Function and Regulation of Septins
During Mammalian Cell Division

by

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A thesis submitted in conformity with the requirements
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Department of Biochemistry
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Abstract

Septins are a family of GTP-binding proteins implicated in mammalian cell division. Since these proteins form heterologous complexes and filaments in interphase cells, it has been assumed that depletion of any or all septins in a given cell type will give rise to the same phenotype. I demonstrate that while all septins expressed in HeLa cells localize to the cleavage furrow and midbody during cytokinesis, and co-immunoprecipitate throughout cell division, they do not all have identical roles during this process. Specific depletion of SEPT2 or SEPT11 caused defects in the early stages of cytokinesis, ultimately resulting in binucleation. Similar results were observed upon simultaneous depletion of all septins. In sharp contrast, SEPT9 was dispensable for the early stages of cell division, but was critical for the final separation of daughter cells. I demonstrate that SEPT9 mediates the localization of the vesicle-tethering exocyst complex to the midbody. Immunofluorescence microscopy suggests that SEPT9 may act to compartmentalize the exocyst at the site of abscission, analogous to the role performed by septins in...
*Saccharomyces cerevisiae*. I provide evidence that the N-terminal region of SEPT9, which is absent from the shorter SEPT9 isoforms, plays an important role in abscission. I describe a long-anticipated link between a mammalian septin and the cell cycle machinery by showing that the N-terminal region of SEPT9 is phosphorylated at threonine 24 upon mitotic entry by cyclin-dependent kinase 1. This creates a binding site for the WW domain of the peptidyl-prolyl isomerase Pin1. I provide evidence that Pin1 induces a conformational change in the N-terminal region of SEPT9 that is important for the completion of cytokinesis. I propose that mitotic regulation of SEPT9 by Cdk1 and Pin1 regulates an interaction between SEPT9 and an unidentified protein that is critical for abscission.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Alix</td>
<td>apoptosis-linked gene-2-interacting protein X</td>
</tr>
<tr>
<td>Amp</td>
<td>ampicillin</td>
</tr>
<tr>
<td>APC</td>
<td>anaphase promoting complex</td>
</tr>
<tr>
<td>APP</td>
<td>amyloid precursor protein</td>
</tr>
<tr>
<td>ARF6</td>
<td>ADP-ribosylation factor 6</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CENP-E</td>
<td>centromere-associated protein E</td>
</tr>
<tr>
<td>Cep55</td>
<td>centrosomal protein of 55 kDa</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CHMP1B</td>
<td>charged multivesicular body protein 1B</td>
</tr>
<tr>
<td>CIP</td>
<td>calf intestinal phosphatase</td>
</tr>
<tr>
<td>CRIK</td>
<td>citron kinase</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>EGTA</td>
<td>ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>ESCRT</td>
<td>endosomal sorting complex required for transport</td>
</tr>
<tr>
<td>Emi1</td>
<td>early mitotic regulator 1</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FIP3</td>
<td>family of Rab11-interacting protein 3</td>
</tr>
<tr>
<td>FRAP</td>
<td>fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>G1 phase</td>
<td>gap 1 phase</td>
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<tr>
<td>G2 phase</td>
<td>gap 2 phase</td>
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<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>-------------</td>
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<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine-5′-diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GppNHp</td>
<td>guanosine-5′- (βγ-imino) triphosphate</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
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<td>GTP</td>
<td>guanosine-5′-triphosphate</td>
</tr>
<tr>
<td>GTPase</td>
<td>guanosine-5′-triphosphatase</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's Buffered Salt Solution</td>
</tr>
<tr>
<td>HIT</td>
<td>hamster insulinoma tumor</td>
</tr>
<tr>
<td>HMEC</td>
<td>human mammary epithelial cell</td>
</tr>
<tr>
<td>HNA</td>
<td>Hereditary Neuralgic Amyotrophy</td>
</tr>
<tr>
<td>HPMI</td>
<td>RPMI powder supplemented with 15mM sodium chloride and 20mM HEPES, pH 7.4</td>
</tr>
<tr>
<td>HRP</td>
<td>horse radish peroxidase</td>
</tr>
<tr>
<td>HSP</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-beta-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>Kan</td>
<td>kanamycin</td>
</tr>
<tr>
<td>KD</td>
<td>knockdown</td>
</tr>
<tr>
<td>M phase</td>
<td>mitosis phase</td>
</tr>
<tr>
<td>Map4</td>
<td>microtubule-associated protein 4</td>
</tr>
<tr>
<td>MCAK</td>
<td>mitotic centromere-associated kinesin</td>
</tr>
<tr>
<td>mDia</td>
<td>mammalian homolog of <em>Drosophila</em> diaphanous 2</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MKLP1</td>
<td>mitotic kinesin-like protein 1</td>
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<tr>
<td>MLCK</td>
<td>myosin light chain kinase</td>
</tr>
<tr>
<td>MLL</td>
<td>mixed lineage leukemia</td>
</tr>
<tr>
<td>MSF</td>
<td>MLL septin-like fusion</td>
</tr>
<tr>
<td>MYPT</td>
<td>myosin phosphatase targeting</td>
</tr>
<tr>
<td>Nedd5</td>
<td>neural precursor cell expressed developmentally down-regulated protein 5</td>
</tr>
<tr>
<td>NEM</td>
<td>n-ethylmaleimide</td>
</tr>
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</table>
PBD  polobox domain
PBS  phosphate buffered saline
PBS-T phosphate buffered saline with 0.05% Tween-20
PCR  polymerase chain reaction
PMSF phenylmethylsulfonyl fluoride
pS-P  phosphoserine-proline
pT-P  phosphothreonine-proline
PI(4)P  phosphatidylinositol 4-phosphate
PI(5)P  phosphatidylinositol 5-phosphate
PI(4,5)P\(_2\)  phosphatidylinositol 4,5-bisphosphate
PI(3,4,5)P\(_3\)  phosphatidylinositol 3,4,5-trisphosphate
Plk1 polo-like kinase 1
PVDF polyvinylidene fluoride
rMLC  myosin regulatory light chain
RNA  ribonucleic acid
ROCK rho-associated kinase
S phase  synthesis phase
SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM  standard error of the mean
siRNA  small interfering RNA
SNARE soluble N-ethylmaleimide-sensitive factor attachment protein receptor
TAP  tandem affinity purification
TB  terrific broth
TBS-T Tris buffered saline with 0.1% Tween-20
Tsg101 tumor susceptibility gene 101
VAMP8 vesicle-associated membrane protein 8
YNB  yeast nitrogen base
1 Introduction

1.1 The septin family of proteins

1.1.1 Discovery of septins

Septin genes were first described in *Saccharomyces cerevisiae* by Leland Hartwell, as part of his groundbreaking work that identified genes involved in the cell division cycle (Hartwell, 1971). He described temperature-sensitive mutations in four genes (*cdc3, cdc10, cdc11, and cdc12*) that prevented proper cytokinesis. When grown at the restrictive temperature, these mutants underwent DNA replication, nuclear separation, and bud emergence, but the bud was unable to separate from the mother cell. After several cycles, these mutants became multinucleate, as a result of developing several elongated buds that remained connected to the mother cell. Analysis of the mother-bud neck by electron microscopy revealed the presence of filamentous structures in close proximity to the plasma membrane (Byers and Goetsch, 1976). Subsequent work showed that these structures were absent in the *cdc3, cdc10, cdc11, and cdc12* mutants, and that the *CDC12* gene product (Cdc12p) localized to the mother-bud neck (Haarer and Pringle, 1987). Similar results were later obtained for Cdc3p, Cdc10p, and Cdc11p (Ford and Pringle, 1991; Kim et al., 1991). Collectively, this work suggested that Cdc3p, Cdc10p, Cdc11p, and Cdc12p are major components of the filamentous structures at the mother-bud neck. Sequence analysis revealed that these proteins form a related family, the septins.

1.1.2 Septins are a highly conserved family of proteins

Since their initial discovery in yeast, it has been demonstrated that septins are highly conserved amongst eukaryotes, although they are notably absent in plants. The number of septin genes varies considerably between different species. For example, there are 14 septin genes in *H. sapiens* (called SEPT1-SEPT14), 13 in *M. musculus*, 2 in *C. elegans*, and 5 in *D. melanogaster* (Cao et al., 2007). However, septins from all species have the same general architecture, as shown in Figure 1.1.
Figure 1.1 General septin architecture.

Septins contain a central conserved GTP binding domain and a polybasic domain. These are flanked by N and C-terminal extensions of varying length and divergent sequence. Many septins contain a predicted coiled-coil in their C-terminal extension.
Septins bear a central conserved GTPase domain, and belong to the superclass of P-loop GTPases (Leipe et al., 2002). The GTPase domain has been shown to mediate interactions between different septin family members (see 1.1.5 Insights into septin structure; (Sirajuddin et al., 2007)). While the precise role of GTP hydrolysis and exchange remains unclear, recent work suggests that the nature of the bound guanine nucleotide may influence septin-septin interactions (see 1.1.5 Insights into septin structure; (Sirajuddin et al., 2009)).

The polybasic region is a short stretch of basic residues that is located immediately N-terminal to the GTPase domain, and is thought to mediate the association of septins with certain phospholipids. The polybasic region of mammalian SEPT4 directly binds phosphatidylinositol 4,5-bisphosphate [PI(4,5)P$_2$] and phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P$_3$] (Zhang et al., 1999). Likewise, yeast septins associate with phosphatidylinositol 4-phosphate [PI(4)P] and phosphatidylinositol 5-phosphate [PI(5)P] via the polybasic domain (Casamayor and Snyder, 2003). In both cases, interfering with the availability of these phospholipids disrupted the cellular distribution of septins.

Septins also contain N and C-terminal extensions of varying length. Although many septins contain a predicted coiled-coil in their C-terminal extension, the overall sequences of these extensions are highly divergent. Information on the significance of the N and C-terminal extensions is limited, but recent analyses suggest that they may be important for septin-septin interactions and/or interactions with other proteins (see 1.1.5 Insights into septin structure; (Sirajuddin et al., 2007; Sirajuddin et al., 2009; Zhu et al., 2008)). Given their divergent sequences, the N and C-terminal extensions may confer unique properties to individual septins.

1.1.3 **Septin splicing and tissue distribution**

Many of the transcripts that encode the 14 human septin proteins undergo alternative splicing, making the number of septin polypeptides in humans even greater (Russell, 2008). The most well-characterized case is that of SEPT9, which contains six 5’ splice sites and three 3’ splice sites (described further in Chapter 4). This yields a total of 18 distinct transcripts that encode 15 different polypeptides (McIlhatton et al., 2001). Whether other septin family members undergo the same degree of complex alternative splicing remains unclear.
Human septin proteins exhibit different tissue expression profiles. Some septins, including SEPT2, SEPT7, and SEPT9 appear to be ubiquitously expressed (Cao et al., 2007; Hall et al., 2005). Others, such as SEPT3 and SEPT5 are predominantly expressed in the brain, whereas SEPT12 and SEPT14 are expressed mainly in the testis (Peterson et al., 2007; Steels et al., 2007). In some instances, even different isoforms of a given septin exhibit distinct tissue expression profiles (Hall et al., 2005; Tsang, 2007).

1.1.4 Septins associate with each other to form complexes and filaments

A general feature of septins from all species is that they associate with each other to form complexes. Given the diverse tissue expression profiles of septins and their isoforms, the composition of septin complexes varies depending on cell type. For example, immunoprecipitation of SEPT5 from mouse brain lysate resulted in the co-precipitation of SEPT2, SEPT3, SEPT4, SEPT6, SEPT7, SEPT8, SEPT9 and SEPT11 (Tsang et al., 2008). Immunoprecipitation of SEPT9 from HeLa cells resulted in the co-precipitation of SEPT2, SEPT6, and SEPT7 (Surka et al., 2002). However, it is not clear whether these septins were all present in the same complex, or whether multiple complexes with different compositions existed. To complicate matters further, septin complexes are thought to associate with each other, acting as the building blocks to form larger structures like filaments and rings (Ihara et al., 2005; Kinoshita et al., 2002; Kissel et al., 2005).

Upon simultaneous expression of SEPT2, SEPT6, and SEPT7 in insect cells, these septins co-fractionated by gel filtration chromatography and co-purified with equal stoichiometry (Kinoshita et al., 2002). This suggested that SEPT2, SEPT6, and SEPT7 can form a complex. In addition, electron and immunofluorescence microscopy analysis revealed that these septins were present in filaments and rings. Consequently, subsequent work has focused on the SEPT2/SEPT6/SEPT7 complex and it is now routinely described as the ‘core septin complex’ (even though there is no evidence suggesting that this is in fact the case in vivo, and a detailed analysis of other potential septin complexes is lacking).
1.1.5 **Insights into septin structure**

The crystal structure of human SEPT2 (lacking the final 46 amino acids) in the presence of GDP was recently solved to a resolution of 3.4Å (Sirajuddin et al., 2007). The overall structure of SEPT2 was very similar to the canonical GTPase domain defined by Ras. However, SEPT2 contained additional structural elements, including an extra α-helix at both the N and C-terminal end of the GTPase domain (referred to as α0 and α6, respectively), and two extra antiparallel strands (called β7 and β8). These elements contain residues that are highly conserved amongst members of the septin family. The crystal structure showed two distinct dimerization interfaces (Figure 1.2): one composed of the nucleotide binding site and the β7 and β8 strands (called the G interface), and the other mediated primarily by the α0 and α6 helices (called the NC interface, which should not be confused with the variable N and C terminal extensions of the GTPase domain shown in Figure 1.1). Gel filtration analysis of SEPT2 bound to GDP revealed that SEPT2 exists as a dimer in solution. Mutational analysis of the two dimerization interfaces observed in the crystal structure suggested that it is the G interface that mediates SEPT2 dimerization.
Figure 1.2 Crystal structure of human SEPT2 bound to GDP.

Ribbon representation of human SEPT2 (lacking the final 46 amino acids) bound to GDP. Three SEPT2 monomers are shown (colored in green, red, and green from left to right), and disordered regions are represented by dashed lines. The G and NC dimerization interfaces are indicated. Adapted from (Sirajuddin et al., 2007).
The same study also reported the crystal structure of the SEPT2/SEPT6/SEPT7 complex solved to 4 Å resolution (Sirajuddin et al., 2007). While this was not high enough to resolve individual amino acid side chains, the crystal structure revealed several important insights into the overall architecture of the SEPT2/SEPT6/SEPT7 complex (Figure 1.3). The complex was a linear hexamer (with the order SEPT7-SEPT6-SEPT2-SEPT2-SEPT6-SEPT7), and contacts between adjacent septins were made through the same conserved G and NC interfaces identified in the SEPT2 crystal structure. The hexameric units associated end to end, thus forming a nonpolar filament. Electron microscopy analysis showed that the purified complex formed filaments and rings under low salt conditions, but dissociated into a hexamer (with the order SEPT7-SEPT6-SEPT2-SEPT2-SEPT6-SEPT7) under high salt conditions. This suggested that the SEPT7-SEPT7 G interface may be the weakest interaction in the septin filament, and filament formation and disassembly is therefore mediated by this interface. The N-terminal extension of SEPT6 (which is longer than that of SEPT2 and SEPT7) made contacts with SEPT7, and may play a role in stabilizing the SEPT6-SEPT7 NC interface. The C-terminal extensions of SEPT2, SEPT6, and SEPT7 all contain a predicted coiled coil. Although no electron density was observed for these regions, they appear to extend at a 90° degree angle from the septin filament axis. Therefore, they could be important for stabilizing interactions with adjacent septins in the filament. Alternatively, they could be involved in lateral associations with other proteins (including other septin filaments).
Figure 1.3  Crystal structure of the SEPT2/SEPT6/SEPT7 complex.
Surface representation of the SEPT2/SEPT6/SEPT7 complex, with SEPT7 in cyan, SEPT6 in pink, and SEPT2 in blue. The SEPT2/SEPT6/SEPT7 hexamer can then associate with other SEPT2/SEPT6/SEPT7 hexamers via the G interface of SEPT7 (shown in grey), thus forming a non-polar filament. The nature of the nucleotide bound to each septin is indicated, and the predicted positions of the C-terminal extensions are indicated by arrows. Adapted from (Sirajuddin et al., 2007).
The SEPT2/SEPT6/SEPT7 crystal structure also showed that these septins were not all bound to the same guanine nucleotide: SEPT2 and SEPT7 were bound to GDP, whereas SEPT6 was bound to GTP (Sirajuddin et al., 2007). This suggested that the nature of the bound nucleotide may play a role in septin complex and/or filament assembly. Consistent with this hypothesis, the crystal structure of SEPT2 bound to a non-hydrolyzable GTP analogue (GppNHp) showed structural differences compared to the GDP-bound form (Sirajuddin et al., 2009). This study used SEPT2 lacking the N-terminal 32 amino acids (and thus part of the α0 helix that mediates the SEPT2-SEPT2 NC interface). GTP binding caused conformational changes in the G interface that induced tighter binding between SEPT2 subunits at this interface. At the same time, conformational changes that may destabilize the NC interface were also observed. Therefore, the nature of the bound nucleotide appears to influence the structure and stability of both the G and NC interfaces, at least in the case of SEPT2. This study also demonstrated that SEPT6, SEPT8, SEPT10, SEPT11 and SEPT14 all lack a threonine residue that is critical for GTP hydrolysis. Therefore, these septins are likely unable to hydrolyze GTP, possibly explaining why SEPT6 is bound to GTP in the SEPT2/SEPT6/SEPT7 crystal structure.

### 1.1.6 Human septins can be divided into 4 groups

The 14 human septin proteins can be organized into 4 distinct groups based on sequence similarity (Figure 1.4): the SEPT2 group contains SEPT2, SEPT1, SEPT4, and SEPT5; the SEPT7 group contains SEPT7 and SEPT13; the SEPT3 group contains SEPT3, SEPT9, and SEPT12; the SEPT6 group contains SEPT6, SEPT8, SEPT10, SEPT11, and SEPT14 (Peterson et al., 2007). As mentioned above, members of the SEPT6 group are unable to hydrolyze GTP (Sirajuddin et al., 2009). It has been proposed that septins from a given group may be able to substitute for the corresponding group member in the SEPT2/SEPT6/SEPT7 complex (Kinoshita, 2003). This would mean that different septin complexes would form in different tissues (depending on the septin expression profile), possibly to perform different functions. In cell types where multiple members of a given group are expressed (or multiple isoforms of a given septin are expressed), these septins may be redundant, or could form different complexes with different functions. This model remains to be tested experimentally. It addition, it remains unclear how members of the SEPT3/SEPT9/SEPT12 group fit into the SEPT2/SEPT6/SEPT7 complex.
Figure 1.4 Classification of human septins based on sequence similarity.
Phylogenetic tree of human septin proteins, generated using Clustal W. Adapted from (Steels, 2008).
1.2 Cell division and the roles of septins

Cell division is the complex process by which two daughter cells are generated from a single mother cell. Consequently, this process is critical for both the development of multicellular organisms and the proliferation of unicellular organisms. Many complicated events must be properly coordinated to ensure proper cell division. First, the entire genome must be faithfully replicated. Second, all cellular materials, including the replicated DNA and organelles, must be equally partitioned into each nascent daughter cell. This must be properly coupled with cytokinesis (the physical division of the cytoplasm), thus ensuring that each daughter cell receives the correct complement of chromosomes and cellular material. Improper separation of the genome or cytokinesis failure results in aneuploidy, which can ultimately contribute to cancer (Fujimori et al., 1999).

The septin family of proteins was first identified as being critical for the final stages of cell division (Hartwell, 1971). Before reviewing what is known about the roles of septins in cell division, I will first describe this complex process in detail.

1.2.1 Overview of the cell cycle

The cell cycle can be divided into four different phases: gap1 (G1), synthesis (S), gap2 (G2), and mitosis (M) (Figure 1.5). G1, S, and G2 are collectively referred to as interphase, which corresponds to the time between successive mitosis phases (Vermeulen et al., 2003). During G1, the cell grows and prepares to replicate its DNA, which occurs during S phase. This is followed by G2, during which time the cell prepares to divide. The duplicated DNA separates during M, at which point the mother cell is physically separated into two daughter cells through the process of cytokinesis.

Transition through the different stages of the cell cycle is controlled by a family of serine/threonine kinases called cyclin-dependent kinases (CDKs) (Malumbres and Barbacid, 2005). CDKs are activated at specific phases of the cell cycle, where they mediate phosphorylation events that are crucial for cell cycle progression. Therefore, activation of CDKs must be tightly regulated in order to ensure proper cell cycle progression. This is achieved
primarily through the association of CDKs with activating subunits called cyclins, which are expressed and degraded at specific stages of the cell cycle. The activity of CDKs is also modulated by activating and inhibitory phosphorylation and dephosphorylation events.

In *Saccharomyces cerevisiae*, the cell cycle is driven by a single CDK called Cdc28 (Bloom and Cross, 2007). This protein associates with different cyclins at different times in order to ensure proper cell cycle progression. In contrast, mammalian cells express multiple CDKs, and it was thought that different CDKs and their corresponding cyclins are important for driving specific stages of the cell cycle. For example, CDK2, CDK4, and CDK6 were thought to be responsible for driving cells through interphase, whereas CDK1 was thought to be crucial for entry into and progression through mitosis (Malumbres and Barbacid, 2005). However, recent analysis of various CDK knockout mice suggests that this may not be the case (reviewed in (Malumbres and Barbacid, 2009)). Disruption of the *Cdk2, Cdk4*, or *Cdk6* gene did not result in major cell cycle defects in most cell types. Likewise, even simultaneous disruption of the *Cdk2, Cdk4*, and *Cdk6* genes did not cause cell cycle arrest, as these embryos developed up to day 12.5 (Santamaria et al., 2007). Interestingly, Cdk1 interacted with all cyclins in these embryos, similar to what is observed with Cdc28 in yeast. In sharp contrast, disruption of the *Cdk1* gene resulted in cell cycle arrest, suggesting that CDK1 is essential for driving the cell cycle in mammalian cells (Santamaria et al., 2007).

In mammalian cells, CDK1 is activated at the G2/M transition by several mechanisms. First, the expression of its activating cyclin (cyclinB) peaks at this point in the cycle, allowing for the formation of a CDK1/cyclinB complex. In addition, CDK1 is held in an inactive state until the onset of mitosis by inhibitory phosphorylation at T14 and Y15 by Myt1 and Wee1 kinases (Mueller et al., 1995; Parker and Piwnica-Worms, 1992). Upon entry into mitosis, these kinases are inactivated. Concurrently, a phosphatase called Cdc25 is activated and removes the inhibitory phosphates on CDK1, thus facilitating Cdk1 activation (Hoffmann et al., 1993). Once active, CDK1/cyclinB phosphorylates at least 70 different proteins at the consensus motif S/T–P–X–K/R, where X is any amino acid (Malumbres and Barbacid, 2005; Songyang et al., 1994). These phosphorylation events mediate many different key mitotic events, such as breakdown of the nuclear envelope and chromosome condensation. Consequently, CDK1/cyclinB mediated phosphorylation is critical for both the initiation of, and progression through mitosis.
Figure 1.5 Schematic overview of the cell cycle.

Dividing cells cycle through rounds of interphase (which is composed of G₁, S, and G₂) and mitosis. During interphase, the cell replicates its genome and cellular material, and prepares to divide. During mitosis, the cellular material is equally partitioned into two daughter cells.
1.2.2  Overview of mitosis and cytokinesis

As mentioned above, the replicated genome is separated during mitosis, which can be broken down into distinct phases called prophase, metaphase, anaphase, and telophase, based on DNA morphology. Once the two copies of the genome have been separated, the mother cell physically divides into two daughter cells through the process of cytokinesis. Overall, these processes are similar in animals, plants, and budding yeast. However, important differences exist, and are discussed below (Barr and Gruneberg, 2007).

1.2.2.1  Animals

An overview of mitosis and cytokinesis in animals is depicted in Figure 1.6. In prophase, the nuclear envelope breaks down and the chromosomes start to condense in response to CDK1/cyclinB-mediated phosphorylation events. As the cell progresses into metaphase, the chromosomes are attached to the mitotic spindle microtubules and align at the centre of the cell. In anaphase, the spindle poles migrate towards opposite ends of the cell, thus separating the DNA. A bundle of microtubules, called the spindle midzone (also referred to as the central spindle), forms between the separating chromosomes. At the same time, the onset of cytokinesis is triggered, and components of the actomyosin contractile ring assemble at the cortex of the cell. In telophase, the replicated genome has been separated, the nuclear envelope re-forms, and the DNA begins to decondense. Concurrently, the contractile ring constricts, generating an invagination in the membrane called the cleavage furrow. As the furrow ingresses, the microtubules of the spindle midzone are compacted. At this point, the nascent daughter cells remain joined solely by a dense bundle of microtubules called the midbody. In the final stage of cytokinesis, which is called abscission, the midbody is severed and two distinct daughter cells are generated.
Figure 1.6 Overview of mitosis and cytokinesis in animals.
Schematic representation of the coordinated events of mitosis and cytokinesis in animals. DNA is shown in blue, microtubules are shown in green, and the contractile ring is shown in red. The mitotic stage is indicated at left, and the corresponding cytokinetic events are shown at right. Adapted from (Eggert et al., 2006).
1.2.2.2 Plants

While plant mitosis is very similar to that described above for animals, the process of cytokinesis varies considerably between plants and animals. In plants, the physical division of the cytoplasm is not mediated by an actomyosin-based contractile ring, but involves the generation of a new membrane, called the cell plate, between the separated chromosomes (Nishihama and Machida, 2001). This is mediated by a microtubule and actin-based structure called the phragmoplast, which directs the fusion of endosome and/or Golgi derived vesicles between the daughter nuclei (Barr and Gruneberg, 2007). Consequently, no intercellular microtubule bridge is formed, and plants do not undergo abscission.

