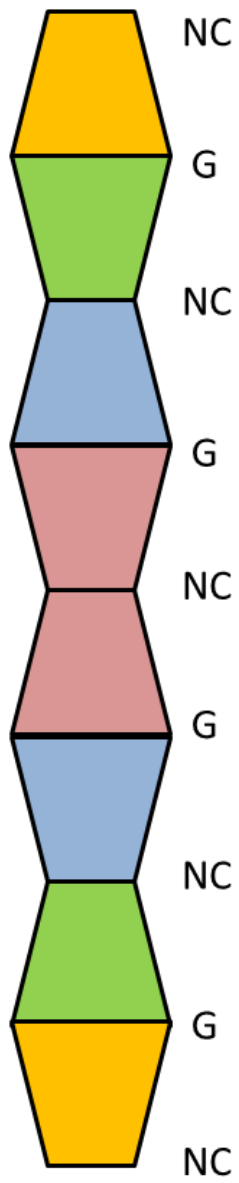
interaction with spindle microtubules of other nuclei [36]. In addition, the nucleolus is excluded and disassembled in the cytoplasm during telophase and as nuclei progress into G1 the new nucleolus is reassembled in both daughter nuclei [36].

Figure 01.1: Schematic representation of septin subunits, filaments, and higher order (HO) structures. (A) Septins in *S. cerevisiae* (orange, Cdc11), (blue, Cdc3), (green, Cdc12), (red, Cdc10) and orthologs in *A. nidulans* (orange, AspA), (blue, AspB), (green, AspC), (red, AspD) (B) The different septins interact either via the G or NC interface to form heterooctamers. (C) Heterooctamers form HO structures by interactions of the coiled coil domains of Cdc3 (AspB), Cdc11(AspA) and Cdc12 (AspC).

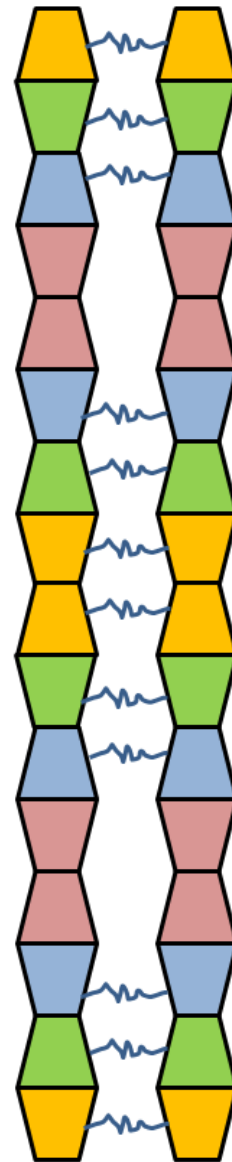
A



B



C



CHAPTER 2

FUNGAL CELL CYCLE: A UNICELLULAR VERSUS MULTICELLULAR COMPARISON¹

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INTRODUCTION

DNA is the master instruction set, encoding everything that makes cells work. So it is no surprise that faithful duplication of DNA and proper distribution of new copies to daughter cells is highly choreographed and tightly controlled. The appearance of nuclei changes in predictable patterns during the cell cycle passing through the morphologically uneventful interphase into the dramatic rearrangement of mitosis. The core cell cycle machinery is highly conserved among eukaryotes. Many of the mechanisms that control the cell cycle were worked out in fungal cells, taking advantage of their powerful genetics and rapid duplication times.

The critical insight made by researchers working with fungi in the late 70s and early 80s was that distinct cell morphologies could serve as landmarks indicating cell cycle stage. This insight combined with the elegant use of conditional mutants allowed dissection of the control of cell cycle progression, the dependency relationships and checkpoints that ensure fidelity [37]. Hartwell and co-workers used temperature-sensitive mutants arrested in the emergence and enlargement of the bud, the cell division cycle (*cdc*) mutants, to follow the state of the single nucleus of *S. cerevisiae* [38]. Similarly Nurse and co-workers used conditional mutants arrested at specific cell sizes, also called cell division cycle (*cdc*) mutants, to follow the nucleus of the fission yeast *S. pombe* [39, 40]. Morris and co-workers used conditional mutants with abnormal nuclear number and morphology, the blocked in mitosis (*bim*) and never in mitosis (*nim*) mutants, to follow the multiple nuclei of the filamentous fungus *A. nidulans* [41, 42]. In this review we will focus on an overview of the cell cycle in the unicellular budding yeast *S. cerevisiae* and compare and contrast it with the cell cycle of the multicellular filamentous fungus *A. nidulans*. For both fungi, we will only consider the cell cycle during vegetative growth because this is where most studies have been done. It should be noted however that in

reproductive growth (sporulation) basic cell cycle modules are undoubtedly regulated in different ways.

Though much of the basic cell cycle machinery is evolutionarily conserved, the fact that *S. cerevisiae* is uninucleate and unicellular while *A. nidulans* is multinucleate and multicellular leads to significant differences in cell cycle regulation. In *S. cerevisiae* the single nucleus moves to the neck for division, and septum formation and cytokinesis occur as mitosis ends [43]. This tight coupling of mitosis, septation, and cytokinesis means that budding yeast cells always have a single nucleus. In contrast, septation does not follow every mitosis in the filamentous fungus *A. nidulans*, and cytokinesis results in partitioning of cells rather than separation (Fig. 02.1). Typically cell cycle studies in *S. cerevisiae* begin with vegetatively budding cells. In contrast, cell cycle studies in *A. nidulans* begin with dormant, asexual spores (conidia). The first two or three rounds of mitosis take place in the initial cellular compartment formed when the conidium breaks dormancy and establishes polarity. After the cell passes a critical size threshold, the next mitosis (typically the 3rd or 4th division depending on conditions) triggers formation of the first septum dividing the hypha asymmetrically into two compartments [44]. Subsequent mitotic divisions are also followed by septation with the tip cell remaining mitotically active and subapical cells arresting in interphase until branch formation [45].

Many of the cellular events underlying these morphological shifts in *S. cerevisiae* and *A. nidulans* have been described and are themselves landmarks, that is they always occur with the same stage of the cell cycle and so can be used to anchor and order observations.

SACCHAROMYCES CEREVISIAE LANDMARKS

The unicellular yeast *S. cerevisiae* propagates by budding and the cell must grow to a critical size before it initiates a new cycle. Attaining this critical size in the G1-phase (gap1) of the cycle allows the cell to pass START, which is defined as the point at which the cell irreversibly commits to the mitotic cell cycle [46]. The size threshold at START prevents cell size decrease through premature cell cycle progression [47]. Though the mechanisms are not clear, START appears to involve some of the same CDKs and cyclins that drive the cell cycle (see below) [48]. After passing START, the cell enters the S phase (synthesis phase), where DNA and the spindle pole body (SPB) are duplicated (Fig. 02.2). The SPBs are microtubule organizing centers, functionally equivalent to centrosomes of higher organisms [43]. However, unlike higher eukaryotes, the nuclear envelope does not break down in *S. cerevisiae*, so the SPBs are embedded in the nuclear envelope. During S phase the SPBs separate forming short microtubule spindles between them. The SPBs continue to separate eventually reaching the opposite poles of the nucleus where they anchor the medial spindles thought to function in metaphase [49].

The presence of short spindles during S phase is unusual. In most organisms, spindle formation is one of the defining features of M phase [35]. The presence of short spindles during S phase has led to disagreement about whether *S. cerevisiae* has a true G2 phase; consequently many *S. cerevisiae* researchers use the designation “G2/M”. However, the evolutionary conservation of cell cycle processes and careful measurements suggesting that DNA replication finishes before medial spindle assembly has led others to designate a separate G2 phase [43]. To make comparisons between *S. cerevisiae* and *A. nidulans* easier, we will use the convention of separate G2 and M phases in this review.

Late in S phase, the actin cytoskeleton polarizes toward a region on the cell surface and directs secretory vesicles to the site of future polarity establishment [50]. This leads to the formation of a bud, certainly the easiest S phase landmark to spot in *S. cerevisiae* (Fig. 02.2).

The G2-phase (gap 2) follows the successful completion of S phase and is characterized by rapid cell growth and protein synthesis, which prepare the cell for mitosis. The M phase (mitotic phase) is subdivided into prophase, metaphase, anaphase, and telophase (Fig. 02.2). During the closed mitosis of *S. cerevisiae* regulated active transport through the nuclear pore complexes still occurs [35]. During prophase the chromatin condenses and the microtubules of the mitotic spindle attach to the kinetochores. In contrast to higher eukaryotes, yeast chromosomes do not line up at the midzone of the spindle forming a metaphase plate [51]. The absence of a metaphase plate can make metaphase difficult to detect, but it is generally defined as the point when all chromosomes show bipolar attachment to the spindle microtubules [49]. During anaphase sister chromatids separate and travel to opposite poles and are equally distributed between the mother and daughter cell [52]. In telophase chromosomes decondense and cytokinesis rapidly follows, separating mother and daughter cells by contraction of the actomyosin ring and synthesis of a new cell wall between the cell bodies [53].

ASPERGILLUS NIDULANS LANDMARKS

While a START commitment point has not been characterized in *A. nidulans*, it seems likely that an analogous size threshold is present because G1-arrested dormant conidia [54] all expand to a very similar size before germ tube emergence (M. Momany, personal observations). Any such size threshold in conidia would only be used once before emergence of the first germ tube, as opposed to START in yeast which occurs in every cell cycle. It is possible that other

commitment points gate entry into subsequent cell cycles in *A. nidulans*. Certainly a critical size threshold must be crossed in hyphae before septum formation (cytokinesis) can be triggered [55]. In S phase (Fig. 02.2), the nucleus undergoes replication and the spindle pole body is duplicated [35, 56]. The two SPBs do not separate until the cell passes the G2/M boundary [57]. As the cell enters mitosis the cytoplasmic microtubules depolymerize [58] and the nuclear pore complexes (NPCs) partially disassemble [35]. The partial disassembly of the NPCs allows tubulin molecules to enter and form spindle microtubules originating from the nuclear face of the SPBs [34, 57]. In prophase the SPBs move apart [59]. Similarly to *S. cerevisiae* the bipolar attachment of chromosomes to the spindle does not trigger the formation of a distinct metaphase plate. During anaphase as the sister chromatids begin to move toward the poles, microtubules between the two SPBs elongate, astral microtubules form on the cytoplasmic face of the SPBs [60] and the segregating DNA is separated from the nucleolus. The nucleolus is excluded to the cytoplasm within telophase and nucleolar proteins are reimported into the daughter nuclei during G1 [36].

CYCLIN DEPENDENT KINASES AND CYCLINS DRIVE CELL CYCLE

The cell cycle is driven by the interaction of the cyclin dependent kinases (CDKs) with cyclins [43]. Cyclins, so named because of their periodic, cyclical expression [61], bind CDKs and regulate their phosphorylation of target proteins. The CDKs and cyclins are themselves regulated via phosphorylation. One of the best-studied CDK/cyclin pairs is CDK1 and Cyclin B which controls entry into mitosis. Many of the details of CDK/cyclin interaction were worked out in *S. pombe* and so the *S. pombe* naming conventions are used. CDK1/Cyclin B is phosphorylated by the Wee1 kinase inhibiting entry into mitosis and dephosphorylated by the Cdc25 phosphatase,

allowing mitosis to proceed (Fig. 02.3). As might be expected for such critical control pathways, CDK-cyclin complexes are also regulated by other mechanisms including binding by inhibitors, transcription, and proteolytic degradation. Targeted proteolysis of cell cycle regulators by the ubiquitin proteasome system, including cyclins, helps makes the cell cycle irreversible and unidirectional, which imposes a further control mechanism. The degradation takes place at three different time points: during the transition between G1 and S phase, before anaphase, and at mitotic exit [62].

SACCHAROMYCES CEREVISIAE CELL CYCLE

In *S. cerevisiae* the CDK regulating cell cycle events is Cdc28 [43]. Yeast cyclins are divided into two classes: the G1 cyclins Cln1-3, which predominantly regulate events at the end of G1 and are essential to pass START [63], and the B-type cyclins Clb1-6 that are expressed between START and mitotic exit [64]. CDK-cyclin complexes are themselves regulated by phosphorylation and dephosphorylation (Fig. 02.3).

G1 phase. Cln3 is expressed early in G1 together with the Cdc28-Cln inhibitor Far1 [65]. As G1 progresses levels of Far1 fall below the threshold needed to inhibit the Cdc28-Cln3 complex [66]. This allows Cdc28-Cln3 to stimulate the transcription of G1 cyclins including Cln1 and Cln2, pass START and trigger entry into S-phase [67].

S phase. The Cdc28-Cln1 and Cdc28-Cln2 complexes initiate DNA replication and the events that lead to bud emergence and SPB duplication, two main S phase landmarks [68]. In addition, the Cdc28-Cln2 complex stimulates the degradation of Sic1, a repressor of B-type cyclins [69, 70]. This enables the activation of the Cdc28-Clb5 and -Clb6 complexes which induce DNA replication and ensure entry into S phase [71, 72]. Cdc28-Clns trigger their own

degradation via autophosphorylation [73]. S phase ends when DNA synthesis is correctly completed (see DNA checkpoint below).

G2 phase. As described in the discussion of *S. cerevisiae* landmarks above, the presence of short spindles during S phase in *S. cerevisiae* has led to disagreement about precisely when M phase starts and the existence of G2 phase.

M phase. To enter into M phase the Cdc28-Clb2 (CDK-cyclin B) complex must be activated (Fig. 02.3). In G2 the Cdc28-Clb2 complex is inhibited by phosphorylation by the Swe1 kinase [74]. The inhibitory phosphate is removed by the phosphatase Mih1. The degradation of Swe1, mediated by the morphogenesis checkpoint (see below), creates a positive feedback loop that is responsible for a rapid increase of active Cdc28-Clb complexes. This ensures the entry into M phase and subsequent bud growth [75, 76]. In conjunction with Cdc28 the mitotic cyclins Clb1 – Clb4 control the initiation and the progression through the cell cycle. Cdc28-Clb3 and Cdc28-Clb4 trigger the formation and elongation of the mitotic spindle, whereas Cdc28-Clb1 and Cdc28-Clb2 activate the anaphase promoting complex APC/C.

The APC/C degrades G1 cyclins, B-type cyclins, and securin, whose loss initiates the separation of sister chromatids [77]. The expression of the Cdc28-Clb inhibitor Sic1 and activation of the phosphatase Cdc14 is also required for mitotic exit. Sic1 represses B-type cyclins and Cdc14 dephosphorylates substrates of Cdc28, which reinitiates the G1-phase [78, 79].

The activity of Cdc14 is controlled by the inhibitor Net1, which keeps Cdc14 anchored in the nucleolus and inactive throughout the G1, S, G2, and early M phase [80]. Two pathways control the release of Cdc14: the FEAR (Cdc Fourteen Early Anaphase Release) and MEN (Mitotic Exit Network) networks (Fig. 02.4). The FEAR network releases Cdc14 from the nucleolus during early anaphase and the MEN network maintains Cdc14 in the released state during late anaphase

and telophase. The MEN network includes several components that are also part of the spindle assembly and position checkpoints. These are discussed in more detail in the checkpoint section of this review.

The FEAR network includes the separase Esp1, the kinetochore protein Slk19, the nucleolar proteins Spo12 and Fob1, and the polo kinase Cdc5 [81]. The protease Esp1 is responsible for the cleavage of cohesins at the onset of anaphase. Slk19 is also a target of this protease, but its role in the FEAR network is still unclear. Cdc5 promotes the activity of the spindle position checkpoint protein Dbf2 and probably activates Bub2 and Bfa1 [80]. Cdc5 is essential for the release of Cdc14 from the nucleolus, and for its maintenance in the released state [82].

The MEN network (Fig. 02.4) consists of several proteins that are part of the spindle assembly checkpoint including the GTPase Tem1, its negative regulators Bub2 and Bfa1, and its positive regulator the GDP-GTP exchange factor (GEF) Lte1. After interacting with Lte1, Tem1 activates Cdc15. The protein kinase Cdc15 then activates the kinase Dbf2 through phosphorylation. Phosphorylated Dbf2 activates proteins involved in septum formation and cytokinesis [83]. Tem1 localizes to the SPB and migrates into the daughter cell during anaphase, whereas Tem1's exchange factor Lte1 localizes to the bud. Tem1 and Lte1 are in the same cellular compartment when the nucleus enters the daughter cell. This ensures that mitotic exit only occurs when the genetic material is distributed between mother and daughter cell [84].

ASPERGILLUS NIDULANS CELL CYCLE

In *A. nidulans* NimX is the CDK equivalent to the *S. cerevisiae* Cdc28 [85] (Fig. 02.3). The only characterized mitotic cyclin is NimE, a homolog of cyclin B, though cyclins involved in

development have been investigated [86] and putative cyclin orthologues have been identified based on sequence conservation [87]. In addition to the CDK NimX, *A. nidulans* requires a second kinase, NimA, for entry into mitosis. The NimX and the NimA kinases are responsible for the initiation of mitosis in *A. nidulans*, and their inactivation is required for progression through mitosis [88, 89]. Interestingly, the *S. cerevisiae* homolog of NimA, Kin3, is not essential and appears to play a role in DNA damage response.

G1 phase. *A. nidulans* orthologs of the G1 cyclins have been computationally predicted, but they have not been characterized [87]. The Ca^{2+} /calmodulin-regulated kinases CMBK and CMKC have both been reported to have roles in progression through G1/S, though how they interact with the other cell cycle machinery is not yet clear [90].

S phase. The CDK cyclin machinery that drives *A. nidulans* entry into and exit from S phase is not well-understood, though more is known about the DNA replication checkpoints that allow exit from S phase when DNA synthesis is correctly completed (see checkpoints below).

G2 phase. During interphase NimX is associated with NimE (cyclin B) and phosphorylated by AnkA, the *A. nidulans* homolog of the *S. cerevisiae* phosphatase Swe1 [85, 91, 92]. NimX is inhibited (G2 arrest) by AnkA phosphorylation, and activated by NimT dephosphorylation [93]. However, activation of NimX alone is not sufficient to trigger mitotic entry. A temperature-sensitive mutant version of the NimA kinase (*nimA5*) causes a late G2 arrest at restrictive temperature [94], while the NimX/NimE complex is fully active at the *nimA5* arrest point [95]. This demonstrates that NimA is required for the G2/M transition by a mechanism independent from that of NimX. NimX and NimE are excluded from nuclei in G2 arrested *nimA* mutants [92] suggesting that NimA regulates mitotic entry by affecting the localization of NimX/NimE. NimA is located in the cytoplasm throughout G2 [34].

M phase. To exit from G2 and enter into M phase the NimX-NimE (CDK-cyclinB) complex must be activated (Fig. 02.3). In G2 NimX is inhibited by phosphorylation by the AnkA kinase. To move into M the NimX CDK must be activated by the NimT phosphatase [93]. Once activated the NimX-NimE (CDK-cyclin B) complex phosphorylates and activates the NimA kinase. Subsequently NimA moves into the nucleus, locates at the spindle poles during mitosis and is degraded at mitotic exit [96, 97].

Mitotic exit in *S. cerevisiae* is mediated by the MEN. The equivalent network in the fission yeast *S. pombe*, known as the Septation Initiation Network (SIN), has been extensively studied. As noted above, in *S. cerevisiae* mitotic exit and cytokinesis are tightly coupled, but they are temporally separated in *A. nidulans*. Mitotic exit in *A. nidulans* is more similar to the *S. pombe* SIN than to the *S. cerevisiae* MEN [98]. Both the polo kinase Plo1 and the cyclin-dependent kinase Cdc2/Cdc13 activate the SIN in *S. pombe* and coordinate it with mitotic events [99]. Once activated the upstream GTPase Spg1 activates the downstream protein kinase cascade Cdc7 to Sid1 to Sid2 [100]. Ultimately the Sid2/Mob1 complex is relocated from the spindle poles to the contractile actin ring, where it is required for ring contraction and septum deposition (i.e. cytokinesis) [101].

