Probing Septin Function Through Interaction Screens: Identification of Novel Septins and Possible Regulatory Mechanisms

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

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Abstract

Septins are a family of guanine nucleotide-binding proteins that function in eukaryotic cell division, where they form a high-order cortical structure at the site of division, which is essential in most eukaryotes. Expanded roles have evolved for septins in metazoans, where they also have essential functions in terminally-differentiated cell types, such as neurons and spermatozoa. Specific details of septin function are lacking in most roles described, due at least in part to the limited number of characterized binding partners. In this work, yeast two-hybrid screens and pull-downs from tissue homogenate were used to identify novel septin binding partners for subsequent characterization.

The neuron-enriched septin, SEPT5, interacted directly with SUMO E3 ligases of the PIAS family. However, I was not able to demonstrate endogenous sumoylation of SEPT5 and SUMO isoforms did not concentrate with the septins during cytokinesis. SEPT5 also interacted with a novel septin, SEPT12, which I further characterized to be testis-specific and localized to the annulus in mature spermatozoa. Further, using SEPT12-specific reagents, I determined that the annulus forms via sequestration and subsequent segregation from the Golgi during spermiogenesis. SEPT9 pull-downs identified another novel testis-specific septin, SEPT14.
Reagents specific to SEPT2 and SEPT9 also revealed a septin-rich structure in the seminiferous epithelium in close association with the ectoplasmic specialization. The specific role of septins in this structure awaits further characterization. Several other intriguing candidate septin-interaction partners were identified and the further study of their possible *in vivo* interaction with septins may provide substantial insight into the mechanisms of septin function in eukaryotes.
Acknowledgments

I am very grateful to Dr. William Trimble for his unwavering support over the duration of my graduate studies. He was open-minded and accepting, available, interested, and engaged in my work. Through his supervisory style, I was able to take a very self-directed approach to my studies and thoroughly examine my own motivators – through teaching, supervision of summer students, and participation in international conferences of the highest calibre. I am so grateful for this flexibility within the graduate program because it has provided me with an extremely well-rounded graduate experience and has ultimately provided the platform for transition into what I believe will be a long-lived and fulfilling career in University administration.

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<th>Full Form</th>
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<tr>
<td>AEBSF</td>
<td>4-(2-Aminoethyl) Benzenesulfonyl Fluoride Hydrochloride</td>
</tr>
<tr>
<td>AES</td>
<td>Apical Ectoplasmic Specialization</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<td>BES</td>
<td>Basal Ectoplasmic Specialization</td>
</tr>
<tr>
<td>bp</td>
<td>Base Pairs</td>
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<tr>
<td>BLAST</td>
<td>Basic Local Alignment and Search Tool</td>
</tr>
<tr>
<td>CDC</td>
<td>Cell Division Cycle</td>
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<tr>
<td>CDNA</td>
<td>Complimentary Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese Hamster Ovary</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>DUB</td>
<td>De-ubiquitinating</td>
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<td>E1</td>
<td>Enzyme 1</td>
</tr>
<tr>
<td>E2</td>
<td>Enzyme 2</td>
</tr>
<tr>
<td>E3</td>
<td>Enzyme 3</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced Chemiluminscence</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced Green Fluorescent Protein</td>
</tr>
<tr>
<td>EM</td>
<td>Electron Microscopy</td>
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<tr>
<td>ES</td>
<td>Ectoplasmic Specialization</td>
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<tr>
<td>EST</td>
<td>Expressed Sequence Tag</td>
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<tr>
<td>GDP</td>
<td>Guanosine Diphosphate</td>
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<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<td>GST</td>
<td>Glutathione S-transferase</td>
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<td>GTP</td>
<td>Guanosine Triphosphate</td>
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<td>Abbreviation</td>
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<td>GTPase</td>
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<td>HA</td>
<td>Hemagglutinin</td>
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<td>HECT</td>
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<td>Beta-D-galactosidase Gene</td>
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<tr>
<td>MAGI</td>
<td>Membrane Associated Guanylate Kinase with Inverted Domain Structure</td>
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<tr>
<td>MALDI-TOF</td>
<td>Matrix-assisted Laser-desorbed Ionization Time-of-flight</td>
</tr>
<tr>
<td>mM</td>
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</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<td>MYST</td>
<td>MOZ, Ybf2/Sas3, Sas2 and Tip60</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>NSF</td>
<td>N-ethylmaleimide Sensitive Fusion Protein</td>
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<tr>
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<tr>
<td>PIAS</td>
<td>Protein Inhibitor of Activated STAT</td>
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<td>PIMT</td>
<td>Protein-L-isoaspartate methyltransferase</td>
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<tr>
<td>PIN1</td>
<td>Protein Interactor of NIMA-1</td>
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<td>PIP</td>
<td>Phosphatidylinositol Phosphate</td>
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<td>PMSF</td>
<td>Phenylmethylsulfonylfluoride</td>
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<tr>
<td>PVDF</td>
<td>Polyvinyl Difluoride</td>
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<tr>
<td>RanBP2</td>
<td>Ran Binding Protein-2</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>----------</td>
<td>-----------------------------------------------------------</td>
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<tr>
<td>RanGAP</td>
<td>Ran GTPase Activating Protein</td>
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<tr>
<td>R-HPLC</td>
<td>Reverse-phase High Pressure Liquid Chromatography</td>
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<td>Really Interesting New Gene</td>
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<td>Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis</td>
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<td>SENP</td>
<td>Sentrin-specific Protease</td>
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<td>SNAP</td>
<td>Soluble NSF Attachment Protein</td>
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<td>Soluble NSF Attachment Protein (SNAP) Receptor</td>
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<td>SUE</td>
<td>Septin Unique Element</td>
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<td>Tight Junction</td>
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<td>YPD</td>
<td>Yeast Peptone Dextrose</td>
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1. INTRODUCTION

1.1. The Septin Family of Proteins

1.1.1. Discovery of the Septins in Saccharomyces cerevisiae

The septin family of genes was first characterized in the budding yeast, *Saccharomyces cerevisiae*. In a landmark paper which identified many key proteins involved in the regulation of the cell cycle, Hartwell and colleagues identified a sub-grouping of cell division cycle (CDC) genes essential for the progression of cytokinesis and further defined the role of four specific genes: CDC3, CDC10, CDC11 and CDC12 (Hartwell 1971). Temperature sensitive mutants of any of these genes resulted in defective cytokinesis: at the restrictive temperature, mutants underwent multiple rounds of bud emergence, initiation of DNA synthesis and nuclear division, attaining up to an octonucleate stage. These mutants failed to complete cytokinesis and remained attached together. Shortly thereafter, Byers and Goetsch (1976) published a report detailing a highly ordered circumferential ring of filaments, each 10nm in diameter, lying in direct apposition to the inner surface of the plasma membrane at the mother-bud neck. While the diameter of this ring was observed to increase with the diameter of the mother-bud neck during bud enlargement, it was not observed to constrict, but to disappear from the mother-bud neck at cytokinesis. Actin was then shown to localize to the bud site during early stages of bud formation, after which time it was shown to disappear from the mother-bud neck, only to reappear again during the final stages of cytokinesis (Kilmartin and Adams 1984). This suggested that the identity of the mother-bud neck filaments observed by Byers and Goetsch was not actin. These filaments were not tubulin, either, because in the same study it was demonstrated that tubulin forms a separate structure through the centre of the mother-bud neck, independent of the membrane (Kilmartin and Adams 1984). In pioneering work by John Pringle and colleagues, the septin gene products were pinpointed to an hourglass structure at the mother-bud neck throughout cytokinesis, until late in this process, when the septin ring structure was observed to split and recede into the respective mother and daughter cells (Haarer and Pringle 1987; Kim et al. 1991). These researchers then demonstrated loss of the same filaments seen by Byers and Goetsch in cytokinesis-defective septin mutants (Frazier et al. 1998). Collectively,
this data suggested that Cdc3p, Cdc10p, Cdc11p and Cdc12p were the major constituents of the 10nm filaments found at the mother-bud neck in yeast. There are now seven known septin genes in *S. cerevisiae*: SPR3 (Fares et al. 1996) and SPR28 (De Virgilio et al. 1996) are expressed only during sporulation and a seventh homologue of septin (SHS1) was later discovered (Mino et al. 1998) to function in vegetative growth.