1.2.2.3 Budding yeast

In contrast to animals and plants, budding yeast form a bud (nascent daughter cell) in S phase, which continues to grow throughout the cell cycle (Herskowitz, 1988). Mitosis in budding yeast is very similar to mitosis in animals, with the exception that it occurs inside the nucleus. After one of the daughter nuclei has translocated into the bud, cytokinesis begins. Contraction of an actomyosin ring guides the formation of a chitin-based primary septum at the mother-bud neck (Bi, 2001). The mother and daughter cells then both generate a secondary septum, whose composition is similar to the cell wall (Cabib et al., 2001). The final separation of these cells is achieved via the partial breakdown of the primary septum. As a result, budding yeast does not form a midbody or undergo abscission in the same sense as mammalian cells.

The details of mitosis will not be discussed further, as this thesis focuses on the roles of septins in mammalian cytokinesis. As such, I will now focus on the details of contractile ring assembly and constriction, and abscission in mammalian cells.
1.2.3 **Contractile ring assembly and constriction**

Once the replicated genome has been separated to opposite ends of the mother cell, the cytoplasm and its contents must be divided in order to generate two distinct daughter cells. This is achieved through the assembly and constriction of a contractile ring that is composed predominantly of the motor protein nonmuscle myosin II (hereafter referred to as myosin) and actin (Glotzer, 2005).

1.2.3.1 **The actomyosin contractile ring drives cytokinesis**

Myosin is a hexameric protein, consisting of a dimer of heavy chains that each associate with an essential light chain and a regulatory light chain (rMLC) (Glotzer, 2005). These hexamers associate with each other to form myosin filaments, which use the energy of ATP hydrolysis to translocate actin filaments. At anaphase, actin and myosin filaments assemble into a contractile ring at the cortex of the cell, between the separated DNA. The translocation of actin filaments by myosin causes the contractile ring to constrict, leading to the formation of the cleavage furrow. The contractile ring continues to constrict, causing ingression of the cleavage furrow, until the cells remain joined solely by the midbody.

Myosin is normally held in an autoinhibited state. However, phosphorylation of the rMLC relieves this autoinhibition, permitting the formation of myosin filaments, allowing association with actin, and activating myosin’s ATPase activity (Matsumura, 2005). In *Drosophila*, substitution of wild type rMLC for a non-phosphorylatable mutant caused severe defects in cytokinesis, whereas a phosphomimetic mutant largely compensated for the loss of wild type rMLC (Jordan and Karess, 1997). Likewise, expression of a non-phosphorylatable rMLC mutant in mammalian cells impaired cytokinesis (Komatsu et al., 2000). Therefore, phosphorylation of rMLC is thought to play a critical role in both the assembly and constriction of the cleavage furrow during cytokinesis.

Several cleavage furrow components have been shown to regulate phosphorylation of rMLC. Rho-associated kinase (ROCK) is thought to promote rMLC phosphorylation by two mechanisms. First, it phosphorylates rMLC directly (Amano et al., 1996). Second, ROCK inhibits myosin phosphatase by phosphorylating the myosin phosphatase targeting (MYPT)
subunit, thereby indirectly promoting rMLC phosphorylation (Kimura et al., 1996). While ROCK inhibition delayed the time it took to complete ingression of the cleavage furrow, it did not block cytokinesis (Kosako et al., 2000; Madaule et al., 1998). These results suggest that ROCK cannot be the sole kinase mediating rMLC phosphorylation during cytokinesis. Citron kinase (CRIK) also localizes to the cleavage furrow and directly phosphorylates rMLC (Madaule et al., 1998; Yamashiro et al., 2003). Overexpression of CRIK mutants in HeLa cells resulted in abnormal ingression of the cleavage furrow, consistent with a role for CRIK protein in contractile ring assembly and/or constriction (Madaule et al., 1998). While CRIK knockout mice exhibited severe cytokinetic defects in specific neuronal precursor cells, no such defects were observed in many other cell types (Di Cunto et al., 2000). A third cleavage furrow component, called myosin light chain kinase (MLCK), also phosphorylates rMLC (Poperechnaya et al., 2000; Sellers et al., 1981). However, MLCK knockout mice developed to full size before expiring shortly after birth (Somlyo et al., 2004). Given the number of kinases that mediate rMLC phosphorylation, the exact mechanism of contractile ring assembly and constriction may vary in different cell types and different species.

The other major component of the contractile ring is the cytoskeletal protein actin. The nucleation and polymerization of unbranched actin filaments is mediated by a family of proteins called formins (Higgs, 2005). Recent work demonstrated that the formin mammalian homolog of Drosophila diaphanous 2 (mDia2) plays an important role in the assembly of actin filaments at the contractile ring (Watanabe et al., 2008). Depletion of mDia2 by siRNA and injection of mDia2 antibodies into NIH 3T3 cells induced binucleation, indicating cytokinetic failure. Time-lapse microscopy revealed that this reflects abnormal contraction at multiple sites in dividing cells. Importantly, the amount of filamentous actin at the contractile ring was decreased in the absence of mDia2. Like myosin, formins are also held in an autoinhibited state via intramolecular interactions (Higgs, 2005). Consequently, formin activation is another prerequisite for the proper assembly and constriction of the contractile ring.

### 1.2.3.2 RhoA is a key contractile ring regulator

The small GTPase RhoA acts as the master regulator of contractile ring assembly and constriction (Glotzer, 2005). Like other small GTPases, RhoA exists in an active, GTP-bound

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form, and an inactive, GDP-bound form. Conversion between these two forms is mediated by guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP for GTP, and GTPase activating proteins (GAPs), which promote the hydrolysis of GTP to GDP. In its active form, RhoA mediates assembly and constriction of the contractile ring by multiple mechanisms. First, RhoA activates ROCK (Matsui et al., 1996), which mediates myosin activation and filament assembly through rMLC phosphorylation. Second, RhoA mediates the localization of CRIK at the cleavage furrow of dividing cells (Eda et al., 2001). Lastly, RhoA is thought to interact with formins (including mDia2) and relieve their autoinhibition, thus permitting the nucleation and polymerization of unbranched actin filaments (Higgs, 2005).

1.2.3.3 RhoA activation at the cell cortex triggers the onset of cytokinesis

Given the multiple roles of RhoA in mediating cleavage furrow assembly and constriction, it must be activated at the right place and the right time in order to ensure proper cytokinesis. The GEF that is thought to be responsible for RhoA activation during cytokinesis is called epithelial cell transforming protein 2 (ECT2) (Tatsumoto et al., 1999). Inhibition of ECT2 by antibody injection or siRNA strongly impaired cytokinesis, resulting in the accumulation of multinucleated cells (Kim et al., 2005; Tatsumoto et al., 1999). Time-lapse microscopy suggested that cells are unable to form a cleavage furrow in the absence of ECT2 (Yuce et al., 2005). Consistent with this hypothesis, the contractile ring components actin, myosin, and RhoA all failed to accumulate at the cell cortex during cytokinesis upon ECT2 depletion.

During cell division, ECT2 localizes to the central spindle, where it associates directly with CYK-4 (also known as MgcRacGAP) (Tatsumoto et al., 1999; Yuce et al., 2005). Along with mitotic kinesin-like protein 1 (MKLP1), CYK-4 forms the centralspindlin complex that is thought to be critical for the formation of the central spindle (Mishima et al., 2002). Depletion of CYK-4 prevented the accumulation ECT2 at the central spindle, and the localization of actin, myosin, and RhoA at the cell cortex (Yuce et al., 2005). Consequently, it was proposed that CYK-4 directly recruits ECT2 to the central spindle, where it locally activates RhoA to trigger the assembly and constriction of the contractile ring.
1.2.3.4 Plk1 regulates the onset of cytokinesis by controlling the ECT2-CYK-4 interaction

Polo-like kinase 1 (Plk1) is a serine/threonine kinase that was recently shown to play an essential role in cytokinesis (Burkard et al., 2007; Petronczki et al., 2007). Anaphase-specific inhibition of Plk1 prevented the formation of the cleavage furrow, and abolished localization of RhoA at the cell cortex and ECT2 at the central spindle. Intriguingly, inhibition of Plk1 also severely impaired the interaction between ECT2 and CYK-4 (Petronczki et al., 2007). Subsequent work demonstrated that phosphorylation of CYK-4 by Plk1 regulates the association between CYK-4 and ECT2 (Burkard et al., 2009; Wolfe et al., 2009).

A summary of the molecular events that mediate assembly of the contractile ring are summarized in Figure 1.7. Plk1 phosphorylates CYK-4, allowing CYK-4 to recruit ECT2 to the central spindle. ECT2 then locally activates RhoA. RhoA mediates the recruitment of CRIK to the cell cortex, and activates ROCK. These kinases (and possibly MLCK) mediate myosin activation and filament assembly through phosphorylation of rMLC. At the same time, active RhoA relieves mDia2 autoinhibition, permitting the nucleation and elongation of unbranched actin filaments.
Figure 1.7 Molecular mechanism of contractile ring assembly.
Pathways leading to myosin activation and filament formation, and actin nucleation and polymerization at the contractile ring are depicted. ‘p’ denotes phosphorylation. As mentioned in the text, the mechanisms leading to rMLC phosphorylation may be organism and cell-type specific.
1.2.3.5 Anillin acts as a contractile ring scaffold

Another component that is thought to be critical for the proper assembly and constriction of the contractile ring is the scaffolding protein anillin. Depletion of anillin by siRNA resulted in an accumulation of binucleate cells as a consequence of abnormal contractile ring constriction (Straight et al., 2005). The nascent daughter cells underwent multiple rounds of growing and shrinking as the cytoplasm was propelled back and forth through the contractile ring. In some cells, the cleavage furrow ingressed to one side of both daughter nuclei, whereas in other cells it regressed, giving rise to binucleate cells in both instances. Anillin interacts with RhoA (Piekny and Glotzer, 2008), myosin (Straight et al., 2005), actin (Field and Alberts, 1995), and mDia2 (Watanabe et al., 2010), suggesting that it acts as a scaffold for contractile ring components. In addition, anillin binds to the homologue of CYK-4 in Drosophila, suggesting that it may also link CYK-4/ECT2 to contractile ring components (D’Avino et al., 2008).

It should be noted that some proteins involved in contractile ring formation and constriction (such as Plk1, ROCK, and anillin) also localize to the midbody. However, the role of these proteins in abscission (if any) remains unclear.

1.2.4 Abscission

In the final stage of cytokinesis, called abscission, the midbody bridge that connects the two nascent daughter cells must be resolved. This involves severing and/or disassembling the midbody microtubule network, and physically breaking the membrane that connects the two nascent daughter cells. While many questions remain unanswered, recent work has identified many of the players involved in abscission, and the molecular mechanisms of this complex process are beginning to be understood.
1.2.4.1 Vesicle trafficking and fusion

A large body of work has demonstrated that proteins involved in vesicle trafficking and membrane fusion are critical for abscission. In fact, approximately one third of the midbody proteome represents proteins involved in these processes (Skop et al., 2004). A simplified overview of vesicle trafficking and fusion at the plasma membrane is depicted in Figure 1.8. After being delivered to the vicinity of the plasma membrane, vesicles are tethered at the site of fusion by a highly conserved protein complex called the exocyst (He and Guo, 2009). This complex is composed of eight subunits called Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84. The exocyst complex is thought to associate with the plasma membrane via direct binding to PI(4,5)P$_2$ through the Sec3 and Exo70 subunits (Liu et al., 2007; Zhang et al., 2008). Concurrently, the exocyst associates with vesicles (through other regulatory proteins such as Rab GTPases), thus acting as a physical link between vesicles and the plasma membrane (He and Guo, 2009). Vesicle fusion with the plasma membrane is driven by the assembly of a complex between soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins present on the plasma membrane (t-SNAREs) and the vesicle (v-SNAREs).
Figure 1.8 Simplified overview of vesicle trafficking and fusion at the plasma membrane. Once delivered to the plasma membrane, vesicles are tethered by the exocyst complex (subunits shown in green and blue; 15 = Sec15, 84 = Exo84, 10 = Sec10, 8 = Sec8, 6 = Sec6, 5 = Sec5, 3 = Sec3, 70 = Exo70). Sec3 and Exo70 associate with PI(4,5)P₂ (shown in red) via their polybasic domains (denoted by ++++). At the same time, the exocyst complex associates with the vesicle via other regulatory factors such as Rab GTPases. It should be noted that the organization of exocyst subunits within the complex remains poorly understood. Vesicle fusion is catalyzed by the assembly of a SNARE complex between t-SNAREs present on the plasma membrane (purple and blue) and a v-SNARE present on the vesicle (orange). Adapted from (He and Guo, 2009).
Early studies on the role of the vesicle trafficking machinery in abscission showed that the v-SNARE vesicle-associated membrane protein 8 (VAMP8) and the t-SNARE syntaxin 2 localized to the midbody (Low et al., 2003). Overexpression of dominant negative forms of these proteins impaired abscission, consistent with a potential role for SNARE proteins in the completion of cytokinesis (Low et al., 2003). Subsequent work identified a protein named centriolin, whose depletion led to the accumulation of cells with long intercellular bridges (Gromley et al., 2003). Interestingly, these cells continued to cycle, leading to the formation of multicellular syncytia. Centriolin was later found to mediate the localization of VAMP8, syntaxin 2, and the exocyst complex to the midbody (Gromley et al., 2005). Importantly, depletion of exocyst subunits by siRNA also impaired abscission, leading to the accumulation of cells with long midbodies that sometimes persisted for multiple rounds of division. This was accompanied by an accumulation of secretory vesicles at the midbody, suggesting that exocyst-mediated vesicle fusion is important for abscission. Other factors also appear to play a role in mediating the proper localization of the exocyst complex at the midbody. Sec5 and Exo84 both interact with the active form of the small GTPase RalB (Moskalenko et al., 2002; Moskalenko et al., 2003). Depletion of RalB also led to the accumulation of cells with persistent intercellular bridges (Cascone et al., 2008). A RalB mutant uncoupled from Sec5 could rescue this cytokinetic defect, whereas a mutant uncoupled from Exo84 could not, suggesting that only the RalB-Exo84 interaction is important for abscission. In addition, RalB depletion impaired the accumulation of Sec6 at the midbody, suggesting that RalB is important for mediating the proper localization of the exocyst complex at the terminal stage of cytokinesis.

Two different pools of vesicles have been shown to accumulate at the midbody prior to abscission. The first pool originates from the recycling endosomes, and contains the small GTPase Rab11 (Wilson et al., 2005). This protein plays a critical role in membrane trafficking through endosomes. Consistent with a role for recycling endosomes at the terminal stage of cytokinesis, depletion of Rab11 by siRNA impaired abscission. While ingression of the cleavage furrow and formation of the midbody appeared normal in Rab11-depleted cells, the midbody persisted and eventually collapsed, giving rise to binucleated cells (Yu et al., 2007). Similar results were observed upon depletion of Rab35, which mediates membrane trafficking between endosomes and the plasma membrane (Kouranti et al., 2006). Rab11 interacts with an effector protein called family of Rab11-interacting protein 3 (FIP3) that is also present on recycling
endosomes, and FIP3 depletion causes similar defects in midbody abscission (Wilson et al., 2005). Both FIP3 and Rab11 co-immunoprecipitate with the vesicle tethering exocyst complex, and depletion of the exocyst subunit Exo70 impairs the accumulation of FIP3 and Rab11 at the midbody (Fielding et al., 2005). Since recruitment of FIP3 to the midbody occurs independently of Rab11 (Wilson et al., 2005), FIP3 has been proposed to serve as a link between recycling endosome vesicles and the exocyst complex. This may occur indirectly via another GTPase called ADP-ribosylation factor 6 (Arf6), which interacts with the exocyst (Prigent et al., 2003) and forms a ternary complex with FIP3 and Rab11 (Fielding et al., 2005). In support of this notion, Arf6 localizes to the midbody, and expression of a GDP-restricted mutant of Arf6 impaired the accumulation of FIP3 and Rab11 at the midbody (Fielding et al., 2005). However, depletion of Arf6 by greater than 95% did not impair midbody abscission (Yu et al., 2007). It should be noted that other mechanisms may also contribute to the recruitment of recycling endosome vesicles to the midbody. For example, Rab11 has been shown to interact directly with Sec15 (Zhang et al., 2004). In addition, both Rab11 and the exocyst complex co-immunoprecipitate with BRUCE, a large multidomain protein that bears ubiquitin-conjugating activity (Pohl and Jentsch, 2008). Accordingly, depletion of BRUCE by siRNA impaired abscission and led to the accumulation of Rab11-containing vesicles close to the midbody.

Secretory vesicles originating from the Golgi are the second pool of vesicles that accumulate at the midbody at the terminal stage of cytokinesis. These vesicles can be identified by fluorescently labeled secretory proteins (Goss and Toomre, 2008; Gromley et al., 2005). Although the mechanism of targeting of these vesicles to the midbody and their contribution to abscission is not well understood, it has been demonstrated that disruption of the Golgi with brefeldin A impairs abscission (Gromley et al., 2005; Skop et al., 2001).

While it is clear that trafficking of endosome and Golgi-derived vesicles to the midbody is important for abscission, the precise role of these vesicles remains unclear. One possibility is that fusion of vesicles with each other and with the plasma membrane is responsible for physically severing the midbody and generating two distinct daughter cells (as depicted in the targeted secretion model in Figure 1.9). Alternatively, vesicle fusion could simply narrow the midbody, thus facilitating the final separation by some other means (discussed below). It is also possible that vesicle fusion events are simply required to deliver certain factors to the midbody that are critical for abscission.
1.2.4.2 Membrane fission

It remains to be determined whether vesicle fusion events are sufficient to physically separate the membrane connecting nascent daughter cells at the end of cytokinesis. Increasing evidence suggests that endosomal sorting complex required for transport (ESCRT) proteins may also be important for abscission (McDonald and Martin-Serrano, 2009). These proteins play a key role in membrane fission events, such as the formation of multivesicular bodies and viral budding. Importantly, membrane fission during abscission would be topologically equivalent to these fission events. The ESCRT protein tumor susceptibility gene 101 (Tsg101) and the ESCRT-associated protein apoptosis-linked gene-2-interacting protein X (Alix) both localize to the midbody through direct interaction with the microtubule bundling protein centrosomal protein of 55 kDa (Cep55) (Carlton and Martin-Serrano, 2007; Morita et al., 2007). Depletion of any of these proteins by siRNA impaired abscission. Several other ESCRT and ESCRT-associated proteins also localize to the midbody and are important for abscission, consistent with the notion that ESCRT-mediated membrane fission may play an important role in midbody abscission (Agromayor et al., 2009; Bajorek et al., 2009; Carlton and Martin-Serrano, 2007; Dukes et al., 2008; Morita et al., 2007).

1.2.4.3 Microtubule remodeling

Midbody abscission not only requires physical separation of the membrane connecting the two daughter cells, but also requires that the midbody microtubules be severed and / or disassembled. Recent work suggested that the microtubule severing protein spastin may play a critical role in this process. Spastin is recruited to the midbody via a direct interaction with the ESCRT protein charged multivesicular body protein 1B (CHMP1B) (Connell et al., 2009; Reid et al., 2005; Yang et al., 2008). Depletion of spastin by siRNA or expression of catalytically inactive spastin led to the accumulation of cells with elongated intercellular bridges (Connell et al., 2009). Live imaging of HeLa cells expressing fluorescently labeled tubulin suggested that midbody microtubules break down abruptly, approximately two hours after the onset of anaphase, but before the final separation of daughter cells. Depletion of spastin by siRNA caused either a severe delay in microtubule breakdown, or prevented it from occurring altogether. Thus, it
appears that spastin plays an important role in mediating microtubule severing and/or breakdown prior to abscission.

### 1.2.4.4 Models for midbody abscission

While much progress has been made over the last few years in identifying many of the components that contribute to abscission, a detailed understanding of how these components work together to break the midbody remains elusive. Several models attempting to explain the mechanism of abscission have been proposed, and are outlined in Figure 1.9 (Steigemann and Gerlich, 2009).

In the mechanical rupture model (Figure 1.9, left), the nascent daughter cells pull apart, generating enough force to physically tear the midbody. This model therefore does not require membrane fusion or fission events, nor does it require specialized machinery to sever or depolymerize the midbody microtubules. However, the daughter cells need to be highly motile to generate sufficient force to mechanically break the midbody.

In the targeted secretion model (Figure 1.9, middle) vesicles are delivered to the intercellular bridge, where they fuse with each other and the plasma membrane to separate the daughter cells. Midbody microtubules would need to be severed and/or depolymerized before vesicle fusion could break the midbody membrane. This model does not require the daughter cells to be highly motile, nor does it require membrane fission.

In the membrane ingestion model (Figure 1.9, right), the midbody microtubules depolymerize and the plasma membrane of the intercellular bridge narrows. This could result from fusion of recycling endosome and Golgi-derived vesicles with the plasma membrane. ESCRT-mediated fission of the midbody membrane then leads to the final separation of daughter cells.

It should be noted that these models are not mutually exclusive. In addition, different cell types may rely more heavily on specific components of the abscission machinery to achieve final separation of daughter cells.
Figure 1.9 Proposed models for midbody abscission.
Three proposed models for abscission of the midbody are shown. Midbody microtubules are shown in blue, the plasma membrane is shown in dark green, and vesicles are depicted in light green. Adapted from (Steigemann and Gerlich, 2009).
1.2.5 Roles of septins in mammalian cell division

The first evidence that mammalian septins play a role during cytokinesis came from a study on SEPT2, which was called neural precursor cell expressed developmentally down-regulated protein 5 (Nedd5) at the time (Kinoshita et al., 1997). SEPT2 localized to the cleavage furrow during anaphase and the midbody during telophase in a variety of cell lines. Injection of anti-SEPT2 antibodies into HeLa cells at late anaphase/early telophase impaired the completion of cytokinesis. In some cases, the cleavage furrow immediately regressed, giving rise to a binucleated cell. In other instances, the daughter cells remained joined by a persistent midbody, which in some cases collapsed and gave rise to a binucleated cell.

Similar results were later reported for SEPT9, which was called mixed lineage leukemia (MLL) septin-like fusion (MSF) at the time (Nagata et al., 2003; Surka et al., 2002). Injection of anti-SEPT9 antibodies into synchronized HeLa cells 4 hours before the onset of division or treatment of HeLa cells with SEPT9 siRNA led to an accumulation of binucleate cells and cells connected by persistent midbodies (Surka et al., 2002). Likewise, SEPT9 depletion in human mammary epithelial cells (HMEC) caused similar defects (Nagata et al., 2003). It should be noted that many groups have demonstrated that depletion of one septin by siRNA can also cause depletion of other septin family members, a phenomenon that may result from destabilizing the septin complex (Kinoshita et al., 2002). Consequently, it is unclear whether the expression levels of other septins were affected in the above-described studies, or whether SEPT9 was depleted specifically.

Most subsequent studies on the role of septins in mammalian cell division have treated the septin family as a whole, rather than examining the function of individual septins. In other words, the field has assumed that because septins co-exist in complexes and filaments, depletion of any or all septins in a given cell type should give rise to the same phenotype. Conveniently, several groups have demonstrated that treatment with siRNA directed against SEPT7 results in decreased levels of all septin family members (Kinoshita et al., 2002; Kremer et al., 2005; Tooley et al., 2009).

When septin localization was assessed during the early stages of cell division, it was found that SEPT2 and SEPT6 localize to the microtubules of the mitotic spindle during metaphase (Spiliotis
et al., 2005). Simultaneous depletion of SEPT2, SEPT6 and SEPT7 (and possibly others) in Madin-Darby canine kidney (MDCK) cells led to an accumulation of binucleate cells. However, this appeared to result from defects in chromosome congression and segregation. This group also argued that septin depletion prevented the proper localization of CENP-E, a mitotic motor protein that mediates stable kinetochore-microtubule attachment, at the kinetochores of congressing chromosomes. It was later shown that the C-terminal extension of SEPT7, but not that of SEPT2 or SEPT6 directly interacts with CENP-E and mediates its localization to kinetochores (Zhu et al., 2008). Whether other septins directly interact with CENP-E via their C-terminal extensions / coiled-coils remains to be tested.

Other work showed that simultaneous depletion of SEPT2, SEPT6, and SEPT7 (and likely others) in HeLa cells led to an accumulation of cells with multiple and/or fragmented nuclei (Kremer et al., 2005). This study also argued that septin depletion causes a pronounced increase in microtubule stability. SEPT2, but not a SEPT6/SEPT7 dimer was found to interact directly with microtubule-associated protein (Map4). This association prevented Map4 from binding and bundling microtubules in vitro. The region of SEPT2 that mediates this interaction was not identified, nor is it clear whether other septin family members interact directly with Map4. However, depletion of Map4 rescued the cytokinetic defects observed upon simultaneous depletion of SEPT2, SEPT6, and SEPT7. This study therefore suggested that septin-mediated regulation of microtubule stability through Map4 may be important for cell division.