In *A. nidulans* repeated mitotic divisions without cell separation lead to the formation of a mycelium of multinucleate cells that are separated by multiple septa. Strains carrying a temperature-sensitive mutation in the Ser/Thr protein kinase SepH resulted in a viable strain that failed to septate at restrictive temperature [102]. Further studies suggested that SepH is required for the construction of the actin ring [103]. It is likely that SepH, which catalyzes the initial event of septum formation, collaborates with other proteins like SidB and MobA [103, 104]. In *S. pombe* Mob1, the ortholog of MobA, is associated with Sid2, the ortholog of SidB. Mutant

versions of SepH, SidB or MobA do not impair hyphal growth or colony formation in *A. nidulans*, whereas their respective orthologs Cdc7, Sid2 or Mob1 are essential for cytokinesis in *S. pombe*. This shows that cytokinesis is essential in the uninucleate fission yeast but not in the vegetative growth of the multinucleate, filamentous fungus *A. nidulans* [104].

CHECKPOINTS GUARD CELL CYCLE TRANSITIONS

As described above, there are several CDK-cyclin-dependent mechanisms that establish the correct order of cell cycle events including size control at START, phase-specific gene expression, post-translational modification (especially phosphorylation and dephosphorylation), and targeted proteolysis. In addition, critical cell cycle events are monitored by a series of ‘checkpoints’ that halt progression to the next phase until key events are complete and error free [105]. A checkpoint generally consists of sensors which monitor the completion of a cell cycle event, transducers which forward information from the sensor, and effectors which halt cell cycle progression [106] (Fig. 02.5). Checkpoints are usually identified through the use of toxic agents that cause major damage and so impose an exaggerated cell cycle delay while damage is repaired; but, surveillance of major cell cycle events by checkpoints occurs under normal (nonstressed) growth conditions too [107, 108]. For example, the cell uses many of the same proteins to perform routine DNA repair and to trigger the DNA damage checkpoint [109].

DNA Damage and Replication Checkpoints

The evolutionarily conserved DNA damage checkpoint ensures that DNA is correctly and completely replicated before mitosis is triggered. This checkpoint is one branch of the DNA

damage response pathway (or network) that also includes branches that trigger DNA repair, damage-induced transcription and, in multicellular organisms, apoptosis [109]. The DNA damage checkpoint is triggered by double-strand breaks (DSBs) or replication fork stalls. Depending on when the checkpoint is triggered, cell cycle progression can be delayed or halted at the G1/S or G2/M boundaries by downregulating the appropriate CDK or the checkpoint can slow down replication by preventing more origins from firing (sometimes called the “intra-S” or “slowed-S” checkpoint) [110-112]. The G2/M block in the DNA damage checkpoint is covered below with an emphasis on checkpoint specific components rather than replication and repair machinery that also play roles in the checkpoint.

***S. cerevisiae*.** In *S. cerevisiae* the sensors for DNA damage can include repair and replication complex proteins (which can also be effectors) along with phosphatidylinositol-3-OH-like kinases (PIKKs) [112]. The sensor for processed DSBs and replication fork stalls is the PIKK Mec1 (ATR in mammals) and for unprocessed DSBs is Tel1 (ATM in mammals) [113, 114]. Both of these DNA damage sensors and their interacting proteins are physically associated with the DNA repair machinery and replication forks (Fig. 02.6).

Before DNA is replicated in S phase, the double helix is opened up by a multi-protein complex. DNA binding proteins, helicases, polymerases and other specialized proteins are recruited to form the replisome which makes new DNA strands bidirectionally at the fork. Replisomes can stall when they hit obstacles or when nucleotide pools are low. When replisomes stall, helicase and polymerase activities are uncoupled which produces excess single stranded DNA. It is this excess ssDNA along with the RPA (Replication Protein A) that coats it which appears to be the physical trigger for the DNA damage checkpoint. Ddc2, the *S. cerevisiae* ATR interacting

Protein (ATRIP) ortholog, binds to RPA and associates with Mec1, thus targeting it to sites of DNA damage.

To repair DSBs, multiple proteins are recruited to the lesion, including the MRX complex (Mre11, Rad50, Xrs2; called MRN in mammals). The MRX complex in turn recruits the *S. cerevisiae* ATM ortholog Tel1. In the process of repairing DSBs, the DNA repair machinery makes 3' ssDNA tails. The RPA complex coats these ssDNA tails, which leads to recruitment of Mec1 and DNA damage checkpoint activation. RPA is later displaced by Rad51 and Rad52.

Several activators of Mec1 (the ATR kinase) are needed for its full activity including Rad17-Mec3-Ddc1 (the “9-1-1 complex” in mammals) [114]. Activated Mec1 phosphorylates DNA Damage Response kinases Rad53, Chk1, and Dun1. In *S. cerevisiae* the DNA damage checkpoint ultimately blocks sister chromatid separation by phosphorylation of Pds1. In most other organisms including mammals and *S. pombe* the DNA damage checkpoint acts by phosphorylation of the CDK1 and a block at the G2/M boundary, often through inhibition of the Cdc25 phosphatase.

A. nidulans. The *A. nidulans* DNA damage checkpoint appears to have the same basic structure as that of *S. cerevisiae*. *A. nidulans* has an ATR kinase ortholog UvsB and an ATM kinase ortholog AtmA. Based on sensitivity of deletion mutants to genotoxic agents the *A. nidulans* ATM appears to be mainly involved in repair of DS breaks while ATR recognizes both DSB and replication fork stalls [85, 115-119]

Unlike *S. cerevisiae*, but like most other organisms, the DNA damage checkpoint in *A. nidulans* triggers an arrest at the G2/M boundary by inhibitory phosphorylation on NimX, the CDK1/Cdc28 ortholog. It is not yet clear how the Cdc25 phosphatase (NimT) and Wee1 kinase (AnkA) are involved in this process. Also different from *S. cerevisiae*, the *A. nidulans* ATM

(AtmA) and ATR (UvsB) appear to be partially redundant since the double ATM/ATR mutant is nonviable [119]. In addition to its role in regulating the DNA damage checkpoint UvsB/ATR inhibits septation in predivisional hyphae that have sustained DNA damage [118, 120, 121]. Similarly, in addition to its role in the damage response, AtmA is required for polarized growth [122, 123].

Another possible DNA damage checkpoint in *A. nidulans* includes the nuclear pore protein SonB. The *nimA* G2 arrest can be suppressed by mutations in *sonB*, first identified as a suppressor of *nimA* [124]. Certain *sonB* mutants are hypersensitive to DNA damage, but have a functional G2 DNA damage checkpoint. These same mutants also show synthetic lethality with mutants in the MRE complex member *scaA* leading to the suggestion that SonB might play a novel role in the DNA damage response pathway in *A. nidulans* [85, 115].

Spindle Assembly and Spindle Position Checkpoints

For proper chromosome segregation at anaphase, sister chromatids must be stably attached to opposing spindle microtubules so that they will be pulled in opposite directions. The spindle assembly checkpoint (SAC) monitors the attachment of chromosomes to microtubules at kinetochores and prevents the transition from metaphase to anaphase until all sister chromatids are properly attached [125, 126]. The transition from metaphase to anaphase is driven by the anaphase promoting complex/cyclosome (APC/C), a multisubunit E3 ubiquitin ligase, which targets securin, along with other cell cycle proteins, for degradation. The anaphase promoting complex/cyclosome (APC/C) is activated by binding to Cdc20. The presence of even a single unattached kinetochore triggers the assembly of the mitotic checkpoint complex (MCC) which

binds to Cdc20 and prevents it from activating the APC, thereby halting anaphase and cell cycle progression.

S. cerevisiae. The main components of the SAC were discovered in the early 1990s through *S. cerevisiae* mutants that did not arrest in metaphase in the presence of spindle toxins. These include the products of the *mitotic arrest deficient* (MAD) genes *MAD1*, *MAD2*, *MAD3* and the *budding uninhibited by benzimidazole* (BUB) genes *BUB1* and *BUB3*, which are highly conserved in all eukaryotes [127].

Kinetochores are decorated with multiple KMN complexes (Knl1, Mis12 and the Ncd80 complex) that work together like “molecular Velcro” to bind microtubules [126] (Fig. 02.7). The SAC kinase Mps1 associates with kinetochores in early mitosis, an association that requires the Ncd80 complex and is regulated by the Ilp1 Aurora kinase. Ilp1 detects improper tension between sister chromatids and phosphorylates microtubule binding sites, thereby destabilizing the connection between kinetochores and spindle microtubules. As a consequence the SAC is activated and SAC components are recruited to the kinetochore to form the MCC (Mad2, Mad3, Bub3 and Cdc20) [125]. Mad2 can exist in two conformations: the inactive open form (Mad2-O) and the active closed form (Mad2-C). Through binding to Mad1, Mad2 undergoes a conformational change from inactive (Mad2-O) to active (Mad2-C) forming a stable Mad1-Mad2-C complex [128]. This complex is recruited to the unattached kinetochore and serves as a receptor for the recruitment of more inactive Mad2-O protein from the cytoplasm. After binding to the Mad1-Mad2-C receptor the inactive Mad2-O is converted into an active Mad2-C, which facilitates the binding of Cdc20 [129]. The resulting MCC binds the APC/C and inhibits its activity [130, 131]. The MCC itself is unstable and must continually be renewed to maintain the SAC block on cell cycle progression in the presence of unattached kinetochores.

APC/C inhibition is released once all sister chromatid kinetochores are properly associated with microtubules. Properly attached kinetochores no longer trigger high levels of Mps1 kinase activity and the resulting Mad1 and Mad 2 recruitment. Quickly inactivating the SAC signal also requires dynein, which transports previously activated SAC proteins toward the microtubule poles, and the protein phosphatase 1 (PP1) which dephosphorylates kinetochore substrates necessary for the formation of the MCC [126, 132, 133].

After anaphase is triggered, another checkpoint must be satisfied before *S. cerevisiae* cells are permitted to exit mitosis. The spindle position checkpoint (SPOC) prevents cells with misaligned spindles from exiting mitosis [134] (Fig.02.8). Tem1, a RAS-like GTPase that controls the mitotic exit network, is the key protein in this process. In response to spindle alignment defects the GTPase-activating dimer complex Bub2/Bfa1 accelerates GTP-hydrolysis of Tem1, keeping it in its GDP-bound inactive form. Support is provided by the Ser/Thr kinase Kin4, which protects Bub2/Bfa1 from an inhibitory modification by the polo-like kinase Cdc5 [135]. The subcellular localization of these proteins changes during the cell cycle. From G1 to metaphase the inactive Tem1 and Bub2/Bfa1 complex are located at both SPBs. The amount of Tem1 increases at the SPB of the daughter cell once the correctly positioned spindle passes the bud neck. Here Bub2/Bfa1 is inhibited by the polo kinase Cdc5. Lte1, which is exclusively localized at the cortex of the daughter cell, activates Tem1, which phosphorylates the protein kinase Dbf2 and initiates the mitotic exit network (MEN) [135].

A. *nidulans*. Much of the SAC machinery is conserved between *A. nidulans* and *S. cerevisiae* [57]. Consistent with *A. nidulans* undergoing a semi-open mitosis rather than a closed mitosis as in *S. cerevisiae*, it possesses a layer of additional spatial regulation. In interphase, An-Cdc20 and SldA (the *A. nidulans* homolog of Bub1) are in the cytoplasm, An-Mps1 is at SPBs, An-Mad1

and An-Mad2 are localized to the nuclear periphery, and SldB (the *A. nidulans* homolog of Bub3) is in the nucleoplasm [57, 136]. After the partial disassembly of the NPCs at mitosis, An-Mad1 and An-Mad2 enter the nucleus and concentrate at unattached kinetochores. Subsequently SldA (Bub3) and An-Cdc20 enter the nucleus and accumulate at the SPB/kinetochore complex. An-Cdc20 spreads through the nucleus while SldB leaves it [57]. The SAC mitotic arrest in *A. nidulans* has a finite life span and is actively turned off, even when cells cannot make a mitotic spindle [137, 138].

No spindle position checkpoint (SPOC) has been identified in *A. nidulans*. This is not surprising since in *S. cerevisiae* determination of the cytokinetic division plane precedes mitosis and the spindle must later be aligned to deposit one set of chromosomes each in mother and daughter [134]. Because conidiophore formation in *A. nidulans* requires multiple coordinated budding steps, it is possible that an SPOC might be involved in asexual reproduction.

Morphogenesis Checkpoint

In *S. cerevisiae* the morphogenesis checkpoint delays mitosis until the bud is of a suitable size to receive the daughter nucleus. The morphogenesis checkpoint has not been found in other organisms, but it would be surprising if the principle of assuring proper morphology before mitosis is not eventually found elsewhere.

***S. cerevisiae*.** Yeast cells contain a highly polarized actin cytoskeleton that is responsible for polarized growth and therefore bud formation [139]. Perturbation of actin polymerization was shown to delay bud formation, but not to uncouple the budding cycle from the cell cycle [140]. This suggested the presence of a checkpoint mechanism that monitored bud formation. Mutant strains lacking the protein kinase Swe1 display a functional cell cycle under normal conditions

[74], but do not arrest when actin polarization is impaired [141]. Under normal conditions, septins, filament-forming cytoskeletal proteins encoded by *CDC3*, *CDC10*, *CDC11*, and *CDC12*, create an hourglass-shaped collar at the bud neck between the mother and daughter cell [5] (Fig. 02.9). After bud emergence the “bud sensor” Elm1 and the protein kinase Hsl1 are recruited to the bud side of the septin collar and recruit the methyltransferase Hsl7 [142, 143]. As a consequence a subpopulation of Swe1 is transported from the nucleus to the neck region [144], where Swe1 is degraded. This leads to a depletion of nuclear Swe1, which promotes cycle progression since Swe1 normally inhibits Cdc28 via phosphorylation. Actin depolymerization and delayed budding activate the morphogenesis checkpoint, which stabilizes Swe1 by blocking its degradation [145]. Swe1 inhibits the Cdc28 CDK1 [146].

***A. nidulans*.** Though no morphogenesis checkpoint has been identified yet in *A. nidulans*, it is possible that an analogous mechanism exists, especially in the budlike process of conidiophore formation. Conidiophore development is severely disorganized in septin deletion mutants consistent with a possibility of a septin-dependent morphogenesis checkpoint operating at this developmental stage [25, 147].

Reboot Regulation

Classic checkpoints halt cell cycle progression when requisite events are not correctly completed to give time for repair. But, what happens when errors in cell cycle key events cannot be repaired regardless of time? In recent work Osmani and colleagues have uncovered a situation where cells appear to back-up and retry cell cycle transitions multiple times, a mechanism they dubbed “reboot regulation” [148].

***S. cerevisiae*.** Reboot regulation has not yet been reported in *S. cerevisiae* or any other organism other than *A. nidulans*.

***A. nidulans*.** The eukaryotic nucleus is enclosed by a nuclear envelope (NE), which consists of an outer (ONM) and an inner nuclear membrane (INM) separated by a perinuclear space [149]. Nuclear pore complexes (NPCs) are large assemblies that span pores in the NE and control the nuclear transport of macromolecules across the double-membraned NE [150]. *A. nidulans* undergoes a semi-open mitosis in which the NE remains largely intact, the NPCs partially disassemble, and the nucleolus is expelled from the nucleus [34, 36]. After mitotic exit, functional G1 nuclei once more contain intact NPCs that gate transport and contain nucleoli.

By following NPC gating of RFP fused with a nuclear organization signal (NLS-RFP) and nucleolar markers, a recent study uncovered an unexpected cell cycle regulatory mechanism [148]. In the absence of the INM protein Src1, nuclei exit mitosis, move into G1, but then cycle back into mitosis based on NPC modifications and disassembly of nucleoli. Nuclear transport appears to be properly controlled leading to the suggestion that there is a defect in G1 architecture. This cycle of M to G1 can repeat multiple times, indicating that the G1 nucleus is not stable. Osmani et al. coined the term “reboot regulation” to describe this cycling between M and G1 [148]. They suggest that there are errors in nuclear architecture that cannot be repaired by simply allowing more time and, in these cases, the cell must dismantle and rebuild the nucleus. As the authors point out, a precedent for the cell dismantling and rebuilding incorrectly formed nuclear structures is seen in Aurora kinase action on improper spindle/kinetochore attachments. Reboot regulation promotes repetition of defective cell cycle transitions until the architecture defect is corrected [148].

CONCLUSION

The successful cell cycle must accomplish two critical tasks: duplicating DNA and partitioning it to daughter nuclei. CDK-cyclin complexes and their regulators reliably drive the cycle forward between phases, but the timing of several critical events within the cycle are at least somewhat stochastic. The length of time it takes polymerases to make new copies of DNA can be influenced by the state of DNA condensation, the supply of nucleotides, and the temperature. Similarly, kinetochore capture depends upon collisions of spindle microtubules with the appropriate kinetochore region of chromosomes. It is not surprising then that surveillance (checkpoint) mechanisms have evolved to ensure that pre-requisite events occur before the cycle proceeds.

We certainly have not yet found all of the surveillance mechanisms that ensure the integrity of the cell cycle. Undiscovered checkpoints seem especially likely in the case of filamentous fungi and higher organisms where nuclei must be coordinated across multiple compartments. How cell cycle signals are integrated across multiple compartments in differing developmental states is a particularly exciting question and one that filamentous fungi are poised to answer.

Table 02.1: Cell cycle proteins discussed in text

Cell cycle machinery		
S. cerevisiae	A. nidulans	Function
Cdc28	NimX	CDK catalytic subunit required for cell cycle progression
Cln1	-	Initiate entry into S phase
Cln2	-	
Cln3	-	Activates transcription of Cln1 and Cln2
Clb1	-	B-type cyclins required for transition from G2 to M phase
Clb2	NimE	
Clb3	-	Trigger formation and elongation of the mitotic spindle
Clb4	-	
Clb5	-	

Clb6	-	
Far1	-	Inhibitor of Cdc28-Cln complex
Kin3	NimA	Not essential in <i>S. cerevisiae</i> , possible role in DNA damage response; essential for entry into mitosis in <i>A. nidulans</i>
Mih1	NimT	Regulate phosphorylation state of CDK and entry into M phase
Sic1	-	Represses B-type cyclins and prevents premature entry into S phase
Swe1	AnkA	Inhibit Cdc28/Nimx and entry into mitosis
DNA damage and replication checkpoint		
<i>S. cerevisiae</i>	<i>A. nidulans</i>	Function
Chk1	ChkA	<i>S. cerevisiae</i> : mediates cell cycle arrest via phosphorylation of Pds1
Ddc2	UvsD	Proteins of the DNA damage checkpoint targeting sites of DNA damage

Mec1	UvsB	<i>S. cerevisiae</i> : DNA damage sensor for processed DSBs; <i>A. nidulans</i> : regulates DNA damage checkpoint and inhibits septation in hyphae w/ DNA damage
Mre11	MreA	<i>S. cerevisiae</i> : MRX subunit, part of the intra-S phase checkpoint
Pds1	-	Phosphorylated Pds1 blocks sister chromatid separation
Rad50	SldI	<i>S. cerevisiae</i> : MRX subunit involved in DSB repair
Rad51	UvsC	<i>S. cerevisiae</i> : replaces RPA in DSB repair
Rad53	ChkC	<i>S. cerevisiae</i> : required for cell cycle arrest in response to DNA damage
Tel1	AtmA	<i>S. cerevisiae</i> : DNA damage sensor for unprocessed DSBs; <i>A. nidulans</i> : regulates DNA damage checkpoint and is required for polarized growth

Xrs2	ScaA	<i>S. cerevisiae</i> : MRX subunit
Spindle assembly and spindle position checkpoint		
<i>S. cerevisiae</i>	<i>A. nidulans</i>	Function
Bfa1	-	Negative regulator of Tem1
Bub1	SldA	SAC protein kinase prevents progression into anaphase in presence of spindle damage
Bub2	-	Forms complex with Bfa1 accelerating GTP hydrolysis of Bfa1
Bub3	SldB	SAC protein kinase prevents progression into anaphase in presence of unattached kinetochores
Cdc20	Cdc20	Activator of APC/C complex
Ipl1	Aurora	Correction of mono-oriented kinetochore attachment
Kin4	KfsA	<i>S. cerevisiae</i> : Prevents inhibitory modification of Bub2/Bfa1 when SPOC is active; <i>A. nidulans</i> : possible checkpoint kinase, present at septa but not SPBs

Kn11	-	Acts as a scaffold for Bub1 and Bub3
Lte1	-	Positive regulator of Tem1
Mad1	Mad1	<i>S. cerevisiae</i> : Forms complex with Mad2 and inhibits APC/C upon SAC activation
Mad2	Mad2	<i>S. cerevisiae</i> : Forms complex with Mad2 and inhibits APC/C upon SAC activation
Mps1	Mps1	<i>S. cerevisiae</i> : required for SPB duplication and SAC function
Ndc80	Ndc80	Acts as a scaffold for Mad1/Mad2 complex
Tem1	-	GTP-binding protein of SPOC involved in termination of M phase and control of actomyosin and septin dynamics
FEAR and MEN network		
<i>S. cerevisiae</i>	<i>A. nidulans</i>	Function
Cdc5	PlkA	Polo-like kinase; <i>S. cerevisiae</i> : promotes Cdc14 release from nucleolus in FEAR and

		relieves inhibition of Tem1 by inhibiting Bub2/Bfa1 in MEN
Cdc14	CdcA	Phosphatase; <i>S. cerevisiae</i> : inactivates Cdc28
Cdc15	SepH	Protein kinase; <i>S. cerevisiae</i> : promotes mitotic exit; <i>A. nidulans</i> : involved in septation
Dbf2	SidB	<i>S. cerevisiae</i> : MEN component; <i>A. nidulans</i> : SIN component
Esp1	-	Separase, promotes sister chromatid separation
Fob1	-	Negative regulator of FEAR network, prevents dissociation of Cdc14 from Net1
Hct1	-	Activator of APC/C complex
Mob1	MobA	<i>S. cerevisiae</i> : required for Cdc15 dependent phosphorylation of Dbf2; <i>A. nidulans</i> : SIN component required for septation and conidiation

Net1	-	Inhibitor of Cdc14, keeps Cdc14 in nucleolus
Slk 19	-	Promotes Cdc14 release from nucleolus
Spo12	-	Promotes Cdc14 release from nucleolus by antagonizing Fob1 function
Swi5	-	Transcription factor that triggers the transcription of the CDK inhibitor Sic1
Morphogenesis checkpoint		
S. cerevisiae	A. nidulans	Function
Cdc3	AspB	S. cerevisiae: Component of septin ring required for cytokinesis; A. nidulans: Core septin required for sexual and asexual development
Cdc10	AspD	
Cdc11	AspA	
Cdc12	AspC	

Elm1	-	Bind septin ring and recruit Swe1 to the neck region for degradation
Hsl1	-	
Hsl7	-	
Swe1	AnkA	Regulates G2/M transition; <i>S. cerevisiae</i> : Negative regulator of Cdc28

Figure 02.1: Mitosis, septation and cytokinesis in *S. cerevisiae* and *A. nidulans*. Three rounds of mitosis in *S. cerevisiae* (top) vs. *A. nidulans* (bottom). Black dots are nuclei. M1, M2 and M3 denote the indicated mitotic divisions.