### 1.1.2. Metazoan Septins and General Septin Structure

It is now known that, with the exception of plants, septins are highly conserved in eukaryotic species. Here, they function in complexes of remarkably variable size and composition. The number of molecules present in functional septin complexes varies considerably between organisms or specific tissue types in mammals, owing to the number of septin genes and corresponding protein products that a specific organism has, or the subset of expressed septins in a given cell/tissue type. For example, in a recent phylogenetic and evolutionary analysis, it was found that there are 14 septins in *Homo sapiens* (human), 13 in *Mus musculus* (mouse), 10 *Gallus gallus* (chicken), 9 in *Xenopus laevis* (frog), 17 in *Danio rario* (zebrafish), 4 in *Strongylocentrotus purpuratus* (sea urchin), 4 in *Ciona intestinalis* (Ciona), 2 in *Caenorhabditis elegans* (nematode) and 5 in *Drosophila melanogaster* (fruit fly) (Cao et al. 2007). Interestingly, these researchers determined that since evolutionary divergence of chordates from other multicellular eukaryotes, septins in all chordates are observed to separate into four major sub-families, where each sub-family member likely originated from a gene duplication event earlier in evolution. The phylogenetic relationship of the human septins is presented in Figure 1, as well as the basic septin structure. When the amino acid sequence of the core highly conserved GTP-binding and septin unique element (SUE) is compared between all septins, four major septin sub-families are observed, as expected (Cao et al. 2007).
Figure 1. Phylogenetic Relationship of the Human Septins and Key Structural Features.

(A) For each septin, the amino acid sequence of the highly conserved GTP-binding domain through to the end of the septin unique element (SUE) was entered. This unrooted dendritic tree was created using ClustalW at the Kyoto University Bioinformatics Institute (http://align.genome.jp), using the default settings. The distance between two septins indicates the degree of similarity between their core conserved primary amino acid sequences. (B) The core septin structure consists of variable N- and C-terminal sequences; in all septins but SEPT3, 9 and 12 the C-terminus is predicted to form a coiled-coil structure. There is a short polybasic sequence (PB) directly N-terminal to the G1 sequence of the nucleotide binding domain (composed of G1, G3 and G4 sequences shown in red). Directly following the G4 sequence is the SUE, a sequence highly conserved in septins, but of unknown function.
1.1.3. Biochemical Properties of Septins

Septins in all organisms possess a conserved GTP-binding domain and fall into the large superclass of P-loop GTPases (Leipe et al. 2002). Sequence comparison indicates that the GTP-binding domain is distantly related to the Ras family of small GTPases. The P-loop is defined by a Walker A motif (GXXGXGKST); a Walker B motif (DXXG); and the GTP-specificity motif (XKXD). These three sequences are also referred to as G1, G3 and G4, respectively (see Figure 1, B). Septins differ from most other members of this subclass because they lack an asparagine at position 1 of the GTP-specificity motif (AKAD or GKAD instead of NKXD).

Most septins have been observed to have little or no GTPase activity; likewise, they have not been observed to exchange nucleotide. However, there are variable results in the literature (Huang et al. 2006; Oegema et al. 1998; Steels et al. 2007; Vrabioiu et al. 2004). In any case, nucleotide hydrolysis and/or exchange is not thought to play a regulatory role in the assembly of septin hetero-oligomers beyond the initial folding of septin monomers (Kinoshita et al. 2002; Macara et al. 2002; Mitchison and Field 2002; Sheffield et al. 2003). Although GTPase activity has been demonstrated to be dispensable for the assembly of the S. cerevisiae septin collar, GTP binding is required for the establishment of higher ordered polymer (Versele et al. 2004).

Septins also contain a short (10-15 amino acids) polybasic region just N-terminal to the G1 sequence of the P-loop and this has been postulated to bind to membranes due to its similarity to a domain found in a variety of phosphoinositol-binding proteins (Joo et al. 2005; Zhang et al. 1999). Indeed, S. cerevisiae septins have been found to preferentially bind to phosphatidylinositol 4-phosphate (PI(4)P) and phosphatidylinositol 5-phosphate (PI(5)P) with their polybasic domains; binding to PI(4)P was determined to be essential for proper localization of septins to the mother-bud neck during cytokinesis (Casamayor and Snyder 2003). Mammalian SEPT4 was shown to bind to phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) and phosphatidylinositol 3,4,5-triphosphate (PI(3,4,5)P₃). PI(4,5)P₂ is produced at the cleavage furrow during mammalian cytokinesis, raising the possibility that mammalian septins are also recruited to membranes by localized lipid production (Emoto et al. 2005; Field et al. 2005; Zhang et al. 1999).
1.1.4.  The Basic Septin Unit Complex

Biochemical purification of septins from a variety of organisms has revealed that the simplest septin unit complex is made up of two copies of each of the different septin gene products it contains. Consistent with this, the *C. elegans* unit septin complex contains two copies of each of its two septins (tetramer) (John et al. 2007), *D. melanogaster* and some mammalian septin complexes contain two copies of three different septins (hexamer) (Farkasovsky et al. 2005; Field et al. 1996; Joberty et al. 2001; Kinoshita et al. 2002) and fungal complexes tend to contain 4-5 septins (octamer or decamer) (Field and Kellogg 1999; Frazier et al. 1998; Longtine et al. 1996; Versele and Thorner 2005). Higher order septin complexes containing many different septin gene products have also been isolated from brain tissue homogenates, suggesting more complex structures also form in mammals.

Recently, elegant studies using purified *C. elegans* septins have shown that the unit septin complex is apolar, unlike other higher-order structures composed of polymerized monomers, such as actin or tubulin (John et al. 2007). These researchers found that the two *C. elegans* septins, Unc59 and Unc61, polymerize in the linear order (Unc59:Unc61):(Unc61:Unc59). This finding has been supported by the recent crystal structure of the core mammalian septin complex, where the basic unit was found to consist of (SEPT7:SEPT6:SEPT2):(SEPT2:SEPT6:SEPT7), arranged in a linear order (Sirajuddin et al. 2007). Studies in *S. cerevisiae* have determined that the basic subunit in this organism is likely to be organized similarly (Gladfelter and Montagna 2007; Versele and Thorner 2005).