SEPT2 was also shown to directly interact with myosin (Joo et al., 2007). Disrupting this interaction through the use of an inhibitory peptide impaired the full activation of myosin, and caused regression of the cleavage furrow and subsequent binucleation. Immunofluorescence analysis showed that upon inhibition of the SEPT2-myosin interaction, the myosin kinases CRIK and ROCK remained associated with SEPT2. It was therefore proposed that SEPT2-containing septin filaments act as a scaffold for myosin and its kinases, thus facilitating the full activation of myosin that is required for contractile ring assembly and ingression. While it was demonstrated that the SEPT2-myosin interaction was not mediated through the C-terminal extension / coiled coil of SEPT2, the interacting domain was not mapped further. In addition, whether other septin family members are also capable of interacting with myosin, or whether they mediate the recruitment of CRIK and ROCK to SEPT2-containing septin filaments remains to be elucidated.
Septins may also interact with the contractile ring scaffolding protein anillin, since anillin recruits septins to actin filaments \textit{in vitro} (Kinoshita et al., 2002). In addition, SEPT7 and anillin colocalize at the cleavage furrow (Oegema et al., 2000). However, evidence for an \textit{in vivo} interaction between septins and anillin is lacking.

1.3 Other functions of mammalian septins

1.3.1 Vesicle trafficking

Numerous studies indicate that mammalian septins play a role in vesicle trafficking. Early work demonstrated that several septins co-purify with the vesicle tethering exocyst complex when immunoprecipitated from rat brain (Hsu et al., 1998). In addition, SEPT5 (which is predominantly expressed in the brain) was shown to interact directly with syntaxin, a SNARE protein that mediates membrane fusion (Beites et al., 1999). Overexpression of SEPT5 in HIT-T15 cells impaired exocytosis, suggesting that septins may regulate vesicle trafficking via direct association with syntaxin. Surprisingly, Sept5 knockout mice displayed no obvious developmental defects, and both synaptic function and hippocampal neuron growth appeared normal (Peng et al., 2002). However, Sept5 deficient mice had altered levels of other septin family members in the brain, raising the possibility that such changes may functionally compensate for the lack of SEPT5. Indeed, we have demonstrated that acute perturbation of SEPT5 levels by RNA interference leads to defects in axon outgrowth in hippocampal neurons (Tsang et al., submitted), which could be a consequence of perturbed vesicle trafficking. Subsequent work has suggested that SEPT5 may act as a barrier to regulate the proximity of synaptic vesicles to the presynaptic membrane in the immature calyx of Held synapse (Yang et al., 2010).

While SEPT5 was initially thought to be predominantly expressed in the brain (Beites et al., 1999), it was later shown that it is also highly expressed in platelets (Dent et al., 2002). Since a critical role of platelets is to secrete molecules from storage granules, this study assessed whether SEPT5 may play a role in exocytosis in platelets. SEPT5 was shown to interact with syntaxin 4, a SNARE protein involved in platelet secretion. In addition, platelets from Sept5 knockout mice
exhibited enhanced secretion compared to platelets from wild type mice. Therefore, SEPT5 appears to act as a negative regulator of secretion in platelets.

1.3.2 Membrane diffusion barrier

Analysis of Sept4 knockout mice shed significant light on the role of SEPT4 in vivo (Ihara et al., 2005; Kissel et al., 2005). SEPT1, SEPT4, SEPT6 and SEPT7 were found to be the major components of a ring-like membrane associated structure called the annulus, which forms a barrier between the middle and principal piece of the sperm tail. We later discovered SEPT12, and showed that it is also a component of this structure (Steels et al., 2007). Sperm from male Sept4 knockout mice had no detectable annulus, leading to severe bending of the sperm tail, drastic defects in mobility, and complete sterility. In addition, the annulus was found to be absent in some infertile human males (Ihara et al., 2005). Subsequent work showed that the annulus acts as a diffusion barrier, preventing the diffusion of membrane proteins between the middle and principal piece of the sperm (Kwitny et al., 2010).

Septins were recently found to play a similar role at the base of the primary cilium (Hu et al., 2010). This organelle senses extracellular signals and transmits them into the cell through the action of specific membrane proteins. Fluorescence recovery after photobleaching (FRAP) analysis demonstrated that these membrane proteins are mobile within the primary cilium. However, their movement past the base of the primary cilium was restricted by a diffusion barrier. SEPT2 was found to localize to the base of the primary cilium. Depletion of SEPT2 by siRNA impaired the accumulation of membrane proteins at the primary cilium, and increased the mobility of these proteins across the base of the primary cilium. Likewise, signal transduction through the primary cilium was also impaired upon SEPT2 depletion. Whether other members of the septin family localize to the base of the primary cilium or are important for the diffusion barrier remains to be tested.

SEPT5, SEPT7, and SEPT11 were similarly found to localize to the base of dendritic protrusions and branch points in cultured hippocampal neurons (Tada et al., 2007; Xie et al., 2007). Consequently, it was proposed that septins may also form a diffusion barrier at the base of
dendritic protrusions, thus restricting the movement of membrane proteins within these structures. However, this hypothesis remains to be tested experimentally.

1.3.3 Septin knockout mice

In addition to those described above, several other septin knockout mice have been generated (Kinoshita, 2008). Sept6 knockout mice displayed no obvious phenotype (Ono et al., 2005). The authors reasoned that members of the SEPT6 group (i.e., SEPT6, SEPT8, SEPT10, SEPT11, and SEPT14) are likely functionally redundant, and that simultaneous knockout of several septins within this group may be required to discern its functions in vivo. However, recent work suggests that disruption of Sept11 causes embryonic lethality (B. Zieger, personal communication). This not only argues against the notion that the SEPT6 group members are redundant, but also suggests that SEPT11 (and not SEPT6) may be a critical component of septin complexes. Likewise, disruption of Sept9 causes embryonic lethality, and Sept7 knockout mice were never born, although the embryos were not examined (Kinoshita, 2008). These studies demonstrate that SEPT7 and SEPT9 are critical for mouse development, and are consistent with the notion that SEPT7 and SEPT9 play important roles during cell division (Kremer et al., 2005; Nagata et al., 2003; Spiliotis et al., 2005; Surka et al., 2002; Zhu et al., 2008). Sept3 knockout mice and Sept3/Sept5 double knockout mice displayed no obvious phenotype (Fujishima et al., 2007; Tsang et al., 2008) beyond a subtle delay in endocytosis (C. Tsang, personal communication).

1.3.4 Septins and disease

1.3.4.1 Cancer

Alterations in SEPT9 expression have been linked to a variety of cancers (Scott et al., 2005), including breast (Gonzalez et al., 2007; Kalikin et al., 2000; Montagna et al., 2003) and ovarian carcinomas (Burrows et al., 2003; Kalikin et al., 2000; Russell et al., 2000; Scott et al., 2006), and leukemia (Kreuziger et al., 2007; Kurosu et al., 2008; Osaka et al., 1999; Saito et al., 2010; Santos et al., 2010; Strehl et al., 2006; Yamamoto et al., 2002). Some of these instances appear to involve a loss of SEPT9 function, either through fusion of the SEPT9 gene with the MLL gene.
(Kreuziger et al., 2007; Kurosu et al., 2008; Osaka et al., 1999; Saito et al., 2010; Santos et al., 2010; Strehl et al., 2006; Yamamoto et al., 2002) or via loss of heterozygosity (Russell et al., 2000). In other cases, SEPT9 is overexpressed, either through amplification of the SEPT9 gene (Montagna et al., 2003) or increased expression of individual SEPT9 isoforms. Several studies have demonstrated that SEPT9_i4 is overexpressed in many tumours (Burrows et al., 2003; Scott et al., 2006). This SEPT9 isoform is encoded by two distinct transcripts, SEPT9_v4 and SEPT9_v4*, with the_v4* transcript being translated more efficiently (McDade et al., 2007). Of these two, the_v4 transcript is the major form in normal cells, whereas the_v4* transcript is the predominant form in tumours, which accounts for the increased levels of SEPT9_i4 in neoplasia. Others studies have shown that SEPT9_i1 is overexpressed in ovarian tumours and breast cancer cells (Gonzalez et al., 2007; Scott et al., 2006).

In addition, it has been demonstrated that the SEPT9 gene is hypermethylated in colorectal cancer tissue compared to non-pathologic tissue, suggesting that SEPT9 methylation may serve as a biomarker for the detection of colorectal cancer (Lofton-Day et al., 2008). Subsequent work has established a minimally invasive blood-based test for methylation of the SEPT9 gene, which has been shown to detect colorectal cancer with a sensitivity of 70% and a specificity of 90% (deVos et al., 2009; Grutzmann et al., 2008).

Several studies have reported links between other septin family members and cancer. SEPT2 (Cerveira et al., 2006; Cerveira et al., 2008b; van Binsbergen et al., 2007), SEPT5 (Tatsumi et al., 2001), SEPT6 (Borkhardt et al., 2001; Cerveira et al., 2008a; Fu et al., 2003; Kadkol et al., 2006; Kim et al., 2003; Ono et al., 2002; Strehl et al., 2006), and SEPT11 (Kojima et al., 2004; Stevens et al., 2010) have all been found as MLL fusions in patients with leukemia. In addition, the expression levels of SEPT1, SEPT2, SEPT4, SEPT6, SEPT8, SEPT10, SEPT11, and SEPT14 have all been shown to be altered in certain types of cancer (Hall and Finger, 2008; Liu et al., 2010).

1.3.4.2 Hereditary Neuralgic Amyotrophy

Hereditary Neuralgic Amyotrophy (HNA) is a rare autosomal dominant disorder characterized by the sudden onset of severe pain in the shoulder and/or arm, accompanied by weakness and
wasting of arm muscles. It was recently demonstrated that HNA is caused by mutations in the SEPT9 gene (Kuhlenbaumer et al., 2005). Some families with this disease have a single point mutation in the coding region of the SEPT9 gene (Hannibal et al., 2009; Kuhlenbaumer et al., 2005). Others bear duplications within the SEPT9 gene, resulting in larger transcripts and protein products (Collie et al., 2010; Landsverk et al., 2009). Interestingly, all of these mutations involve the N-terminal extension of SEPT9, suggesting that this region is important to the pathogenesis of HNA.

1.3.4.3 Other Diseases
SEPT4 has been found to associate with α-synuclein and localizes to Lewy bodies in the brains of Parkinson’s disease patients (Ihara et al., 2003; Ihara et al., 2007). SEPT1, SEPT2, and SEPT4 have been found in neurofibrillary tangles in the brains of Alzheimer’s disease patients (Kinoshita et al., 1998). Proteomic screens have also shown expression of septin family members to be altered in a variety of other diseases, including Down’s syndrome, Schizophrenia, Bipolar Disorder, and Epilepsy (Hall and Finger, 2008).

1.4 Regulation of mammalian septins
There is emerging evidence that septin family members are subject to various types of post-translational modifications. Although the details are limited in most instances, such modifications may play an important role in regulating septin function.

Several septin family members have been showed to be phosphorylated in a variety of cellular contexts. SEPT3 is phosphorylated at Ser-91 by cGMP-dependent protein kinase-I in nerve terminals (Xue et al., 2004). This post-translational modification was postulated to regulate the subcellular localization of SEPT3 in neurons. SEPT2 was found to be phosphorylated at Ser-218 by Casein Kinase II in HeLa cells (Huang et al., 2006; She et al., 2004). It was proposed that this modification decreases the affinity of SEPT2 for guanine nucleotide (Huang et al., 2006). SEPT5 is phosphorylated at Ser-17 (in the N-terminal extension) by Cdk5-p35 in mouse brain (Taniguchi et al., 2007). This amino acid is not present in human SEPT5; however, it was
subsequently shown that Cdk5-p35 also phosphorylates human SEPT5 (Amin et al., 2008).
Interestingly, the phosphorylation site in human SEPT5 was mapped to Ser-327, which is present in the C-terminal extension. In both mouse and human, phosphorylation of SEPT5 by Cdk5-p35 inhibited the interaction between SEPT5 and syntaxin. Consequently, this post-translational modification may play an important role in regulating vesicle traffic.

High-throughput phosphorylation screens have also identified several putative phosphorylation sites in septin family members, although these sites have not been validated experimentally. Some of these sites were identified from mitotic cell lysates and represent putative recognition sites for Cdk1 (Beausoleil et al., 2006; Dephoure et al., 2008). This raises the intriguing possibility that some septin family members may be regulated by phosphorylation at the onset of mitosis.

There is limited evidence that mammalian septins may also be regulated by other types of post-translational modifications, such as acetylation (Choudhary et al., 2009), and sumoylation (Zhu et al., 2010). However, the significance of these modifications remains elusive.

1.5 The peptidyl prolyl isomerase Pin1

1.5.1 Pin1 is highly conserved protein important for cell division

Pin1 is a highly conserved and ubiquitously expressed nuclear protein of approximately 18kDa (Lu et al., 1996). The Pin1 gene was first identified in Saccharomyces cerevisiae (where it is referred to as ESS1), where it was shown to be essential for cell viability (Hanes et al., 1989). Analysis of a temperature sensitive mutant demonstrated that cells lacking ESS1 become multibudded, without any apparent defects in nuclear division. These results suggested that Ess1 may play a critical role in cytokinesis in Saccharomyces cerevisiae. In contrast, subsequent work argued that inactivation of ESS1 leads to mitotic arrest and nuclear fragmentation, and similar results were observed upon depletion of Pin1 in HeLa cells using antisense RNA (Lu et al., 1996). More recent work has suggested that depletion of Pin1 in HeLa cells using siRNA does not lead to cell cycle arrest (van der Horst and Khanna, 2009; Xu and Manley, 2007). The different phenotypes reported in these studies could be a consequence of variable depletion or inactivation efficiencies.
1.5.2 Pin1 contains a C-terminal peptidyl prolyl isomerase domain and an N-terminal WW domain

The C-terminal region of Ess1 and Pin1 encodes a peptidyl prolyl cis/trans isomerase (PPIase) domain, which catalyzes the conversion of peptide bonds preceding proline between the cis and trans conformations (Lu et al., 1996; Ranganathan et al., 1997). This has the effect of locally and/or globally changing the conformation of the target protein (Figure 1.10), which can regulate a variety of properties including enzymatic activity, subcellular localization, binding affinity, and stability (Lu and Zhou, 2007). Screening of peptide libraries revealed that Pin1 specifically isomerizes phosphoserine-proline (pS-P) and phosphothreonine-proline (pT-P) peptide bonds (Yaffe et al., 1997). Given that Pin1 had been implicated in proper cell division (Hanes et al., 1989; Lu et al., 1996), this work raised the intriguing possibility that Pin1 may act in a mitosis-specific manner in response to mitotic phosphorylation at S/T-P motifs. Indeed, subsequent work demonstrated that Pin1 interacts with many proteins in a mitosis-specific and phosphorylation dependent manner. It should be noted that Pin1 does not interact with all pS/T-P motifs, suggesting that additional determinants for recognition exist. These could include preferences for specific amino acids flanking the pS/T-P motif (Yaffe et al., 1997) and/or specific structural determinants (Lu and Zhou, 2007).
Figure 1.10 Regulation of pS/T-P motifs by Pin1-mediated isomerization.

Upon phosphorylation by a proline-directed kinase (such as CDK1), pT-P (and pS-P) motifs can be recognized by Pin1, which catalyzes the interconversion between the trans and the cis conformations. These conformations may have diverse functions due to different enzymatic activities, subcellular localizations, stabilities, or affinities for binding partners. Adapted from (Lu and Zhou, 2007).
Ess1 and Pin1 also contain an N-terminal WW domain, which is characterized by two invariant tryptophan residues (Lu et al., 1996). This domain is found in many proteins, where it serves to mediate protein-protein interactions. This usually occurs via binding to proline rich motifs in target proteins (Ilsley et al., 2002). However, the WW domain of Pin1 acts as a phosphoserine/phosphothreonine recognition module that specifically interacts with pS/pT-P motifs (Lu et al., 1999b). Nearly all mitotic Pin1-binding proteins interacted with the isolated WW domain but could not bind the isolated PPIase domain, arguing that the WW domain of Pin1 serves as the substrate recognition module (Lu et al., 1999b). Consistent with this notion, single Y23A or W34A point mutations within the WW domain of Pin1 (which did not have drastic effects on PPIase activity) completely abolished its ability to interact with mitotic proteins. However, some point mutations within the PPIase domain (that inactivated the catalytic activity of Pin1) also significantly impaired the ability of Pin1 to associate with mitotic proteins. This suggests that the PPIase domain may contribute to substrate binding in the context of full length Pin1 (Shen et al., 1998).

In substrates that only contain a single pS/pT-P motif, substrate recognition and isomerization are thought to occur at the same site (Lu and Zhou, 2007). In contrast, the WW and PPIase domains may act on different sites in proteins that contain more than one pS/pT-P motif. Likewise, these domains may act on different proteins within a multiprotein complex if multiple pS/pT-P motifs are present. Structural studies suggest that the WW domain only recognizes pS/pT-P motifs in the trans conformation (Verdecia et al., 2000; Wulf et al., 2005). Therefore, Pin1 is thought to catalyze the trans to cis conversion in substrates with only one pS/pT-P motif. The presence of multiple pS/pT-P motifs would allow Pin1 to be targeted to the substrate via one pS/pT-P motif in the trans conformation, and catalyze the cis to trans (or trans to cis) conversions at other pS/pT-P motifs.

1.5.3 Regulation of Pin1 activity

Since Pin1 interacts with its substrates in a phosphorylation dependent manner, substrate phosphorylation provides a key means of regulating Pin1 activity. In addition, Pin1 is predominantly sequestered in the nucleus until the breakdown of the nuclear envelope at the
onset of mitosis, providing an additional layer of regulation (Lu et al., 1996). Pin1 itself is also regulated by post-translational modifications. Phosphorylation at Ser16 of the WW domain impairs the ability of Pin1 to interact with its substrates (Lu et al., 2002). This post-translational modification appears to be most prominent during interphase, and decreases during mitosis. In addition, Plk1 phosphorylates Pin1 at Ser65, which is thought to increase Pin1 stability (Eckerdt et al., 2005).

1.5.4 Pin1 and cell division

Pin1 associates with several key mitotic regulators at the onset of mitosis. Pin1-mediated isomerization is thought to regulate the activation of Cdc25, the phosphatase that plays a key role in CDK1 activation (Shen et al., 1998; Stukenberg and Kirschner, 2001). Likewise, Pin1 plays an important role in the inactivation of Wee1 kinase at the beginning of mitosis (Okamoto and Sagata, 2007). Pin1 is also thought to stabilize early mitotic inhibitor 1 (eml1), which plays an important role in facilitating the accumulation of cyclinB in G2 via inhibition of the anaphase promoting complex (APC) (Bennis et al., 2007). Finally, Pin1 mediates chromosome condensation by stimulating the mitotic phosphorylation of topoisomerase IIα (Xu and Manley, 2007).

Recent work has also implicated Pin1 in the late stages of cell division (van der Horst and Khanna, 2009). Depletion of Pin1 in HeLa cells impaired abscission of the midbody at the terminal stage of cytokinesis, and MEFs from Pin1 knockout mice exhibited similar defects. This study also argued that Pin1 associates with the midbody component Cep55 and mediates its phosphorylation by Plk1. This may be important for the stability of Cep55 during mitosis (van der Horst et al., 2009).

1.5.5 Pin1 and other cellular processes

Pin1 has also been implicated in the regulation of other stages of the cell cycle (reviewed in (Yeh and Means, 2007)). In addition, it plays key roles in numerous other cellular processes, which is not surprising given that proline-directed phosphorylation occurs in many contexts other than
cell cycle regulation. For example, Pin1 facilitates the stabilization and activation of the tumor suppressor p53 in response to genotoxic insult (Zacchi et al., 2002; Zheng et al., 2002). Pin1 is highly expressed in the brain, where it regulates a variety of neuronal proteins. These include tau (Lu et al., 1999a) and amyloid precursor protein (APP) (Pastorino et al., 2006), both of which play key roles in the pathogenesis of Alzheimer’s disease. Since the primary focus of this thesis is cell division, the role of Pin1 in other cellular processes will not be discussed in detail.

1.5.6 **Pin1 knockout mice**

Pin1 knockout mice are viable (Fujimori et al., 1999), which was quite surprising given that disruption of Pin1 in *Saccharomyces cerevisiae* and human cells caused defects in cell division (Hanes et al., 1989; Lu et al., 1996). However, more recent work has demonstrated that mice have a second functional Pin1 isoform, called Pin1L, which is encoded by a different gene (Zhu et al., 2007). In humans, Pin1L is non-functional due to a shift in the reading frame (Campbell et al., 1997). Consequently, it has been suggested that Pin1L may at least partially compensate for Pin1 in Pin1 knockout mice (Lu and Zhou, 2007; Zhu et al., 2007). This could account for the lack of a more severe phenotype upon Pin1 disruption in mice compared to *Saccharomyces cerevisiae* and human cells. Subsequent work demonstrated that Pin1 deficient mice exhibit severe fertility defects as a consequence of impaired proliferation of primordial germ cells (Atchison et al., 2003). In addition, Pin1 null mice develop age-dependent neurodegeneration and exhibit both motor and behavioural defects (Liou et al., 2003).

1.5.7 **Pin1 and disease**

As in the case with SEPT9, alterations in Pin1 expression are associated with several types of cancers (Bao et al., 2004; Wulf et al., 2004; Yeh and Means, 2007). How Pin1 contributes to cancer appears to be quite complex and is not completely understood. Some studies suggest that Pin1 acts as an oncogene, whereas others propose that it acts as a tumor suppressor (Yeh and Means, 2007).
Pin1 has also been implicated in neurodegenerative diseases. Like SEPT4 (Ihara et al., 2003; Ihara et al., 2007), Pin1 forms a complex with α-synuclein, and localizes to Lewy bodies in the brains of Parkinson’s disease patients (Ryo et al., 2006). As is seen with SEPT1, SEPT2, and SEPT4 (Kinoshita et al., 1998), Pin1 accumulates in neurofibrillary tangles in the brains of Alzheimer’s disease patients (Lu et al., 1999a). Pin1 knockout mice exhibit age-dependent neurodegeneration and their neurons have characteristics of neurofibrillary tangles, suggesting that Pin1 plays an important role in the pathogenesis of Alzheimer’s disease (Liou et al., 2003). This likely reflects the ability of Pin1 to regulate tau (Lu et al., 1999a) and APP (Pastorino et al., 2006).

1.6 Hypotheses

While several studies have implicated the septin family of proteins in mammalian cell division, it remains unclear whether these proteins work together to perform the same function or whether they have different roles during this process. I hypothesized that individual septins, and perhaps even individual isoforms of a given septin, do not play identical roles during cell division.

A link between mammalian septins and the cell cycle machinery has been postulated for some time. Recent high-throughput screens to identify mitotic phosphoproteins suggested that some septins may be phosphorylated at putative CDK1 sites upon mitotic entry. I therefore hypothesized that mitosis-specific phosphorylation by CDK1 serves as a mechanism to regulate septin function during cell division.
2 Materials and Methods

2.1 Nomenclature

Septin gene and protein names are presented according to the current conventions for naming human septins (Hall et al., 2008). That is, human protein names are capitalized, whereas human gene names are capitalized and italicized. For septin genes that produce multiple transcripts, an underscore and a ‘v’ followed by a number is added as a suffix to the gene name, with ‘_v1’ corresponding to the largest variant. For the protein product of a given transcript, an underscore and an ‘i’ followed by the transcript number is added as a suffix to the protein name. For example, $\text{SEPT9}_v1$ encodes $\text{SEPT9}_i1$. Nomenclature for mouse genes is identical to that described above, except only the first letter is capitalized.

2.2 Cell Culture

HeLa, human embryonic kidney (HEK293), and retinal pigment epithelial (ARPE-19) cells were obtained from ATCC. All cells were grown at 37°C in a humidified incubator with 5% CO₂. HeLa and HEK293 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Wisent) with 10% fetal bovine serum (FBS) (Wisent), whereas ARPE-19 cells were grown in DMEM/F-12 (Lonza) with 10% FBS (Lonza). SF21 insect cells were cultured in suspension in a 1:1 mixture of Grace’s media and Sf-900II SFM (Gibco) at room temperature. Cells were arrested in mitosis by adding nocodazole (Sigma) to the culture medium to a final concentration of 50 ng/mL, and incubating for approximately 16 hours. To enrich for the later stages of cell division, nocodazole-arrested cells were plated on Poly-D-lysine coated dishes, washed extensively after attachment, and lysed at the indicated time after release. For Cdk1 inhibition, roscovitine (Sigma) was added to a concentration of 25 µM for the final three hours of the nocodazole treatment.
2.3 Western Blotting

Samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. Blots were blocked in phosphate buffered saline (PBS) with 0.05% Tween-20 (PBS-T) containing 5% milk for at least one hour, and incubated with the following primary antibodies diluted in PBS-T containing 1% milk: LexA (Invitrogen), SEPT9 (Surka et al., 2002), SEPT11 (Huang et al., 2008), SEPT2 (Xie et al., 1999), SEPT6 (Huang et al., 2008), SEPT7 (a gift from Dr. B. Zieger), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Millipore), Flag (Sigma), His (Covance), CyclinB (Santa Cruz), green fluorescent protein (GFP) (Santa Cruz), Cdk1 (Calbiochem), Pin1 (Santa Cruz), Mpm-2 (Upstate), pThr-Pro (Cell Signaling), and RalB (Cell Signaling). All primary antibodies were incubated for at least one hour, and the RalB and pThr-Pro antibodies were incubated overnight at 4°C. For the pThr-Pro antibody, all solutions were diluted in Tris buffered saline with 0.1% Tween-20 (TBS-T) instead of PBS-T. Following extensive washing, blots were incubated for one hour with appropriate horse radish peroxidase (HRP) - conjugated secondary antibodies (Biorad or Jackson) diluted in PBS-T, washed thoroughly, incubated with Western Lightning Chemiluminescence Reagent Plus (PerkinElmer), and exposed to film.