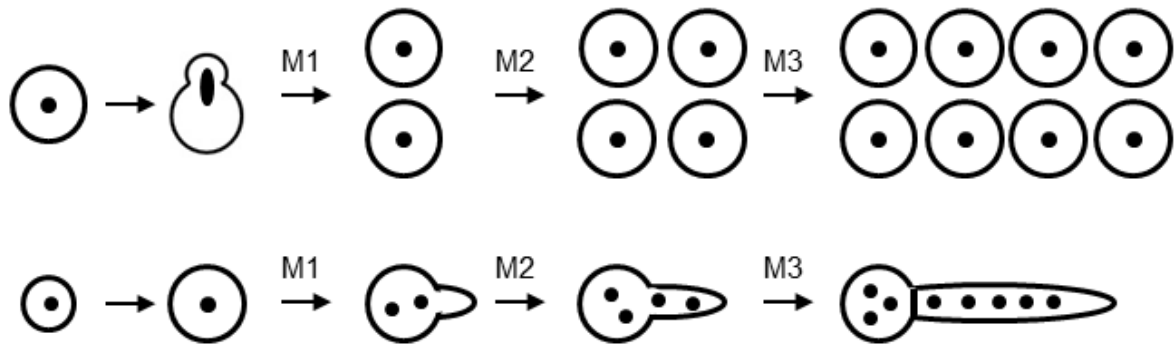
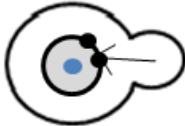


Figure 02.2: Cell cycle landmarks in *S. cerevisiae* and *A. nidulans*. *S. cerevisiae*: within G1 the cell grows to a critical size and passes the START point upon which it irreversibly enters the mitotic cell cycle. DNA and SPB are duplicated in S phase. The SPBs move to the opposite poles of the nuclear envelope and form a spindle between them towards the end of S phase. Also late in S phase the cell triggers the formation of the bud. G2 involves rapid cell growth and protein synthesis which prepares the cell for mitosis. During prophase the chromatin condenses and spindle microtubules attach to the kinetochores. The sister chromatids separate and travel to the opposite poles during anaphase. The chromosomes decondense during telophase and mother and daughter cell separate during cytokinesis. *A. nidulans*: DNA and SPB are duplicated in S phase. As the cell enters mitosis the nucleus partially disassembles and cytoplasmic microtubules depolymerize. During prophase the SPBs move to the opposite poles of the nuclear envelope and intranuclear microtubules form the spindle. The sister chromatids are separated in anaphase. The nucleolus is detached from the chromatids and excluded into the cytoplasm within telophase. Nucleolar proteins are reimported into the daughter nuclei during G1. Gray circle, nucleus; solid border, intact nuclear envelope; broken-line border, partial nuclear pore complex disassembly; small black dots, spindle pole bodies; blue dots, nucleolus; lines, microtubules. Data from references [148] and [151].

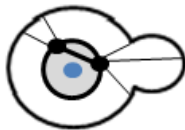
S. cerevisiae



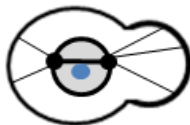
G1



S



G2



Prophase



Metaphase



Anaphase



Telophase



G1

A. nidulans

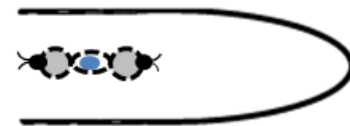
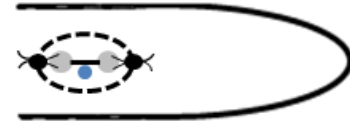
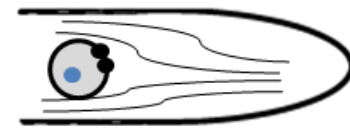


Figure 02.3: Comparison of CDK and cyclin activity at G2/M in *S. cerevisiae* and *A. nidulans*. Data from reference [1].

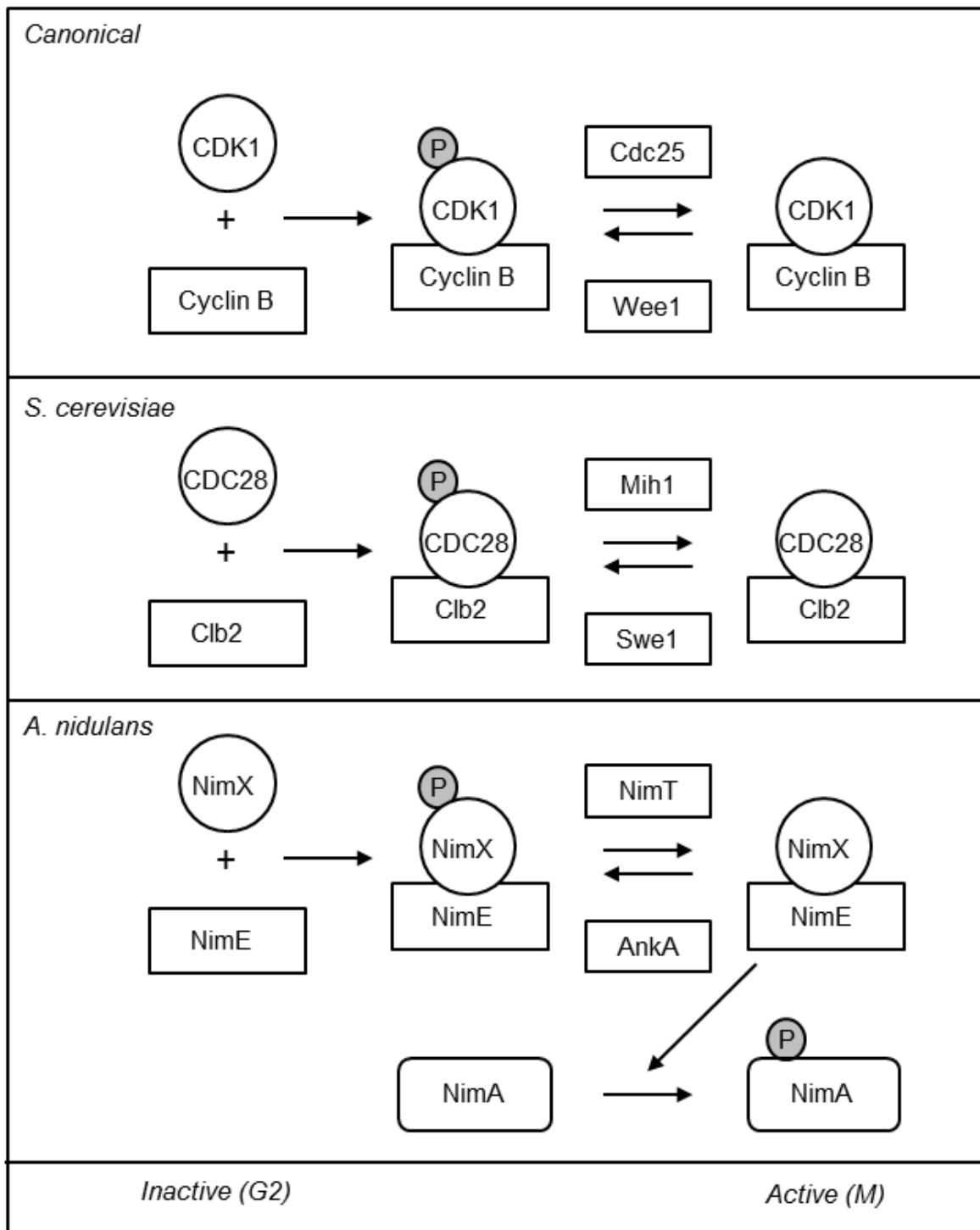


Figure 02.4: Model of the FEAR and MEN network towards the end of mitosis. In early anaphase the FEAR network promotes the release of Cdc14 from the nucleolus. In late anaphase the MEN releases Cdc14 from the nucleus into the cytoplasm, where it activates both Swi5 and Hct1. Swi5 triggers the transcription of the CDK inhibitor Sic1. Hct1 binds APC, and this complex marks Cdk for degradation. These events cause the cell to exit mitosis and enter into a new cell division cycle. Modified from reference [152] with permission.

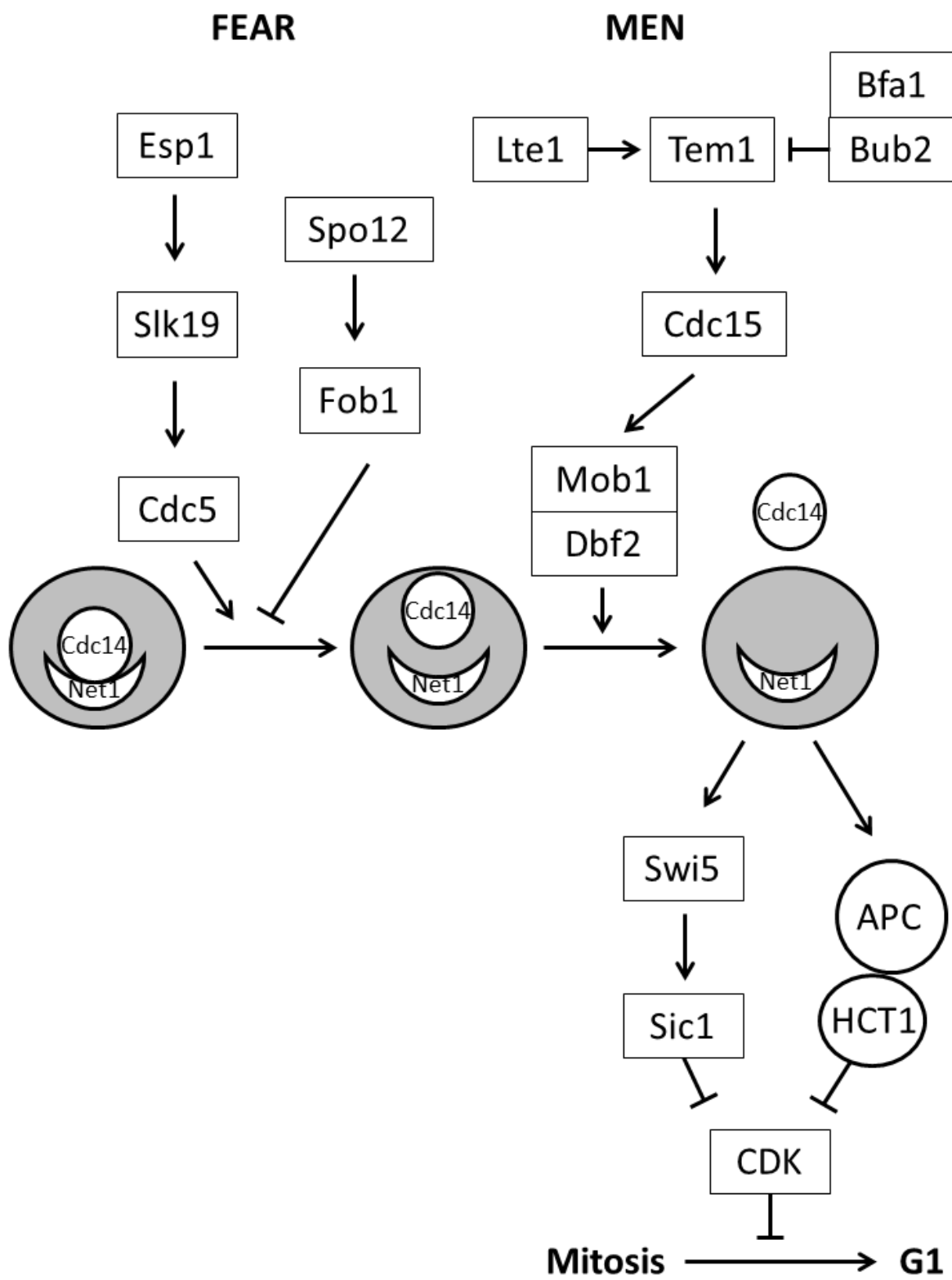


Figure 02.5: Cell cycle checkpoints in *S. cerevisiae* and *A. nidulans*. Sensors (depicted as rectangles) monitor the completion of specific cell cycle events (depicted as diamonds) and forward the information to effectors which halt or reboot the cell cycle. G1: Reboot regulation (not reported in *S. cerevisiae*). S phase: DNA damage network includes branches that are responsible for DNA repair and damage-induced transcription. Depending on when the checkpoint is triggered, cell cycle can be delayed or halted at G1/S or G2/M boundaries. S/G2: Morphogenesis checkpoint (not reported in *A. nidulans*). M phase: Spindle assembly- and spindle position checkpoint (SPOC not reported in *A. nidulans*).

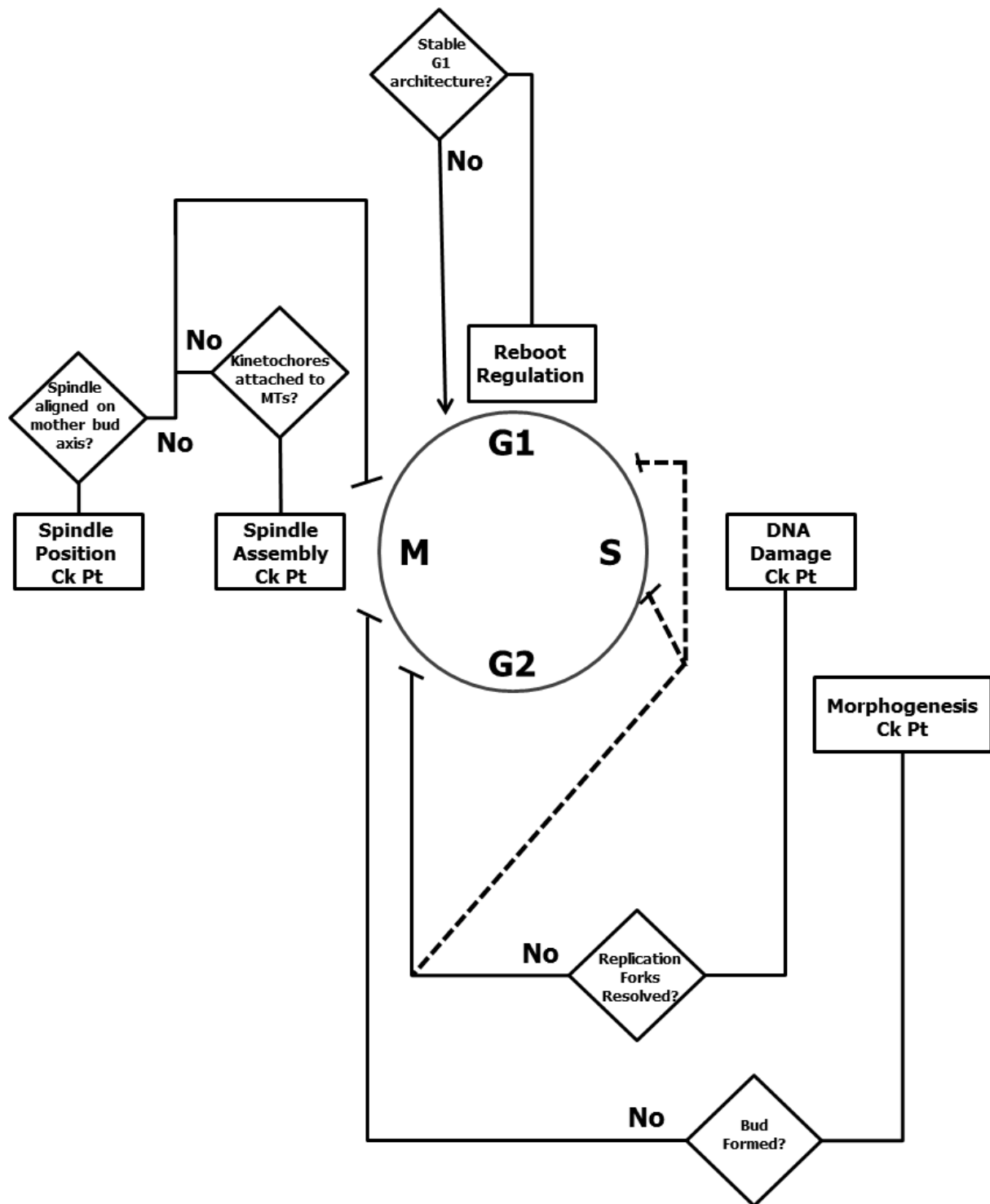


Figure 02.6: DNA damage checkpoint in *S. cerevisiae* and *A. nidulans*. Sensor proteins monitor DNA damage and transmit the information to transducers and effectors that ultimately modulate the cell cycle. Only proteins discussed in text are shown. DSB, double strand break; RPA, replication protein A; ATRIP, ATR interacting protein. Data from reference [113].