1.1.5.  Septin Filaments

Substantial evidence supports the notion that polymerization of the basic septin unit complex drives the formation of higher order septin filaments. First, septins biochemically purify from a variety of organisms in a basic unit complex that is consistently found to be approximately 25nm in length and 8-10nm in diameter (Field et al. 1996; Frazier et al. 1998; Hsu et al. 1998; Sirajuddin et al. 2007). Second, this unit complex is sufficient to form linear filaments, and in many cases, has been induced to re-assemble into filaments *in vitro* (Farkasovsky et al. 2005; Field et al. 1996; Frazier et al. 1998; Versele et al. 2004). This seems to require the minimum septin complement for formation of the unit septin complex, although notable exceptions include
X. laevis Sep2 (Mendoza et al. 2002) and H. sapiens SEPT2 (Huang et al. 2006), which are capable of forming homomeric filaments in vitro. Third, using electron microscopy and other high-resolution imaging techniques, higher-order septin assemblies are visible in S. cerevisiae at the mother-bud neck as distinct ultrastructures of rings and gauzes (Rodal et al. 2005). Fourth, the recent crystal structure of a mammalian septin complex more clearly explains the specific septin:septin interactions in the core septin unit complex, reconciles many conflicting septin:septin interaction results in the literature and suggests how higher order filament formation is likely to occur.

1.1.6. Crystal Structure of the Mammalian Septin Unit Complex

The structure of SEPT2 was recently solved to 3.4Å, demonstrating that the core of SEPT2 folds like the small GTPase Ras, as predicted from its primary amino acid sequence (Sirajuddin et al. 2007). Interestingly, SEPT2 interfaces were made up of two different conserved regions in septins, which these researchers term the “G-dimer” (whose interface consists of the guanine nucleotide binding pockets and the β7/8 strands) and the “NC-dimer” (whose interface consists of the α0/2/6 helices near the N- and C-termini). This is shown in Figure 2, A. In addition, SEPT2 has four characteristic extensions that contact adjacent monomers. As with Ras, SEPT2 appears to hold GDP by coordinating the β-phosphate with the G1 motif (GXXXXGKST), while contacting the guanine ring with the G4 domain (AKAD). Unfortunately, this study did not provide structural information about the coiled-coil, the details of the five ambiguous loop regions and the GTP-bound conformer. However, the model is suggestive of how individual septins may form homomeric filaments during overexpression and/or during in vitro experiments.

In the same report, the crystal structure of the mammalian SEPT2/SEPT6/SEPT7 hexamer was solved to 4Å, providing substantial information about the molecular details of unit septin complex formation (Sirajuddin et al. 2007). The crystal structure provided a picture of the unit complex consistent with previous observations: approximately 25nm in length and 5nm in diameter, as shown in Figure 2, B. The minimal asymmetric unit consists of a SEPT2/6/7 trimer with SEPT6 sitting in the middle, whose interfaces with SEPT2 and SEPT7 were similar to those of G- and NC-dimers, respectively (as seen with SEPT2). Remarkably, coiled-coils were
invisible in the crystal and apparently are not involved in inter-subunit interactions. Electron microscopy indicates that coiled-coils of adjacent monomers project out of the filament at a common location between subunits, 90-degrees relative to the length-wise axis of the filament (and are therefore likely to promote interaction between subunits and contribute to the diameter of the filament). In addition, SEPT6 was observed bound to GTP while SEPT2 and SEPT7 were bound to GDP. This model is highly consistent with others’ findings: (1) that some septins are capable of self-association while others are not; (2) that coiled-coils from two different septins can interact; (3) that there seem to be distinct subsets of septin binding partners for a given septin, suggesting an ordering within the unit septin complex and (4) that septin filaments contain a mixed ratio of GTP and GDP (Farkasovsky et al. 2005; Field et al. 1996; Frazier et al. 1998; Garcia et al. 2006; Hu et al. 2006; Joberty et al. 2001; Kinoshita et al. 2002; Low and Macara 2006; Mendoza et al. 2002; Sheffield et al. 2003; Versele et al. 2004; Vrabioiu et al. 2004).

Both the X-ray and electron microscopy data revealed that septin complexes are non-polar along their polymerization axis, unlike actin and tubulin. However, along the length of the filament, the disposition of the coiled-coils provides lateral polarity to the complex. As such, the ordered arrangement of septin filaments into sheets (presumably leading to the formation of observed gauzes (Rodal et al. 2005)), may allow higher order septin structures to possess planar polarity, where sheets or gauzes have two structurally or functionally distinct faces. For example, one face may interact with membrane components, while the other face may expose coiled-coils which then contribute to the recruitment of additional binding partners. As such, coiled-coils may also facilitate filament pairing/bundling and/or interaction with non-septin molecules. This model does not account for non-coiled-coil containing septins, such as SEPT3, 9 and 12 or S. cerevisiae Cdc10p; the latter of which has been suggested to laterally pair filaments thereby stabilizing the septin hourglass structure at the mother-bud neck (Versele et al. 2004).
Figure 2. Crystallization of Mammalian Septins Reveals Structural Insight.

(A) Ribbon representation of three SEPT2 monomers found in the crystal structure with two possible interfaces labeled “G-dimer” to denote the interface between two nucleotide binding domains and “NC-dimer” to denote the interface between two N-/C-terminal domains. Relevant helices are noted, as well as the position of GDP/GTP; dotted lines represent disordered regions.

(B) Surface representation of the basic hexameric unit. The hexameric unit makes contact with an adjacent hexameric unit using SEPT7, thereby making filaments. The presumed orientations of the coiled-coil sequences are shown schematically (arrows). Adapted from (Sirajuddin et al. 2007).
1.1.7. **Septin Function in S. cerevisiae Mitosis**

In *S. cerevisiae*, the septins function prominently in mitosis and sporulation. Early in the cell cycle, they coalesce at the presumptive bud site and then form a prominent collar resembling an hourglass at the bud-neck throughout cytokinesis. Near the end of this process, the septin structure splits into two separate rings, which digress into the respective mother and daughter cells and are disassembled. As such, specific roles for the septins in bud site selection, the establishment and maintenance of polarized bud growth, the switch from polarized to isotropic bud growth, and spindle positioning have been demonstrated (Faty et al. 2002; Gladfelter et al. 2002; Kusch et al. 2002).

During *S. cerevisiae* division, there seem to be two major roles for septins. First, they function as a scaffold for the recruitment of many proteins, such as components of the bud site selection machinery, the actomyosin cleavage apparatus, kinases, and chitin synthase enzymes. A well-characterized scaffold role includes the so-called morphogenesis checkpoint pathway: in this elaborate scheme involving the recruitment and differential phosphorylation of many components, the proper assembly of septin filaments directs efficient passage into mitosis. Briefly, the kinase Swe1p accumulates in the cell prior to mitosis, during DNA synthesis. During this time, it inhibits the mitosis-promoting cyclin-dependent kinase Cdc28p by phosphorylating it on a conserved tyrosine (Booher et al. 1993). Normally, Swe1p is gone from the cell by the time nuclear division has occurred and mitosis begins, releasing this brake. This occurs because the septin scaffold recruits the remaining cytoplasmic Swe1p, the kinase Hsl1p and the methyl transferase Hsl7p, which then form a complex together. Somehow formation of this trimeric complex requires scaffolding by the septins in order to then effect the degradation of Swe1p via its phosphorylation by subsequently recruited kinases, Cla4p and Cdc5p (Hanrahan and Snyder 2003; Lew 2003; Longtine et al. 2000; Shulewitz et al. 1999; Versele and Thorner 2005).