2.4 Constructs

All constructs were verified by sequencing after polymerase chain reaction (PCR) amplification or mutagenesis. All constructs obtained from others were also verified by sequencing.

2.4.1 SEPT9 Constructs

pFB-HTA SEPT9_v3 (accession number NM_006640), pFBΔH SEPT9_v1 (accession number AF189713), and pFBHTA SEPT9_v4 (accession number NM_001113494.1) were obtained from Dr. C. Froese. Each of these constructs bears a silent point mutation that destroys the single EcoR1 site present in all SEPT9 isoforms.
2.4.1.1 Generation of SEPT9_i3 T24A constructs

Threonine 24 of human SEPT9_i3 was mutated to alanine by site directed mutagenesis using pFB-HTA SEPT9_v3 as the template and the following primers:

5’ GAG ACA CCC AAC TCC GCC CCA CCC CGG AGG GTC 3’
5’ GAC CCT CCG GGG TGG GGC GGA GTT GGG TGT CTC 3’

The DNA sequence encoding SEPT9_i3 WT and T24A was then subcloned into FB-HTA, pEGFP-C, pcDNA3.1 Flag-N, and pcDNA3.1 Myc-N using EcoR1 and XhoI.

2.4.1.2 Generation of pEGFP-C SEPT9_i3 T24D

Threonine 24 was also mutated to aspartic acid by site directed mutagenesis using pFB-HTA SEPT9_v3 as the template and the following primers:

5’ GAGACACCCCAACTCCGACCCACCCCGAGGGTC 3’
5’ GACCCTCCGGGTGAGTTGGGTGTCTC 3’

The DNA sequence encoding SEPT9_i3 T24D was then subcloned into pEGFP-C using EcoR1 and XhoI.

2.4.1.3 Generation of pEGFP-C SEPT9_i3 T24E

Threonine 24 was also mutated to glutamic acid by site directed mutagenesis using pFB-HTA SEPT9_v3 as the template and the following primers:

5’ GAGACACCCCAACTCCGACCCACCCCGAGGGTC 3’
5’ GACCCCTCCGGGTGAGTTGGGTGTCTC 3’

The DNA sequence encoding SEPT9_i3 T24E was then subcloned into pEGFP-C using EcoR1 and XhoI.

2.4.1.4 Generation of constructs used to make inducible stable cell lines

Sequences encoding human SEPT9_i3, SEPT9_i3 T24A, SEPT9_i1, and SEPT9_i4 were made siRNA-resistant by introducing three silent point mutations into the siRNA target sequence by
site-directed mutagenesis using pEGFP-C \textit{SEPT9} v3, pEGFP-C \textit{SEPT9} v3 T24A, pFBΔH \textit{SEPT9} v1, or pFBHTA \textit{SEPT9} v4 as the template and following primers:

5’ GATCAAGTCCATCACGCACGACATCGAAGAAAGGCGTCCGGATG 3’
5’ CATCCGGACGCCTTTCTCTTTCGATGTCGTGCGTGGACTTGATC 3’

The Kozak, Flag, and \textit{SEPT9} v3 sequences were amplified from Flag-N \textit{SEPT9} v3 by PCR using the following primers:

5’ GGTACGCGGCGCCGCCACCATGGACTACAAGGACGAC 3’
5’ GCCGCCAATTGCTGAGCTACATCTCCGGAGGTTC 3’

This sequence was then cloned into pRetroX-Tight-Pur with Not1 and Mfe1, generating pRetroX-Tight-Pur Flag-\textit{SEPT9} v3 (diagrammed below).

\textbf{Figure 2.1 Schematic of pRetroX-Tight-Pur Flag-SEPT9_v3.}

The single EcoR1 and Sbf1 sites are indicated, as is T24 and the siRNA target sequence. Numbers indicate amino acid position of \textit{SEPT9}_i3.
pRetroX-Tight-Pur Flag-SEPT9_v3 was then cut with EcoR1 and Sbf1, and this sequence was replaced with the corresponding EcoR1-Sbf1 fragment that was cut out of pEGFP-C SEPT9_v3 siRNA resistant, pEGFP-C SEPT9_v3 T24A siRNA resistant, pFBΔH SEPT9_v1 siRNA resistant, or pFBHTA SEPT9_v4 siRNA resistant. This generated pRetroX-Tight-Pur-Flag-SEPT9_v3 siRNA-resistant, pRetroX-Tight-Pur-Flag-SEPT9_v3 T24A siRNA-resistant, pRetroX-Tight-Pur-Flag-SEPT9_v1 siRNA-resistant, and pRetroX-Tight-Pur-Flag-SEPT9_v4 siRNA-resistant.

2.4.1.5 Generation of pEGFP-C SEPT9 N-term

The DNA sequence encoding amino acids 8-146 of SEPT9_i3 (which corresponds to the sequence common to SEPT9_i1 and SEPT9_i3 that is absent from SEPT9_i4) was amplified by PCR using the following primers:

5’ GCGCGAATTCGCCTTGAAAAGATCTTTTGAGGTCG 3’
5’ TATACTCGAGTTACCTCCGGTGGGCTGACTCCTG 3’

This sequence was cloned into pEGFP-C using EcoR1 and Xho1.

2.4.2 Pin1 Constructs

GST-Pin1, GST-Pin1^{Y23A}, and GST-Pin1^{WW} were obtained from Dr. D. Litchfield (Messenger et al., 2002).

2.4.3 Plk1 Constructs

Human Plk1 cDNA was obtained from ATCC (IMAGE ID 3854860), and the Polobox domain (PBD, amino acids 371-603; (Elia et al., 2003)) was amplified by PCR using the following primers:

5’ GGTACGAATTCGACTGCCACCTCAGTGACATGCTG 3’
5’ GCCGCCTCGAGTTAGGAGGCCTTGAGACGGTTGCT 3’
This sequence was then cloned into pGEX-6P using EcoRI and XhoI. H538A and K540M mutations were introduced by site-directed mutagenesis using the following primers (generating Plk1 PBD mut, which cannot bind to phosphorylated ligands; Lowery et al., 2007):

5’ GATCAACTTTCCAGGATGCCACCATGCTCATCTTGTGGCCCACTG 3’
5’ CAGTGGGCCCAAGATGACATGGTGCCATCCTGGAAGAAGTTGATC 3’

2.4.4 RalB Constructs

pRK5Myc-RalB and pRK5Myc-RalB S28N were obtained from Dr. J. Camonis (Cascone et al., 2008).

The long isoform of human RalGPS1A (GI # 21708077) was obtained from SIDNET at the Hospital for Sick Children, and amplified by PCR using the following primers:

5’ GGTACGAATTCCATGTACAAGAGGAATGGTCTGATG 3’
5’ GCCGCCTCGAGTCAAGTGATCCTCCTGCTTCGGCT 3’

This sequence was cloned into Flag-N and pEGFP-C using EcoRI and XhoI.

2.5 Antibody Specificity

Yeast expressing LexA-tagged mammalian SEPT2 to SEPT12 and SEPT14 were described previously (Steels et al., 2007). Mouse SEPT1 (NP_059489.2) was cloned into pEG202, and a yeast strain that expresses LexA-tagged mouse SEPT1 was generated as described previously (Steels et al., 2007). Each strain was grown overnight in 3 mL of YNB media at 30°C with shaking. The cultures were then diluted to an optical density (600 nm) of 0.1 with yeast nitrogen base (YNB), and grown at 30°C with shaking to an optical density of 0.5. Cells were pelleted and resuspended in 500 µL of 2X SDS-PAGE loading buffer with dithiothreitol (DTT), frozen on dry ice, and boiled. The freeze-thaw procedure was repeated twice. Cell lysates were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted as indicated.
2.6 siRNA Treatment

The following double stranded RNAs were ordered from Dharmacon with a dTdT overhang and dissolved in diethyl pyrocarbonate (DEPC)-treated water to a concentration of 40 pmol/μL and stored at -80°C in working aliquots:

- Human SEPT2: GAATATTGTGCCTGTCATTG
- Human SEPT6: CCTGAAGTCTCTGACCTAGT
- Human SEPT7: TATATGCTGCCTGAATGGAA
- Human SEPT9: GCACGATATTGAGGAGAAA
- Human SEPT11: CAAGAGGAATTGAAGATTTAAA
- Human Pin1 (Xu and Manley, 2007): GCCATTTGAAGACGCCTCG
- Control siRNA: GCAGCGACCATGAGTATCA

The following double stranded RNAs were obtained from Qiagen and stored as above:

- Human Cdk1 (hs_Cdc2_10 HP): AAGGGGTTCTTAGTACTGCAA
- Human Pin1 #2 (hs_PIN1_5 HP): CCGCCAGAT TCTCCCTAA

Cells grown to 60-80% confluence in 6-well plates were transfected with double stranded siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Briefly, 3 μL of Lipofectamine 2000 was added to 200 μL of serum free DMEM and allowed to incubate for 5 min. This was then added to a mixture of 200 μL of serum free DMEM and 120 pmol double stranded RNA, incubated for another 20 min, and was subsequently added to one well of a 6-well plate. Five hours later, cells were washed and replated as required. Knockdown was achieved after at least 56 hours for septins and Cdk1, and 72 hours for Pin1. For siRNA treatment of stable cell lines, antibiotics were removed prior to transfection.

2.7 Immunofluorescence Microscopy

For analysis of cytokinetic defects, cells were washed with IsoNaCl (125 mM NaCl, 20 mM HEPES (pH 7.4), 3 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂ and 5 mM glucose) warmed to 37°C, and fixed with 4% paraformaldehyde in IsoNaCl for 30 minutes at room temperature. After washing four times with PBS, cells were incubated in PBS with 100mM glycine for 10 minutes,
washed extensively, permeabilized for 20 minutes with 0.1% TX-100 in PBS with 1% bovine serum albumin (BSA), and blocked for at least one hour with 3% BSA in PBS. Cells were then incubated with α-tubulin (Sigma) antibody diluted 1:100 in PBS with 0.5% BSA for at least an hour. Following extensive washing, cells were incubated in the dark with goat anti-mouse Cy3 (Jackson) diluted 1:1000 in PBS with 0.5% BSA for at least one hour, followed by incubation with 2µg/mL Hoescht (Fluka) in PBS for two minutes. After washing, coverslips were mounted on slides using DAKO fluorescent mounting medium. Any cells connected via a microtubule bridge, as judged by α-tubulin staining (Sigma), were counted as being connected by a midbody. Any cell having more than one nucleus, as judged by Hoescht staining, was counted as being multinucleated. For analysis of the localization of septins and midbody components, cells were fixed for 5 minutes at 37°C with 2% paraformaldehyde in PBS, inactivated for 15 minutes at 37°C with 25 mM glycine and 25 mM ammonium chloride in PBS, permeabilized for 15 minutes at room temperature with 0.2% Triton X-100 in PBS, and blocked for one hour in PBS with 0.1% saponin, 1% horse serum and 2% BSA. Cells were then incubated with the following primary antibodies: Sec8 (Abcam), Map4 (Transduction Laboratories), Plk1 (Zymed), SEPT2 (Xie et al., 1999), SEPT7 (Santa Cruz), SEPT9 (Surka et al., 2002), SEPT11 (Huang et al., 2008), VAMP8 (a gift from Dr. T. Weimbs), RalB (Cell Signalling), and α-tubulin (Sigma) or α/β-tubulin (Cell Signalling). For Cep55, cells were transfected with GFP-Cep55 (a gift from Dr. K. Kutsche) two days after treatment with control or SEPT9 siRNA. For ROCKII, cells were fixed with methanol, blocked in PBS with 10% FBS, and incubated with ROCKII antibody (Upstate). All steps after incubation with the primary antibody were the same as those described above. Cells were imaged using a Leica DMIRE2 inverted fluorescence microscope equipped with a Hamamatsu Back-Thinned EM-CCD camera and spinning disk confocal scan head. The unit was equipped with 4 separate diode-pumped solid state laser lines (Spectral Applied Research: 405 nm, 491 nm, 561 nm, 638 nm), an ASI motorized XY stage, an Improvision Piezo Focus Drive and a 1.5X magnification lens (Spectral Applied Research). The equipment was driven by Volocity acquisition software, and powered by an Apple Power Mac G5.
2.8 Time-Lapse Microscopy

HeLa and ARPE-19 cells treated with the indicated siRNA were imaged in HPMI (RPMI powder [Gibco/Invitrogen] supplemented with 15 mM sodium chloride and 20 mM HEPES, pH 7.4) at 37°C on a Nikon TE2000 inverted microscope adapted with a Solent environmental chamber. A Nikon Plan-Fluor 40X/0.6NA objective was employed in combination with a Hamamatsu Orca AG CCD camera. The Improvision Volocity 4.2 software suite was used for image acquisition, analysis and manipulation. The time from the beginning of chromosome segregation to midbody abscission was determined for each cell that started division within the first 24 hours of imaging. Cells that were multinucleated prior to division were excluded from analysis.

2.9 Generation and Characterization of Stable Cell Lines

Stable cell lines were generated using the Retro-X Tet-On Advanced Inducible Expression System (Clontech). pRetroX-Tet-On-Advanced was transfected into FLYRD18 packaging cells (a gift from Dr. C. Tailor) using the calcium phosphate method, and the media was changed 24 hours later. After an additional 24 hours, the virus containing media was filtered (using a Pall 0.2 µm HT Tuffryn filter), and polybrene (Sigma) was added to 8µg/mL. The virus containing media was then added to wild type HeLa cells, and the media was subsequently changed 4-6 hours after infection. Two days later, G418 was added to a final concentration of 500 µg/mL. Individual colonies were expanded and screened for the presence of the Tet-Advanced transactivator by Western blotting with the TetR antibody (Clontech). This yielded the parent HeLa Tet-On cell line. Transcripts encoding Flag-SEPT9_i3 and Flag-SEPT9_i4 were made siRNA-resistant by introducing three silent point mutations into the siRNA target sequence, and were subsequently cloned into pRetroX Tight Pur (Clontech). These plasmids were then individually transfected into FLYRD18 packaging cells and the resulting viruses were used to infect the parent HeLa Tet-On cell line (which was maintained in 250 µg/mL G418), as described above. Two days after virus infection, puromycin was added to a final concentration of 1 µg/mL. Individual colonies were expanded and screened for the presence Flag-SEPT9_i3 or Flag-SEPT9_i4 by induction with various amounts of doxycycline and Western blotting with an anti-Flag (Sigma) or anti-SEPT9 antibody. The resulting cell lines, which inducibly express
siRNA-resistant SEPT9_i3, or SEPT9_i4 were maintained in DMEM with 10% FBS, 1% antibiotics, 500 µg/mL G418, and 1 µg/mL puromycin.

2.10 Generation and Testing of a SEPT9 Monoclonal Antibody (10C10)

Human septins 1, 2, 5, 6_i2, 7, 8, 9_i1, 11 and 12_i2 were cloned into pFastBacHTa (Invitrogen) using the 5’ EcoRI and 3’ XhoI sites. Silent mutations were first introduced into any internal EcoRI or XhoI sites in the septins. Baculoviruses were prepared according to manufacturer’s instructions and used to infect 50 mL of suspended SF21 cells for 144 hours. These cells were pelleted and resuspended in lysis buffer (50 mM HEPES, pH 7.5, 200 mM magnesium sulfate, 200 mM ammonium sulfate, 150 mM sucrose (Low and Macara, 2006) and 25 mM imidazole). Immediately before French pressing, protease inhibitors were added. The lysates were centrifuged at 20,000 g for 20 minutes and the supernatants were incubated with 250 µl nickel-nitrilotriacetic acid agarose (Qiagen) for 1 hour rotating end over end. The beads were pelleted and washed three times in lysis buffer before being transferred to a column, and the proteins were eluted with lysis buffer containing 500 mM imidazole. All steps were performed on ice or at 4°C. The proteins were quantified using Biorad protein reagent and examined for purity by SDS-PAGE and Coomassie staining. Monoclonal antibodies were prepared by the Monoclonal Antibody Facility at the Hospital for Sick Children, using 5 mL of a 0.5 mg/ml septin mixture containing each of the septins described above. Sixteen hybridomas recognizing the antigens were screened for reactivity with individual septins, the His-S tag present on each of the septins, and SF21 cell lysate. One hybridoma (10C10) produced antibody specific to SEPT9. For Western blotting, approximately 10 ng of each recombinant septin was analyzed using 10C10 at 1:500.
2.11 Mapping of the 10C10 epitope

The DNA sequence encoding amino acids 8-39 of SEPT9_i3 (which I call Epia) was amplified by PCR using the following primers:

5’ GCGCGAATTCCGCTTGAAGAAGATCTTTTGAGGTCG 3’
5’ TATACTCGAGCTAGGCCACAGTGCGTCGGAGTAGG 3’

The DNA sequence encoding amino acids 8-95 of SEPT9_i3 (which I call Epiab) was amplified by PCR using the following primers:

5’ GCGCGAATTCCGCTTGAAGAAGATCTTTTGAGGTCG 3’
5’ TATACTCGAGCTAGTCAATGGACAGCTCAGTGCGC 3’

The DNA sequence encoding amino acids 40-95 of SEPT9_i3 (which I call Epib) was amplified by PCR using the following primers:

5’ GCATGAATTCAGCTCCACCCAGAAATTCCAGGACC 3’
5’ TATACTCGAGCTAGTCAATGGACAGCTCAGTGCGC 3’

The DNA sequence encoding amino acids 40-146 of SEPT9_i3 (which I call Epibc) was amplified by PCR using the following primers:

5’ GCATGAATTCAGCTCCACCCAGAAATTCCAGGACC 3’
5’ TATACTCGAGTTACCTCCGGTGGGCTGACTCCTG 3’

The DNA sequence encoding amino acids 96-146 of SEPT9_i3 (which I call Epic) was amplified by PCR using the following primers:

5’ GCATGAATTCATCTCGTCCAAGCAGTGGAAGACG 3’
5’ TATACTCGAGTTACCTCCGGTGTTGCTGACTCCTG 3’

Each sequence was cloned into His-Myc-SUMO pQE30 with EcoR1 and Xho1, and transformed into BL21 magic cells. Bacteria expressing the fusion protein of interest were grown overnight in terrific broth (TB) containing Amp and Kan. 2.5 mL of this starter culture was diluted to 50 ml of TB containing Amp and Kan and grown for 30 min at 37°C. 100µL of uninduced culture was collected, spun down, and resuspended in 50 µL of SDS-PAGE loading buffer containing
DTT. The remaining culture was induced with 0.4 mM IPTG for 2.5 hours. 50 µL of induced culture was collected, spun down, and resuspended in 50 µL of SDS-PAGE loading buffer containing DTT. 10 µL of each lysate was subjected to SDS-PAGE and Coomassie staining, whereas 5 µL of each sample was subjected to Western blotting with 10C10.

2.12 Immunoprecipitation

Cells were harvested by scraping (interphase) or shakeoff (mitotic) in fresh culture media, pelleted by centrifugation, and resuspended in TX-100 lysis buffer (30 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM ethylene glycol tetraacetic acid (EGTA), 1% TX-100, 20 mM NaF) with phosphatase (1 mM sodium orthovanadate, 100 nM okadaic acid, and 100 nM calyculin A) and protease (1 µg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM benzamidine, and 1 µg/mL pepstain A) inhibitors. Cells were lysed by passing through a 27.5-gauge needle 3-5 times and rotated for 15 minutes at 4°C. Lysates were clarified by centrifugation at 13000 g for 10 minutes, and the supernatant was used for immunoprecipitations. 1-4 µg of antibody was added to approximately 1 mg of lysate and incubated at 4°C with constant mixing for at least one hour. 30-50 µL of protein A sepharose (Sigma) or protein G sepharose (GE Healthcare) was washed twice with TX-100 lysis buffer, added to the antibody-lysate mixture, and incubated at 4°C with constant mixing for at least one hour. The beads were then washed three times with TX-100 lysis buffer, resuspended in SDS-PAGE loading buffer containing DTT or n-ethylmaleimide (NEM), and subjected to SDS-PAGE and Western blotting.

2.13 Protein Purification from Bacteria

Bacteria expressing the glutathione S-transferase (GST) fusion protein of interest were inoculated in 5 mL of LB containing 100 µg/mL ampicillin (LB Amp) or LB containing 100 µg/mL ampicillin and 50 µg/mL kanamycin (LB Amp Kan - for BL21 Magic) and grown overnight at 37°C (BL21 for GST-Pin1 and its variants, and BL21 Magic for GST-Plk1 and its variants). This starter culture was then diluted into 500 mL of LB Amp (or Amp Kan for BL21 Magic) and grown at 37°C until the O.D.\textsubscript{600nm} reached 0.8-1. Expression was induced by adding isopropyl-beta-D-thiogalactopyranoside (IPTG) as follows: 1 mM IPTG for 4 hours at 30°C for
GST-Pin1 and its variants; 0.1 mM IPTG for 3 hours at 37°C for GST-Plk1 and its variants. Bacteria were centrifuged at 6000 g for 10 minutes. For long term storage bacterial pellets were frozen at -80°C at this stage. Bacterial pellets were suspended in 20 mL of resuspension buffer (25 mM HEPES, pH 7.8, 100 mM NaCl, 5 mM MgCl₂, 0.05% Tween-20, 1 mM DTT, and protease inhibitors), and lysed using a French Press. Lysates were centrifuged at 15,000 g for 10 minutes, and the supernatant was added to 1 mL of glutathione agarose beads (50% slurry in resuspension buffer). This mixture was rotated end-over-end for at least 1.5 hours. The beads were then washed 3 times with resuspension buffer (lacking protease inhibitors), and stored at 4°C as a 50% slurry (in resuspension buffer) for up to 4 weeks. To elute GST-Pin1 for Far Western analysis, beads were first washed three times with 50 mM Tris, pH 8.0, 1 mM DTT, and then incubated with 1 mL elution buffer (50 mM Tris, pH 8.0, 1 mM DTT, 20 mM reduced glutathione) for one hour at 4°C with constant mixing. The sample was dialyzed with 50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM DTT, and frozen at -80°C until use.

### 2.14 Pulldown Analysis

Interphase cells were washed with TBS, and harvested by scraping in Tris lysis buffer (50 mM Tris, pH 8.0, 140 mM NaCl, 10% glycerol, 1% TX-100, 1 mM DTT, 100 mM NaF) containing phosphatase (1 mM sodium orthovanadate, 100 nM okadaic acid) and protease (1 μg/mL leupeptin, 1 mM PMSF, 2 mM benzamidine, and 1 μg/mL pepstatin A) inhibitors. Fresh media was added to mitotic cells, which were subsequently harvested by shakeoff, washed with TBS, and resuspended in Tris lysis buffer. Cells were then lysed by passing through a 27.5-gauge needle 4-5 times and rotated for 45 minutes at 4°C. Lysates were clarified by centrifugation at 4000 g for 5 minutes, and the supernatant was used for pulldown experiments. In some cases, 50 U/mL calf intestinal phosphatase (CIP, New England Biolabs) was added to the lysate for 30 minutes at 30°C prior to pulldown analysis. Equal amounts of glutathione-agarose beads complexed with GST, GST-Pin1, GST-Pin₁ᵀ₂₃⁻, GST-Plk1 PBD, or GST-Plk1 PBD mut were added to 0.5-1 mg of cell lysate, and incubated for 1.5 hours at 4°C with continuous mixing. The beads were subsequently washed four times with Tris lysis buffer, resuspended in SDS-PAGE loading buffer and subjected to Western blotting.
2.15 Transfections

GFP, Myc, and Flag-tagged constructs were transfected into HeLa cells using Fugene6 (Roche) or JetPrime (PolyPlus) according to the manufacturer’s instructions. Protein expression was permitted for approximately 24 hours before treatment with or without nocodazole.

2.16 Protein Purification from Insect Cells

Sf21 insect cells growing in suspension were infected with baculovirus expressing His\textsubscript{6}-SEPT9\textsubscript{i3}, His\textsubscript{6}-SEPT9\textsubscript{i4}, or His\textsubscript{6}-SEPT9\textsubscript{i3} T24A, and incubated for approximately six days. Cells were pelleted by centrifugation, resuspended in isolation buffer (40 mM Tris, pH 7.4, 100 mM NaCl, 2 mM β-mercaptoethanol, 20% glycerol, and 25 mM imidazole) with protease inhibitors (1 µg/mL leupeptin, 1 mM PMSF, 2 mM benzamidine, and 1 µg/mL pepstatin A), and lysed by French Press. Lysates were clarified by centrifugation at 22,000 g for 30 minutes at 4˚C, and the supernatant was added to washed Ni-NTA agarose beads (Qiagen) and incubated for 1.5 hours at 4˚C with constant mixing. The beads were then washed extensively with isolation buffer and bound protein was eluted in 40 mM Tris, pH 7.4, 100 mM NaCl, 2 mM β-mercaptoethanol, 20% glycerol, and 500 mM imidazole. Purified protein was stored at -80˚C until use.