Sensors/ mediators

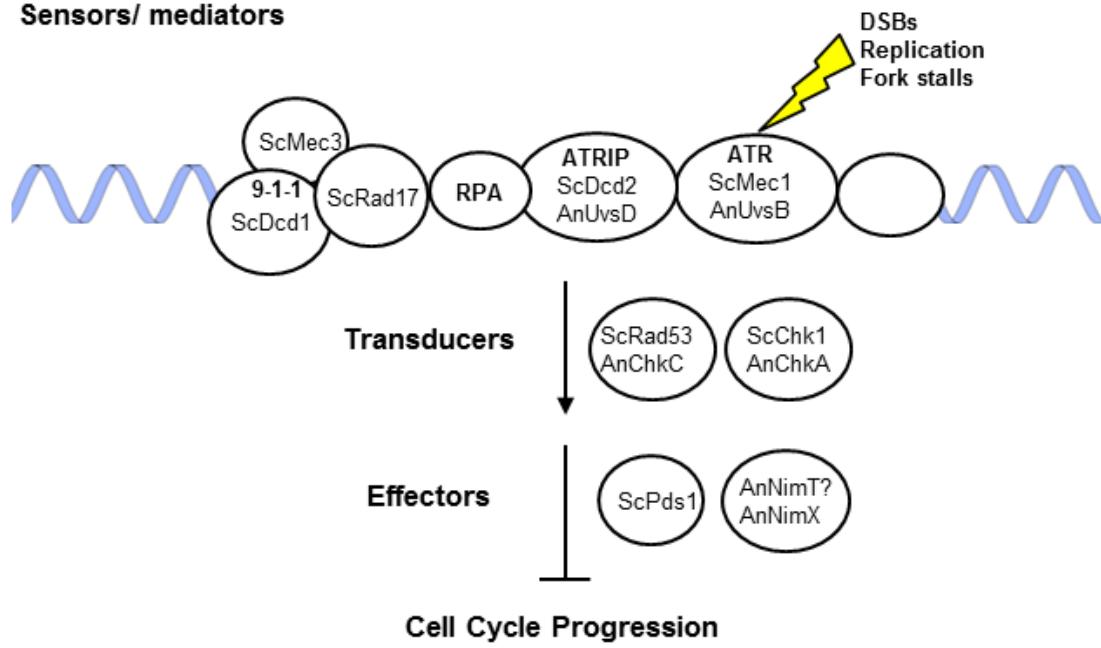


Figure 02.7: The pathway of the spindle assembly checkpoint (SAC) in *S. cerevisiae*.

Unattached kinetochores activate the SAC response by recruiting MPS1 which phosphorylates Knl1. The KMN (Knl1-Mis12-Ndc80) network serves as a scaffold for the recruitment of checkpoint proteins. Binding of Bub1/3 promotes the recruitment of the Mad1/Mad2 core complex. Kinetochore bound Mad1/Mad2 catalyzes the conformational change of the open Mad2-O to the closed Mad2-C form. Mad2-C interacts with Cdc20 and forms the mitotic checkpoint complex (MCC) consisting of Cdc20, Mad2-C, BubR1 and Bub3. This inhibits the APC activator Cdc20 and blocks the metaphase to anaphase transition. Data from reference [125].

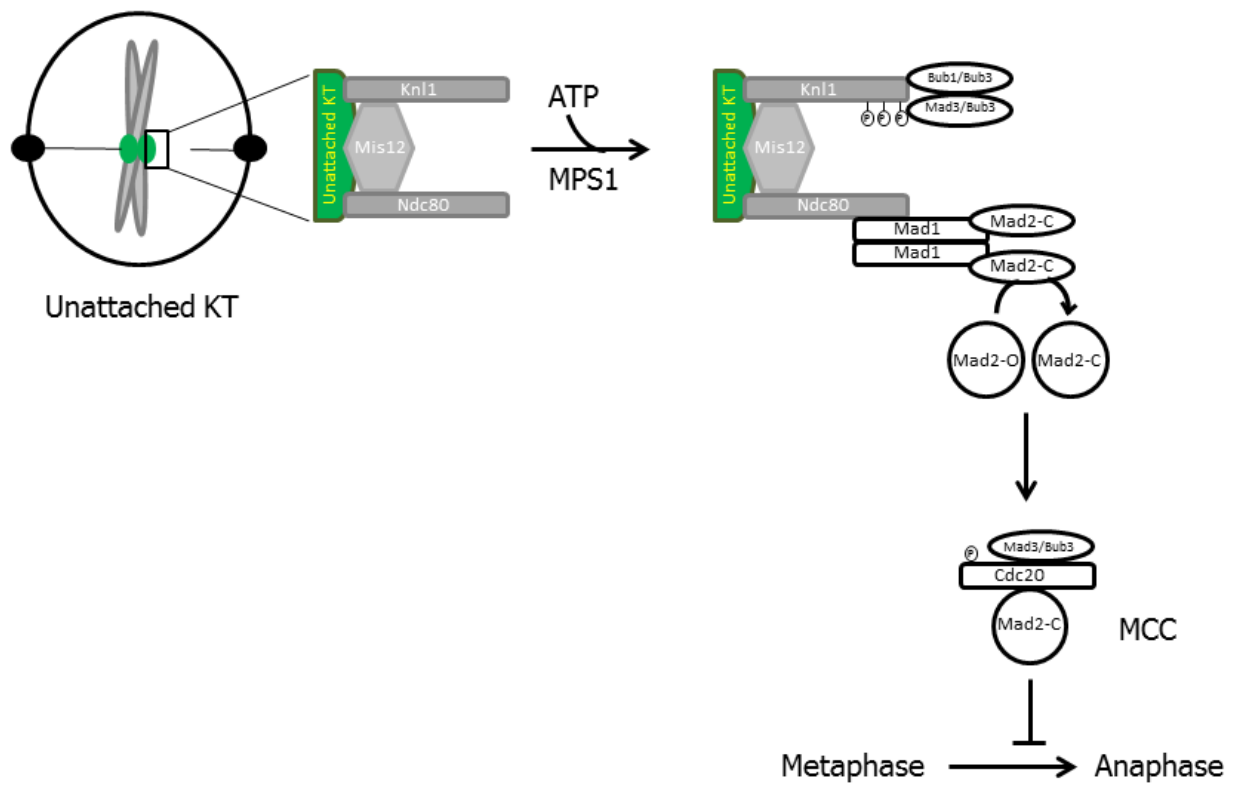


Figure 02.8: Spindle position checkpoint controls mitotic exit in *S. cerevisiae*. When the spindle is misaligned Bub2/Bfa1 keeps Tem1 in its inactive form. Kin4 protects Bub2/Bfa1 itself from an inhibitory modification. Bub2/Bfa1 disappears from the mother cell once one SPB is correctly positioned in the daughter cell. At the same time the amount of Tem1 and Bub2/Bfa1 increases at the SPB of the daughter cell. Lte1, which is located at the cortex of the daughter cell, activates Tem1 and Bub2/Bfa1 is phosphorylated by the polo kinase Cdc5. Bub2/Bfa1 disappears from the daughter cell, whereas Kin4 is removed from the SPB of the mother cell. Simultaneously Lte1 diffuses into the cytoplasm of mother and daughter cell. It is possible that all these events contribute equally to the activation of the MEN. The nucleus is depicted as a gray circle, and the pink highlighted part of the cell shows Lte1 in the cytoplasm. The blue empty circle represents the GTPase Tem1, and the blue filled circle is Tem1 associated with the dimer complex Bub2/Bfa1. Data from reference [153].

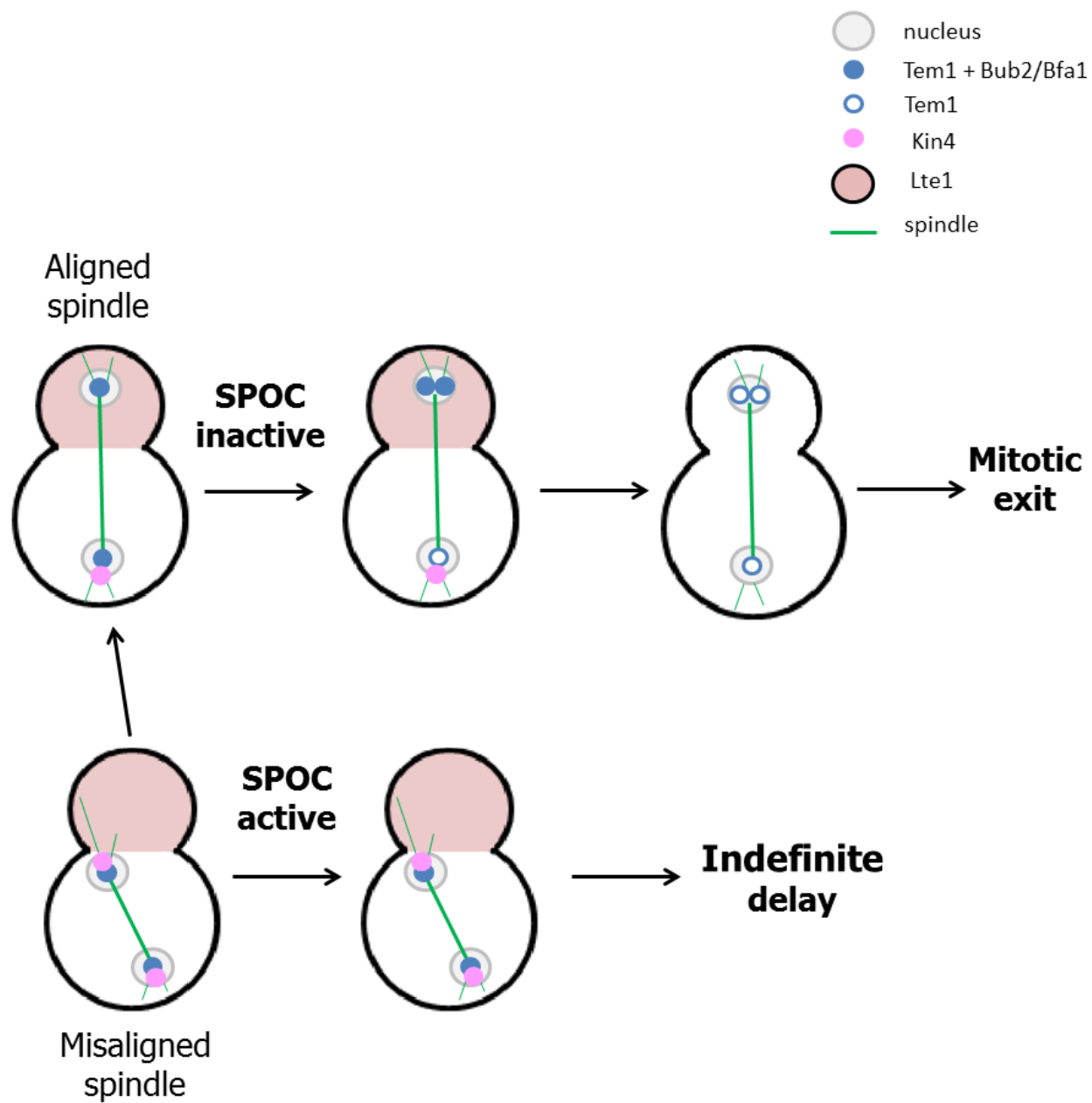
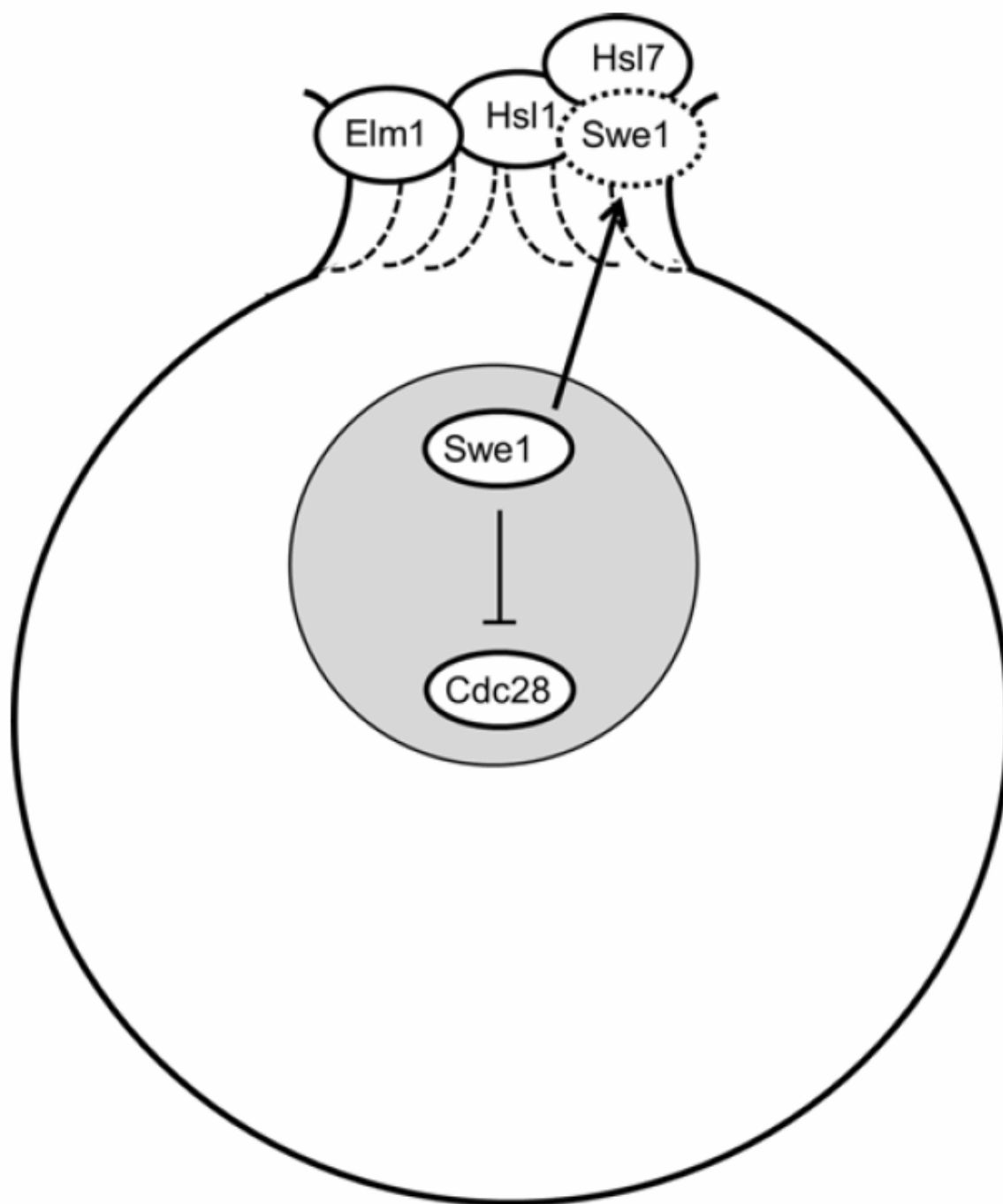


Figure 02.9: Summary of the morphogenesis checkpoint in *S. cerevisiae*. In the nucleus the kinase Swe1 inhibits Cdc28 via phosphorylation. When the bud emerges, Elm1, Hsl1, and Hsl7 are recruited to the bud side of the septin collar and in turn recruit a subpopulation of Swe1 from the nucleus to the bud side of the septin collar, where it is degraded. This depletes nuclear Swe1 and allows progression into mitosis. Actin depolymerization and delayed budding activate the morphogenesis checkpoint, which stabilizes Swe1. Dotted lines represent the septin collar between mother and bud. The gray circle represents the nucleus. Swe1 with a solid border is stable and active. Swe1 with a broken line border is destabilized. The arrow shows recruitment of Swe1 from the nucleus.



CHAPTER 3

INTERPLAY BETWEEN SEPTIN ASPD, NUCLEAR DIVISION AND THE NUCLEOLUS IN THE EARLY VEGETATIVE GROWTH OF *ASPERGILLUS NIDULANS*²

² Dorter, Mela, Kang, and Momany. To be submitted to *Molecular Biology of the Cell*

INTRODUCTION

The filamentous ascomycete *A. nidulans* is a soil-living organism, significantly involved in the remineralization of organic materials. Filamentous fungi grow as long cylinders with a slightly conical apex called hyphae [154]. The hyphal growth of filamentous fungi occurs at the tip [155, 156]. Therefore, these organisms are ideal for studying polar growth, which also occurs in neurons, root cells or pollen tubes [157].

Polarity is a fundamental property for the development of the cell. The polarity in filamentous fungi is established by constant synthesis and selective targeting of proteins, lipids and cell wall building components, which are transported to specific domains on the apex of the hyphal plasma membrane [154]. Microtubules, actin and septins are required for the polar growth of *A. nidulans* [158-160]. Septins can be found in all eukaryotes except land plants. They form heteropolymers, which assemble into higher order structures. Septins have a variety of functions, including cell cycle regulation, formation of diffusion barriers in plasma membranes, labeling of the budding site, directed secretion, and septation [16]. *A. nidulans* has four core septins AspA^{Cdc11}, AspB^{Cdc3}, AspC^{Cdc12} and AspD^{Cdc10}, which form heteropolymeric complexes. The deletion of *aspA^{CDC11}*, *aspB^{CDC3}* or *aspC^{CDC12}* results in perturbations of early developmental landmarks, such as delayed septation, increased emergence of germ tubes and branches, and greatly reduced conidiation. The Δ *aspD^{cdc10}* mutants grow similar to wild type (Wt) [27].

Two predominant morphological forms in fungi are yeast and hyphae. Unicellular fungi like *S. cerevisiae* or *S. pombe* divide through a process of budding or symmetric division in which the daughter cell, after successful mitosis, pinches off the mother cell by septation and cytokinesis.

In contrast, filamentous growth like that of the ascomycete *A. nidulans*, is characterized by elongated tubular cells, which are separated by septa and form a branched mycelium. Dimorphic fungi, such as *Candida albicans*, switch from unicellular to filamentous growth under certain environmental conditions [161]. Once conidiospores of the filamentous fungus *A. nidulans* land on a suitable growth medium, they undergo a period of isotropic swelling, which is followed by a switch to apical growth of the germ tube [162]. An axis of polarity is established and the germ tube emerges. The first nuclear division takes place after approximately four hours, whereas the subsequent nuclear divisions will occur about every 90 minutes. Apical growth is maintained and the first septum is formed at the base of the germ tube after the third round of mitosis [163].

Previous studies in our lab identified at least two different septin heteropolymer populations in *A. nidulans*. Heterooctamers containing all four core septins ($\text{AspA}^{\text{Cdc11}}$, $\text{AspB}^{\text{Cdc3}}$, $\text{AspC}^{\text{Cdc12}}$ and $\text{AspD}^{\text{Cdc10}}$) co-exist with heterohexamers ($\text{AspA}^{\text{Cdc11}}$, $\text{AspB}^{\text{Cdc3}}$ and $\text{AspC}^{\text{Cdc12}}$) lacking $\text{AspD}^{\text{Cdc10}}$ [27].

These two populations might play different roles during the numerous mitotic events in *A. nidulans*. Mitosis includes karyokinesis in which two identical daughter nuclei are formed. Mitoses in the cells of eukaryotic organisms follow a similar scheme, but not all occur in the same form. Higher eukaryotes undergo an open mitosis, in which the nuclear envelope disintegrates, nuclear pores disassemble, and the nucleolus disappears. Many fungi, like *S. cerevisiae*, undergo a so called closed mitosis, in which the nucleus remains intact during nuclear division. In filamentous ascomycetes, like *A. nidulans*, karyokinesis is not connected to cellular division, but to partitioning. These fungi undergo a so called semi-open mitosis. Here, peripheral nucleoporins (Nups) dissociate from core Nups in the nuclear pore complex, which creates an opening in the nuclear envelope allowing the transport of macromolecules, such as tubulin. The

tubulin molecules associate with the spindle pole body (SPB) and initiate the formation of spindle microtubules. At the same time, so called astral microtubules are formed, which position the spindle in the cell. The spindle microtubules adhere to the centromeres of the chromosomes and separate the chromatids. *A. nidulans* hyphae has multiple nuclei since its nuclear division is not linked to cellular division. The multinucleate hyphae are separated by septa, which are also cell cycle regulated: they are opened during interphase but closed during mitosis [164]. During the telophase of *A. nidulans* mitosis the nucleolus is excluded in the cytoplasm, where it is disassembled and subsequently reassembled in both daughter nuclei. These events occur in both normal mitosis and spindle independent mitosis (SIM), indicating that they are independent of microtubules [36].

The goal of this project was to elucidate the role of AspD^{Cdc10} in the early vegetative growth of *A. nidulans* by performing fluorescence microscopy on septins, nuclei and nucleoli of Wt and $\Delta aspD^{cdc10}$ mutants.