The second major role described for septins during *S. cerevisiae* cytokinesis is in establishing discrete subcellular compartments. It is now known that the septin collar serves to compartmentalize the membrane at the cleavage furrow. Sitting in direct apposition to the inner leaflet of the plasma membrane, sandwiching the actomyosin ring, the septins have been demonstrated to contain diffusible cortical factors at the site of cleavage. Such factors include
the Sec6/8 complex (*S. cerevisiae* exocyst) and polarisome, both of which are required for cytokinesis (Dobbelaere and Barral 2004). The endoplasmic reticulum is also compartmentalized by the septins during *S. cerevisiae* polarized growth and this is predicted to restrict pre-synthesized material from the bud, favouring only the insertion of new material into buds (Gladfelter and Montagna 2007; Luedeke et al. 2005). Finally, the septin collar has also been described to function more broadly as a diffusion barrier by restricting actin-based transport of mRNA between the mother and bud (Takizawa et al. 2000).

1.1.8. **Septin Function in Metazoan Mitosis**

Septins are essential for most forms of cytokinesis in *D. melanogaster*, where disruption of the gene encoding the septin Pnut results in a lethal phenotype with multinucleated cells (Neufeld and Rubin 1994) and where another *D. melanogaster* septin, Sep1 has been demonstrated to localize to the contractile ring in dividing cells (Fares et al. 1995; Neufeld and Rubin 1994). Similarly, *C. elegans* septins Unc59 and Unc61 are required for post-embryonic cell division (Nguyen et al. 2000), although not for embryonic divisions.

Not surprisingly, septins are also essential for mammalian cytokinesis (Kinoshita et al. 1997; Spiliotis et al. 2005; Surka et al. 2002), although their distribution is not restricted to a single robust structure, as in *S. cerevisiae*. During division of mammalian cells, septins localize to three distinct regions: 1) the kinetochore microtubules, where they have been postulated to scaffold centromere-associated protein E (CENP-E) and other effectors to coordinate cytokinesis with chromosome condensation and congression (Spiliotis et al. 2005); 2) the cortex of the cleavage furrow, where they work in concert with the actin-binding protein anillin and the actomyosin cleavage apparatus to effect ingression efficiently, much like in *S. cerevisiae* (Joo et al. 2007; Maddox et al. 2007); and 3) the mitotic spindle, although the role of septins at this last structure has not been explored in detail (Surka et al. 2002). While more specific details of septin function and dynamics during mammalian cytokinesis remain to be elucidated, it is speculated that there will be many similarities revealed between *S. cerevisiae* and mammalian cytokinesis. This is supported by the requirement of septins for this process in both organisms and the conserved ability to bind such essential components of cytokinesis as anillin and the exocyst complex, the latter of which has been suggested to function in the delivery of membranes to the site of

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absission within the narrow ring of plasma membrane partitioned by the split septin ring, late in cytokinesis (Boyd et al. 2004; Finger and Novick 1998; Hsu et al. 1998; Joo et al. 2005).

1.1.9. Expanded Roles for Metazoan Septins

While septins are critical for cytokinesis, in metazoans they have also been implicated in a vast number of other cellular processes. Interestingly, while septin assemblies in *S. cerevisiae* are limited to distinct ultrastructures of rings and gauzes (Kinoshita 2006), metazoan septins are often found dispersed in the cortical cytoplasm or integrated with actin- or tubulin-based structures.

Interestingly, septins localize to various distinct locations at the cleavage furrow during cytokinesis. They are also found dispersed along stress fibres and microtubular networks in interphase cells, where they appear to influence the integrity and stability of these structures (Joo et al. 2007; Kinoshita et al. 1997; Kremer et al. 2005; Spiliotis et al. 2008; Surka et al. 2002).

While most septins are expressed in the brain, SEPT5 was the first septin for which a role was suggested in synaptic vesicle exocytosis in neurons (Beites et al. 1999). In this and subsequent studies, SEPT5 was demonstrated to interact directly with the essential SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor) component, syntaxin-1.

The current version of the SNARE hypothesis (Sudhof 2004) suggests that a vesicle (v)-SNARE associates with its cognate target membrane (t)-SNARE, and syntaxin-1, to form a ternary complex with a sedimentation value of 7S. These SNAREs, localized on opposing membranes, form a tight connection between the membranes in a *trans* conformation. This is proposed to provide the driving force for membrane fusion. During membrane fusion, the SNARE transmembrane domains become aligned in parallel in a *cis* configuration on the same membrane, requiring disassembly in order for further rounds of fusion to take place. Thus, once membrane fusion has occurred, this 7S complex serves as a target for soluble NSF (N-ethylmaleimide-sensitive fusion protein) through its attachment receptor (SNAP), and together they form a 20S complex. NSF is an ATPase, whose catalytic activity leads to the disassembly of the SNARE complex (Haas 1998), freeing the SNAREs for subsequent rounds of exocytosis. Interestingly, overexpression of point mutants of SEPT5 augmented normal synaptic vesicle
exocytosis in pheochromocytoma cells (PC-12), suggesting that SEPT5 plays some role in regulation of exocytosis. A more recent study demonstrates that SEPT5 can bind to syntaxin-1 while it is part of the trans SNARE complex, but not once NSF (N-ethylmaleimide-sensitive fusion protein) is recruited to the complex (presumably in the cis conformation) to effect its disassociation for subsequent rounds of fusion. Thus SEPT5 has been implicated to function directly in the regulation of exocytosis, although this process is not well understood and is limited to two studies (Beites et al. 2005; Beites et al. 1999).

In neurons, septins have also been shown to localize to growth cones and axon tips and have been demonstrated to function in neurite outgrowth and axon guidance, in addition to being implicated in the synaptic vesicle cycle (Beites et al. 2005; Beites et al. 2001; Beites et al. 1999; Finger et al. 2003; Hsu et al. 1998; Kinoshita et al. 2000; Xue et al. 2004). Most recently, septins have been localized to the base of dendritic spines, where they are suggested to function as a scaffold and/or diffusion barrier during neurotransmission and synaptic plasticity (Tada et al. 2007; Xie et al. 2007).

Septins are also found in mature spermatozoa, where they contribute to the cytoskeletal structure of the sperm by forming the annulus, a prominent proteinaceous barrier between the middle and principal portions of the tail. Recently, through targeted disruption of the Sept4 gene in mice, SEPT4 was found to be an essential component of the annulus. In the absence of SEPT4, the annulus was completely missing: sperm tails were bent back on themselves, kinesin-mediated transport within the tail was defective, and the sperm were immotile (Ihara et al. 2005; Kissel et al. 2005). Since several other septins were localized to the annulus in wildtype animals and subsequently mislocalized in the absence of SEPT4, septins are now suspected to be the principal protein components of this cytoskeletal structure.

Additional roles described for mammalian septins include: phagosome formation (Huang et al. 2008); epithelial cell polarity (Spiliotis et al. 2008); platelet function (Dent et al. 2002); mitochondrial function and apoptosis (Larisch et al. 2000); regulation of gene expression (Amir et al. 2006); cardiomyocyte function (Ahuja et al. 2006); retinal cell function (Pache et al. 2005; Xin et al. 2007); and many more. Aberrant septin expression is associated with many disease states, including Alzheimer disease, Parkinson disease, schizophrenia and many cancers, although the reasons for this remain elusive. Thorough reviews addressing the implication of
septin misregulation in various disease states have been presented by Hall and Russell (Hall and Russell 2004; Russell and Hall 2005). Subcellular septin localization reported to date is summarized in a recent review by Lindsey and Momany (Lindsey and Momany 2006).