2.17 Kinase Assays

5 µL of 5X reaction buffer (40 mM MOPS, pH 7.0, 1 mM ethylenediaminetetraacetic acid (EDTA)), 10 µL of adenosine-5'-triphosphate (ATP) cocktail (25 mM MgAc, 0.25 mM ATP, 1 µCi \(\text{32P}^\gamma\)–ATP (Perkin Elmer) per 10 µL of cocktail), and 2.5 µg of substrate were mixed to a final volume of 22.5 µL and warmed to 30˚C. Reactions were initiated by adding 10 ng recombinant cdk1/cyclinB (Upstate) in kinase dilution buffer (20 mM MOPS, pH 7.0, 1 mM EDTA, 5% glycerol, 0.01% Brij-35, 0.1% 2-mercaptoethanol, 1 mg/mL BSA). After incubating for 10 minutes at 30˚C with vortexing every minute, reactions were stopped by adding 5 µL of 3% phosphoric acid, spotted on MF-Membrane filters (Millipore), washed extensively with 0.75% phosphoric acid, and subjected to scintillation counting.
2.18 Far Western Analysis

HeLa cells were transfected with GFP-SEPT9_v3 or GFP-SEPT9_v3 T24A, and treated with 100 nM calyculin A (Sigma) for the final 30 minutes of the nocodazole arrest. Exogenously expressed SEPT9 was immunoprecipitated as above, subjected to SDS-PAGE, and transferred to PVDF. After overnight incubation at 4°C in PBS with 5% milk, 0.2% Tween-20, 1 mM DTT and 1 mM PMSF, the membrane was treated with 10 µg/mL GST-Pin1 in PBS with 0.05% Tween-20, 1 mM DTT and 1 mM PMSF for two hours at 4°C. Following extensive washing with PBST, GST-Pin1 bound to the membrane was detected by Western blotting.

2.19 Cell Cycle Analysis

HeLa cells (1-2 x 10^6) were suspended by trypsinization or shakeoff (for nocodazole-arrested cells), washed with Hank's Buffered Salt Solution (HBSS), and resuspended in 100 µL of staining media (HBSS + 10mM HEPES, pH 7.2, 2% FBS, 10mM NaN₃). This was added dropwise to 1 mL of ice cold 80% ethanol and allowed to fix for a minimum of 30 minutes on ice. Cells were then pelleted by centrifugation (500 g, 5 minutes) and resuspended in 500 µL of 2 mg/mL RNAse (Sigma, prepared as per manufacturers instructions and diluted in HBSS]) and allowed to incubate at room temperature for 5 minutes. Subsequently, 500 µL of 0.1 mg/mL propidium iodide (Sigma, prepared as per manufacturer’s instructions and diluted in HBSS with 0.6% NP-40) was added to cells and allowed to incubate for an additional 30 minutes at room temperature. Cells were then pelleted (500 g, 5 minutes) and resuspended in 500 µL of ice cold HBSS. Flow cytometry was performed on a Becton Dickinson FACScan and analysis was done using FloJo v8.1.0 software.

2.20 Statistical Analysis

Two-tailed student’s t-tests were applied to determine statistical significance.
3 Examination of the Role of Individual Septins During Mammalian Cell Division

The work presented in this Chapter (with the exception of Figure 1) is published in the Journal of Cell Biology (November 2010, 191:741-9). I am extremely grateful to Dr. Caterina DiCiano-Oliveira, with whom I collaborated to generate the data presented in Figures 2, 3, 7, 8, 10, and 11. The SEPT9 monoclonal antibody was generated by Dr. Carol Froese, and Margaret Bejide assisted with the analysis presented in Figure 11.

3.1 Rationale

Several studies have demonstrated that the septin family of proteins is important for mammalian cell division. However, most of these studies have treated the septin family as a whole, thus neglecting the possibility that individual septins may have diverse functions. Simultaneous depletion of SEPT2, SEPT6, and SEPT7 (and possibly others) from HeLa cells resulted in accumulation of cells with multiple or fragmented nuclei (Kremer et al., 2005; Spiliotis et al., 2005). These studies took advantage of the observation that depletion of one septin can result in the depletion of other septin family members, a phenomenon that is thought to result from destabilizing the septin complex (Kinoshita et al., 2002; Kremer et al., 2005; Tooley et al., 2009). SEPT9 depletion from HMEC and HeLa cells caused cytokinetic defects (Nagata et al., 2003; Surka et al., 2002), although it was not clear whether the expression of other septins was affected. Recent work demonstrated that SEPT2 acts as a scaffold for myosin and its kinases at the cleavage furrow, thus facilitating the full activation of myosin that is required for cleavage furrow stability (Joo et al., 2007). SEPT7 has been shown to mediate the localization of CENP-E to kinetochores, which is important for proper chromosome segregation (Zhu et al., 2008). However, it is unclear whether other septin family members within the septin complex are also important for these processes. Consequently, while it is well established that the septin family is important for mammalian cell division, it remains elusive whether these proteins act together to perform the same role, or whether they have different functions during cell division.
Since septins associate with each other to form complexes, it is generally accepted that depletion of any or all septins in a given cell type will give rise to the same phenotype. However, there are two major problems with this assumption. First, it remains to be determined whether septins stay associated in a complex during cell division. Since many proteins undergo dramatic changes in localization, structure, and function during cell division, it is possible that the septin complex breaks apart and individual septins perform diverse roles during this stage of the cell cycle. More importantly, even if the septin complex remains intact during cell division, it is possible that different proteins within the complex may have different functions.

To address these problems, I first defined the expression profile of septins in HeLa cells using a comprehensive set of septin antibodies. I then analyzed septin complex composition throughout cell division by immunofluorescence and immunoprecipitation. Lastly, I individually depleted each septin family member expressed in HeLa cells by siRNA, and assessed their contribution to cell division by immunofluorescence and time-lapse microscopy.

### 3.2 Results

#### 3.2.1 Specificity of mammalian septin antibodies

Before commencing a thorough analysis of the roles of individual septins during cell division, I needed to ensure that all of our septin antibodies are specific for the individual septin that they were designed to recognize. To this end, I generated yeast strains that express LexA-tagged fusions of SEPT1-SEPT12 and SEPT14 (Peterson et al., 2007; Steels et al., 2007). Lysates of these strains were subjected to SDS-PAGE and the specificity of each of our septin antibodies was assessed by Western Blotting. As shown in Figure 3.1, all of our septin antibodies are highly specific, and do not cross react with any other septin.
Figure 3.1 Specificity of mammalian septin antibodies.

Equal amounts of LexA-septin fusion proteins (indicated on top) were separated on a 10% SDS-PAGE gel and probed with the specified septin antibodies (indicated at right). Similar loading was confirmed by probing with a α-LexA antibody.
3.2.2 Expression profile of septins in HeLa cells

To examine the role of individual septins in cell division, I first determined the expression profile of septins in HeLa cells. Western blotting with our septin antibodies (which may not recognize all septin isoforms) showed that HeLa cells contain SEPT2, SEPT6, SEPT7, SEPT9 and SEPT11 (Figure 3.2). We did not test SEPT1, SEPT10, SEPT13, and SEPT14, as we do not have antibodies that recognize these proteins (our SEPT1 antibody is mouse-specific and does not recognize human SEPT1). SEPT12 was not tested as it has been previously shown to be exclusively expressed in testis (Steels et al., 2007), and our SEPT12 antibody is rat-specific.

![Figure 3.2 Analysis of septin expression in HeLa cells.](image)

HeLa lysate was probed for the indicated septins, and brain lysate was used as a positive control. Numbers to the right of the blot represent molecular mass standards in kilodaltons.
3.2.3 Septin distribution during cell division

While it is well established that septins form a complex in interphase cells (Kinoshita et al., 2002; Surka et al., 2002), it remains to be determined whether septin complex composition is altered upon mitotic entry. To begin to address this, I assessed the distribution of SEPT2, SEPT6, SEPT7, SEPT9, and SEPT11 during cell division (Figure 3.3). In prophase and metaphase, SEPT2 showed cortical enrichment, whereas the others had a more diffuse cytosolic distribution. All septins accumulated at the cleavage furrow during anaphase, and appeared at both sides of the intercellular bridge and the midbody in telophase. While our SEPT6 antibody had high background staining, it showed a distribution similar to SEPT11. Occasionally, septins were visualized on spindle microtubules, as reported by others (Spiliotis et al., 2005).
Figure 3.3  Septin distribution during cell division.

HeLa cells were stained for α-tubulin (red), DNA (blue), and either SEPT2, SEPT7, SEPT9 or SEPT11 (green). Confocal microscopy was performed and a single z-slice is shown (scale bar represents 17 μm).
3.2.4 Generation and characterization of a mouse monoclonal antibody that specifically recognizes SEPT9

In order to facilitate co-staining of multiple septins within the same cell, we generated a SEPT9 mouse monoclonal antibody (which we call 10C10). To test its specificity, His-tagged SEPT1, SEPT2, SEPT5-9, SEPT11 and SEPT12 were purified from insect cells and subjected to SDS-PAGE and Western Blotting with 10C10 (Figure 3.4A). This antibody specifically recognizes SEPT9 and does not cross react with any other septin tested. To provide further evidence of the specificity of 10C10 for SEPT9, HeLa cells were co-stained with Hoescht (blue), 10C10 (green), and our well-characterized SEPT9 rabbit polyclonal antibody (red) (Surka et al., 2002). As shown in Figure 3.4B, these two antibodies recognize the same structures.

Figure 3.4 Characterization of 10C10, a monoclonal antibody that specifically recognizes SEPT9.

(A) His-tagged SEPT1, SEPT2, SEPT5-9, SEPT11 and SEPT12 were purified from insect cells using a baculovirus expression system. Equal amounts of each recombinant septin were subjected to SDS-PAGE and Coomassie staining (top) or Western blotting with 10C10 (bottom). As shown, this antibody specifically recognizes SEPT9. Numbers to the right of the blots represent molecular mass standards in kilodaltons. (B) HeLa cells were co-stained with Hoescht (blue), 10C10 (green), and our SEPT9 polyclonal antibody (red). Scale bar represents 17 µm.
3.2.5 Septins colocalize at cytokinetic structures during cell division

The results presented in Figure 3.3 suggested that septins would likely colocalize throughout cell division. To address whether this is the case, I double labeled HeLa cells for SEPT9 (using 10C10) and SEPT2 or SEPT11 (Figure 3.5A-B). SEPT9 colocalized with both SEPT2 and SEPT11 at the cleavage furrow during anaphase, and at the midbody during telophase. Therefore, septins are present at the same cytokinetic structures at the same time, consistent with the possibility that they associate in a complex during cell division.

Figure 3.5 SEPT9 colocalizes with other septins at the cleavage furrow in anaphase and the midbody in telophase.

HeLa cells were double stained for SEPT9 (using 10C10) and SEPT2 (A) or SEPT11 (B). Scale bar represents 17 µm.
3.2.6 **Characterization of mitotic septin complex composition**

I next immunoprecipitated SEPT2 and SEPT9 from interphase and mitotic (nocodazole-arrested) HeLa cells, and probed for all septins. Cell cycle analysis by flow cytometry revealed that mitotic arrest was >90% efficient. As shown in Figure 3.6, all septins co-immunoprecipitated with SEPT2 and SEPT9 respectively. Therefore, septin complex composition is largely unaltered upon entry into mitosis.

![Figure 3.6](image)

**Figure 3.6 Septin complex composition is largely unaltered upon mitotic entry.**

SEPT2 (A) or SEPT9 (B) was immunoprecipitated from interphase or mitotic (nocodazole-arrested) HeLa cells, and the precipitates were probed for SEPT2, 6, 7, 9 and 11. Numbers to the right of the blots represent molecular mass standards in kilodaltons.
3.2.7 Septin complex composition at the later stages of cell division

I next sought to determine whether septin complex composition remains the same throughout cell division, as is suggested by the localization data presented in Figure 3.3. To address this, I used human retinal pigment epithelial cells (ARPE-19), which have a normal karyotype (Dunn et al., 1996) and release well from nocodazole arrest (Figure 3.7A). As shown in Figure 3.7B, no obvious differences in septin complex composition were observed when immunoprecipitations were performed from cells enriched at the later stages of cell division. However, I cannot rule out the possibility that subtle rearrangements of septin subunits or septin polymerization state occur during cell division.

Figure 3.7 Analysis of septin complex composition at the later stages of cell division.

(A) Nocodazole-arrested ARPE-19 cells were released into mitosis, and the percentage of cells at each stage of cell division was determined by α-tubulin and Hoescht staining. Approximately 10-25% of cells were either not arrested by nocodazole or could not be conclusively identified as being in a specific stage of cell division, and were excluded from the count. (B) SEPT2 was immunoprecipitated from interphase, mitotic (nocodazole arrested), or nocodazole-released ARPE-19 cells and the precipitates were probed for SEPT2, 6, 7, 9 and 11. Numbers to the right of the blot represent molecular mass standards in kilodaltons.
3.2.8 **Roles of individual septins during cell division**

While I have demonstrated that septin complex composition is largely unaltered upon mitotic entry and throughout cell division, it remains possible that different septins within the septin complex may contribute different functions. To address this, I selectively depleted each septin expressed in HeLa cells by siRNA (Figure 3.8A) and assayed for cytokinesis defects. SEPT6 knockdown (KD) had no adverse effects (Figure 3.8C), although this could reflect incomplete silencing. Depletion of SEPT2 and SEPT11 increased the number of multinucleated cells to 11% and 15% respectively, compared to 3% of cells transfected with control siRNA (Figure 3.8C). Treatment with SEPT7 siRNA reduced the levels of all septins, as reported previously (Kinoshita et al., 2002; Kremer et al., 2005; Tooley et al., 2009), and increased the number of multinucleated cells to 26% (Figure 3.8A, C). While SEPT9 KD also resulted in an increase in multinucleated cells (8%), I observed a second, more prominent cytokinetic defect (Figure 3.8B, C). Strikingly, 23% of SEPT9 KD cells remained joined by a microtubule bridge (midbody), compared to 6% of cells transfected with control siRNA. Expression of siRNA-resistant SEPT9 rescued these cytokinetic defects (see Chapter 4), demonstrating that this phenotype is specifically due to depletion of SEPT9 and is not the result of off-target effects of the siRNA. Intriguingly, this late telophase arrest was not observed upon depletion of any other septin (Figure 3.8C). These results suggest that although septins co-exist in complexes, they may have distinct functions during cell division.
Figure 3.8  Roles of individual septins in cell division.

(A) Efficiency of septin depletion by siRNA. HeLa cells were treated with the indicated siRNA, and lysates were probed as specified. Numbers to the right of the blot represent molecular mass standards in kilodaltons. (B) Representative examples of cells treated with control siRNA, and of multinucleated cells and cells attached by persistent midbodies following SEPT9 KD (red, α-tubulin; blue, DNA). Scale bar represents 17 µm. (C) Quantification of effect of septin KD on cytokinesis. The percentage of cells exhibiting multinucleation or midbody attachment was determined upon treatment with the indicated siRNA. Data are represented as mean +/- SEM (n ≥ 300 cells from 3 or more independent experiments). Asterisks indicate differences between control and septin KD cells; *p < 0.01 and **p < 0.0001 (t-test).
3.2.9 **Depletion of SEPT9 in HEK293 cells causes similar cytokinetic defects**

To rule out the possibility that the late telophase arrest caused by SEPT9 depletion is merely a HeLa cell-specific phenomenon, I depleted SEPT9 in HEK293 cells and assayed for cytokinetic defects as described above (Figure 3.9A, B). As was observed in HeLa cells, SEPT9 depletion in HEK293 cells also caused an increase in multinucleated cells and cells with persistent midbodies.

![Figure 3.9 Depletion of SEPT9 in HEK293 cells.](image)

(A) HEK293 cells were treated with SEPT9 siRNA, and lysates were probed for SEPT9 and GAPDH. Numbers to the right of the blot represent molecular mass standards in kilodaltons. (B) Quantification of effect of SEPT9 KD on cytokinesis in HEK293 cells. The percentage of cells exhibiting cytokinesis defects (multinucleation or persistent midbodies) was determined upon treatment with SEPT9 siRNA. Data are represented as mean +/- SEM (n ≥ 300 cells from 3 independent experiments). Asterisks indicate differences between control and SEPT9 KD cells; *p < 0.01 (t-test).
3.2.10 **SEPT2 and SEPT11 are important for the early stages of cytokinesis, whereas SEPT9 mediates midbody abscission**

To conclusively determine whether septins play different roles during cell division, I followed septin-depleted cells through cell division by time-lapse microscopy. Separation of the DNA, ingression of the cleavage furrow, and formation of the midbody appeared normal upon control KD (Figure 3.10A). However, SEPT2 and SEPT11 (Figure 3.10B) depleted cells exhibited abnormal cleavage furrow contraction at the early stages of cytokinesis. While cleavage furrow ingression began normally, the size of the nascent daughter cells then fluctuated dramatically. In most cases, the cleavage furrow did eventually fully ingress; however, it did so to the side of both daughter nuclei, thus generating a multinucleated cell and an enucleated cell. In a few cases, cleavage furrow regression was observed, as described previously (Spiliotis et al., 2005).

Depletion of all septins with SEPT7 siRNA had the same effects.

Despite the increase in multinucleated cells observed upon SEPT9 KD (Figure 3.8C), these cells showed no defects in DNA separation, cleavage furrow constriction, or midbody formation (Figure 3.10C). Instead, SEPT9-depleted cells exhibited dramatic defects in midbody abscission (Figure 3.10C). The midbody of control KD cells abscised an average of 3.9 hours after the onset of cytokinesis, and all of these cells successfully completed abscission (Figure 3.10D). In contrast, only 70% of SEPT9 KD cells successfully abscised, with the average abscission time being 9 hours after the initiation of cytokinesis (Figure 3.10D; p < 0.0001). Remarkably, in 20% of SEPT9 KD cells, the midbody either persisted until the next division (Figure 3.10C-D), or had not broken 40 hours after the onset of cytokinesis. In some cases, the midbody broke when both daughter cells re-entered mitosis, whereas in other cases it persisted even after a second round of division. The remaining 10% of SEPT9 KD cells exhibited one of two phenotypes: either the midbody regressed after failing to abscise, giving rise to a multinucleated cell (thus accounting for the fixed-cell results), or one or both daughter cells underwent apoptosis after being unable to complete abscission. Collectively, these results suggest that septins contribute distinct functions at different stages of cell division: SEPT9 is important for mediating midbody abscission at the terminal stage of cytokinesis, whereas SEPT2, SEPT7, and SEPT11 have roles earlier in cytokinesis. It remains unclear whether other septins are also involved in abscission, as such a role may be obscured by the early cytokinesis defects that occur upon their depletion.
Figure 3.10  Distinct roles of septins during cell division.

HeLa cells were transfected with siRNA as indicated and randomly selected cells were followed through division by time-lapse microscopy. The time (in hours:minutes) since the beginning of DNA segregation is shown. Black arrows point to intact midbodies, whereas white arrows denote abscission. Scale bar represents 32 μm. (A) Division of HeLa cells after treatment with control siRNA. (B) SEPT11 KD causes defects early in cytokinesis. Similar defects were observed upon SEPT2 and SEPT7 KD. (C) SEPT9 KD causes defects in midbody abscission. (D) Quantification of effect of SEPT9 KD on midbody abscission. The time from DNA segregation to midbody abscission was determined, and the cumulative percentage of cells that have abscised is plotted as a function of time. n = 45 cells for control KD, n = 30 cells for SEPT9 KD.
3.2.11 **SEPT9 mediates abscission in non-cancerous cells**

Impaired midbody abscission upon SEPT9 KD was also observed in noncancerous human retinal pigment epithelial cells (ARPE-19) (Figure 3.11A, B). In these cells, SEPT9 KD increased the average abscission time to 4.2 hours, compared to 2.3 hours for cells transfected with control siRNA (p < 0.01). These cells are highly motile, and may therefore complete abscission by mechanical rupture of the midbody (Steigemann and Gerlich, 2009), possibly explaining the faster abscission time and less penetrant SEPT9 KD phenotype compared to HeLa cells.

**Figure 3.11 SEPT9 KD causes abscission defects in ARPE-19 (diploid) cells.**

(A) Efficiency of SEPT9 KD in ARPE-19 cells. Numbers to the right of the blot represent molecular mass standards in kilodaltons. (B) Quantification of effect of SEPT9 KD on midbody abscission in ARPE-19 cells. The time from DNA segregation to midbody abscission was determined by live cell imaging, and the cumulative percentage of cells that have abscised is plotted as a function of time. n = 34 cells for control KD, n = 25 cells for SEPT9 KD.
3.3 Discussion

3.3.1 Septin complex composition is unaltered during cell division

Western blotting with our septin antibodies suggests that HeLa cells express SEPT2, SEPT6, SEPT7, SEPT9, and SEPT11. Since septins undergo complex alternative splicing (Russell, 2008), I cannot rule out the possibility that these cells also express septin isoforms not recognized by our antibodies. In addition, it is not clear whether SEPT1, SEPT10 and/or SEPT13 are expressed in these cells. SEPT2, SEPT6, SEPT7, SEPT9 and SEPT11 all localized at the cleavage furrow in anaphase and both sides of the intercellular bridge and midbody in telophase. Double labeling demonstrated that SEPT9 colocalizes with other septins at these important cytokinetic structures. In addition, immunoprecipitation of SEPT2, or SEPT9 from either interphase or mitotic lysates resulted in the co-precipitation of all other septins. Similar results were observed when immunoprecipitations were conducted from lysates of cells enriched at the later stages of cell division. Collectively, these results suggest that septin complex composition remains unaltered during cell division.

Prior to the discovery of SEPT11, it was reported that SEPT2, SEPT6, SEPT7, and SEPT9 form a complex in interphase HeLa cells (Surka et al., 2002). The composition of this complex fits well with the hypothesis that septin complexes are composed of one septin from each of the septin groups (Kinoshita, 2003; Peterson et al., 2007). However, the work presented here demonstrates that SEPT11, which is in the same group as SEPT6, also co-immunoprecipitates with these septins in HeLa cells. This could reflect the presence of two separate septin complexes - one composed of SEPT2, SEPT6, SEPT7 and SEPT9, and the other composed of SEPT2, SEPT11, SEPT7 and SEPT9. Alternatively, a single complex containing all these septins could exist.

3.3.2 SEPT6 and SEPT11

Although SEPT6 is regarded as being a member of the core septin complex, Sept6 knockout mice displayed no obvious phenotype (Ono et al., 2005). This could be due to functional redundancy with other members of the SEPT6 group. In particular, SEPT6 and SEPT11 share
greater than 80% sequence identity at the amino acid level. Depletion of SEPT6 did not have any adverse effects on cytokinesis, although I cannot rule out the possibility that this reflects incomplete silencing. In contrast, I have provided the first evidence that SEPT11 localizes to important cytokinetic structures and plays an important role in the early stages of cytokinesis. This suggests that at least in HeLa cells, SEPT6 cannot compensate for the loss of SEPT11. It is possible that HeLa cells express higher levels of SEPT11 than SEPT6, such that the SEPT6 levels are insufficient to compensate for SEPT11 depletion. An alternative and more intriguing possibility is that cell division requires a septin complex containing SEPT11 instead of SEPT6. Consistent with this possibility, disruption of the Sept11 gene in mice results in embryonic lethality (B. Zeiger, personal communication). More work will be required to elucidate the relative contribution of SEPT6 and SEPT11 (and the other members of this septin group) to other septin-dependent processes.

### 3.3.3 SEPT2, SEPT7, and SEPT11 are important for the early stages of cytokinesis

The early cytokinetic defects observed upon depletion of SEPT2, SEPT7 and SEPT11 are very similar to those observed upon depletion of the septin-binding protein anillin (Straight et al., 2005). Previous work has shown that septins are not important for mediating proper localization of anillin (D'Avino et al., 2008), but the results presented here raise the possibility that SEPT2, SEPT7 and SEPT11 may mediate anillin function during cytokinesis. Recent work has demonstrated that stabilization of astral microtubules (either via depletion of the microtubule depolymerizer mitotic centromere-associated kinesin (MCAK) or via treatment with the microtubule-stabilizing drug taxol) also triggers early cytokinetic defects similar to those observed upon depletion of SEPT2, SEPT7, and SEPT11 (Rankin and Wordeman, 2010). Interestingly, it has been suggested that the septin family regulates microtubule stability through interacting with the microtubule-associated protein Map4 (Kremer et al., 2005). This study demonstrated that simultaneous depletion of SEPT2, SEPT6, SEPT7 (and likely SEPT9 and SEPT11) increased microtubule stability. It will therefore be of great interest to determine whether the early cytokinetic defects observed upon individual depletion of SEPT2 and SEPT11 are a result of stabilized astral microtubules, and how these septins influence the stability of astral microtubules during cell division.
3.3.4 Distinct roles of septins in cytokinesis

Unlike depletion of SEPT2, SEPT7, and SEPT11, SEPT9 knockdown did not result in detectable defects in the early stages of cytokinesis, but caused severe defects in midbody abscission at the terminal stage of cytokinesis. These results therefore demonstrate that depletion of any or all septins expressed in a given cell type does not always give rise to the same phenotype. Since I have shown that septin complex composition is largely unaltered during cell division, it appears that different septins within the septin complex have diverse roles: SEPT2, SEPT7, and SEPT11 are important for the early stages of cytokinesis, whereas SEPT9 mediates midbody abscission. While persistent midbodies were not observed upon depletion of SEPT2, SEPT7, or SEPT11, defects in abscission could be masked by the earlier cytokinesis defects that occur in the absence of these septins. Consequently, it is possible that SEPT2, SEPT7, and/or SEPT11 are also important for abscission. Consistent with this notion, these septins colocalized with SEPT9 at both sides of the intercellular bridge and midbody, and co-immunoprecipitated with SEPT9 from a telophase-enriched population of cells. In addition, injection of SEPT2 antibodies into HeLa cells in late anaphase or early telophase caused both regression of the cleavage furrow, and impaired midbody abscission (Kinoshita et al., 1997). This suggests that SEPT2 may also play an important role in midbody abscission at the terminal stage of cytokinesis.