MATERIAL AND METHODS

Slide preparations and microscopy

A. nidulans spores (Table 03.1) were incubated on coverslips in a petri dish containing 10 ml of CM and corresponding auxotrophic markers. After incubation for 4 - 10 h at 30° C, the coverslips were placed for 30 minutes in a coplin jar with fixer (3.7% formaldehyde, 0.2% TritonX-100, 50 mM phosphate buffer). For visualization of nuclei, fixed coverslips were placed for 5 minutes hyphae-side down onto a drop of Hoechst-stain (Sigma Hoechst 33258 [1mg/ml]) and covered with a box lid to protect the fluorescent dye from light. After the fixation process,

the coverslips were mounted with the hyphae facing down on an immersion oil coated slide. The microscope slides were air dried and sealed with nail polish to preserve the specimens. Fluorescence images were taken either with the Zeiss ELYRA S1 (SR-SIM) Super Resolution Microscope and the EM-CCD (Andor iXon) Camera or the DeltaVision II Microscope System and the CoolSnap HQ2 Camera using a 100x or 63x immersion oil lens respectively. The images were edited with the imaging software Zen or ImageJ.

Characterization of nuclear attributes

A. nidulans spores were incubated for 7 h at 30° C, fixed and stained with Hoechst 33258 as described above. Fluorescence images were taken with the DeltaVision II Microscope System and the CoolSnap HQ2 Camera using a 60x immersion oil lens. 200 cells of each $\Delta aspD^{cdc10}$, $\Delta aspB^{cdc3}$ and Wt were picked from randomly chosen field of views for quantitative analyses. The length of the Hoechst stained nuclei and the distance between two neighbored nuclei was quantified with the help of the imaging software ImageJ.

Colony sectoring assay

Fresh conidia of $\Delta aspA^{cdc11}$, $\Delta aspB^{cdc3}$, $\Delta aspC^{cdc12}$, $\Delta aspD^{cdc10}$ and Wt were harvested in ddH₂O and washed to eliminate mycelial debris. 10 ml of supplemented CM were inoculated with 1×10^6 cells and cultured at 30° C for four hours in a shaking incubator. The cultures were subsequently treated with 2.5 µg/ml benomyl (or DMSO as a control) and incubated for another two hours under the same conditions. 10 µl of spores were then spotted on supplemented CM and grown

for three days in the dark at 22° C, 30° C and 37° C. Colonies exhibiting sectors were quantified by eye.

DNA damage assay

Spore dilutions of *ΔaspA^{cdc11}*, *ΔaspB^{cdc3}*, *ΔaspC^{cdc12}*, *ΔaspD^{cdc10}* and Wt were spotted on MM containing 0.03% MMS and grown for three days at 30° C. The plates were photographed with a plate imaging system and the images were edited with the imaging software ImageJ.

Time-lapse analyses

For *in vivo* time-lapse studies 1×10^5 conidia (Table 03.1) were inoculated in Poly-L-Lysine coated, glass bottom μ -well slides (ibidi, Martinsried, Germany) containing 300 μ l of CM. For visualization, cells were incubated for a specific time-period at 30 °C and images were taken with the DeltaVision II Microscope System and the CoolSnap HQ2 Camera using a 100x immersion oil lens. General RFP acquisition settings were 30 s interval time, 0.2 s exposure time, 32 % illumination transmission, and z-axis planes were spaced by 0.2 μ m. General GFP acquisition settings were 30 s interval time, 0.2 s exposure time, 5 % illumination transmission, and z-axis planes were spaced by 0.2 μ m. The background of the acquired images was normalized and the two fluorescence planes were then overlaid by using the imaging software ImageJ. The individual images of 56 time-lapse analyses were then assembled to a movie in .wmv format using the Windows Movie Maker software.

RESULTS

***ΔaspD^{cdc10}* has abnormally shaped nuclei and reveals asynchronous nuclear division**

Previous studies have shown that AspA^{Cdc11}, AspB^{Cdc3} and AspC^{Cdc12} interact with each other and are important for normal development. Deletion of any one of the *aspA^{CDC11}*, *aspB^{CDC3}* and *aspC^{CDC12}* core septins led to a dramatic increase in the numbers of germ tubes and branches in the early vegetative development of *A. nidulans*. Surprisingly, the *ΔaspD^{cdc10}* strain did not show the same obvious changes; its vegetative morphology was similar to Wt [27]. To more closely examine *ΔaspD^{cdc10}* we used the fluorescent probe Hoechst 33258 to label the DNA. Hoechst staining and fluorescence microscopy revealed nuclear anomalies in *ΔaspD^{cdc10}* (Fig. 03.1A). Compared to the nuclei of Wt and *ΔaspB^{cdc3}* (also representative for *ΔaspA^{cdc11}* and *ΔaspC^{cdc12}*) the nuclei of *ΔaspD^{cdc10}* were on average longer and closer together (Fig. 03.1B). This phenotype became more prevalent with each mitotic division giving *ΔaspD^{cdc10}* nuclei in more mature hyphae a stringy appearance (data not shown). This observation raised the question if AspD^{Cdc10} is connected to the regulation of nuclear division. Surprisingly, a proportion of *ΔaspA^{cdc11}*, *ΔaspB^{cdc3}*, *ΔaspC^{cdc12}* and *ΔaspD^{cdc10}* cells revealed odd numbers of nuclei (Fig. 03.2A + B). The uneven number of nuclei suggests that the coordination of nuclear division within a hyphal compartment is impaired in the absence of septins.

***ΔaspD^{cdc10}* colonies exhibit high frequency of sectoring, but are resistant to DNA damage**

When growing *ΔaspD^{cdc10}* on complete medium (CM), we observed spontaneous sectors in *ΔaspD^{cdc10}* colonies. In regard to the abnormal nuclear morphology of *ΔaspD^{cdc10}* (Fig. 03.1), we

hypothesized that the color sectoring phenotype might be a result of chromosome missegregation. In order to investigate if septins play a role in nuclear division, we treated isotropically expanding $\Delta aspA^{cdc11}$, $\Delta aspB^{cdc3}$, $\Delta aspC^{cdc12}$, $\Delta aspD^{cdc10}$ and Wt cells with benomyl and transferred them to supplemented growth medium. After benomyl treatment, the number of sectors within $\Delta aspD^{cdc10}$ colonies was drastically increased compared to Wt and the three other core septin mutants (Fig. 03.3).

Since the microtubule depolymerizing drug benomyl interferes with mitotic spindle assembly we asked if the sectoring phenotype might be the result of an impaired spindle assembly checkpoint (SAC) machinery. The SAC monitors the attachment of chromosomes to microtubules at kinetochores and prevents the transition from metaphase to anaphase until all spindle microtubules are properly attached to the kinetochores [126]. We reasoned that if $AspD^{Cdc10}$ is part of the SAC then its absence should impair the machinery and mitosis should progress in the presence of benomyl, i.e. spindle damage. We grew Wt, $\Delta aspD^{cdc10}$ and $\Delta sldA$ for a specific period of time in the presence and absence of benomyl and quantified their nuclei. The *sldA* gene encodes a SAC protein kinase that prevents the onset of anaphase if the spindle does not assemble correctly [165]. In the absence of benomyl the majority of $\Delta aspD^{cdc10}$ cells showed either four or eight nuclei whereas most of the $\Delta sldA$ cells had only one or two (Fig. 03.4A). In the presence of benomyl $\Delta sldA$ cells displayed two or four nuclei confirming the idea that cell cycle continued in the absence of a critical SAC component (Fig. 03.4B), whereas in the absence of $AspD^{Cdc10}$ the cell cycle still seemed to be arrested. The majority of $\Delta aspD^{cdc10}$ cells had four nuclei (Fig. 03.4B). This result suggested that SAC response does not depend on $AspD^{Cdc10}$. Colonial sectors can also be the result of a change in genetic content. Thus, we wanted to test if $\Delta aspD^{cdc10}$ is part of the DNA damage checkpoint. When grown in the presence of methyl

methansulfonate (MMS), $\Delta aspD^{cdc10}$ mutants grew similar to Wt suggesting that $AspD^{Cdc10}$ is not a critical component of the DNA damage checkpoint (Fig. 03.5).

$AspD^{Cdc10}$ and $AspB^{Cdc3}$ localize differently during the early vegetative growth of *A. nidulans*

To investigate the *in vivo* localization of $AspD^{Cdc10}$ and $AspB^{Cdc3}$ throughout the early vegetative growth, we constructed strains in which a single copy of $AspD^{Cdc10}$ -GFP or $AspB^{Cdc3}$ -GFP was integrated in the endogenous native locus, driven by the native promoter. Proper integration of cassettes was verified by PCR and southern hybridization [26]. Time-lapse microscopy was used to separately visualize $AspD^{Cdc10}$ -GFP and $AspB^{Cdc3}$ -GFP containing HO structures, hereafter referred to as $AspD^{Cdc10}$ or $AspB^{Cdc3}$, within the early vegetative growth of *A. nidulans*. During isotropic expansion $AspD^{Cdc10}$ is highly dynamic. It circulates in the cell and alternately contacts the nucleus and the cell cortex in short and regular intervals (Fig. 03.6A). At the onset of mitosis the $AspD^{Cdc10}$ is in close vicinity to the condensing nucleus (Fig. 03.6A; 7m 30s). We were able to observe the process of nuclear division. During telophase we could observe $AspD^{Cdc10}$ successively contacting mother and daughter nucleus (Fig. 03.6A; 9m – 10m 30s). Right after mitotic exit, when cells enter the G1 phase, $AspD^{Cdc10}$ localizes in between the two nuclei (Fig. 03.6B; 3m). With germ tube emergence $AspD^{Cdc10}$ switches to a linear movement, gliding along the elongating germ tube (Fig. 03.6C). $AspB^{Cdc3}$ -HO structures were more compact and globular than the $AspD^{Cdc10}$ counterparts. During isotropic expansion $AspB^{Cdc3}$ is highly dynamic. It circulates in the cell like $AspD^{Cdc10}$ and alternately contacts cell cortex and nucleus. However, in contrast to $AspD^{Cdc10}$, $AspB^{Cdc3}$ predominantly locates to the cell membrane (Fig. 03.7A) and

does not contact either nucleus during telophase of mitosis (Fig. 03.7A; 9m – 13m 30s). $\text{AspB}^{\text{Cdc3}}$ also does not localize between the two nuclei after mitotic exit (data not shown). However, like $\text{AspD}^{\text{Cdc10}}$, $\text{AspB}^{\text{Cdc3}}$ glides along the plasma membrane of the growing germ tube (Fig. 03.7B).

***A. nidulans* septin HO structures are composed of various septin polymers**

According to our model, *A. nidulans* harbors two septin populations: heterooctamers containing $\text{AspA}^{\text{Cdc11}}$, $\text{AspB}^{\text{Cdc3}}$, $\text{AspC}^{\text{Cdc12}}$ and $\text{AspD}^{\text{Cdc10}}$ and heterohexamers lacking $\text{AspD}^{\text{Cdc10}}$ [27]. We have generated a strain that expresses both $\text{AspD}^{\text{Cdc10}}$ -GFP and $\text{AspB}^{\text{Cdc3}}$ -CFP in order to observe both septin populations simultaneously. Both $\text{AspD}^{\text{Cdc10}}$ -GFP and $\text{AspB}^{\text{Cdc3}}$ -CFP were integrated respectively in the endogenous native locus of $\text{AspD}^{\text{Cdc10}}$ or $\text{AspB}^{\text{Cdc3}}$. The constructs are driven by the native $\text{AspD}^{\text{Cdc10}}$ or $\text{AspB}^{\text{Cdc3}}$ promoter respectively. In regard to our hypothesized model we would expect to see heterooctamers showing a merged GFP and CFP signal, and heterohexamers showing only CFP. We could detect septin HO structures displaying a merged signal. However, we could see that the HO structure is composed of heterooctamers (GFP+CFP), heterohexamers (CFP) and a possible unknown polymer (GFP) (Fig. 03.8A). The polymers seem to alternate in regular intervals. We could also observe ring-like HO structures composed of heterohexamers (CFP) and the unknown polymer (GFP) (Fig. 03.8B). At some point the unknown polymer detached from the ring-like heterohexamer. We have not further characterized this observation, but it suggests that different septin polymers have different functions during the early vegetative growth of *A. nidulans*.

Nucleoli are disorganized in $\Delta aspD^{cdc10}$ cells

In order to investigate the nuclear integrity in Wt and $\Delta aspD^{cdc10}$ we used cells, which were expressing Bop1-mCherry. Bop1 is a nucleolar protein, which has a role in rRNA processing and ribosomal large subunit biogenesis [36]. In isotropically expanding cells we could detect no differences between the nucleoli of Wt and $\Delta aspD^{cdc10}$. After the first nuclear division the nucleoli in both strains were still elliptical and exhibited the same fluorescence profile (Fig. 03.9). The overlap of both Bop1-mCherry and Hoechst fluorescence profiles showed that the nucleoli in both strains are integrated in the respective nuclei. However, after the second round of mitosis, differences became obvious. From a phenotypical point of view the nucleoli lost their elliptical shape and looked more spherical in $\Delta aspD^{cdc10}$ (Fig. 03.10). In addition the nucleoli also seemed to have lost their nuclear integrity in the absence of $AspD^{Cdc10}$. We could detect Bop1-mCherry in the cytoplasm of $\Delta aspD^{cdc10}$, whereas it was absent in the cytoplasm of Wt (Fig. 03.11). After the third round of mitosis the Bop1-mCherry signal overlapped with the Hoechst-DNA signal in Wt, whereas Bop1-mCherry fluorescence was mainly in the cytoplasm of $\Delta aspD^{cdc10}$, suggesting that the nucleolus is not reintegrated properly into the nucleus after mitotic exit (Fig. 03.12).

DISCUSSION

Septins are evolutionarily conserved proteins with essential functions in cytokinesis, and roles throughout the cell cycle. Much of our knowledge about septins originates from studies with *S. cerevisiae*, where they form a ring-like protein scaffold at the mother-bud neck. We have asked

what functions the septins may perform in an organism in which daughter cells do not completely separate from each other. Interestingly, all budding yeast core septins are conserved in *A. nidulans*. Previous studies in our lab revealed two septin populations: presumed heterooctamers that contain all four core septins (AspA^{Cdc11}, AspB^{Cdc3}, AspC^{Cdc12} and AspD^{Cdc10}) and presumed heterohexamers (AspA^{Cdc11}, AspB^{Cdc3} and AspC^{Cdc12}) that lack AspD^{Cdc10} [27]. The deletion of *aspA^{CDC11}*, *aspB^{CDC3}* or *aspC^{CDC12}* led to drastic changes in the vegetative phenotype of *A. nidulans* (abnormal septation, early germ tube emergence, hyperbranching) [166], whereas the deletion of *aspD^{CDC10}* led to growth that was similar to Wt, except for a subtle thickening of germ tube bases and swollen hyphal tips [27].

Time-lapse analyses of GFP-tagged AspD^{Cdc10} and AspB^{Cdc3} allowed better insight into the localization of the higher order structures during the early vegetative growth stages of *A. nidulans*. In contrast to the rod shaped AspD^{Cdc10}-GFP heterooctamer HO structures (Fig. 03.6), AspB^{Cdc3}-GFP heterohexamer HO-structures were more compact and globular (Fig. 03.7). AspB^{Cdc3}-CFP also partially overlapped with AspD^{Cdc10}-GFP in a form of a rod or a ring (Fig. 03.8). The heterohexamers containing AspB^{Cdc3}-GFP were mainly found at the cell cortex, whereas heterooctamers containing AspD^{Cdc10}-GFP moved around freely, either contacting the nucleus or cell cortex. It could be shown that the *A. nidulans* specific septin AspE is required for proper heterohexamer localization and vice versa. Heterohexamers might stabilize cytoskeletal elements that are required for the delivery of AspE to the plasma membrane. In contrast, AspD^{Cdc10}-GFP was still visible in cortical HO structures in *ΔaspE* mutants, indicating that heterooctamers do not rely on AspE and take another route to the plasma membrane [27]. According to our studies heterooctamers include AspD^{Cdc10} and AspB^{Cdc3}, but dual-color microscopy revealed HO structures, which appeared to contain only AspD^{Cdc10}-GFP (Fig.

03.8B). This is the first report of monomeric septin higher order structures *in vivo*, though bacterially expressed and purified yeast septins showed that self-association of a single septin can occur *in vitro* in absence of the preferred partner septin [167]. Thus the unknown polymer might be a heteropolymer lacking AspB^{Cdc3} or a homopolymer composed of AspD^{Cdc10} only. These observations suggest that AspD^{Cdc10} containing heterooctamers and AspB^{Cdc3} containing heterohexamers might share certain functions, but also have independent roles.

All core septins seem to share a function during the parasynchronous nuclear division in *A. nidulans*. Here mitosis is initiated at the hyphal tip and a linear wave of mitoses spreads backwards down the hyphae so that nuclei divide sequentially, doubling the nuclear number [168]. About 15% of $\Delta aspA^{cdc11}$, $\Delta aspB^{cdc3}$, $\Delta aspC^{cdc12}$ and $\Delta aspD^{cdc10}$ mutants contained an odd number of nuclei (Fig. 03.2). This irregularity might be the result of an uncoordinated division pattern in which nuclei divide independent of their neighbors [169] or it might be the result of degradation of individual nuclei. In contrast to the nuclei of Wt, $\Delta aspA^{cdc11}$, $\Delta aspB^{cdc3}$ and $\Delta aspC^{cdc12}$, the nuclei of $\Delta aspD^{cdc10}$ exhibited abnormal morphologies. The neighboring nuclei were closer together and the nuclei themselves were elongated and irregular (Fig. 03.1), a phenotype that worsened with each round of mitosis (data not shown). This suggested that AspD^{Cdc10} might be involved in a cell cycle checkpoint. However, our experiments showed that the SAC machinery and the DNA damage checkpoint are still active in the absence of AspD^{Cdc10} (Fig. 03.4 and Fig. 03.5). The $\Delta aspD^{cdc10}$ nuclei looked similar to the nuclei in $\Delta src1$ mutants. Src1 is an inner nuclear membrane protein whose absence causes changes in nuclear architecture, causing nuclei to oscillate between a G1-like and mitotic-like state. This newly observed phenomenon, termed “reboot regulation”, is thought to act cause repeats the M to G1 transition to be repeated until the defect is repaired [148]. One of the hallmarks of reboot regulation is the

failure of the nucleolar protein Bop1 to stably reintegrate into daughter nuclei at the M/G1 boundary. Our results are consistent with such a failure, though we have not yet demonstrated oscillation between M and G1. Another study showed that the nuclei are misshaped in the absence of the nucleoporin Nup2 [170]. The nuclei of the *Δnup2* mutants looked very similar to the nuclei of *ΔaspD^{cdc10}*: the average distance between two neighboring nuclei was greatly reduced, whereas the nuclei themselves were slightly elongated. It is possible that septins might act as a scaffold recruiting nuclear membrane proteins to their proper destination in order to fully reassemble both daughter nuclei after mitotic exit. Another possibility is that septins might be directly involved in the physical separation of daughter nuclei since we could previously locate AspD^{Cdc10}-GFP in between the two nuclei after mitotic exit. Here it co-localized with the nucleolar ribonucleoprotein Bop1. After two rounds of mitoses the nucleolar protein Bop1 was properly reintegrated in the nuclei of Wt, whereas in the absence of AspD^{Cdc10} it was predominantly found in the cytoplasm (Fig. 03.11) The presence of Bop1 in the cytoplasm of *ΔaspD^{cdc10}* suggests that the AspD^{Cdc10} containing HO structures might serve as a scaffold for the proper reintegration of nuclear components into the daughter nuclei.

Table 03.1: *A. nidulans* strains used in this study

Strain	Relevant genotype	Source
A773	pyrG89; wA3; pyroA4	FGSC
ASH5	biA1; argB::trpC B; methG1; veA1 trpC801; <i>aspA</i> ::argB	[27]
AYR32	<i>aspB</i> ::AfpyrG, argB::trpC_B, trpC801	[27]
ARL161	biA1; argB::trpC B; methG1; veA1 trpC801; <i>aspC</i> ::AfpyrG	[27]
AKK3	pyrG89:wA; argB::trpC-B; methG1; pyroA4; lysB5; nicA2D703; <i>aspD</i> ::AfpyrG	[27]
AMC3	biA1; argB::trpC_B; methG1; veA1 trpC801; <i>AspD</i> ::GFP	This study
AYR35	pyrG89; wA3; pyroA4; <i>AspB</i> ::GFP	[26]
AID3	pyrG89; pyroA4; biA1; argB::trpC_B; methG1; veA1 trpC801; <i>AspB</i> ::CFP; <i>AspD</i> ::GFP; H1::mRFP	This study
AID5	Bop1-mCR-pyrG (pyrG89); pyroA4; Δ mus51::argB(argB2); SE15; nirA14; biA1; argB::trpC_B; methG1; veA1 trpC801	This study
AID6	Bop1-mCR-pyrG(pyrG89); pyroA4; Δ mus51::argB(argB2); SE15; nirA14; pyrG89:wA; argB::trpC-B; methG1; pyroA4; lysB5; nicA2D703; <i>aspD</i> ::AfpyrG	This study

Figure 03.1: Nuclei of $\Delta aspD^{cdc10}$ are longer and closer together than in $\Delta aspB^{cdc3}$ and Wt.