In most cases, the disruption of specific septin genes in mice has not allowed for elucidation of functions attributable to specific septin gene products. Frequently, such gene disruptions result either in embryonic lethality or a minimal/lack of phenotype, speculated to arise due to functional complementation by highly similar septins of the same sub-family (Peng et al. 2002). As such, meticulous tissue-specific gene disruption using conditional knockout mice and carefully selected spatio-temporally regulated promoters will be essential for further elucidation of septin function.

1.1.10. Variable Tissue Expression of Mammalian Septins

While some septins are broadly expressed, others are found only in specific tissues or cell types. For example, SEPT3 and SEPT5 appear to be expressed almost exclusively in the brain, while SEPT7 appears to be ubiquitously expressed. As such, it is expected that certain septins (such as SEPT7) are universal components of septin unit complexes, while others within the same sub-family (see Figure 1) are interchangeable (such as SEPT4 and SEPT5) and impart tissue or cell-type specific properties to septin structures. In this manner, specific localizations, functions and binding partners may be regulated, potentially allowing for a huge variety of additional properties beyond the basic higher order septin structure. The generation of multiple splice forms introduces another level of complexity to septin expression and function. Dr. Christopher Tsang, a recent graduate of the Trimble Lab generated and/or acquired antibodies specific to all septins known at the time of his work. He generated data for the tissue specificity plot, shown in Figure 3, which is generally consistent with literature reports for the expression distribution of the mammalian septins (Cao et al. 2007; Hall et al. 2005).
Figure 3. Tissue Expression of SEPT1-9, SEPT11.

Tissue homogenates were probed with antibodies specific to the septins listed. Note the presence of multiple splice forms (SEPT4, 9) and broad (SEPT9) versus specific (SEPT5) tissue distribution. Adapted from (Tsang 2007).
1.1.11. **Septin Binding Partners**

Despite a vast body of literature on septins and descriptions of their involvement in a vast array of cellular functions, very few direct binding partners have been identified, making it difficult to elucidate specific cellular functions of septins. While several key proteins involved in cytokinesis have been identified, as well as phosphoinositides, this list is limited to a handful of proteins in mammals (see Table 1). The list is more extensive in *S. cerevisiae*, but includes many proteins specific to this organism, such as chitin synthases. Many binding partners are listed in a recent review (Kinoshita 2006), although this list is limited to well-established binding partners for which functional consequences of interaction with septins have been assigned in many cases. While septins have long-been observed at places such as the site of cell division, the list of observed septin roles in mammals and the characterization of their interaction with other proteins and molecules continues to grow.
Table 1. Septin Molecular Interactors and Possible Functions.

Molecular interaction and possible functions of the septins and septin-containing structures in various eukaryotic organisms. Adapted from (Kinoshita 2006).

<table>
<thead>
<tr>
<th>Subcellular structure</th>
<th>Direct/Indirect Interaction Partner</th>
<th>Presumed or Hypothetical Function</th>
<th>References</th>
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<tr>
<td><strong>S. cerevisiae</strong></td>
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<td>Septin collar</td>
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<td>Division plane organization</td>
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1.1.12. Septin Regulation: Post-translational Modification

Phosphorylation

In *S. cerevisiae*, the septin ring is known to transition between a fluid state that exchanges subunits and a rigid, static structure. The initial septin patch at the presumptive bud site is fluid, but the structure becomes static upon transition into the septin collar structure. When the septin ring splits, however, the septin rings become fluid again (Dobbelaere et al. 2003). The transition into a static collar-shaped structure has been correlated with phosphorylation of the different constituent septins at various residues. However, this process is very poorly understood and in many cases, the specific residues that are modified and the kinases involved remain to be determined (Dobbelaere and Barral 2004; Tang and Reed 2002; Versele et al. 2004).

Interestingly, septin ring splitting is linked to the mitotic exit network and requires the activity of the small GTPase Tem1p. Septins play an essential role in restricting mitotic exit until one spindle pole body has entered the bud by restricting the Tem1p activator, Lte1p, to the bud (Castillon et al. 2003; Lippincott et al. 2001). Active Tem1p in turn activates PP2A phosphatase, which localizes to the septin hourglass structure and dephosphorylates septins.

The activity of these proteins is speculated to drive both septin hourglass splitting and a return of the septin structure to a fluid, diffusible state, presumably allowing for actomyosin ring contraction. Thus, the dynamics of phosphorylation have been correlated to a massive shift in polarity of septin filaments at the mother-bud neck upon ring splitting and the distinct change in cellular machinery recruited to the septin scaffold at this time in cell division (Steels and Trimble 2006; Vrabioiu and Mitchison 2006). Determination of the specific molecular consequences of septin phosphorylation will be of utmost interest in the further understanding of the dynamics of septin structures and filament dynamics.

While it is now known that many mammalian septins are also phosphorylated, in most cases even less is known of the signaling cascades, kinases and phosphatases involved. For example, phosphorylation of recombinant SEPT2 produced in insect cells has been demonstrated (She et al. 2004); as well as SEPT9 isolated from mitotic cell extracts (Beausoleil et al. 2006). Modification of brain-specific SEPT3 at serine-91 appears to play a role in the regulation of this protein’s interaction with membranes (Xue et al. 2004) and phosphorylation of SEPT5 at serine-
327 has been suggested to regulate its interaction with syntaxin, thereby contributing to the regulation of exocytosis (Amin et al. 2008).

**Ubiquitination**

Although it has been demonstrated in *S. cerevisiae* that septins are not degraded during the cell cycle (Gladfelter and Montagna 2007; Johnson and Blobel 1999), misregulation of ubiquitin-mediated degradation of septins has been implicated in various mammalian neuropathologies. This is consistent with the implication that misregulation of septin levels may contribute a common underlying pathology of these disease states. Overexpression of SEPT5 and SEPT4 in neurons has been shown to result in neurotoxicity and SEPT5 has been shown to interact with the ubiquitin ligase parkin, leading to its ubiquitin-mediated degradation (Choi et al. 2003; Dong et al. 2003; Moran et al. 2007; Munoz-Soriano and Paricio 2007; Son et al. 2005). *D. melanogaster* septins Sep1 and pnut have also been shown to interact with the fly orthologue of parkin, suggesting the inter-species conservation of the parkin-mediated degradation of septins plays some important role in the regulation of septin levels (Bae et al. 2007).