Why would SEPT9 be dispensable for the early stages of cytokinesis, when other septin complex members (SEPT2, SEPT7, and SEPT11) are critical for this process? The answer to this question may lie in how SEPT9 fits into the septin complex. Since structural data is only available for the SEPT2/SEPT6/SEPT7 complex, it has remained elusive how members of the SEPT9 group fit into the septin complex (Sirajuddin et al., 2007). However, work in our lab suggests that SEPT9 forms a G domain interface with SEPT7 at the end of the SEPT2/SEPT6/SEPT7 complex (M. Kim, personal communication). This suggests that septin filament assembly and disassembly may be mediated through the SEPT9-SEPT9 NC interface. Consistent with this, co-expression of SEPT2, SEPT6, SEPT7, and SEPT9 led to the formation of more robust septin filaments than expression of any subset of these septins. This was dependent on the $\alpha_0$ helix of SEPT9, which is important in the formation of the SEPT9-SEPT9 NC interface. Therefore, it appears that short septin filaments can form in the absence of SEPT9, but formation of robust filaments requires SEPT9. This raises the possibility that the early stages of cytokinesis do not require robust septin filaments, and can thus proceed in the absence of SEPT9.
The requirement of SEPT9 during abscission could reflect the need for robust septin filaments, and/or could be due to a unique role of SEPT9 that cannot be performed by any other septin. Given that SEPT2, SEPT6, SEPT7, SEPT9, and SEPT11 localize to the midbody, and that septins have been shown to act as scaffolds (Joo et al., 2007), it is possible that robust septin filaments and/or SEPT9 on its own may play a key role in recruiting certain factors that are important for abscission to the midbody. An attractive candidate is the exocyst complex, which tethers vesicles at the midbody to promote abscission (Fielding et al., 2005; Gromley et al., 2005). Intriguingly, septins have been shown to interact with the exocyst complex in the brain (Hsu et al., 1998; Vega and Hsu, 2003), raising the possibility that exocyst recruitment to the midbody may be septin-dependent. This hypothesis is addressed in Chapter 4.

3.3.5 SEPT9 mediates midbody abscission

I have demonstrated that specific depletion of SEPT9 in HeLa, HEK293, and ARPE-19 cells causes defects in midbody abscission. While deletion of the Sept9 gene in mice is embryonic lethal, embryos survive for approximately 10 days before expiring, arguing that successful cell division can occur in the absence of SEPT9 (Kinoshita, 2008). There are several possible explanations for this apparent discrepancy. First, it has been demonstrated that deletion of Sept3, Sept5, and both Sept3 and Sept5 in mice causes alterations in the expression of other septin family members (Tsang et al., 2008). This raises the possibility that developmental compensation by other septins may allow Sept9 null embryos to undergo successful cell division until embryonic day 10. Another possible explanation is that abscission is not an absolute requirement for subsequent rounds of cell division. Consistent with this, SEPT9 KD resulted in some cells that were connected by persistent midbodies to multiple other cells. Similar results have been observed upon depletion of other proteins that are crucial for abscission, such as exocyst subunits (Gromley et al., 2005), and BRUCE (Pohl and Jentsch, 2008). Further, during embryonic development, blastomeres often remain joined by intercellular bridges for several rounds of division (Eggert et al., 2006; Goodall and Johnson, 1984). Consequently, it is quite possible that knocking out a protein required for abscission would not prevent cell division from occurring altogether. This is exactly what is observed upon deletion of BRUCE or the exocyst component Sec8, as knockout embryos are hardly distinguishable from control embryos up to embryonic day
12, and day 6.5, respectively (Friedrich et al., 1997; Lotz et al., 2004). Finally, the SEPT9 KD phenotype is incompletely penetrant so that some cells still do eventually divide normally, while others apparently achieve midbody breakage by mechanical strain caused by the subsequent division of the daughter cells. These observations could also contribute to the survival of SEPT9 knockout embryos.

3.3.6 **Implications for the septin field**

By specifically depleting SEPT9, I was able elucidate that this protein plays an important role in midbody abscission, a phenotype that is masked by simultaneous depletion of other septins. Therefore, while depletion of all septin family members provides a good starting point for probing septin function, a complete understanding of septin function will require analysis of the role of individual septins in septin-dependent processes.
4 Elucidation of the role of SEPT9 during abscission

Figures 1-4 and 7-8 are published in the Journal of Cell Biology (November 2010, 191:741-9). Data presented in Figures 7 and 8 were generated in collaboration with Dr. Caterina DiCiano-Oliveira.

4.1 Rationale

Given the unique phenotype observed upon specific depletion of SEPT9, I decided to focus my attention on the role of SEPT9 during cell division. While I have demonstrated that SEPT9 plays a key role in mediating abscission, the molecular function of SEPT9 during this process remains elusive. Previous work has demonstrated that SEPT2 acts as a scaffold for myosin II and its kinases at the cleavage furrow, thus mediating furrow ingression (Joo et al., 2007). It is therefore possible that SEPT9 may play a similar role at the terminal stage of cytokinesis, recruiting important midbody components to mediate abscission. To address this hypothesis, I examined the localization of several midbody components upon depletion of SEPT9 by siRNA.

Due to complex alternative splicing, there are five N-terminal variants of SEPT9 (Figure 4.1). These are referred to as isoform 1 to 5 (_i1 - _i5) (Hall and Finger, 2008), and HeLa cells express all isoforms except _i2 (Burrows et al., 2003). SEPT9_i1, _i2, and _i3 are all extremely similar, diverging only at the first 7-25 amino acids. SEPT9_i4 is a truncation of the larger isoforms, lacking a significant portion of the SEPT9 N-terminal extension. SEPT9_i5 lacks almost the entire N-terminal extension. Importantly, all SEPT9 isoforms contain the polybasic domain and the α0 helix that mediates the NC interface in the septin crystal structure (Sirajuddin et al., 2007). Given that septins contribute distinct functions during cytokinesis, it is possible that individual isoforms of a given septin may also have diverse roles. To begin to address this, I assessed whether individual SEPT9 isoforms are sufficient to rescue the cytokinetic defects that are observed upon depletion of all endogenous SEPT9 isoforms.
Figure 4.1 Alignment of N-terminal SEPT9 isoforms.

N-terminal sequences of human SEPT9_i1 (NP_001106963.1), SEPT9_i2 (NP_001106965.1), SEPT9_i3 (NP_006631.2), SEPT9_i4 (NP_001106966.1), and SEPT9_i5 (NP_001106968.1). Note that our polyclonal SEPT9 antibody does not recognize SEPT9_i5. The approximate position of the $\alpha_0$ helix that mediates the NC interface in the septin crystal structure (Sirajuddin et al., 2007) is shown in red.
4.2 Results

4.2.1 SEPT9 is not important for the localization of other septins to the midbody

I first sought to elucidate the molecular mechanism by which SEPT9 mediates abscission. Given that SEPT9 localizes to the midbody (Surka et al., 2002), and that septins have been shown to act as scaffolds (Joo et al., 2007), I hypothesized that SEPT9 may be important to help localize specific factors that are important for abscission at the midbody. To begin with, I assessed whether the midbody localization of other septins is altered upon SEPT9 depletion. As shown in Figure 4.2, SEPT9 KD had no effect on the midbody localization of SEPT2, SEPT7, or SEPT11.
Figure 4.2 SEPT9 depletion does not alter the localization of other septins at the midbody.

(A) Quantification of the effect of SEPT9 KD on the localization of other septins at the midbody. The percentage of cells exhibiting enrichment of the protein of interest at the midbody was determined by immunofluorescence microscopy after control or SEPT9 KD. Data are represented as mean +/- SEM (n ≥ 300 cells from 3 independent experiments). (B-D) Representative examples of SEPT2 (B), SEPT7 (C), and SEPT11 (D) localization in cytokinetic cells following treatment with control or SEPT9 siRNA. Scale bar represents 17 μm. Arrows point to the midbody. (E-G) SEPT2 (E), SEPT7 (F), and SEPT11 (G) fluorescence at the Flemming body was quantified using Image J following control or SEPT9 KD.
4.2.2 SEPT9 mediates exocyst complex localization to the midbody

I then examined the localization of several other midbody components upon SEPT9 depletion. These included: Cep55, Map4, Plk1, ROCKII, VAMP8, and Sec8. SEPT9 KD had no effect on the midbody localization of Cep55, Map4, Plk1, ROCKII, or VAMP8 (Figure 4.3A, E-I). In contrast, depletion of SEPT9 resulted in a drastic loss of the exocyst component Sec8 at the midbody (Figure 4.3A-D). In control KD cells, Sec8 was enriched at 68% of midbodies; however, only 25% of SEPT9 KD cells exhibited Sec8 enrichment at the midbody (Figure 4.3A-C). Quantification of Sec8 fluorescence at the midbody verified these observations (Figure 4.3D). Previous work suggests that exocyst-dependent tethering of vesicles at the midbody facilitates their fusion with the plasma membrane and with each other, thus contributing to abscission (Gromley et al., 2005). Depletion of exocyst subunits results in abscission defects remarkably similar to those observed upon SEPT9 depletion (Gromley et al., 2005). Thus, SEPT9 is not important for the localization of most of the midbody proteins tested, but plays an important role in mediating exocyst complex localization to the midbody, where it facilitates vesicle tethering that is important for abscission.
Figure 4.3 SEPT9 mediates exocyst complex localization to the midbody.

(A) Quantification of the effect of SEPT9 KD on the localization of midbody components. The percentage of cells exhibiting enrichment of the protein of interest at the midbody was determined by immunofluorescence microscopy after control or SEPT9 KD. Data are represented as mean +/- SEM (n ≥ 300 cells from 3 independent experiments). Asterisks indicate differences between control and SEPT9 KD cells; **p < 0.001 (t-test). (B-C) Representative example of the localization of the exocyst component Sec8 in cytokinetic cells upon control (B) or SEPT9 KD (C). Scale bar represents 17 µm. Arrows point to the midbody. (D) Sec8 fluorescence at the midbody was quantified using ImageJ upon control or SEPT9 KD, **p < 0.0001. (E-I). Representative examples of Plk1 (E), ROCKII (F), Map4 (G), VAMP8 (H), and GFP-Cep55 (I) localization in cytokinetic cells following treatment with control or SEPT9 siRNA.
SEPT9 and Sec8 did not colocalize at the midbody, but were found directly adjacent to each other (Figure 4.4). These results suggest that it is unlikely that SEPT9 and the exocyst complex interact directly at the terminal stage of cytokinesis. However, similar results have been observed in yeast, where septins act to compartmentalize the exocyst at the site of cleavage by creating a membrane diffusion barrier (see discussion for details) (Dobelaere and Barral, 2004).

Figure 4.4 SEPT9 and Sec8 do not colocalize at the midbody.
Double staining of SEPT9 and Sec8 in cytokinetic cells. Scale bar represents 17 µm.
4.2.3 **SEPT9 may regulate activation of the small GTPase RalB**

Previous work has demonstrated that the small GTPase RalB is important for proper targeting of the exocyst complex to the midbody (Cascone et al., 2008). In its active GTP-bound form, RalB directly binds to the exocyst subunits Sec5 and Exo84 (Moskalenko et al., 2002; Moskalenko et al., 2003). I therefore sought to determine whether SEPT9 regulates exocyst localization to the midbody through RalB. To this end, I first examined the localization of RalB at the midbody in cells treated with control or SEPT9 siRNA. As shown in Figure 4.5, RalB localizes to the midbody in cytokinetic cells upon treatment with control or SEPT9 siRNA. This suggests that SEPT9 is not important for the proper localization of RalB at the midbody.

![Image of RalB and Tubulin localization](image)

**Figure 4.5 SEPT9 is not important for the localization of RalB to the midbody.**

HeLa cells were treated with control or SEPT9 siRNA and stained for RalB and tubulin. Scale bar represents 17 µm.
Intriguingly, expression of wild type RalB in SEPT9 depleted cells significantly reduced the number of cells attached by persistent midbodies (Figure 4.6). This effect was not observed upon expression of a mutant form of RalB that cannot bind GTP (RalB S28N; (Cascone et al., 2008)). Therefore, RalB can rescue the abscission defect that is observed upon SEPT9 depletion, as long as it is capable of binding GTP. This raises the possibility that SEPT9 may be important for RalB activation, which is in turn important for recruitment of the exocyst complex to the midbody. Consistent with this notion, expression of RalGPS1A, a GEF important for RalB activation (Cascone et al., 2008), also rescued the SEPT9 KD abscission defect (Figure 4.6).

Figure 4.6  The SEPT9 KD abscission defect can be rescued by expression of wild type RalB or the RalB GEF RalGPS1A.

HeLa cells were treated with SEPT9 siRNA, and transfected with plasmids expressing Myc-RalB, Myc-RalB S28N (dominant negative), or GFP-RalGPS1A. The percentage of cells exhibiting midbody attachment was determined by immunofluorescence microscopy. Data are represented as mean +/- SEM (n ≥ 300 cells from 3 or more experiments), *p < 0.01 (t-test) compared to SEPT9 KD.
4.2.4 The SEPT9 isoforms are not all functionally equivalent

Since our SEPT9 siRNA targets all SEPT9 isoforms, I next assessed whether individual isoforms are capable of rescuing the cytokinetic defect observed upon SEPT9 depletion. To this end, I generated a stable cell line that expresses siRNA-resistant SEPT9_i3 under the control of an inducible promoter (Figure 4.7A). In the absence of induction, this cell line exhibits low levels of leaky expression comparable to endogenous SEPT9 levels. First I verified that SEPT9 KD in the parent cell line, from which the above described stable line was derived, caused the expected cytokinetic defects (persistent midbodies and multinucleation resulting from abscission failure, which I refer to collectively as ‘unresolved cytokinesis’; Figure 4.7C, E). In contrast, the basal expression of siRNA-resistant SEPT9_i3 significantly reduced these defects (Figure 4.7C, E; compare SEPT9 KD in the parent cell line to SEPT9 KD in the SEPT9_i3 cell line). In fact, there was no significant difference in the percentage of cells exhibiting unresolved cytokinesis when the SEPT9_i3 stable cell line was treated with control or SEPT9 siRNA (Figure 4.7E). This not only demonstrates the specificity of my SEPT9 siRNA, but also argues that SEPT9_i3 is sufficient to drive cytokinesis in the absence of the other SEPT9 isoforms. Similar results were obtained with SEPT9_i1, which differs from SEPT9_i3 only at the extreme N-terminus (Figure 4.1). SEPT9_i2 was not tested, since it is not expressed in HeLa cells (Burrows et al., 2003).

SEPT9_i4 lacks a significant portion of the SEPT9 N-terminal extension (Figure 4.1), and has been shown to be overexpressed in many tumours (Burrows et al., 2003; Scott et al., 2006). To assess whether SEPT9_i4 is also sufficient to drive cytokinesis in the absence of other SEPT9 isoforms, I generated a stable cell line that inducibly expresses siRNA-resistant SEPT9_i4. The basal expression of SEPT9_i4 in this cell line is similar to the expression level of endogenous SEPT9_i1-i3 (Figure 4.7D). Interestingly, this level of SEPT9_i4 expression in itself was capable of inducing persistent midbodies and multinucleation (Figure 4.8 and 4.7E; compare control KD in the parent cell line to control KD in the SEPT9_i4 cell line). These defects were not suppressed by depleting endogenous SEPT9 (Figure 4.8 and 4.7E), suggesting that they are not simply the result of increased total SEPT9 levels. Further, depletion of endogenous SEPT9 in the SEPT9_i4 cell line did not cause an increase in cytokinesis defects, indicating that SEPT9_i4 is acting in a dominant negative fashion. In contrast, inducing SEPT9_i3 expression up to 330% of total endogenous SEPT9 did not cause cytokinetic defects. SEPT9_i5 was not tested as it is not recognized by our SEPT9 antibody; however, I expect that this isoform would have a similar
effect since it is a truncated version of SEPT9_i4 (Figure 4.1). These results point to an important role for the N-terminal region of SEPT9 in cytokinesis, and suggest that even individual isoforms of a given septin can have diverse functions.
Figure 4.7 The SEPT9 isoforms are not all functionally equivalent.

(A), (B) Stable cell lines inducibly expressing siRNA-resistant Flag-SEPT9_i3 (A) or Flag-SEPT9_i4 (B) were treated with increasing amounts of doxycycline, and expression was assayed by Western blotting. (C) The parent and Flag-SEPT9_i3 cell lines were treated with control or SEPT9 siRNA in the absence of doxycycline, and SEPT9 levels were assayed by Western blotting. Note that SEPT9_i4 disappears upon treatment with SEPT9 siRNA, demonstrating efficient KD. Also note the higher SEPT9 (_i1-3) levels in the Flag-SEPT9_i3 stable line upon SEPT9 depletion, when compared to the parent line. (D) The parent and Flag-SEPT9_i4 cell lines were treated as in (C). Numbers to the right of the blot represent molecular mass standards in kilodaltons. (E) The parent, Flag-SEPT9_i3, and Flag-SEPT9_i4 cell lines were treated with control or SEPT9 siRNA and assayed for defects in cytokinesis by immunofluorescence microscopy. Unresolved cytokinesis refers to cells exhibiting midbody attachment or multinucleation. Data are represented as mean +/- SEM (n ≥ 300 cells from 3 or more independent experiments), *p < 0.05, **p < 0.005 (t-test).
Figure 4.8 Expression of SEPT9_i4 induces both persistent midbodies and multinucleation.

Data from Figure 4.7 is expressed in an alternate manner (by distinguishing the number of cells exhibiting multinucleation and persistent midbodies) to demonstrate that SEPT9_i4 expression induces both persistent midbodies and multinucleation. Asterisks indicate differences between the parent and SEPT9_i4 cell lines upon control KD; *p < 0.05, **p < 0.001 (t-test).
The results presented above suggest an important role for the N-terminal region of SEPT9 in cytokinesis. To examine this further, I generated a construct that expresses GFP fused to the N-terminal region of SEPT9 (amino acids 8-146 of SEPT9_i3, which represents the common sequence in SEPT9_i1-3 that is absent from SEPT9_i4; see Figure 4.1; I call this region SEPT9 N-term). Expression of GFP-SEPT9 N-term dramatically impaired abscission, as shown in Figure 4.9. Approximately 22 hours after transfection, nearly half of GFP-SEPT9 N-term expressing cells had persistent midbodies, compared to roughly 10% of untransfected cells (or cells expressing GFP alone). This demonstrates that the N-terminal region of SEPT9 also acts in a dominant negative manner.

Figure 4.9 Expression of the N-terminal region of SEPT9 impairs abscission. HeLa cells were transfected with GFP-SEPT9 N-term and assayed for abscission defects by immunofluorescence microscopy after approximately 22 hours. (A) Representative examples of cells attached by persistent midbodies following expression of GFP-SEPT9 N-term (red, α-tubulin). Scale bar represents 17 μm. (B) Quantification of effect of GFP-SEPT9 N-term on abscission. The percentage of cells exhibiting midbody attachment was determined upon transfection with GFP-SEPT9 N-term (-GFP-SEPT9 N-term represents untransfected cells whereas + GFP-SEPT9 N-term represents transfected cells). Data are represented as mean +/- SEM (n ≥ 300 cells from 3 experiments), **p < 0.001 (t-test).
4.3 Discussion

4.3.1 SEPT9 is not important for the localization of other septins at the midbody

In an effort to gain mechanistic insight into the role of SEPT9 during abscission, I examined the localization of several midbody components upon SEPT9 depletion. The percentage of midbodies exhibiting enrichment of SEPT2, SEPT7, and SEPT11 was unperturbed upon SEPT9 knockdown. Likewise, the fluorescence intensity of these septins at the midbody was not altered by SEPT9 depletion. This argues that SEPT9 does not mediate the localization of other septin family members to the midbody, and confirms that my SEPT9 siRNA depletes SEPT9 without affecting the expression of other septin family members.

4.3.2 SEPT9 mediates the recruitment of the vesicle tethering exocyst complex to the midbody

SEPT9 depletion also had no effect on the localization of several other midbody components, including Plk1, ROCKII, VAMP8, Cep55, and Map4. Therefore, SEPT9 does not mediate the proper localization of many proteins at the midbody, arguing that it is not important for overall midbody architecture. However, I observed a drastic defect in the recruitment of the exocyst complex component Sec8 to the midbody in cells treated with SEPT9 siRNA. Previous work has shown that the exocyst complex plays a key role in tethering vesicles at the midbody to promote abscission (Fielding et al., 2005; Gromley et al., 2005). Depletion of exocyst complex components by siRNA led to the accumulation of cells with long midbodies that sometimes persisted for multiple rounds of division, a phenotype that is strikingly similar to what I observed upon depletion of SEPT9. Therefore, the work presented here argues that SEPT9 plays an important role in mediating the proper localization of the exocyst complex to the midbody, where it facilitates vesicle tethering that is critical for abscission.

While this work has provided significant insight into the role of SEPT9 during abscission, further studies will be required to elucidate how SEPT9 facilitates proper exocyst complex localization at the midbody. Previous work has shown that immunoprecipitation of Sec8 from rat brain lysate results in the co-immunoprecipitation of septins (Hsu et al., 1998; Vega and Hsu, 2003).
However, it remains unclear whether septins interact directly with the exocyst complex, and which septins and exocyst subunits are involved in the interaction should this be the case. The results presented here argue that SEPT9 is important for the proper localization of the exocyst complex at the midbody, and that the N-terminal region of SEPT9 plays an important role in abscission (see below). This raises the possibility that the N-terminal region of SEPT9 may directly recruit the exocyst complex to the midbody. However, I feel that this is unlikely because immunofluorescence microscopy suggested that SEPT9 and Sec8 do not colocalize at the midbody, but are found immediately adjacent to each other. In addition, I was unable to detect an interaction between SEPT9 and the exocyst complex by immunoprecipitation from unsynchronized or mitotic cell lysates. I cannot rule out the possibility that SEPT9 and the exocyst complex only interact during telophase (just prior to abscission), as such an interaction would not be detected by immunoprecipitation from interphase or mitotic cell lysates. It is also possible that the SEPT9 and/or Sec8 epitopes are partially masked in telophase, which could account for the lack of colocalization between SEPT9 and Sec8 at the midbody.

Previous work has shown that the small GTPase RalB plays an important role in mediating the recruitment of the exocyst complex to the midbody. This likely occurs through direct interactions between the GTP-bound form of RalB and the exocyst subunits Sec5 and Exo84 (Moskalenko et al., 2002; Moskalenko et al., 2003). Depletion of SEPT9 did not affect the localization of RalB at the midbody, suggesting that SEPT9 does not simply mediate exocyst localization by controlling the localization of RalB. However, expression of wild type RalB in SEPT9-depleted cells significantly reduced the percentage of cells attached by persistent midbodies, an effect that was not observed upon expression of RalB S28N (which cannot bind GTP). In addition, expression of RalGPS1A, a RalB GEF that mediates RalB activation, also rescued the SEPT9 KD abscission defect. Therefore, it seems that the SEPT9 knockdown phenotype can be rescued by increasing the levels of active RalB. This suggests that SEPT9 may play a role in RalB activation, thus mediating the proper recruitment of the exocyst complex to the midbody to facilitate abscission. However, I was unable to detect any consistent differences in the levels of active RalB in interphase or mitotic cells after treatment with control and SEPT9 siRNA. Likewise, no interaction between SEPT9 and RalB could be detected by immunoprecipitation. I cannot rule out the possibility that SEPT9 locally activates RalB at the midbody. A cellular probe specific for active RalB will be required to address this possibility. Alternatively, SEPT9 may not be
involved in RalB activation. It is possible that grossly increasing the levels of active RalB could simply result in the recruitment of enough exocyst complex to the midbody (through interactions with active RalB) to support abscission in the absence of SEPT9.

Septins are thought to act as a membrane diffusion barrier at the sperm tail annulus (Kwitny et al., 2010), the base of the primary cilium (Hu et al., 2010), and possibly at the base of dendritic protrusions (Tada et al., 2007; Xie et al., 2007). Double staining of SEPT9 and Sec8 in cytokinetic cells revealed that SEPT9 localizes to each side of the intercellular bridge, whereas Sec8 appears to be confined between these regions. This raises the intriguing possibility that SEPT9 may be required to form a membrane diffusion barrier at each side of the intercellular bridge. This diffusion barrier would prevent the exocyst, and other membrane associated and membrane proteins, from diffusing out of the midbody region. In addition, a diffusion barrier at either side of the intercellular bridge could also confine certain lipids, such as PI(4,5)P2, to the site of abscission. This would further facilitate the proper localization of the exocyst complex to the midbody. The other midbody components that were analyzed in this study (Plk1, ROCKII, VAMP8, Cep55, and Map4) are not associated with the plasma membrane, so their localization at the midbody would be unperturbed by SEPT9 depletion in this model.