(A) Hoechst stained nuclei in Wt, $\Delta aspB^{cdc3}$ and $\Delta aspD^{cdc10}$ cells. The white dashed line shows the outline of the cell. The scale bar represents 2.5 μm . **(B)** Average length of nuclei and distance between nuclei in Wt, $\Delta aspB^{cdc3}$ and $\Delta aspD^{cdc10}$ cells. The dark grey bar represents the average length of 200 nuclei and the light grey bar represents the average distance between 200 neighbored nuclei. The experiment was replicated three times. Error bars represent Standard Deviation.

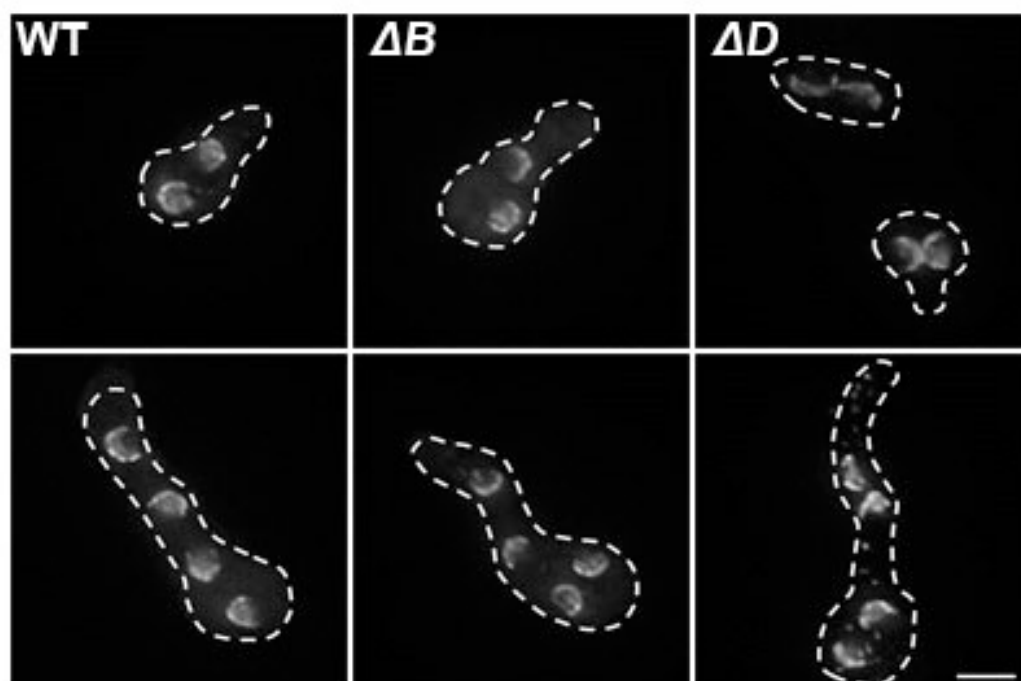
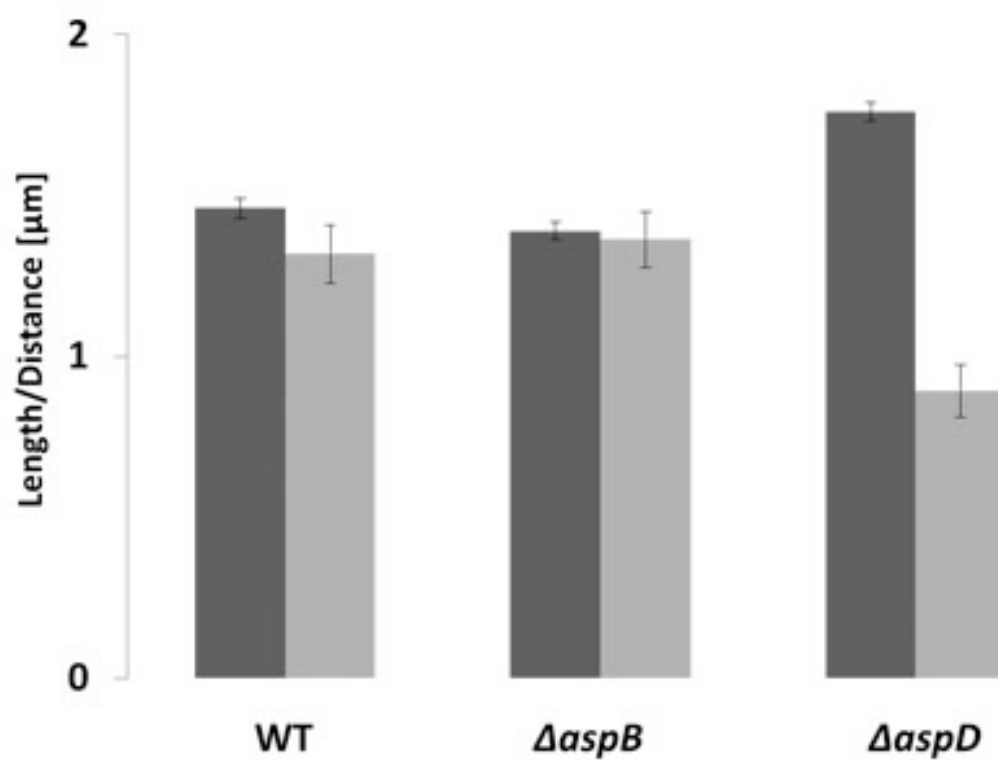
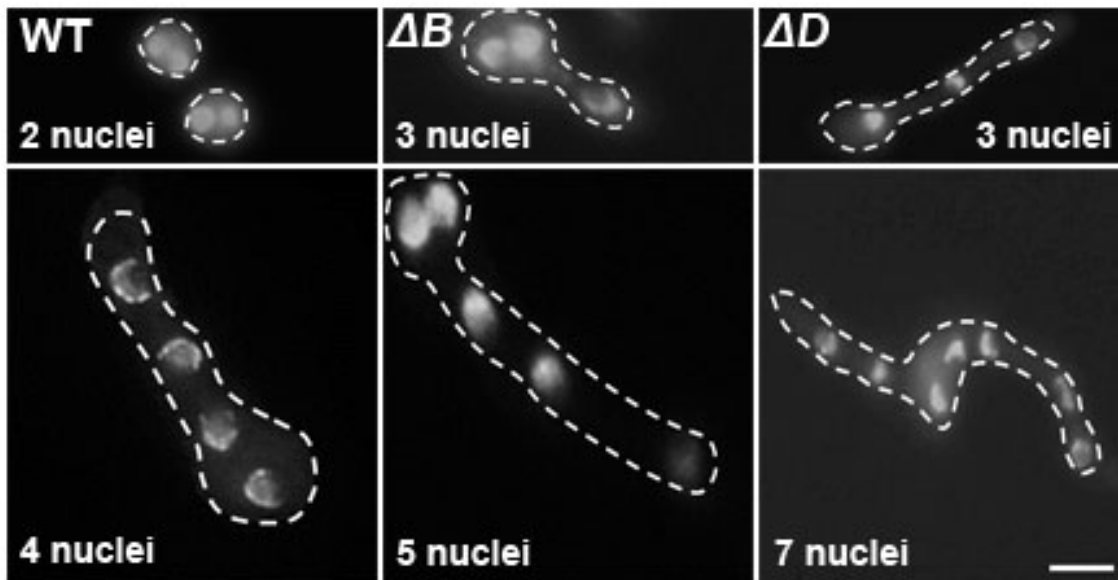
A**B**

Figure 03.2: Nuclear division is misregulated in the absence of septins. (A) Hoechst stained nuclei in Wt, $\Delta aspB^{cdc3}$ and $\Delta aspD^{cdc10}$ cells. The white dashed line shows the outline of the cell. The scale bar represents 2.5 μ m. (B) Misregulation of nuclear division in $\Delta aspB^{cdc3}$ and $\Delta aspD^{cdc10}$ cells (n=200). Cells were incubated for 9 hours and nuclei were counted. Cells with an even number of nuclei (light grey bar), and an uneven number of nuclei (dark grey bar) are shown.

A



B

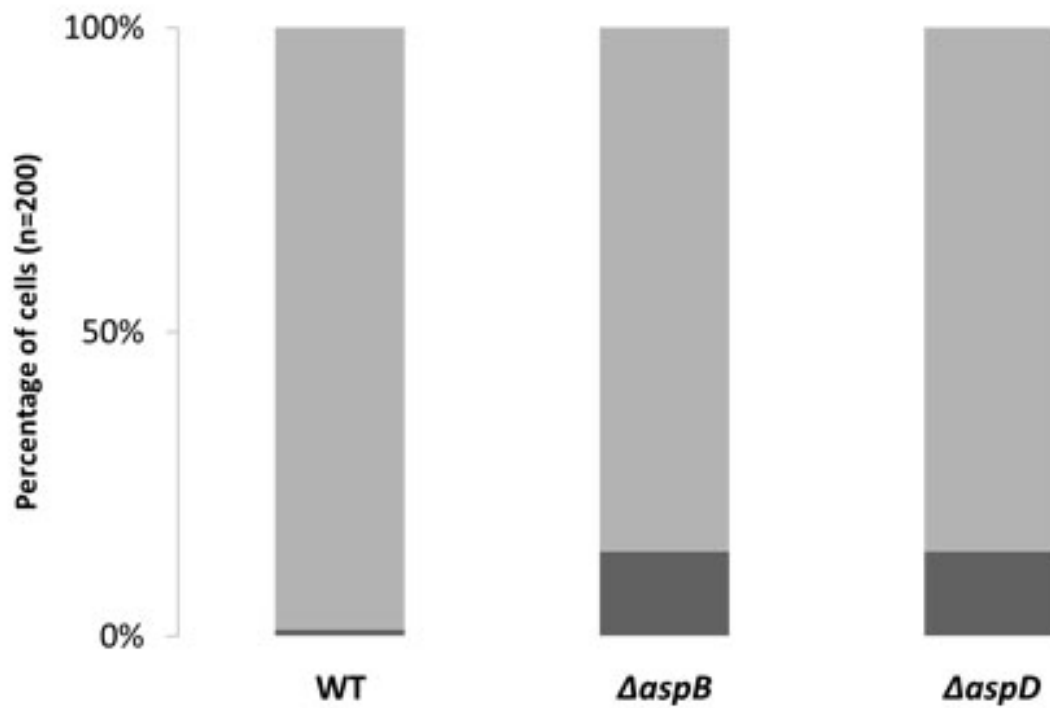
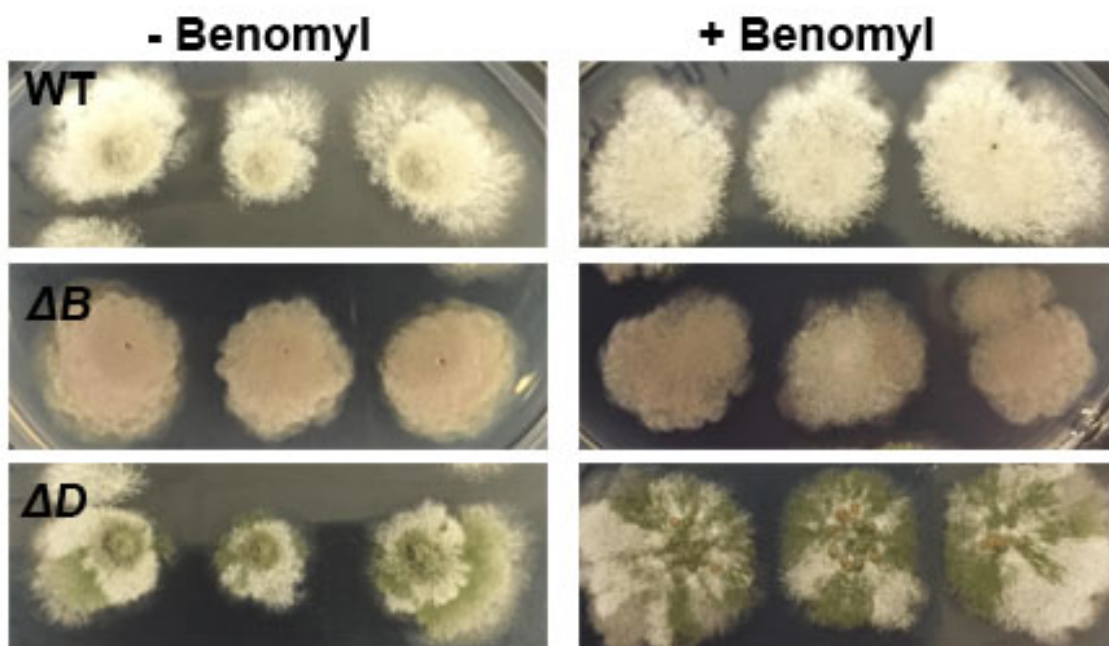


Figure 03.3: $\Delta aspD^{cdc10}$ cells show high frequency of sectoring. (A) Phenotype of Wt, $\Delta aspB^{cdc3}$, $\Delta aspD^{cdc10}$ colonies grown under normal conditions (-Ben) and in the presence of benomyl (+Ben). (B) Number of Wt, $\Delta aspA^{cdc11}$, $\Delta aspB^{cdc3}$, $\Delta aspC^{cdc12}$ and $\Delta aspD^{cdc10}$ colonies exhibiting a sectoring phenotype after benomyl treatment. For each strain 200 cells were treated with benomyl and grown on suitable growth medium for two days at three different temperatures. The experiment was replicated three times. Sectoring colonies (light grey bar), and non-sectoring colonies (dark grey bar) are shown.

A



B

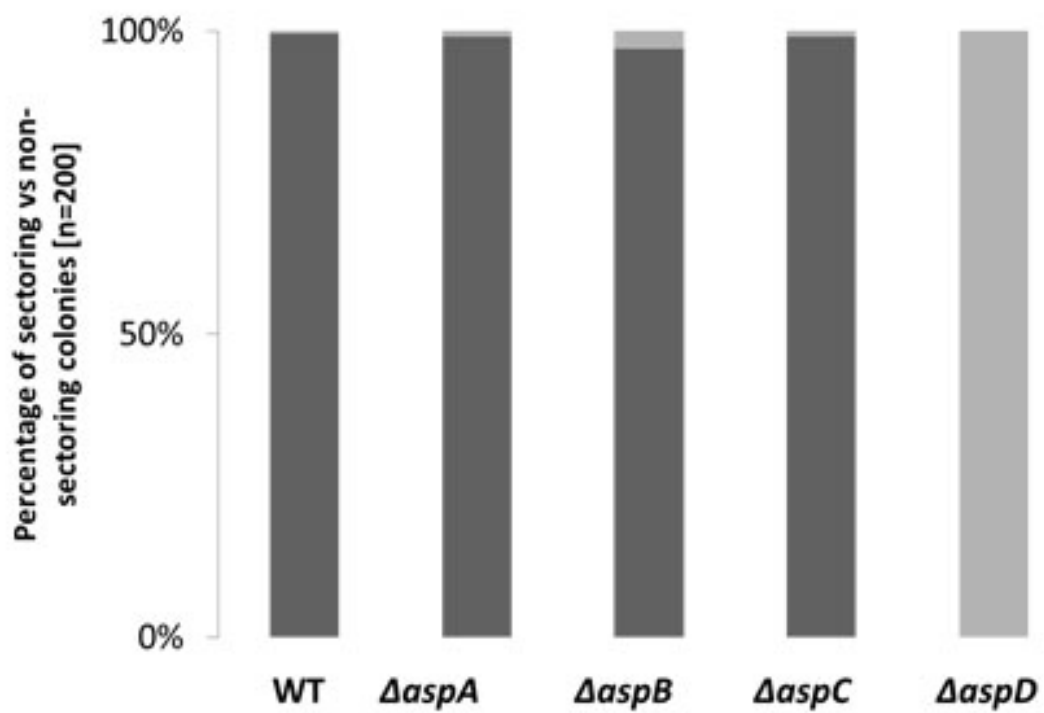


Figure 03.4: AspD^{Cdc10} does not seem to be involved in the spindle assembly checkpoint. Wt, *sldA* and Δ *aspD*^{*cdc10*} cells were grown in the absence (**A**) and presence (**B**) of benomyl. Nuclei of 100 cells were counted. The experiment was replicated three times.

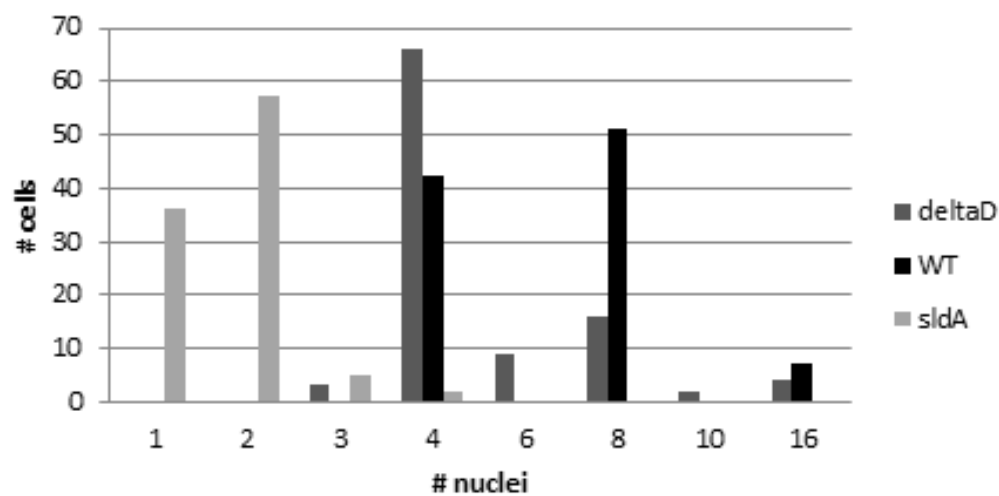
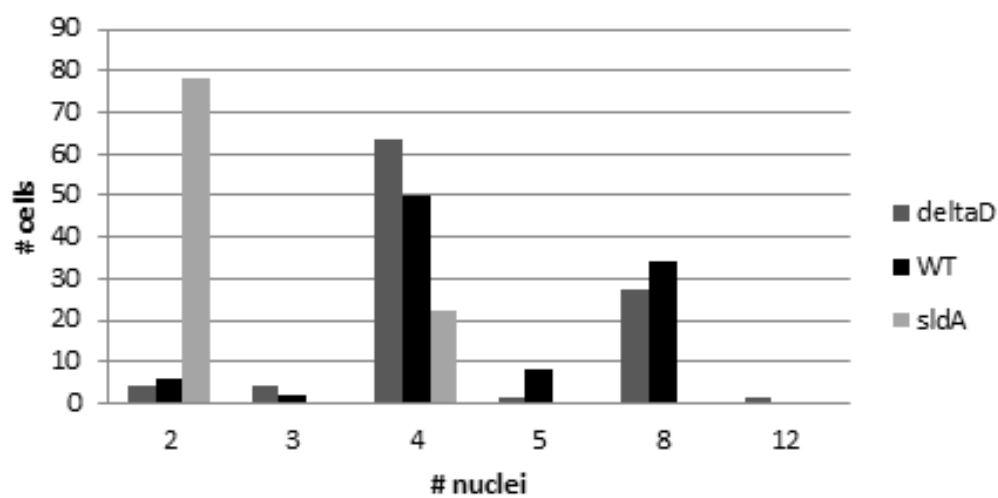
A**No Benomyl****B****Benomyl**

Figure 03.5: AspD^{Cdc10} does not seem to be involved in the DNA damage checkpoint.