**Sumoylation**

Robust sumoylation of *S. cerevisiae* septins also occurs during cytokinesis, although this has not been observed in mammalian cells (Johnson and Blobel 1999; Johnson and Gupta 2001). Small ubiquitin-like modifier (SUMO) is a member of the ubiquitin fold family; in *S. cerevisiae* it is known as Smt3p. It is post-translationally added to specific lysine residues on target proteins which are usually embedded in a consensus sequence. Sumoylation results in an alteration of the surface of a given protein and often changes its interaction specificity and a thorough introduction will be presented in the following section. In addition, it has been reported for some proteins that ubiquitination is antagonized by sumoylation at a common lysine residue (Johnson 2004). Sumoylation of the *S. cerevisiae* septins was observed to occur preferentially on the mother side of the bud-neck and this was originally speculated to facilitate breakdown of the higher-order septin ring following cytokinesis. However, subsequent studies suggest the alteration of ring breakdown dynamics was artificially introduced by generation of many simultaneous point mutations. As such, the function of septin sumoylation in *S. cerevisiae* remains unclear and it is not known if septins are reversibly modified by SUMO under any specific cellular context in mammals.
1.2. The Sumoylation Pathway

1.2.1. Overview

SUMO was discovered to be a reversible post-translational protein modifier more than a decade ago. The gene encoding SUMO was first discovered in *S. cerevisiae* in a genetic screen for suppressors of the centromeric protein, Mif2 (Meluh and Koshland 1995). Initial characterization of SUMO revealed that it was a binding partner of human RAD51 and RAD52, as well as PML (Boddy et al. 1996; Okura et al. 1996; Shen et al. 1996). However, covalent attachment of SUMO was first observed for the target substrate Ran GTPase-activating protein (RanGAP1) (Mahajan et al. 1997; Matunis et al. 1996). These latter studies established SUMO as a reversible modifier and demonstrated that sumoylation can alter the localization of the modified target by altering the capacity for specific protein-protein interactions; while unmodified RanGAP1 is cytosolic, sumoylated RanGAP1 is targeted to the nuclear pore via interaction with the nucleoporin RanBP2.

The discovery of SUMO led to a rapid burst of papers reporting the characterization of the step-wise enzymatic modification pathway and the reversible modification of many sumoylation target proteins. The pattern of SUMO conjugates is dynamic and changes during the cell cycle and in response to various stimuli. A consensus sumoylation sequence has been defined as well as many downstream consequences of sumoylation. Sumoylation shares a characteristic of all dynamic modification pathways: only a small proportion of a target protein is modified, but this can have dramatic effects. While the sumoylation pathway components are localized to the nucleus, the vast majority of SUMO targets are nuclear transcription factors. However, a growing body of literature supports the sumoylation of many cytoplasmic and plasma membrane proteins. It is now known that the sumoylation pathway is highly conserved and is essential in a variety of eukaryotic organisms, including: *S. cerevisiae* (Johnson et al. 1997), *C. elegans* (Fraser et al. 2000), *Arabidopsis thaliana* (Saracco et al. 2007) and *M. musculus* (Nacerddine et al. 2005).
1.2.2. **Sumoylation: An Enzymatic Cascade**

While simple eukaryotes such as *S. cerevisiae*, *D. melanogaster* and *C. elegans* each have only one SUMO gene, higher eukaryotes have an expanded number of SUMO genes. Humans have four SUMO genes: SUMO1, 2 and 3 are ubiquitously expressed, while SUMO4 appears to be expressed mainly in the kidney, lymph node and spleen (Guo et al. 2004).

SUMO proteins are approximately 10kDa in size. The structure has been solved and SUMOs and ubiquitin are virtually superimposable, despite a very low degree of sequence identity (Bayer et al. 1998; Bernier-Villamor et al. 2002; Mossessova and Lima 2000). All the SUMOs are expressed in an immature pro-form which must be proteolytically activated by removal of a C-terminal stretch of 2-11 amino acids, thereby revealing the invariant mature C-terminal di-glycine motif which is required for conjugation. This is achieved by the action of the sentrin-specific proteases (SENPs), so-named because SUMO was originally called sentrin by some research groups.

Much like ubiquitination, sumoylation is a step-wise process involving three enzymes, although there is no overlap in the enzymes used in these conjugation cascades (see Figure 4). Ultimately, sumoylation results in the formation of an isopeptide bond between the C-terminal glycine residue of SUMO and the ε-amino group of a lysine residue in the target protein. After proteolytic cleavage, SUMO is activated by the E1 obligate heterodimer (Aos1-Uba2). This reaction proceeds by formation of a SUMO-adenylate conjugate which functions as an intermediate in the formation of a thioester bond between the C-terminal carboxy group of SUMO and the catalytic cysteine residue of Uba2. SUMO is then transferred to the E2 conjugating enzyme, Ubc9, forming a thioester linkage between the catalytic cysteine residue of Ubc9 and the C-terminal hydroxyl group of SUMO. Ubc9 can then directly interact with substrates, catalyzing sumoylation. In many cases, the activity of a third enzyme (E3) is believed to increase the efficiency of the transfer of SUMO to the substrate.
Figure 4. Sumoylation Cascade.

Schematic of enzymatic cascade leading to covalent modification of target substrates by SUMO. Pro-SUMO is cleaved by SENPs to reveal a free C-terminal di-glycine. SUMO is then activated, consuming ATP, forming a thioester with E1 Aos1-Uba2. SUMO is then passed to Ubc9 in a transesterification reaction, and subsequently forms an isopeptide bond with an acceptor lysine on the substrate. Sumoylation E3s such as members of the PIAS family, RanBP2 and polycomb have been shown to enhance the sumoylation of some targets. Removal of SUMO from substrate targets is also catalyzed by SENPs.
1.2.3. *The Sumoylation Enzymes*

**SUMO**

Of the four human SUMOs, SUMO1, 2 and 3 are ubiquitously expressed and have been most extensively studied; the role of SUMO4 remains enigmatic and it remains uncertain whether or not it is conjugated *in vivo* (Guo et al. 2004; Owerbach et al. 2005). They are all predominantly nuclear: SUMO1 is concentrated on the nuclear envelope and SUMO2/3 are concentrated in nuclear puncta. The SUMO proteins share approximately 18% sequence identity with ubiquitin, although the structures are virtually super-imposable (Johnson 2004); see Figure 5. The surface charge topology of SUMO is also very different to that of ubiquitin, with distinct positive and negative regions (Bayer et al. 1998). SUMOs are approximately 10kDa, but appear larger by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), where they are observed to add approximately 20kDa to the observed molecular weight of most substrates. SUMOs are approximately 20 residues longer than ubiquitin, with the additional residues making up a flexible N-terminal extension which does not seem to be necessary for substrate conjugation or downstream effects in *S. cerevisiae* (Bylebyl et al. 2003). SUMO2 and SUMO3 are approximately 95% identical, yet only approximately 50% identical to SUMO1. Most studies to date do not distinguish between SUMO2 and SUMO3. Indeed, they are also more closely related to *S. cerevisiae* Smt3p than SUMO1, suggesting the later evolution of SUMO1 from a common precursor. Interestingly, cells contain a large pool of free unconjugated SUMO2/3 but there is virtually no pool of free SUMO1 at a given time; the vast majority of SUMO1 is conjugated (Hoege et al. 2002; Matunis et al. 1996). Conjugation of SUMO2/3 is induced by response to various stresses, whereas conjugation of SUMO1 is not (Saitoh and Hinchey 2000). As such, it has been postulated that one function of SUMO2/3 is to provide a reservoir of free SUMO for stress responses. There are examples of substrates that are preferentially sumoylated with one SUMO isoform as well as those that are modified equally by all SUMO isoforms (Johnson 2004). SUMO1 haploinsufficiency has been associated with cleft lip and palate in humans and this is supported by rodent studies (Alkuraya et al. 2006). Embryonic lethality or immediate post-natal death resulting from disruption of the SUMO1 gene supports the essential role of SUMO1 in mammals (Alkuraya et al. 2006)
Another major difference between SUMO1 and SUMO2/3 is that the latter contain consensus sumoylation motifs within their N-terminal extensions which have been demonstrated to function as SUMO-acceptor sites allowing for poly-SUMO chain formation (Bencsath et al. 2002; Bylebyl et al. 2003; Johnson and Gupta 2001). SUMO2/3 chain formation has not been frequently reported, although it has been demonstrated \textit{in vivo} for histone deacetylase HDAC4 (Tatham et al. 2001) and it was recently reported that the essential microtubule motor protein CENP-E is recruited to kinetochores through binding to poly-SUMO2/3 chains (Zhang et al. 2008). These studies are indicative of the important and distinct ability of SUMO2/3 to form chains and are suggestive that additional cellular processes will be revealed to depend critically upon this property.
Figure 5. Alignment of SUMO and Ubiquitin.