Support for the diffusion barrier hypothesis comes from studies performed in *Saccharomyces cerevisiae*. In this organism, septins form a ring at the mother-bud neck (Bi, 2001), which splits into two at the onset of cytokinesis (Lippincott et al., 2001). Several proteins that are critical for cytokinesis, including the exocyst complex, do not colocalize with septins but are found between the two septin rings (Dobbelaere and Barral, 2004). Fluorescence recovery after photobleaching (FRAP) experiments demonstrated that the mobility of the exocyst complex was confined to the region between the septin rings. Experiments with temperature sensitive septin mutants revealed that the localization of the exocyst complex to the cleavage site requires intact, continuous septin rings. Therefore, septins act to delineate the site of cleavage in *Saccharomyces cerevisiae*, by forming a diffusion barrier to restrict the mobility of the exocyst complex and other key proteins involved in cytokinesis. The work presented here raises the possibility that SEPT9 could be important for a similar diffusion barrier at the midbody of mammalian cells that promotes abscission. Consequently, facilitating the proper localization of the exocyst complex during cytokinesis through the formation of a diffusion barrier could be an evolutionarily conserved role of the septin family of proteins.
A diffusion barrier at the midbody of mammalian cells would likely involve other septins, since such a barrier would presumably require robust septin filaments. Consistent with this possibility, SEPT2, SEPT7, and SEPT11 colocalized with SEPT9 at either side of the intercellular bridge, and co-immunoprecipitated with SEPT9 from a population of cells enriched in telophase (Chapter 3). Since SEPT2, SEPT7, and SEPT11 are also important for the early stages of cytokinesis, acute methods of inhibiting these septins will be required to study their role in abscission and exocyst localization at the midbody.

4.3.3 The N-terminal region of SEPT9 is important for the completion of cytokinesis

Rescue experiments demonstrated that expression of siRNA-resistant SEPT9_i3 significantly suppressed the cytokinetic defects observed upon depletion of all SEPT9 isoforms. Similar results were observed upon expression of SEPT9_i1, suggesting that the long isoforms of SEPT9 are sufficient to drive cytokinesis in the absence of other SEPT9 isoforms. In sharp contrast, expression of SEPT9_i4, which lacks a significant portion of the SEPT9 N-terminal extension, induced persistent midbodies and multinucleation. These results argue that SEPT9_i4 is not functionally equivalent to the longer SEPT9 isoforms, and point to an important role for the N-terminal region of SEPT9 in cytokinesis. Interestingly, several studies have demonstrated that SEPT9_i4 is overexpressed in many types of cancer (Burrows et al., 2003; Scott et al., 2006). The results presented here suggest that overexpression of SEPT9_i4 impairs the completion of cell division. This could result in genomic instability, ultimately contributing to cancer (Fujiwara et al., 2005).

Overexpression of the N-terminal region of SEPT9 that is common to SEPT9_i1 and SEPT9_i3, but is absent from SEPT9_i4 (which I call SEPT9 N-term) also caused severe defects in cytokinesis. Strikingly, nearly half of SEPT9 N-term expressing cells were connected by persistent midbodies the day after transfection. This suggests that like SEPT9_i4, SEPT9 N-term also acts in a dominant negative manner. A potential explanation for these observations is that an interaction between the N-terminal region of SEPT9 and some unidentified protein may be important for the completion of cytokinesis. In such an instance, SEPT9_i4 would be unable to interact with this protein, but could presumably still incorporate into the septin complex.
Therefore, overexpression of SEPT9_i4 could prevent the proper recruitment of this protein to the septin complex, resulting in a dominant negative effect. SEPT9 N-term would be able to interact with this protein, but would be unable to incorporate into the septin complex (since it lacks the α0 helix that mediates the NC interface in the septin crystal structure). Therefore, overexpression of SEPT9 N-term could also prevent the proper recruitment of this protein to the septin complex, likewise resulting in a dominant negative effect. The potential identity of this unidentified protein is discussed in Chapter 5.

If SEPT9_i4 is not functionally equivalent to the larger SEPT9 isoforms, then why would cells express this protein in the first place? One must keep in mind that septin complexes associate with each other to form filaments, and these structures are thought to mediate septin function. Therefore, incorporation of SEPT9_i4 into a given septin filament would limit the abundance of the larger SEPT9 isoforms within that filament. This could play an important regulatory role, ensuring that the proper amounts of the longer SEPT9 isoforms are present within the septin filament to mediate the completion of cytokinesis. Such a regulatory mechanism could be applicable to other septin-dependent processes.
5 Mitotic Regulation of the N-terminal region of SEPT9 by CDK1 and Pin1 is Important for the Completion of Cytokinesis

The work presented in this Chapter is unpublished. Dr. Carol Froese mapped the 10C10 epitope in SEPT9 (Figure 9), and data presented in Figures 10, 11, 13, and 14 were generated in collaboration with Dr. Caterina DiCiano-Oliveira.

5.1 Rationale

Given that the N-terminal region of SEPT9 seems to play a critical role during abscission, I turned my attention to this region of the protein. Sequence analysis using the bioinformatic tools PROSITE and SMART did not identify any known protein domains within the N-terminal region of SEPT9. In contexts other than cell division, phosphorylation has been shown to regulate septin function (Amin et al., 2008; Taniguchi et al., 2007), raising the possibility that septins may also be controlled by phosphorylation during cell division. Indeed, a link between mammalian septins and the cell cycle machinery that controls entry into and progression through mitosis via phosphorylation has been speculated for some time, but has never been demonstrated experimentally (Hall and Russell, 2004). Intriguingly, two recent high throughput phosphorylation screens identified a threonine residue within the N-terminal region of SEPT9 as a putative mitotic phosphorylation site (Beausoleil et al., 2006; Dephoure et al., 2008). I therefore hypothesized that phosphorylation of the N-terminal region of SEPT9 is important for its role during cell division.
5.2 Results

5.2.1 CDK1 phosphorylates T24 in the N-terminal region of SEPT9 in a mitosis-specific manner

I first addressed the possibility that SEPT9 may be regulated by phosphorylation during mitosis. Exogenously expressed GFP-SEPT9_i3 was immunoprecipitated from unsynchronized or nocodazole-arrested (hereafter called “mitotic”) HeLa cell lysates and probed with a phospho-Thr-Pro (pT-P) antibody (Figure 5.1A). While I did not detect phosphorylation of SEPT9 in unsynchronized cells, SEPT9 in mitotic cells was recognized by the pT-P antibody. CDK1 phosphorylates at least 70 different proteins critical for both the initiation of, and progression through mitosis (Malumbres and Barbacid, 2005). This kinase preferentially phosphorylates the motif S/T–P–X–K/R, where X is any amino acid (Songyang et al., 1994). The phosphorylation screens mentioned above identified T24 of SEPT9 (numbering according to SEPT9_i3) as a putative phosphorylation site (Beausoleil et al., 2006; Dephoure et al., 2008), and this residue matches the CDK1 consensus motif perfectly. I therefore hypothesized that CDK1 phosphorylates SEPT9 at T24 during mitosis. Consistent with this notion, treatment of mitotic cells with the cyclin-dependent kinase inhibitor roscovitine (Figure 5.1A), or mutation of T24 to alanine (Figure 5.1B) completely abolished SEPT9 phosphorylation detected by the pT-P antibody.
Figure 5.1  SEPT9 is phosphorylated at T24 in a mitosis-specific and CDK1-dependent manner.

(A) SEPT9_i3 is phosphorylated in a mitosis-specific manner. HeLa cells were transfected with a plasmid encoding GFP-SEPT9_i3. Cells were either left untreated, arrested in mitosis with nocodazole, or arrested in mitosis and treated with the CDK inhibitor roscovitine. Left: GFP-SEPT9_i3 was immunoprecipitated with a GFP antibody (GFP IP), and the precipitates were probed with phospho-Thr-Pro (pT-P) or GFP antibodies. Immunoprecipitations were also performed with a non-specific antibody (IgG IP). Right: lysates were probed for pT-P. Unsync, unsynchronized; rosc, roscovitine. Numbers to the right of the blot represent molecular mass standards in kilodaltons. (B) Mitotic phosphorylation of SEPT9_i3 occurs at T24. GFP-SEPT9_i3 WT or GFP-SEPT9_i3 T24A was immunoprecipitated from mitotically arrested HeLa cells and probed for pT-P and GFP.
In vitro kinase assays demonstrated that CDK1 preferentially phosphorylates SEPT9_i3, but not SEPT9_i4, which lacks T24 (Figure 5.2A). Likewise, mutation of T24 to alanine greatly decreased the in vitro phosphorylation of SEPT9_i3 (Figure 5.2B), demonstrating that CDK1 directly phosphorylates SEPT9 at T24. While the in vivo phosphorylation of SEPT9 (Figure 5.1B) was completely abolished by the T24A mutation, the in vitro phosphorylation (Figure 5.2B) was only decreased by approximately 60%. This suggests that CDK1 phosphorylates at least one other site in SEPT9 in vitro that is either not phosphorylated in vivo or is not recognized by the pT-P antibody (as would be the case with a pSer-Pro motif).

Figure 5.2 CDK1 phosphorylates SEPT9 directly at T24.
(A) CDK1/cyclinB preferentially phosphorylates SEPT9_i3 over SEPT9_i4. SEPT9_i3 or SEPT9_i4 was incubated with CDK1/cyclinB in the presence of radiolabeled ATP. Reactions were spotted on nitrocellulose, and radioactivity was counted using a scintillation counter. Data are expressed as a percentage of the counts per minute (cpm) obtained for SEPT9_i3 after normalizing to a no substrate control, and are represented as mean +/- SEM (n = 3).
(B) CDK1/cyclinB phosphorylates SEPT9_i3 at T24. SEPT9_i3 and SEPT9_i3 T24A were subjected to kinase assays as in (A). Data are expressed as a percentage of the cpm obtained for SEPT9_i3 after normalizing to the SEPT9_i4 control, and are represented as mean +/- SEM (n = 3).
To determine whether endogenous SEPT9 undergoes mitotic phosphorylation, I immunoprecipitated SEPT9 from mitotic HeLa cells and probed with the pT-P antibody (Figure 5.3A). As expected, endogenous SEPT9 was also recognized by this antibody, and treatment of mitotic cells with roscovitine completely abolished endogenous SEPT9 phosphorylation. To provide further evidence that CDK1 is the kinase responsible for the mitotic phosphorylation of SEPT9 at T24, I assayed the effect of CDK1 depletion on SEPT9 phosphorylation. As shown in Figure 5.3B, CDK1 knockdown decreased the mitotic phosphorylation of SEPT9. It should be noted that cells treated with control and CDK1 siRNA had similar cell cycle profiles, and arrested in mitosis with similar efficiencies upon treatment with nocodazole (Figure 5.10). This suggests that the decrease in SEPT9 phosphorylation upon CDK1 depletion was not due to an inability to enter mitosis or a delay in mitotic entry as a result of decreased CDK1 levels. Since complete knockdown of CDK1 was not achieved, I believe that sufficient amounts of this protein remained to drive entry into mitosis. Consistent with this hypothesis, recent work has demonstrated that mutant mice exhibiting about a 50% reduction in CDK1 protein levels are fully viable (Santamaria et al., 2007). Collectively, these results demonstrate that CDK1 phosphorylates SEPT9 at T24 during mitosis, and thus identify the first link between a mammalian septin and the cell cycle machinery. I cannot rule out the possibility that CDK1 activates another kinase that in turn phosphorylates SEPT9 at T24, but given the \textit{in vitro} data I feel that this is unlikely.
Figure 5.3 CDK1 phosphorylates endogenous SEPT9 during mitosis.

(A) Endogenous SEPT9 is phosphorylated during mitosis in a CDK1-dependent manner. HeLa cells were arrested in mitosis with nocodazole and treated with or without roscovitine. Left: SEPT9 was immunoprecipitated, and the precipitates were probed for pT-P and SEPT9. Right: lysates were probed for pT-P. (B) CDK1 knockdown abrogates the mitotic phosphorylation of SEPT9. HeLa cells were treated with control or CDK1 siRNA and arrested in mitosis with nocodazole. Left: SEPT9 was immunoprecipitated, and the precipitates were probed for pT-P and SEPT9. Right: lysates were probed for CDK1, pT-P, and GAPDH. Numbers to the right of the blots represent molecular mass standards in kilodaltons.
5.2.2 Mitotic phosphorylation of SEPT9 at T24 does not regulate association with Plk1

I next sought to determine the functional significance of SEPT9 phosphorylation at T24. A recent proteomic screen identified SEPT9 as a putative binding partner of Plk1 (Lowery et al., 2007), which contains a Polo-box domain (PBD) that binds to S-(pS/pT)-P motifs in target proteins (Elia et al., 2003). Since T24 of SEPT9 resides in such a motif, I tested whether mitotic phosphorylation of SEPT9 regulates binding to Plk1. I performed GST pulldowns using recombinant GST-Plk1 PBD and mitotic lysate from HeLa cells that were transfected with a plasmid encoding GFP-SEPT9_i3. SEPT9_i3 interacted with the PBD of Plk1, and this association was impaired upon mutation of residues in the PBD that are required for binding to phosphorylated ligands (Figure 5.4A). However, mutation of T24 to alanine had no effect on the ability of SEPT9_i3 to bind to the PBD of Plk1 (Figure 5.4B). These results suggest that phosphorylation of SEPT9 at T24 does not control association with Plk1.
Figure 5.4 Mitotic phosphorylation of SEPT9 at T24 does not regulate association with Plk1.

(A) The PBD of Plk1 interacts with SEPT9 in a phosphorylation-dependent manner. HeLa cells were transfected with a plasmid encoding GFP-SEPT9_i3 and arrested in mitosis with nocodazole. Lysates were incubated with glutathione-agarose beads complexed with GST, GST-Plk1 PBD, or GST-Plk1 PBD mut (which cannot bind to phosphorylated ligands). Bound proteins were analyzed by Western blotting for SEPT9 or Mpm-2 (which recognizes the motif pS/T-P and thus serves as a positive control). (B) HeLa cells were transfected with plasmids encoding GFP-SEPT9_i3 or GFP-SEPT9_i3 T24A. Cells were arrested in mitosis and subjected to pulldown analysis as in (A).
5.2.3 Mitotic phosphorylation of SEPT9 at T24 regulates binding to Pin1

Previously, I identified the peptidyl-prolyl isomerase, Pin1, as a putative septin-interacting protein in a yeast two-hybrid screen (Steels, 2008). Pin1 is a highly conserved enzyme consisting of two domains: an N-terminal WW domain and a C-terminal peptidyl-prolyl isomerase (PPIase) domain (Lu et al., 1996). The WW domain acts as a substrate recognition module by binding to specific phosphorylated Ser/Thr-Pro (pS/T-P) motifs (Ranganathan et al., 1997). The PPIase domain isomerizes the peptide bond between the pS/T and proline (Yaffe et al., 1997). This conformational change is thought to have various effects on Pin1 substrates, such as regulation of function, stability, and/or localization (Lu et al., 1999a; Ryo et al., 2001). Interestingly, Pin1 interacts with many of its substrates in a mitosis-specific manner (Shen et al., 1998), and has also been implicated in cancer (Bao et al., 2004; Wulf et al., 2001; Yeh and Means, 2007).

I therefore hypothesized that Pin1 may interact with SEPT9 in response to mitotic phosphorylation at T24 by CDK1. To test this, I performed GST pulldowns using recombinant GST-Pin1 and unsynchronized or mitotic HeLa cell lysates. While no interaction was observed between Pin1 and SEPT9 when unsynchronized lysates were used, SEPT9 in mitotic lysates associated with Pin1 (Figure 5.5). Note that SEPT9_i4 did not interact with Pin1. The SEPT9-Pin1 interaction was dependent on the WW domain of Pin1, as inactivation of the WW domain through a Y23A mutation (Lu et al., 1999b) completely abolished the interaction (Figure 5.5).
Figure 5.5 Pin1 interacts with SEPT9 in a mitosis-specific manner via its WW domain. Unsynchronized or mitotic HeLa cell lysates were incubated with glutathione-agarose beads complexed with GST, GST-Pin1, or GST-Pin1$^{Y23A}$ (which has an inactivated WW domain). Bound proteins were analyzed by Western blotting for SEPT9 or Mpm-2 (which recognizes the motif pS/T-P and thus serves as a positive control).
Treatment of lysates with calf intestinal phosphatase (Figure 5.6A) or pre-treatment of cells with roscovitine (Figure 5.6B) prior to pulldown analysis greatly impaired the SEPT9-Pin1 association, arguing that the interaction is dependent on CDK1-mediated phosphorylation.

Figure 5.6 The SEPT9-Pin1 interaction is dependent on CDK1-mediated phosphorylation. (A) The SEPT9-Pin1 interaction is phosphorylation dependent. Mitotic HeLa cell lysates were incubated with or without calf intestinal phosphatase (CIP), and pulldowns were performed as in Figure 5.5. (B) The Pin1-SEPT9 interaction is dependent on CDK1-mediated phosphorylation. Mitotic HeLa cells were treated with or without the CDK1 inhibitor roscovitine prior to lysis, and pulldowns were performed as in Figure 5.5.
To elucidate whether Pin1 binds SEPT9 at T24, I first transfected HeLa cells with plasmids encoding GFP-SEPT9_i3 or GFP-SEPT9_i3 T24A, arrested the cells in mitosis, and performed pulldowns with GST-Pin1. While GFP-SEPT9_i3 interacted with Pin1, the single T24A point mutation greatly inhibited the association, demonstrating that T24 is critical for Pin1 binding (Figure 5.7A). I performed a similar experiment to determine whether Pin1 directly binds to SEPT9 at T24. GFP-SEPT9_i3 or GFP-SEPT9_i3 T24A was immunoprecipitated from mitotically arrested HeLa cells. Far Western analysis using recombinant Pin1 as a probe showed that Pin1 interacts directly with wild type SEPT9, but cannot bind the T24A mutant (Figure 5.7B).

Figure 5.7 Pin1 interacts with SEPT9 directly via T24.
(A) T24 of SEPT9 is required for the association with Pin1. HeLa cells were transfected with plasmids encoding GFP-SEPT9_i3 or GFP-SEPT9_i3 T24A. Cells were arrested in mitosis and subjected to pulldown analysis as in Figure 5.5. (B) Pin1 binds to SEPT9 directly via T24. GFP-SEPT9_i3 or GFP-SEPT9_i3 T24A was immunoprecipitated from mitotically arrested HeLa cells and subjected to Far Western analysis using recombinant Pin1 as the probe.
I next sought to generate a mutant version of SEPT9_i3 that constitutively interacts with Pin1. To this end, I mutated T24 to either aspartic or glutamic acid, since the negative charge of these amino acids has been shown to mimic phosphorylation in some instances (Joo et al., 2007). To determine whether these mutants can associate with Pin1, I transfected HeLa cells with plasmids encoding GFP-SEPT9_i3 WT, GFP-SEPT9_i3 T24A, GFP-SEPT9_i3 T24D, or GFP-SEPT9_i3 T24E, arrested the cells in mitosis, and performed pulldowns with GST-Pin1. While GFP-SEPT9_i3 WT interacted with Pin1, the T24D and T24E point mutations greatly inhibited the association, demonstrating that these mutations do not adequately mimic the Pin1-binding site in SEPT9 (Figure 5.8).

Figure 5.8  SEPT9_i3 T24D and T24E mutants have greatly impaired binding to Pin1. HeLa cells were transfected with plasmids encoding GFP-SEPT9_i3 WT, GFP-SEPT9_i3 T24A, GFP-SEPT9_i3 T24D, or GFP-SEPT9_i3 T24E. Cells were arrested in mitosis and subjected to pulldown analysis as in Figure 5.5.
Intriguingly, our SEPT9 monoclonal antibody (10C10, described in Chapter 3) consistently immunoprecipitates far more SEPT9 from unsynchronized cells than from mitotic cells, even though SEPT9 expression levels do not change upon mitotic entry (Figure 5.10). This raises the possibility that phosphorylation and/or isomerization of SEPT9 by CDK1 and Pin1 (at onset on mitosis) may affect the ability of 10C10 to recognize SEPT9. To begin to address this, I mapped the epitope of SEPT9 that is recognized by 10C10, in collaboration with Dr. Carol Froese. We assessed whether 10C10 can recognize purified His-SEPT9_i1, His-SEPT9_i3, and His-SEPT9_i4 by Western blotting. As shown in Figure 5.9A, 10C10 recognizes His-SEPT9_i1 and His-SEPT9_i3, but not His-SEPT9_i4. This demonstrates that the 10C10 epitope is somewhere within the 139 amino acids at the N-terminal region of SEPT9 that are common between SEPT9_i1 and SEPT9_i3 and are absent from SEPT9_i4 (which I call SEPT9 N-term; Figure 5.9B). To map the epitope further, we generated peptides corresponding to different fragments of this region (called Epia, Epi-a, Epi-b, Epi-c, and Epic; see Figure 5.9B,C), and assessed whether 10C10 can recognize these peptides by Western blotting (Figure 5.9C). Only peptides containing Epic, which corresponds to amino acids 96 to 146 of SEPT9_i3, were recognized by 10C10. This shows that T24 does not lie within the epitope of SEPT9 that is recognized by 10C10. Consequently, the inability of 10C10 to efficiently immunoprecipitate SEPT9 from mitotic cells is not simply a direct consequence of phosphorylation of SEPT9 at T24.
Figure 5.9 Mapping the epitope of SEPT9 that is recognized by 10C10.
(A) 10C10 does not recognize SEPT9_i4. His-SEPT9_i1, His-SEPT9_i3, and His-SEPT9_i4 were purified from insect cells and subjected to Western blotting with His and 10C10 antibodies. (B) Schematic of the 139 amino acids at the N-terminal region of SEPT9 that are common between SEPT9_i1 and SEPT9_i3 and are absent from SEPT9_i4 (SEPT9 N-term). Constructs expressing peptides Epia, Epia-b, Epib, Epib-c, and Epic were generated. The CDK1 phosphorylation site (T24) is shown in red. (C) Lysates of induced bacteria expressing the various peptides were subjected to SDS-PAGE and stained with Coomassie. (D) Lysates of uninduced (U) and induced (I) bacteria expressing the various peptides were subjected to Western Blotting with 10C10. Numbers to the right of the blot represent molecular mass standards in kilodaltons.
I therefore hypothesized that Pin1 induces a conformational change in SEPT9, in response to CDK1-mediated phosphorylation upon mitotic entry, which masks the 10C10 epitope on SEPT9. To test this hypothesis, I depleted CDK1 (Figure 5.10A) or Pin1 (Figure 5.10B) by siRNA, immunoprecipitated SEPT9 with 10C10 from unsynchronized and mitotic cells, and probed the immunoprecipitate with our polyclonal SEPT9 antibody (Surka et al., 2002). Strikingly, both CDK1 and Pin1 depletion rescued the ability of 10C10 to immunoprecipitate SEPT9 from mitotic cells. This demonstrates that the 10C10 epitope on SEPT9 is masked upon mitotic entry in a CDK1 and Pin1-dependent manner, likely as a result of Pin1-mediated isomerization of SEPT9.
Figure 5.10  The 10C10 epitope on SEPT9 is masked upon mitotic entry in a CDK1 and Pin1-dependent manner.

(A) The 10C10 epitope on SEPT9 is masked upon mitotic entry in a CDK1-dependent manner. HeLa cells were treated with control (con) or CDK1 siRNA, and SEPT9 was immunoprecipitated with 10C10 from unsynchronized (U) or mitotic (M) HeLa cells. Precipitates were probed with a polyclonal SEPT9 antibody (left) and lysates were probed for SEPT9, CDK1, and GAPDH (right). All precipitate samples were analyzed on the same blot, as were all lysate samples. (B) Pin1 is required to mask the 10C10 epitope on SEPT9 upon mitotic entry. HeLa cells were treated with control or Pin1 siRNA, and SEPT9 was immunoprecipitated with 10C10 as in (A). Numbers to the right of the blots represent molecular mass standards in kilodaltons.
Consistent with previous work, cells treated with control and Pin1 siRNA had similar cell cycle profiles (Xu and Manley, 2007), and arrested in mitosis with similar efficiencies upon treatment with nocodazole (Figure 5.11). Similar results were observed upon depletion of CDK1, as discussed above. Therefore, the rescue observed upon depletion of CDK1 or Pin1 was not an indirect consequence of an inability to enter mitosis.
Figure 5.11 Depletion of CDK1 or Pin1 does not alter cell cycle profiles or impair mitotic arrest with nocodazole.