Different concentrations of $\Delta aspA^{cdc11}$, $\Delta aspB^{cdc3}$, $\Delta aspC^{cdc12}$, $\Delta aspD^{cdc10}$, $\Delta aspE$ and A773 (Wt) spores were grown for three days on MMS containing medium. The experiment was replicated two times.

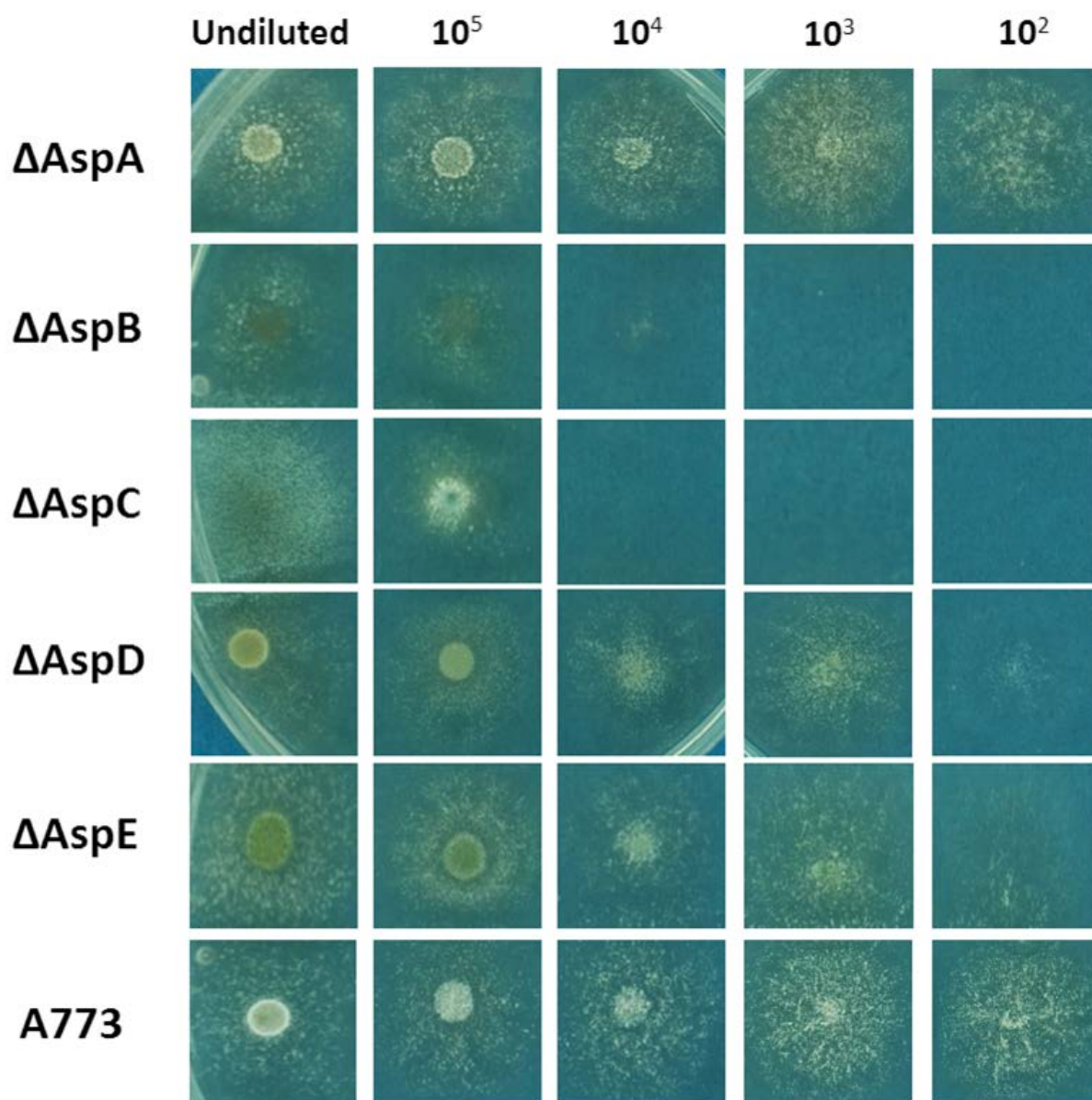


Figure 03.6: Septin AspD^{Cdc10} higher order structure movement changes with cell cycle and cell growth. AspD^{Cdc10}-GFP/H1-RFP was visualized in multiple cells during early developmental stages: (A) isotropic expansion, (B) mitosis, (C) germ tube emergence. A DVII microscope was used to capture images at 3 time points. The scale bar represents 2.5 μm .

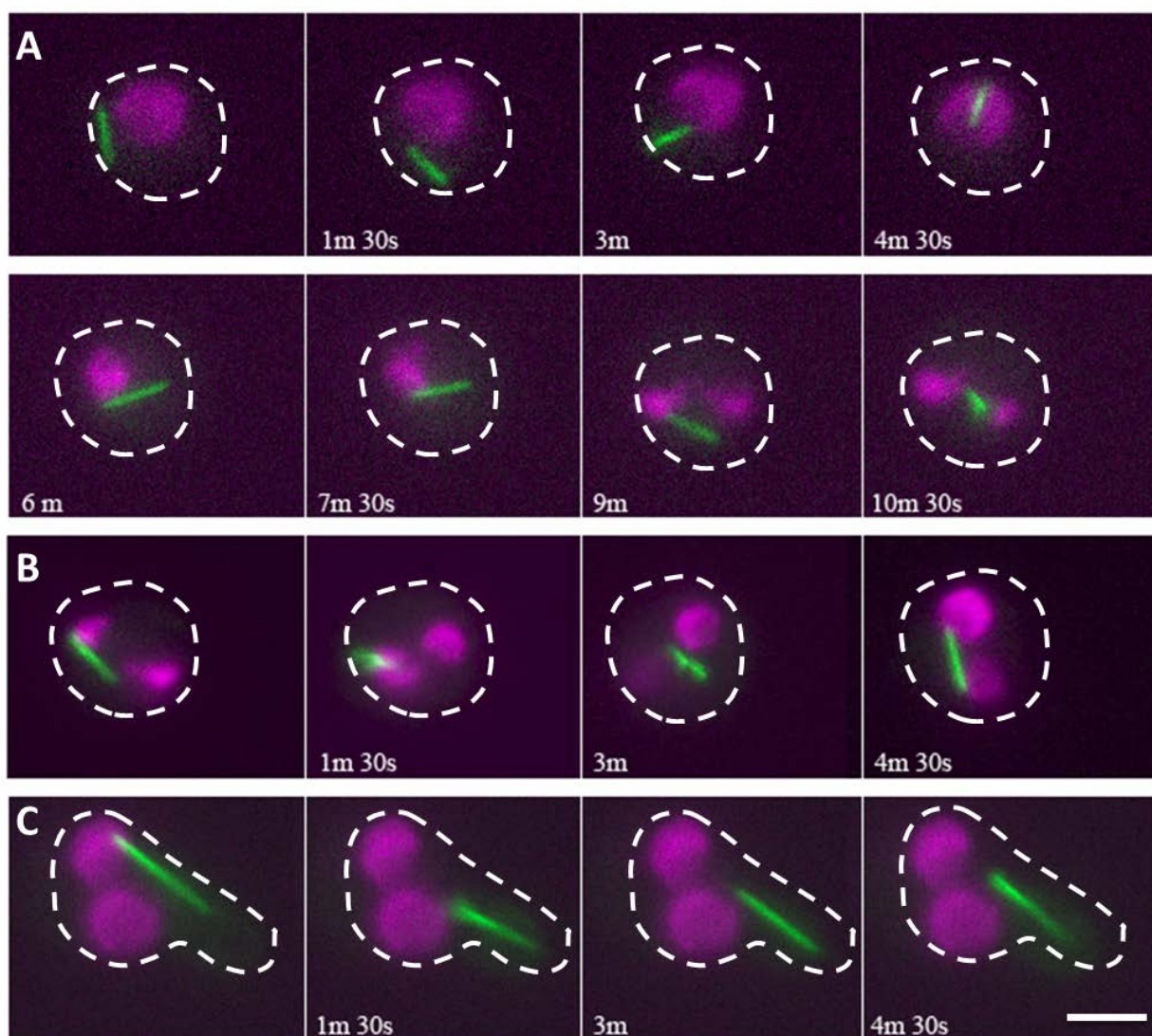


Figure 03.7: Septin AspB^{Cdc3} higher order structure movement changes with cell cycle and cell growth. AspB^{Cdc3}-GFP/H1-RFP was visualized in multiple cells during early developmental stages: **(A)** isotropic expansion and mitosis, **(B)** germ tube emergence. A DVII microscope was used to capture images at 3 time points. The scale bar represents 2.5 μm .

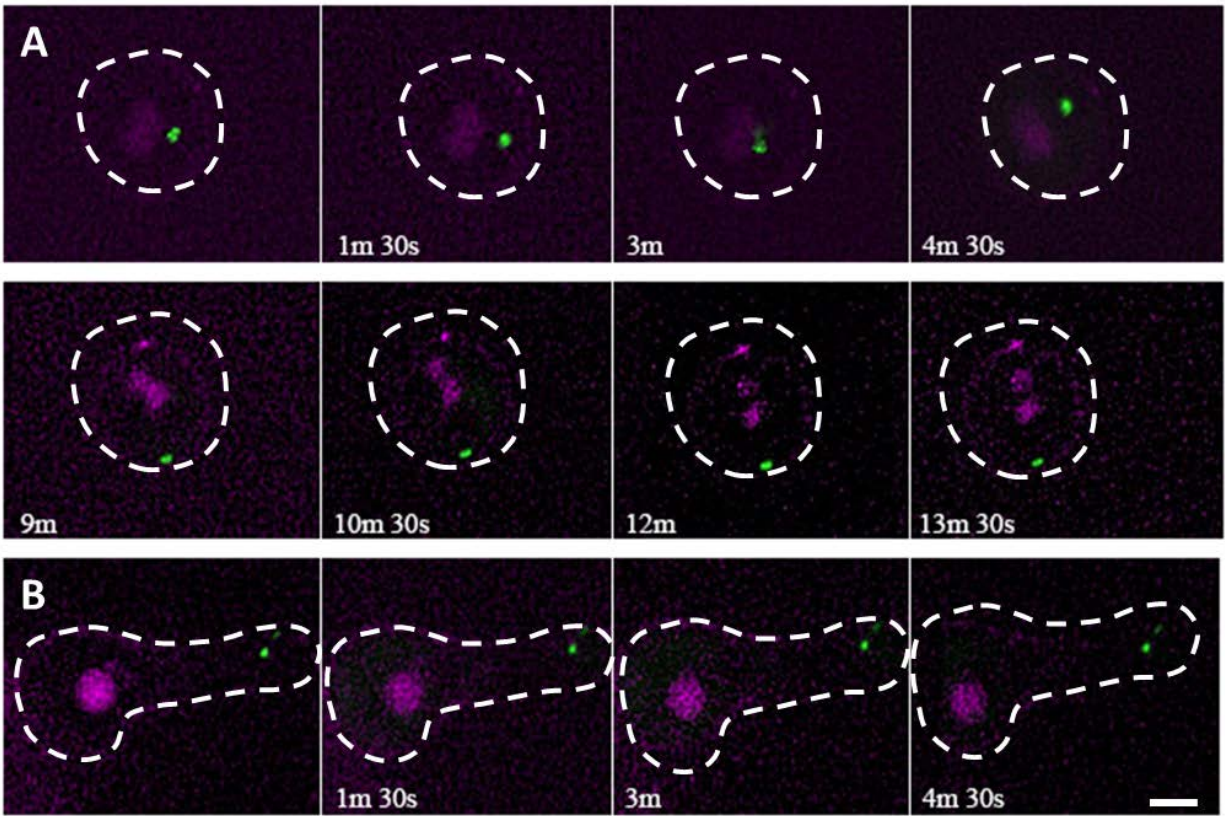


Figure 03.8: A. *nidulans* septin HO structures are composed of various septin polymers.

AspD^{Cdc10}-GFP/AspB^{Cdc3}-CFP/H1-RFP visualized during early developmental stages. A SR-SIM microscope was used to capture images of fixed cells. **(A)** Septin HO structure composed of polymers containing AspD^{Cdc10}-GFP (false color: red) and AspB^{Cdc3}-CFP (false color: green). The scale bar represents 5 μ m. **(B)** Ring-like septin HO structure composed of polymers containing AspD^{Cdc10}-GFP (false color: red) and AspB^{Cdc3}-CFP (false color: green). Scale bar represents 2.5 μ m.

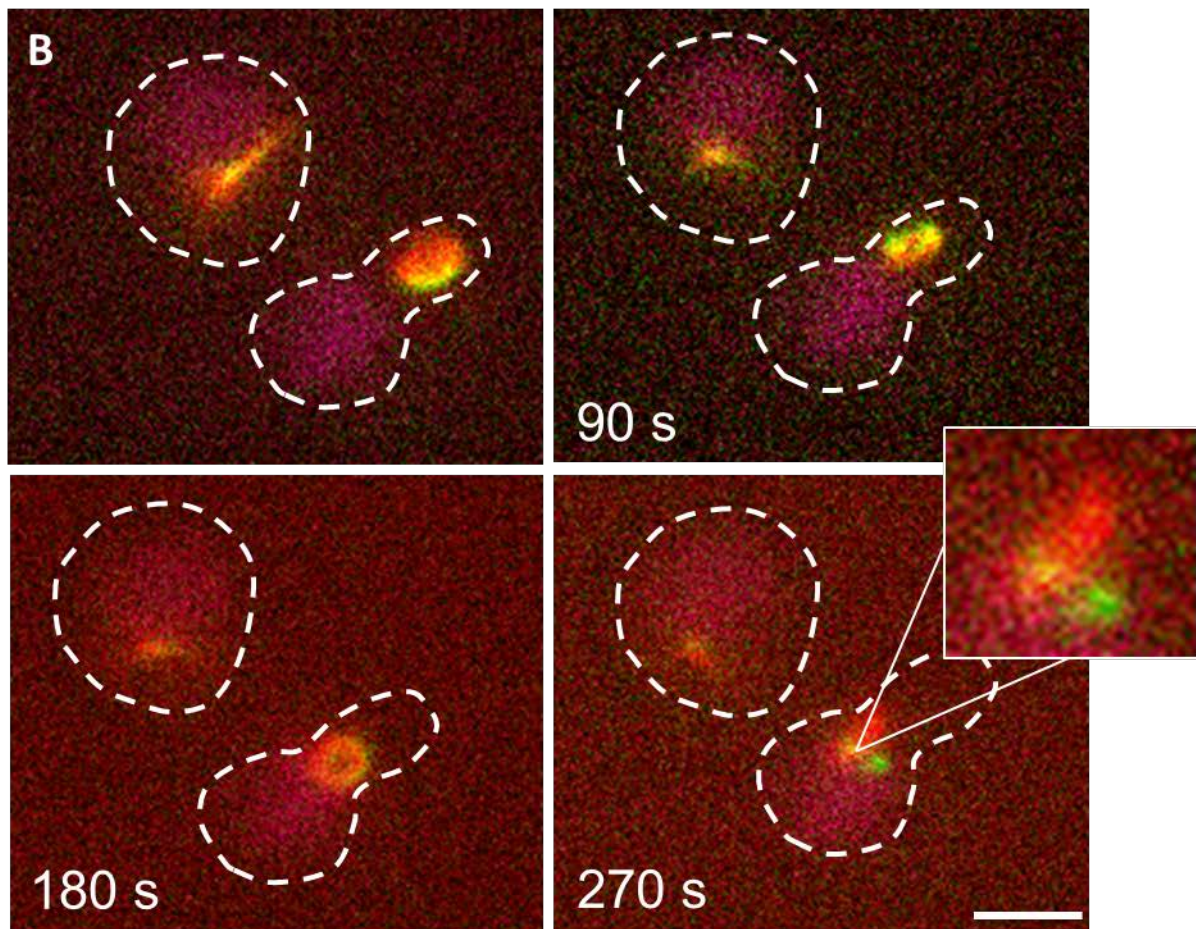
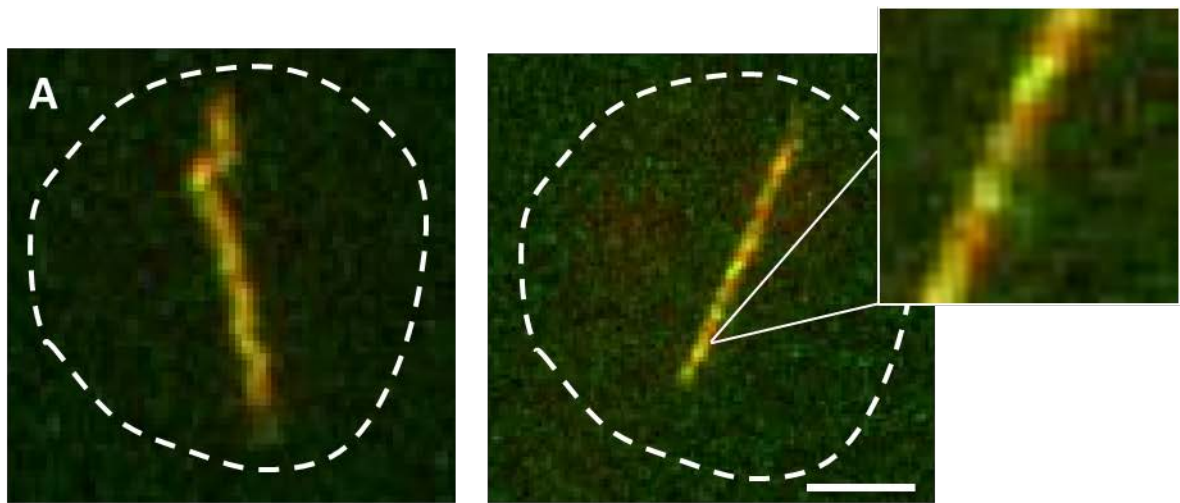


Figure 03.9: Fluorescence profiles of Bop1-mCherry and Hoechst stained DNA in *ΔaspD^{cdc10}* and Wt cells after the first nuclear division. Nucleoli are visualized by expression of Bop1-mCherry. The y-axis represents the fluorescence intensity and the x-axis represents the distance of a line that was imposed on the pictures. The line was manually drawn through the center of each nucleus starting with the left nucleus. The scale bar represents 1 μm .