The primary amino acid sequence of the SUMOs show considerable sequence identity amongst themselves and limited sequence identity with ubiquitin. *H. sapiens* SUMOs 1-3 were aligned against *S. cerevisiae* Smt3p (the only *S. cerevisiae* SUMO) and *H. sapiens* ubiquitin (mature form). Shown in blue highlighting are all identical residues conserved in three or more SUMOs; shown in grey are residues conserved between all proteins; shown in yellow are the C-terminal pro-peptides removed by SENP to reveal the mature di-glycine motif required for conjugation to substrates. Noted below the alignment are (1) residues conserved in all proteins (*) and (2) amino acids with highly similar properties conserved in all proteins (:). Alignment performed using ClustalW default settings at the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/clustalw2/index.html).
E1: Aos1-Uba2

Most organisms contain a single E1, which is an obligate heterodimer. While ubiquitination E1s consist of a single protein, the two components the sumoylation E1 resemble the ubiquitin E1. Aos1 resembles the N-terminus of the ubiquitination E1, while Uba2 corresponds to the C-terminus and contains the active site cysteine. Aos1-Uba2 has been demonstrated to be essential for both viability and for sumoylation (Desterro et al. 1999; Gong et al. 1999; Johnson et al. 1997; Okuma et al. 1999).

E2: Ubc9

Ubc9 was originally assumed to conjugate ubiquitin, but was subsequently revealed to be the only SUMO conjugating enzyme known to exist in eukaryotes (Desterro et al. 1997; Hayashi et al. 2002; Johnson and Blobel 1997; Jones et al. 2002). Thus, the presence of a single sumoylation E2 is in stark contrast to the ubiquitination pathway, where multiple E2s participate in the ubiquitination of distinct sets of substrates. Ubc9 shares substantial sequence identity with the ubiquitin E2s and assumes essentially the same structure (Tong et al. 1997). Overall, Ubc9 has a positive charge and a patch surrounding the active site cysteine has been demonstrated to bind directly to the consensus sequence ΨKX(D/E) in substrates (defined in section 1.2.4) (Bernier-Villamor et al. 2002; Tong et al. 1997). A second region in Ubc9, which is separate from the active site, binds directly to SUMO and is involved in the transfer of SUMO from the E1 (Takahashi et al. 2001b; Tatham et al. 2003). Consistent with the essential role of the sumoylation pathway in eukaryotes and the existence of only one SUMO E2, Ubc9 is essential for eukaryotic viability (Hayashi et al. 2002; Seufert et al. 1995).

E3: PIAS, RanBP2, Polycomb

Three distinct types of SUMO E3 ligases have been discovered: the protein inhibitor of activated stat (PIAS) family, which were originally discovered as inhibitors of STAT transcription factors (Hochstrasser 2001; Jackson 2001; Shuai 2000); RanBP2, a component of the vertebrate nuclear pore complex (Pichler et al. 2002); and polycomb group protein, Pc2 (Kagey et al. 2003). These proteins all (1) bind the sumoylation E2, (2) bind the substrate and (3) promote sumoylation of the substrate. Similar to the RING domain-containing ubiquitin E3 ligases, these proteins do not form covalent interactions with SUMO, but rather, seem to act by bringing together Ubc9 and
the substrate. While sumoylation has been demonstrated to take place in vitro in the absence of an E3 in a lysine-specific manner (Desterro et al. 1999; Okuma et al. 1999), the majority of sumoylation in S. cerevisiae appears to be E3-dependent. In this organism, PIAS orthologues Siz1p and Siz2p have been shown to enhance sumoylation of many substrates, including septins (Johnson and Gupta 2001; Kirsh et al. 2002; Kotaja et al. 2002; Pichler et al. 2002; Sachdev et al. 2001; Schmidt and Muller 2002; Takahashi et al. 2001a). As such, this introduction will focus on E3s of the PIAS family.

PIAS proteins were first identified and named based on their ability to interact with and inhibit STATs (Chung et al. 1997; Liu et al. 1998). Since their discovery, PIAS proteins have been observed to interact with a variety of structurally diverse proteins; the majority of which are nuclear transcription factors, with the notable exception of the S. cerevisiae septins and small, but growing list, of other cytoplasmic and plasma membrane proteins (Johnson and Gupta 2001; Schmidt and Muller 2003). PIAS proteins are evolutionarily conserved in eukaryotes: the H. sapiens genome encodes four members: PIAS1, PIASx (PIAS2), PIAS3 and PIASy (PIAS4), reviewed in (Shuai and Liu 2005). Two isoforms of each of PIASx (PIASxα and PIASxβ) and PIAS3 (PIAS3 and PIAS3β) are produced: variants differ in their extreme C-termini. In addition, there is a second form of PIASy, lacking exon 6 which encodes the “PINIT” motif (see below), referred to as PIASyE6 (Wong et al. 2004). Homologues exist in S. cerevisiae, D. melanogaster and plants. In addition, H. sapiens Zimp10 and Zimp7 may be distantly related members of this family. A schematic of PIASxα is depicted in Figure 6.

The PIAS proteins vary in length from 507 (PIASy) to 650 (PIAS1) amino acids. They are broadly expressed, although PIASx is very highly expressed in testis and almost undetectable in other tissues (Gallagher et al. 1999; Moilanen et al. 1999; Sturm et al. 2000; Valdez et al. 1997). They share a conserved region of approximately 400 amino acids which includes: a scaffold attachment factorA/B/Acinus/PIAS (SAP) domain that has been implicated in binding to A/T-rich DNA sequences (Aravind and Koonin 2000; Kipp et al. 2000; Sachdev et al. 2001; Tan et al. 2002); a “PINIT” motif implicated in substrate specificity and subcellular localization of PIAS proteins (Duval et al. 2003; Kotaja et al. 2002; Reindel et al. 2006; Takahashi and Kikuchi 2005; Wong et al. 2004); a putative RING-type zinc binding structure which binds Ubc9 (Kahyo et al. 2001; Sachdev et al. 2001; Takahashi et al. 2001a); and a SUMO-interacting motif (SIM) which
is implicated in direct binding to SUMO (Hannich et al. 2005; Hecker et al. 2006; Kerscher 2007; Minty et al. 2000; Song et al. 2004)

While deletion of individual *S. cerevisiae* SIZ1 or SIZ2 genes resulted in a lack of observable phenotype, simultaneous deletion of both genes resulted in substantial growth delays (Johnson and Gupta 2001), suggesting a redundancy in Siz protein function. Disruption of the single *D. melanogaster* PIAS gene (dPIAS/Zimp/Su(var)2-10) leads to embryonic lethality due to severe abnormalities in chromosome condensation and congression (Hari et al. 2001), much like what is observed in the absence of Ubc9 (Nacerddine et al. 2005). However, in mice, the deletion of PIAS genes is much less informative: PIASy-deficient mice are phenotypically normal and display only slightly attenuated interferon and wnt signaling (Roth et al. 2004; Wong et al. 2004). PIAS1 deficient mice; especially males; are smaller than wildtype litter mates and are born at a lower than expected Mendelian ratio (Liu et al. 2004). Interestingly, these mice also show increased protection against pathogenic infection and altered interferon-mediated immune responses. PIASx is most highly expressed in the testis. In knockout mice, spermatogenesis proceeds normally and mice are fertile, although testis size is reduced compared to wildtype littermates (Santti et al. 2005). Overall, these results are highly suggestive of considerable functional redundancy among the PIAS proteins *in vivo* (Palvimo 2007).
Figure 6. Schematic of PIAS Domain Structure.