(A), (B), (C), HeLa cells were transfected with control (A), CDK1 (B), or Pin1 (C) siRNA and subsequently treated with nocodazole (mitotic) or without nocodazole (unsynchronized). Cells were then subjected to cell cycle analysis by flow cytometry. The cell cycle profile remains essentially unaltered upon depletion of CDK1 or Pin1. Likewise, cells were efficiently arrested in mitosis upon treatment with nocodazole, even after CDK1 or Pin1 knockdown. The slight shift in the CDK1 KD – mitotic sample is due to differences in acquisition parameters.
5.2.4  **Like SEPT9, Pin1 is also important for midbody abscission**

I next sought to elucidate whether Pin1 is important for midbody abscission, as I have demonstrated for SEPT9. I depleted Pin1 by siRNA (Figure 5.12A) and assayed for defects in cytokinesis by immunofluorescence. Strikingly, Pin1-depleted cells exhibited a similar cytokinetic defect to that observed upon SEPT9 depletion. 19% of Pin1-depleted cells remained joined by a midbody, compared to 7% of the control cells (Figure 5.12B, C; t test, p < 0.005). Similar results were observed with a second siRNA targeting a different region of the Pin1 gene (Figure 5.12C), arguing that this cytokinetic defect was not due to off-target effects of the siRNA. These results suggest that Pin1 may also play a role in mediating midbody abscission.
Figure 5.12  Pin1 is important for midbody abscission.

(A) Efficiency of Pin1 depletion by siRNA. HeLa cells were treated with control siRNA or one of two siRNAs targeting different regions of the Pin1 gene. Lysates were probed for Pin1 and GAPDH. Numbers to the right of the blots represent molecular mass standards in kilodaltons. (B) Representative example of cells attached by persistent midbodies following depletion of Pin1 (red, α-tubulin; blue, DNA). Scale bar represents 17 µm. (C) Quantification of effect of Pin1 depletion on cytokinesis. The percentage of cells exhibiting cytokinesis defects (multiple nuclei (Multinuc) or persistent midbodies) was determined upon treatment with control or Pin1 siRNA. Data are represented as mean +/- SEM (n ≥ 300 cells from 3 or more independent experiments). Asterisks indicate differences between control and Pin1 knockdown cells; **p < 0.005 (t-test).
To confirm these results, I followed HeLa cells treated with control (Figure 5.13A) or Pin1 siRNA (Figure 5.13B) through cell division by time-lapse microscopy. In cells treated with control siRNA, the midbody abscised an average of 3 hours after the onset of cytokinesis, and 97% of these cells successfully completed abscission within 5.5 hours (Figure 5.13C). The slight discrepancy between the control cells here and those presented in Chapter 3 is likely due to variations in experimental conditions. As was observed upon SEPT9 depletion, cells depleted of Pin1 exhibited defects in midbody abscission. Of the cells that successfully abscised, the average abscission time was 4.4 hours (Figure 5.13C). However, only 64% of Pin1-depleted cells abscised within 5.5 hours of cytokinesis onset. The remaining cells either underwent apoptosis after abscission failure, or took longer than 6.25 hours to abscise (in some cases, imaging was terminated at this point). This was somewhat surprising given that mice lacking Pin1 are viable (Fujimori et al., 1999), but the recent discovery of a second functional Pin1 isoform in mice (but not humans) suggests that redundancy may only exist in mice (Zhu et al., 2007). In addition, others reported similar findings while this work was being conducted (van der Horst and Khanna, 2009). Therefore, Pin1 is also important for midbody abscission, thus suggesting that Pin1 acts as a regulator of cytokinesis.
Figure 5.13 Live imaging of Pin1-depleted cells.

(A) Division of HeLa cells after treatment with control siRNA. HeLa cells were transfected with control siRNA and randomly selected cells were followed through division by time-lapse microscopy. The time (in hours:minutes) since the beginning of DNA segregation is shown. Black arrows point to intact midbodies, whereas white arrows denote abscission. Scale bar represents 16 µm. (B) Pin1 depletion causes defects in midbody abscission. HeLa cells were transfected with Pin1 siRNA and imaged as in (A). (C) Quantification of effect of Pin1 depletion on midbody abscission. The time from DNA segregation to midbody abscission was determined for each cell (N = 32 cells for control KD, n = 33 cells for Pin1 KD), and the cumulative percentage of cells that have abscised is plotted as a function of time.
5.2.5 The Pin1-SEPT9 interaction is important for the completion of cytokinesis

Having demonstrated that both SEPT9 and Pin1 are important for midbody abscission, I sought to determine whether the SEPT9-Pin1 interaction is important for this process. To this end, I generated a stable cell line that expresses siRNA-resistant SEPT9_i3 T24A under the control of an inducible promoter. This cell line exhibits low levels of leaky expression in the absence of induction (Figure 5.14A). Similar to what was observed with SEPT9_i4 (Figure 4.7E), the basal expression of siRNA-resistant SEPT9_i3 T24A caused severe cytokinetic defects, even in the presence of endogenous SEPT9 (Figure 5.14C; compare control KD in the parent cell line to control KD in the SEPT9_i3 T24A cell line; t test, p < 0.005). Approximately half of these cells remained joined by midbodies to one or multiple cells, and/or had more than one nucleus (Figure 5.14B, C). Similar defects were observed in multiple independent clones, arguing that this is not a clone-specific phenomenon. These defects were not suppressed by depleting endogenous SEPT9 (Figure 5.14B), suggesting that they are not simply the result of increased total SEPT9 levels. Further, depletion of endogenous SEPT9 in the SEPT9_i3 T24A cell line did not cause an increase in cytokinesis defects. These results suggest that SEPT9_i3 T24A has a dominant negative effect, and argue that the interaction between SEPT9 and Pin1 is important for the completion of cytokinesis.
Figure 5.14 Expression of SEPT9_i3 T24A causes defects in cytokinesis.

(A) Stable cell line inducibly expressing Flag-SEPT9_i3 T24A was treated with increasing amounts of doxycycline, and expression was assayed by Western blotting with a Flag antibody. Note that Flag-SEPT9_i3 T24A is expressed at low levels in the absence of doxycycline.

(B) Expression of SEPT9_i3 T24A causes cytokinetic defects. Representative examples of cells attached by persistent midbodies (in some cases to several cells) and multinucleated cells following basal stable expression of siRNA-resistant SEPT9_i3 T24A (red, α-tubulin; blue, DNA). White arrows point to midbodies, which in some cases appear to have regressed. Scale bar represents 53 μm.

(C) Expression of SEPT9_i3 T24A causes cytokinetic defects. The parent and siRNA-resistant SEPT9_i3 T24A cell lines were treated with control or SEPT9 siRNA and assayed for defects in cytokinesis by immunofluorescence microscopy. Unresolved cytokinesis refers to cells exhibiting midbody attachment or multinucleation. The parent cells are the same as those in Figure 4.7 as these experiments were performed concurrently. Data are represented as mean +/- SEM (n ≥ 300 cells from 3 experiments), **p < 0.005 (t-test).
5.2.6  **Pin1-mediated isomerization of SEPT9 is not important for exocyst recruitment to the midbody**

These results raise the question of whether Pin1-mediated isomerization of SEPT9 is important for the accumulation of the exocyst complex at the midbody, or whether these are independent events. Depletion of Pin1 by siRNA had no effect on the localization of the exocyst component Sec8 at the midbody (Figure 5.15). In addition, I found that Sec8 localization at the midbody appeared normal in the SEPT9_i3 T24A cell line. This suggests that SEPT9 mediates recruitment of the exocyst complex to the midbody in a Pin1-independent manner.
Figure 5.15 Pin1 is not important for the proper localization of the exocyst complex at the midbody.

(A) Representative examples of Sec8 localization in cytokinetic cells following treatment with control or Pin1 siRNA. Scale bar represents 17 μm. Arrows point to the midbody. (B) The percentage of cells exhibiting enrichment of Sec8 at the midbody was determined by immunofluorescence after treatment with control or Pin1 siRNA. Data are represented as mean +/- SEM (n ≥ 300 cells from 3 experiments). (C) Sec8 fluorescence / pixel (arbitrary units) at the midbody was measured using Image J software after treatment with control or Pin1 siRNA.
5.3 Discussion

5.3.1 Mitotic phosphorylation of SEPT9 by CDK1 regulates association with Pin1

Given that mammalian septins undergo dramatic changes in localization and possibly function at the onset of cell division, it has long been speculated that they may be regulated by the cell cycle machinery that controls mitotic entry (Hall and Russell, 2004). However, experimental evidence supporting this hypothesis has been non-existent. The work presented here describes the first link between a mammalian septin and the cell cycle machinery. Specifically, I have demonstrated that CDK1 phosphorylates SEPT9 at T24 at the onset of mitosis. This creates a S-pT-P motif, which is a perfect match to the consensus recognition motif of the Plk1 PBD (Elia et al., 2003). While SEPT9 did interact with the PBD of Plk1, this association was not dependent on phosphorylation at T24. Further work will be required to identify the Plk1 binding site in SEPT9 and elucidate the functional significance of this interaction. I have demonstrated that mitotic phosphorylation of SEPT9 at T24 by CDK1 creates a binding site for the WW domain of Pin1. Immunoprecipitation analysis revealed that the 10C10 epitope on SEPT9 is masked in a CDK1 and Pin1-dependent manner. This is consistent with the notion that Pin1 induces a conformational change in the N-terminal region of SEPT9 at the onset of mitosis, in response to phosphorylation at T24 by CDK1.

5.3.2 The functional significance of Pin1-mediated isomerization of SEPT9

As was observed upon depletion of SEPT9, depletion of Pin1 with two different siRNAs led to an increase in the percentage of cells exhibiting midbody attachment. Time-lapse microscopy confirmed that Pin1-depleted cells exhibit defects in abscission, demonstrating that Pin1 is important for the completion of cytokinesis in HeLa cells. These findings were also confirmed by a recent study, which reported similar results (van der Horst and Khanna, 2009). In addition, detailed analysis of dividing mouse embryonic fibroblasts (MEFs) derived from Pin1 knockout mice revealed that these cells take twice as long to abscise compared to wild type MEFs. Therefore, it appears that Pin1 plays an important role in abscission.
While SEPT9_i3 was sufficient to drive cytokinesis in the absence of the other SEPT9 isoforms, SEPT9_i3 T24A (which cannot interact with Pin1) acted in a dominant negative manner, causing severe defects in cytokinesis. Similar defects were observed upon expression of SEPT9_i4 (Chapter 4), which lacks the Pin1 binding site. These results argue that the SEPT9-Pin1 interaction is important for the completion of cytokinesis. Since I have shown that SEPT9 mediates the recruitment of the exocyst complex to the midbody, it seemed feasible that Pin1-mediated isomerization of SEPT9 may be important for this process. However, both depletion of Pin1 by siRNA and expression of SEPT9_i3 T24A had no effect on exocyst complex localization, suggesting that SEPT9 mediates recruitment of the exocyst complex to the midbody in a Pin1-independent manner. As discussed in Chapter 4, it is possible that recruitment of the exocyst complex to the midbody is not strictly dependent on SEPT9 per se, but is mediated by a diffusion barrier created by robust septin filaments. Pin1-mediated isomerization of the extreme N-terminus of SEPT9 may not affect septin polymerization state, and as a result may have no role in exocyst recruitment. Therefore, it appears that SEPT9 plays dual roles during cell division (Figure 5.16): SEPT9 mediates the recruitment of the exocyst complex to the midbody in a Pin1-independent manner, and performs some other role in a Pin1-dependent manner.

![Figure 5.16 Model for SEPT9 function during cell division.](image)

Upon entry into mitosis, CDK1 phosphorylates SEPT9 at T24, which creates a binding site for the WW domain of Pin1. Pin1 induces a conformational change in the N-terminal region of SEPT9, which is important for midbody abscission via an unknown mechanism. SEPT9 also mediates the recruitment of the exocyst complex to the midbody at the terminal stage of cytokinesis; however, this occurs in a Pin1-independent manner.
Pin1-mediated isomerization of the N-terminus of SEPT9 could unmask a binding site for some other protein (which I will refer to as protein X), and this interaction may be important for abscission. SEPT9_i3 T24A would be unable to interact with protein X, as the binding site would remain masked upon mitotic entry. Consequently, SEPT9_i3 T24A would be expected to act in a dominant negative manner, as was observed. SEPT9_i4 would lack the protein X binding site altogether. As a result, SEPT9_i4 would also be expected to act in a dominant negative manner, as was observed (Chapter 4). Overexpression of SEPT9 N-term resulted in a drastic increase in the percentage of cells with persistent midbodies (Chapter 4). This dominant negative effect could be due to SEPT9 N-term interacting with protein X, and preventing it from associating with endogenous SEPT9. One could argue that overexpression of SEPT9 N-term could impair abscission by simply interacting with Pin1, and preventing it from acting on endogenous SEPT9. However, overexpression of SEPT9 N-term T24A, which cannot interact with Pin1, also impaired abscission. Therefore, the abscission defect observed upon overexpression of SEPT9 N-term is not due to a SEPT9 N-term – Pin1 interaction. This also implies that uncoupling the N-terminal region of SEPT9 from the context of full length SEPT9 may unmask the protein X binding site, thus preventing Pin1 from regulating the availability of this site.

In an effort to identify protein X, I generated a construct that expresses SEPT9 N-term fused to a tandem affinity purification (TAP) tag (5’ TAP SEPT9 N-term). Several proteins with an approximate molecular mass of 75kDa co-purified with SEPT9 N-term after transfection into HeLa cells and large-scale purification. Mass spectrometry identified these proteins as members of the heat shock protein (HSP) family (HSPA5, isoform 1 of heat shock cognate 71kDa protein, heat shock 70kDa protein 1A/1B, and stress 70 protein, mitochondrial). While these interactions could merely reflect the association between heat shock proteins and an overexpressed protein, there is precedent for a potential interaction between septins and HSP70 proteins. Large scale pulldowns using GST-Borg3 identified septins and HSP70 as putative interactors (Joberty et al., 2001). In addition, immunoprecipitation of endogenous SEPT6 resulted in the co-precipitation of HSP70 family members (Kremer et al., 2005).
A more attractive protein X candidate is the scaffolding protein Cbl interacting protein of 85 kDa (CIN85; also known as Ruk or SETA), which was recently identified as a putative interactor of the N-terminal extension of SEPT9 in a yeast two-hybrid screen (Nakahira et al., 2010). This protein contains three SH3 domains that bind the consensus motif PX(P/A)XXR (Kurakin et al., 2003). A proteomic screen to identify binding partners of these SH3 domains yielded SEPT9 as the top candidate, strongly suggesting that SEPT9 may interact with CIN85 (Havrylov et al., 2009). SEPT9 contains two putative CIN85 interaction motifs, which are both located within SEPT9 N-term (Figure 5.16). One of these motifs is located within the 10C10 epitope that is masked upon mitotic entry in a CDK1 and Pin1-dependent manner. This raises the intriguing possibility that isomerization of SEPT9 by Pin1 may open up a binding site(s) for CIN85, thus allowing these proteins to interact upon mitotic entry. Several lines of evidence suggest that such an interaction could be important for the completion of cytokinesis. First, CIN85 associates with the midbody components Alix and anillin (Chen et al., 2000; Havrylov et al., 2009). Second, CIN85 associates with both endosomal membranes (Zhang et al., 2009) and vesicles of the Golgi (Havrylov et al., 2008). Finally, the Drosophila homologue of CIN85 (called Cindr) localizes to the cleavage furrow and midbody, and is thought to be important for stabilizing the intercellular microtubule bridge at the terminal stage of cytokinesis (Haglund et al., 2010).

It should be noted that the R88W and S93F point mutations in SEPT9 that cause Hereditary Neuralgic Amyotrophy (HNA) are located between the two putative CIN85 binding sites (Figure 5.16; (Hannibal et al., 2009; Kuhlenbaumer et al., 2005)). It is therefore tempting to speculate that the HNA point mutations may alter the SEPT9-CIN85 interaction, which could contribute to the pathogenesis of this disease. Duplication of the N-terminal extension of SEPT9 in other HNA patients (Collie et al., 2010; Landsverk et al., 2009) could have similar consequences.
Figure 5.17  Schematic of the N-terminal region of SEPT9 that is common between SEPT9_i1 and SEPT9_i3 but absent in SEPT9_i4.

The Pin1 binding site is shown in red, whereas the 10C10 epitope is shown in blue. Putative CIN85 binding sites, which conform to the consensus motif PX(P/A)XXR (Kurakin et al., 2003) are underlined. Residues mutated in HNA (Kuhlenbaumer et al., 2005) are shown in green. Numbers at left indicate the amino acid position according to the sequence of SEPT9_i3.
5.3.3 SEPT9, Pin1, and cancer

The findings presented in this Chapter shed additional light on how alterations in SEPT9 levels may contribute to carcinogenesis. As discussed in Chapter 3, loss of SEPT9 function impairs the completion of cell division, thus potentially causing genomic instability, which could ultimately contribute to cancer (Fujiwara et al., 2005). Since the interaction between SEPT9 and Pin1 is important for the completion of cytokinesis, increased expression of SEPT9_i4 (Burrows et al., 2003; Scott et al., 2006), which lacks the Pin1 binding site, could lead to cancer in a similar manner. Indeed, I have demonstrated that modest increases in SEPT9_i4 levels, such that SEPT9_i4 is expressed at similar levels to the larger SEPT9 isoforms, cause defects in cytokinesis (Chapter 4). Overexpression of SEPT9_i1 (Gonzalez et al., 2007; Scott et al., 2006), which contains the Pin1-binding site, could overwhelm CDK1 and/or Pin1 and result in SEPT9 that is not isomerized by Pin1, which could have a similar effect. The finding that Pin1 is important for the cytokinetic function of SEPT9 is particularly interesting given the many links between alterations in Pin1 expression and cancer (Yeh and Means, 2007). Since variations in Pin1 levels would presumably directly affect the ability of SEPT9 to mediate abscission, it is possible that alterations in Pin1 expression could contribute to cancer through modulating SEPT9 activity. Future studies will be aimed at addressing this possibility.
6 Conclusions and Future Directions

6.1 All septins are not all created equally

While septin complex composition is largely unaltered at the onset of mitosis and throughout cell division, I have shown that different septin family members are important at different stages of cell division: SEPT2, SEPT7, and SEPT11 are important for the early stages of cytokinesis, whereas SEPT9 mediates midbody abscission at the terminal stage of cytokinesis. These results show that depletion of different septins can give rise to distinct phenotypes. As a result, it will be interesting to determine whether individual septin family members have diverse roles in other cell types such as neurons and sperm. In addition, the work presented here suggests that even different isoforms of a given septin can have diverse functions, as the long isoforms of SEPT9 were able to support cytokinesis, whereas SEPT9_i4 acted in a dominant negative manner. Therefore, a complete understanding of septin function will not only require analysis of the role of individual septins in septin-dependent processes, but will also require consideration of the function of individual septin isoforms.

Despite sharing greater than 80% sequence identity at the amino acid level, depletion of SEPT6 and SEPT11 had very different consequences: depletion of SEPT11 caused defects in the early stages of cytokinesis, whereas depletion of SEPT6 had no adverse effects on cell division. More complete silencing of SEPT6 may reveal a role for this septin during cell division. However, Sept6 knockout mice exhibit no obvious phenotype, arguing that this septin is dispensable for cell division (Ono et al., 2005). In contrast, deletion of the Sept11 gene in mice results in embryonic lethality (B. Zeiger, personal communication), consistent with a critical role for SEPT11 during cytokinesis. Therefore, cell division may require a septin complex containing SEPT11 instead of SEPT6, even though SEPT6 is regarded as a member of the ‘core septin complex.’ Interestingly, the sequences of SEPT11 and SEPT6 are most divergent at the N and C-terminal extensions, suggesting that one or both of these regions of SEPT11 may be important for the early stages of cell division. The work presented here also points to an important role for the N-terminal extension of SEPT9 in the late stages of cytokinesis. Therefore, it seems plausible that the N and possibly C-terminal extensions of individual septin family members could confer unique properties that are important for cell division, and likely other septin-dependent processes (discussed further below).
6.2 SEPT9 is important for midbody abscission and mediates the localization of the exocyst complex at the midbody

The work presented here provides insight into the cellular function of SEPT9. By specifically depleting SEPT9, I demonstrated that this protein plays an important role in midbody abscission, a function that is masked by the simultaneous depletion of other septins. Due to the fact that SEPT2, SEPT7, and SEPT11 have roles earlier in cytokinesis, it remains unclear whether these septin family members are also important for abscission. Addressing this possibility will require specific inhibition of SEPT2, SEPT7, or SEPT11 after the cleavage furrow has completely ingressed. Microinjection of septin antibodies into telophase cells may provide the temporal inhibition required to assess the role of these septins during abscission.

I have demonstrated that SEPT9 (and possibly other septin family members) mediates the localization of the exocyst complex to the midbody, where it promotes vesicle tethering that is important for abscission. The mechanism by which this occurs remains elusive. It is possible that SEPT9 directly interacts with exocyst subunits, and thus directly recruits this complex to the midbody. However, this seems unlikely given that SEPT9 and Sec8 do not colocalize at the midbody.

Alternatively, SEPT9 could be important for activating the small GTPase RalB, which is thought to mediate the recruitment of the exocyst complex to the midbody via direct interactions with the Sec5 and Exo84 subunits (Moskalenko et al., 2002; Moskalenko et al., 2003). I was unable to detect any consistent differences in the levels of active RalB in interphase or mitotic cells after treatment with control and SEPT9 siRNA. However, it is entirely possible that SEPT9 mediates the activation of RalB locally at the midbody. A probe that specifically binds to the active form of RalB in live cells would permit analysis of the levels of active RalB at the midbody in control and SEPT9-depleted cells.

A third possibility is that SEPT9 and other septin family members form a membrane diffusion barrier at either side of the intercellular bridge, thus confining the exocyst complex to the midbody. Since a similar mechanism has been described during cytokinesis in *Saccharomyces cerevisiae* (Dobbelaere and Barral, 2004), this could represent an evolutionarily conserved function of the septin family of proteins during cytokinesis. Therefore, future work should
examine the mobility of membrane proteins at the midbody in cells treated with control or SEPT9 siRNA. As discussed above, assessment of the contribution of SEPT2, SEPT7, and SEPT11 to such a diffusion barrier will require telophase-specific inhibition of these septins.

Since the exocyst complex also mediates vesicle tethering in other cellular contexts, it will be interesting to examine whether SEPT9 (and possibly other septins) are general regulators of exocyst complex localization.

6.3 Mitotic regulation of the N-terminal region of SEPT9 by CDK1 and Pin1 is important for the completion of cytokinesis

I have identified the first link between a mammalian septin and the cell cycle machinery by demonstrating that CDK1 phosphorylates SEPT9 at T24 in a mitosis-specific manner. This post-translational modification regulates association with the WW domain of the peptidyl-prolyl isomerase Pin1. I have further demonstrated that the 10C10 epitope in SEPT9 is masked upon mitotic entry in a CDK1 and Pin1-dependent manner. This is consistent with the notion that Pin1 induces a conformational change in the N-terminal region of SEPT9 in response to CDK1-mediated phosphorylation at T24 upon entry into mitosis. Depletion of Pin1 by siRNA and expression of SEPT9_i3 T24A both caused defects in abscission, suggesting that Pin1-mediated isomerization of the N-terminal region of SEPT9 is important for the completion of cytokinesis. However, isomerization of SEPT9 by Pin1 does not appear to be important for the proper localization of the exocyst complex at the midbody.

While this work has demonstrated that the N-terminal region of SEPT9 plays an important role in the completion of cytokinesis, further studies will be needed to clarify the mechanism by which Pin1-mediated isomerization of this region contributes to abscission. However, it seems quite feasible that this modification may regulate an interaction between SEPT9 and some unidentified protein that is important for abscission. A very attractive candidate is the scaffolding protein CIN85, which was recently identified as a putative interactor of the N-terminal region of SEPT9 (Nakahira et al., 2010). Consequently, examination of this potential interaction may further clarify the importance of the SEPT9 N-terminal region in cytokinesis, and may uncover
the mechanism by which Pin1-mediated isomerization of this region contributes to abscission. In addition, the point mutations in SEPT9 that cause HNA lie between the two putative CIN85 binding sites in the N-terminal region of SEPT9. It will therefore be of great interest to elucidate whether these point mutations affect the SEPT9-CIN85 interaction.

6.4 Implications for the septin family of proteins

Members of the septin family vary greatly in terms of the sequence and length of their N and C-termini (Hall and Russell, 2004). In the crystal structure of the SEPT2/6/7 complex (Sirajuddin et al., 2007), the extreme N and C-termini are disordered. Consequently, the role of these regions is poorly understood. Based on the location of the Pin1-binding site and the putative CIN85-binding sites near the extreme N-terminus of SEPT9, it is tempting to speculate that the N-terminal extension of septins may serve as an important regulatory domain that mediates protein-protein interactions. Consistent with this, phosphorylation near the extreme N-terminus of mouse SEPT5 by CDK5 regulates association with syntaxin-1 in the brain (Taniguchi et al., 2007). In addition, phosphorylation near the extreme C-terminus of the yeast septin Cdc3p by Cdc28p is required for septin ring disassembly (Tang and Reed, 2002), suggesting that the C-terminal domain of septins may also serve an analogous role. Since many septins, like SEPT9, have multiple isoforms with different termini, the various isoforms of a given septin may have different functions and may be differentially regulated. As a result, future work should focus on the unique N and C-terminal extensions of septin family members. Identification of binding partners and post-translational modifications within these regions may provide significant insight into the molecular functions of individual septins and their isoforms.

Intriguingly, the above described septin phosphorylation sites also represent putative Pin1-binding sites. In addition, the yeast Pin1 homologue, Ess1 (Hanes et al., 1989), demonstrates synthetic lethality with the yeast septin Cdc12 (Davierwala et al., 2005). Therefore, Pin1-mediated isomerization, in response to phosphorylation, may serve as an evolutionarily conserved mechanism of regulating septin function in a variety of cellular contexts.
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