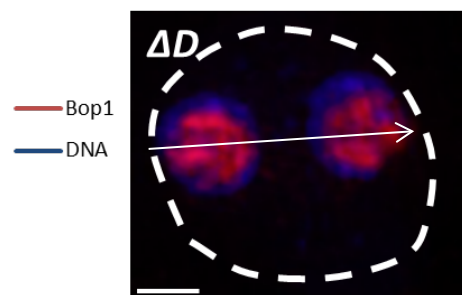
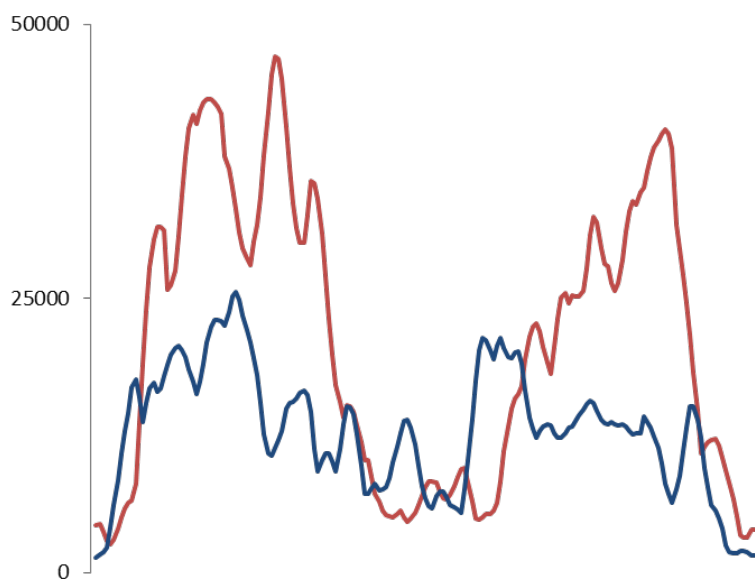
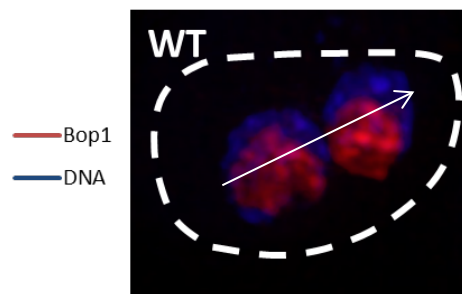
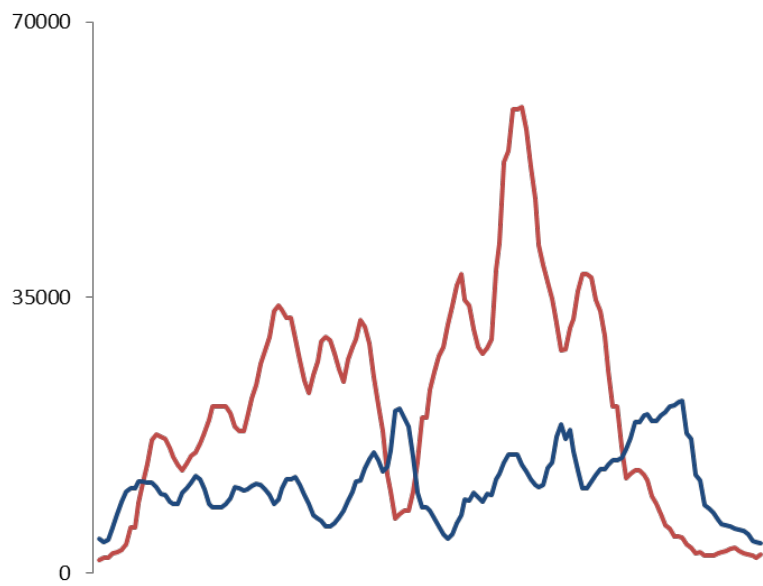


Figure 03.10: Phenotypic differences between nucleoli of $\Delta aspD^{cdc10}$ and Wt cells. Nucleoli are visualized by expression of Bop1-mCherry. After three rounds of mitosis nucleoli in Wt cells have an elliptical shape, whereas nucleoli in $\Delta aspD^{cdc10}$ are more spherical. The scale bar represents 1 μm .

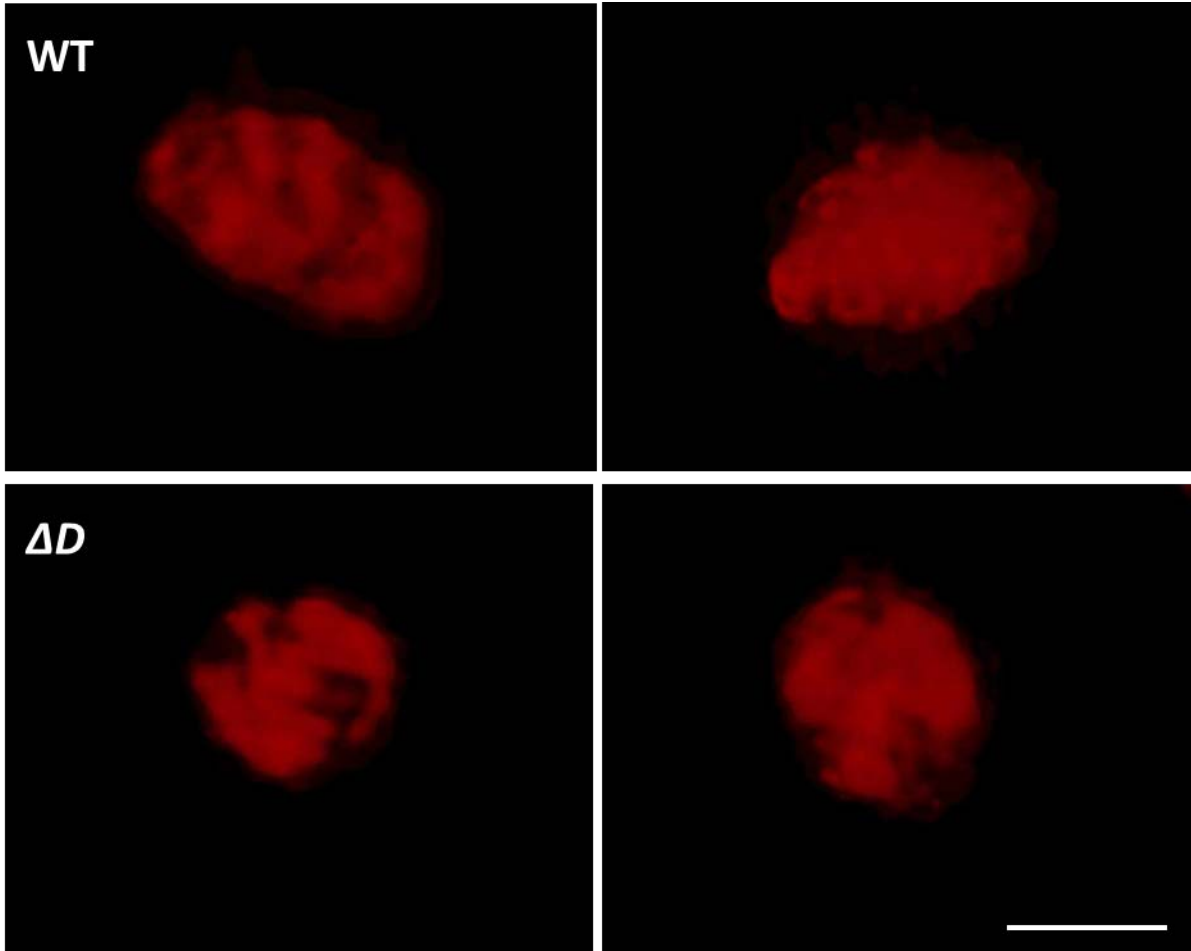


Figure 03.11: Fluorescence profiles of Bop1-mCherry and Hoechst stained DNA in *ΔaspD^{cdc10}* and Wt cells after the second nuclear division. Nucleoli are visualized by expression of Bop1-mCherry. The y-axis represents the fluorescence intensity and the x-axis represents the distance of a line that was imposed on the pictures. The line was manually drawn through the center of each nucleus starting with the first nucleus in the conidium. The scale bar represents 1 μm .

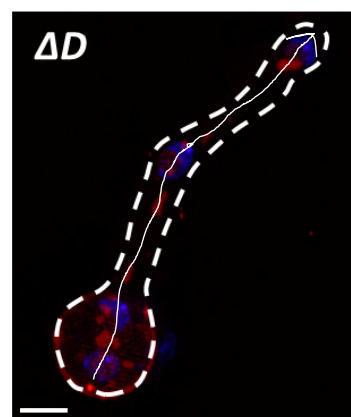
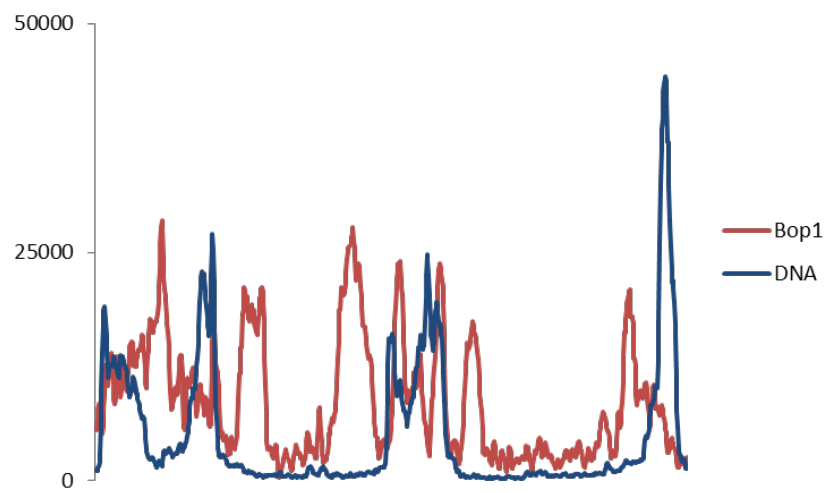
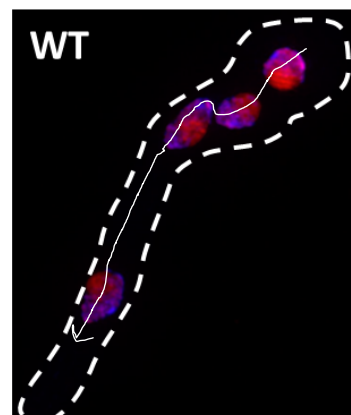
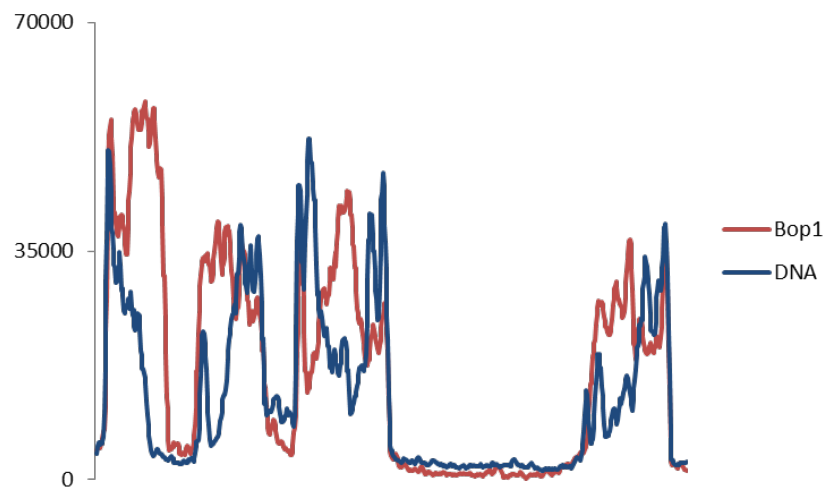
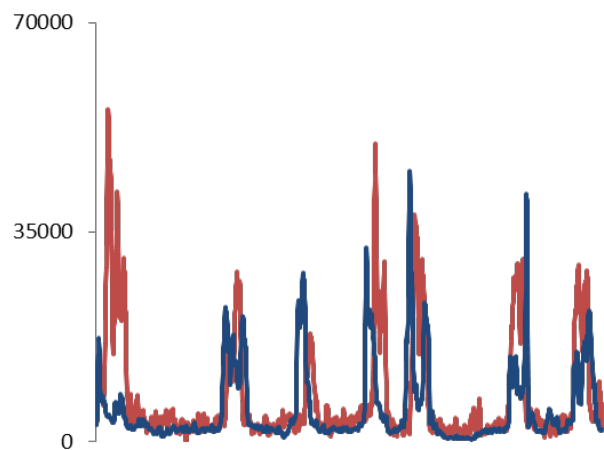
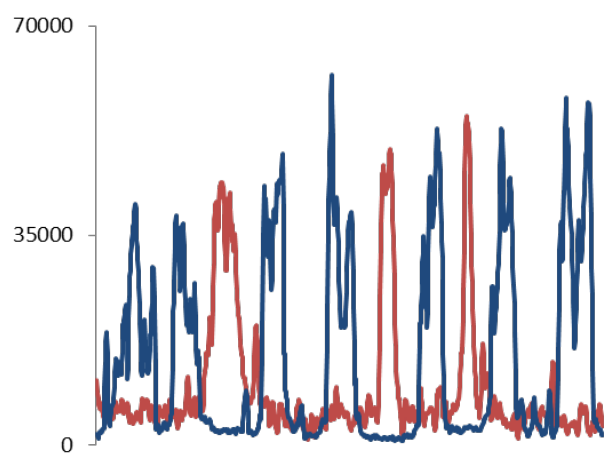
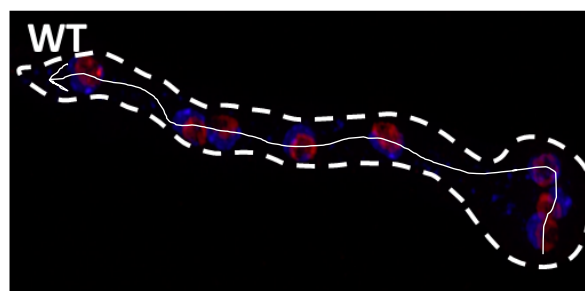


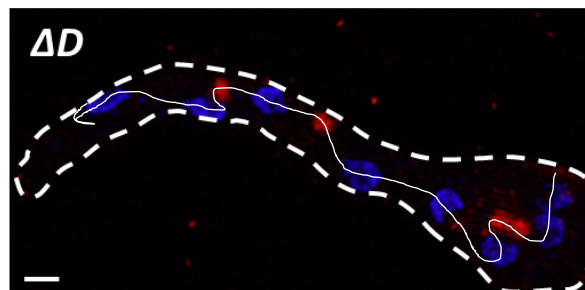
Figure 03.12: Fluorescence profiles of Bop1-mCherry and Hoechst stained DNA in *ΔaspD^{cdc10}* and Wt cells after the third nuclear division. Nucleoli are visualized by expression of Bop1-mCherry. The y-axis represents the fluorescence intensity and the x-axis represents the distance of a line that was imposed on the pictures. The line was manually drawn through the center of each nucleus starting with the first nucleus in the conidium. The scale bar represents 1 μm .



mCherry
Hoechst



mCherry
Hoechst



CHAPTER 4

CONCLUSION

As described in the introduction, it is well known that the septins are essential for cytokinesis in yeast and animal cells. Gaps in the septin framework can destabilize the scaffolding leading to serious diseases. Alzheimer and Parkinson patients have abnormal septin depositions in their brains. Hereditary neuralgic amyotrophy, a painful nerve disorder in shoulder and arm muscles, is caused by mutations in septin genes. An increased level of septin proteins impairs the proper development of the cell leading to formation of cancer cells. Basic research on septins helps elucidate the basic role of these cytoskeletal elements in cell developmental processes. Unlike our own bony skeleton, which keeps the same arrangement, the cellular cytoskeleton is dynamic, continuously morphing in response to cellular signals. It forms a framework for the movement of organelles around the cell and provides important structural support. Even though essential, septins are the far less explored components of the cytoskeleton. They were discovered about 40 years ago in yeast cells. The unicellular yeast *S. cerevisiae* is asexually reproducing by forming buds. In this type of cell division septins form a ring-like structure called septum at the division plane, which recruits cell cycle proteins that ensure proper mitosis and cytokinesis. Unlike *S. cerevisiae*, *A. nidulans* does not undergo cell division. This multicellular, filamentous fungus contains many nuclei in a common cytoplasm. Cytokinesis during filamentous growth is achieved by the formation of a cross wall named septum at a specific point in the hyphal cell.

However, cytokinesis does not follow every nuclear division. The first septum for instance is laid down at the base of the germ tube after the third nuclear division.

We performed single deletions of the genes encoding for the core septins in *A. nidulans*. In the absence of AspA^{Cdc11}, AspB^{Cdc3} or AspC^{Cdc12} we could observe an abnormal increase of lateral branches, which is also known as hyperbranching. The knock-out of the fourth core septin gene *aspD^{CDC10}* did not result in obvious phenotypic changes whereas fluorescence microscopy revealed abnormal nuclear structures that worsened with each mitotic division. This made us think that AspD^{Cdc10} might be involved in the regulation of nuclear division. Thereby, it is possible that various septin polymers play different roles during this process. Previous pulldown analyses in our lab revealed the presence of a septin heterooctamer including two copies of all four core septins and a heterohexamer, which lacks AspD^{Cdc10}. The disruption of the core septins AspA^{Cdc11}, AspB^{Cdc3}, AspC^{Cdc12} and AspD^{Cdc10} revealed an increased number of cells harboring an odd number of nuclei. This may point to an asynchronous nuclear division cycle in the absence of functional heterooctamers, which contain AspD^{Cdc10}. In conclusion, AspD^{Cdc10} might regulate karyokinetic processes as part of the heterooctamer, whereas the other three core septins AspA^{Cdc11}, AspB^{Cdc3} or AspC^{Cdc12} might regulate physiological growth processes as part of the heterohexamer.

Time-lapse analyses of a strain expressing an AspD^{Cdc10}-GFP fusion protein showed septin bars contacting both nucleus and cell cortex. Shortly after nuclear division the septin bar located between the two newly divided nuclei suggesting that septin AspD^{Cdc10} might play a role during mitosis. In contrast to AspD^{Cdc10}-GFP, AspB^{Cdc3}-GFP was mainly found at the cell membrane and did not contact either nucleus during the telophase of mitosis. AspB^{Cdc3} also did not localize between the two nuclei after mitotic exit, supporting the idea of different functional septin

heteropolymers in *A. nidulans*. Further experiments with a strain expressing AspD^{Cdc10}-GFP and AspB^{Cdc3}-CFP revealed an unknown polymer, suggesting that there are also other septin polymers besides heterooctamers and heterohexamers.

In recent work Osmani and colleagues have uncovered a situation where, in the absence of the inner nuclear membrane protein Src1, cells appear to back-up and retry cell cycle transitions multiple times, a mechanism they dubbed “reboot regulation” [148]. Thus, the odd number of nuclei in the core septin deletion mutants might be the result of improper karyokinesis. This raised the question whether septins could be part of a checkpoint that regulates nuclear division by monitoring the nuclear architecture.

In contrast to other cells, $\Delta aspD^{cdc10}$ mutants occasionally showed changes in conidiospore density and color, a sectoring phenotype. This phenotype might be the result of chromosome missegregation events. We wanted to see if AspD^{Cdc10} is part of a checkpoint mechanism that halts the cell cycle in the presence of damage. Therefore, we treated Wt and core septin deletion mutants with MMS to trigger the DNA damage checkpoint. Like Wt, $\Delta aspD^{cdc10}$ was not sensitive to MMS, suggesting that the DNA damage checkpoint still functioned properly. We used benomyl to activate the spindle assembly checkpoint and even though all $\Delta aspD^{cdc10}$ mutants showed a sectoring phenotype on a colony level, nuclear division was halted, suggesting that the SAC checkpoint was working fine. Are septin heteropolymers involved in the regulation of nuclear division? The use of flow cytometry could determine the cell cycle stage of nuclei in core septin deletion mutants. If the mutants harbor nuclei with mitotic features and interphase nuclei, one could say that cells undergo asynchronous nuclear division in the absence of septins. Cyclin proteins are important for the cell cycle clock in eukaryotic cells and drive cell cycle progression when complexed to a CDK [171]. In regard to an asynchronous nuclear division, one

could assume that nuclei are independently expressing and degrading cyclins depending on their cell cycle stage. If the degradation of cyclins does not occur in synchrony then individual nuclei might progress through the cell cycle at different time points. Immunofluorescence or fluorophore tagged cyclins could visualize specific cyclin levels in the individual nuclei. This could also clarify if the asynchronous nuclear division in core septin mutants is due to the absence or disorganization of cyclins.

Furthermore, we wanted to find out if there is a connection between $\text{AspD}^{\text{Cdc10}}$ and the nucleolus, since latter one is excluded into the cytoplasm during mitosis. We could show that during mitosis $\text{AspD}^{\text{Cdc10}}$ -GFP co-localizes with Bop1-mCherry, a nucleolar ribonucleoprotein (data not shown). Even though Bop1 is not restricted to the nucleoplasm, we could detect an increased amount of Bop1-mCherry in the cytoplasm of $\Delta\text{aspD}^{\text{cdc10}}$ mutants after two and three rounds of mitoses. In regard to the previous results we assumed that more Bop1 leaks into the cytoplasm of $\Delta\text{aspD}^{\text{cdc10}}$ due to defects in the nuclear architecture. Dual color microscopy with a strain expressing $\text{AspD}^{\text{Cdc10}}$ -GFP and a peripheral Nup-RFP could show if both of these components co-localize during mitosis, when peripheral Nups dissociate from the NPC. This would suggest that $\text{AspD}^{\text{Cdc10}}$ is involved in the coordination of the nuclear architecture. Alternatively one could check peripheral Nup-fluorophore dynamics within the cell cycle of $\Delta\text{aspD}^{\text{cdc10}}$. Bop1 is also actively transported into interphase nuclei through the NPC and released into the cytoplasm during mitosis. Time-lapse analyses with $\Delta\text{aspD}^{\text{cdc10}}$ expressing Bop1-mCherry could also answer the question if cells undergo ‘reboot regulation’ to fix nuclear architecture defects.

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