PIASxα is shown: amino acid position is noted along the bottom and conserved PIAS domains are noted along the top (see text for explanation; adapted from (Palvimo 2007)).
The first SUMO-specific protease, Ulp1p was isolated from *S. cerevisiae* by Li and Hochstrasser (Li and Hochstrasser 1999). Sequence analysis showed that Ulp1p bears no substantial relationship to deubiquitinating (DUB) enzymes, but is actually related to adenoviral proteases (Li and Hochstrasser 1999; Mossessova and Lima 2000). SENPs possess an approximate 200-amino acid protease fold, defining them as the C48 cysteine protease family. Sequence analysis of this family of proteins does suggest that SENPs diverged from the DUBs early in evolution (Barrett and Rawlings 2001; Li and Hochstrasser 1999; Strunnikov et al. 2001). There are only two SENPs (Ulp1p and Ulp2p) in *S. cerevisiae*, while in humans there are 7 SENPs, named SENP1-3 and SENP5-8. It should be noted that after naming, SENP3 and SENP4 were revealed to be the same protein and SENP8 was found to act on another ubiquitin-like modifier, Nedd8 (Gan-Erdene et al. 2003; Wu et al. 2003). Thus, there are only 6 mammalian SENPs that are SUMO-specific (Mukhopadhyay and Dasso 2007). The various SENPs display differential subcellular localization and enzymatic activity (Table 2). The SENP proteins are very active and upon cell lysis, they are released from their various sub-cellular compartments and cleave SUMO from substrates virtually instantly. This activity, in combination with the small proportion of a given target that is SUMO-modified at a give time, is believed to account for why sumoylation remained undetected for such a long time.

Unlike the PIAS SUMO E3s, even the earliest studies suggested SENP function was not redundant between paralogs. In *S. cerevisiae*, Ulp1p is an essential gene, whereas Ulp2p is not. Interestingly, the predominant phenotype in Δulp1 cells is a defect in the maintenance of 2μm circle copy number, which has been linked to the ability of Ulp1p to process pro-Smt3p (Dobson et al. 2005; Li and Hochstrasser 1999). In the absence of the 2μm, Ulp1p is still essential (Dobson et al. 2005) and has been found to have important roles in nuclear-cytoplasmic trafficking and 60S pre-ribosomal particle export (Panse et al. 2006; Stade et al. 2002). Consistent with these findings, mutants lacking E3 activity are defective in 2μm circle copy maintenance (Chen et al. 2005) and E1 and E2 mutants both show defective ribosomal protein export (Panse et al. 2006). While Ulp2p is not essential, it has been suggested to function in the removal of Smt3p from targets and the disassembly of poly-Smt3p chains (Bylebyl et al. 2003; Schwienhorst et al. 2000). It should also be noted that excess Siz1p is lethal in *S. cerevisiae*,
consistent with the likelihood that uncontrolled desumoylation is toxic (Mossessova and Lima 2000).

In *H. sapiens*, sequence analysis shows that SUMO proteases sub-divide into two major categories: the first containing SENP1, SENP2, SENP3 and SENP5 (most similar to Ulp1p) and the second containing SENP6 and SENP7 (most similar to Ulp2p). Correspondingly, SENP1 and SENP2 are highly related and both localize to the nuclear envelope through association with the nuclear pore complex (Bailey and O'Hare 2004; Hang and Dasso 2002; Zhang et al. 2002). SENP3 and SENP5 are also highly similar and both localize to nucleoli (Di Bacco and Gill 2006; Di Bacco et al. 2006; Gong and Yeh 2006; Nishida et al. 2000). SENP6 and SENP7 are both found in the nucleoplasm (Cheng et al. 2006; Mukhopadhyay et al. 2006). The crystal structure of a transition-state analogue containing Ulp1p linked to the C-terminal glycine of Smt3p shows an extensive hydrophilic interface that does not require substantial conformational change in Smt3p for binding (Mossessova and Lima 2000). The active site within Ulp1p lies within a shallow hydrophobic pocket and conserved tryptophan residues “clamp” down on the conserved di-glycine motif in SUMO. Correspondingly, the active sites in mammalian SENP1 and SENP2 show a similar organization (Reverter and Lima 2004; Shen et al. 2006). An overview of SENP localization and enzymatic properties is presented in Table 2.

Unsurprisingly, residues in the SUMO C-terminal extensions (beyond the di-glycine motif) have been demonstrated to determine a given SENP’s specificity for processing specific pro-SUMOs (SUMO1 vs 2 vs 3) (Reverter and Lima 2004; Shen et al. 2006; Xu and Au 2005).

Likewise, for the deconjugation reaction, SENPs clearly differentiate between SUMO1 and SUMO2/3 paralogs (Di Bacco et al. 2006; Gong and Yeh 2006; Reverter and Lima 2006), although it is still unclear how this discrimination is effected.

The characterization of the specific details of SENP function, as well as the thorough analysis of specific functions attributable to each of the SENPs remains to be revealed. However, it is particularly noteworthy that SUMO2/3-specific SENP5 has been recently demonstrated essential for cell cycle division, although the specific details of its requirement remain unclear (Di Bacco et al. 2006).
Table 2. Characteristics of SENPs.

Comparison of *S. cerevisiae* and mammalian SENPs. Note SUMO paralog subcellular localization preference and specific enzymatic properties. Adapted from (Mukhopadhyay and Dasso 2007).

<table>
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<td>Nuclear pore, nucleoplasm speckles</td>
<td>SUMO1 and SUMO2/3</td>
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1.2.4. Sumoylation Specificity: Consensus Acceptor Sites

The identification of a preliminary consensus SUMO-acceptor site was possible after mapping acceptor lysine residues in just a few substrates, including: RanGAP1, PML, Sp100, IkBα, p53 and c-Jun (Desterro et al. 1998; Kamitani et al. 1998; Mahajan et al. 1998; Matunis et al. 1998; Muller et al. 2000; Sternsdorf et al. 1999). The SUMO-acceptor site was shown to be $\Psi KX(D/E)$, where $\Psi$ is a bulky hydrophobic amino acid, such as valine. This is remarkable, considering that an ubiquitination consensus site has yet to be defined; however this is likely owing to the presence of a single sumoylation E2, Ubc9, in contrast to more than 20 ubiquitin E2s.

The crystal structure of Ubc9 locked into place on the C-terminal domain of RanGAP1 reveals how Ubc9 interacts with this motif: the target lysine is shown to reach into the catalytic pocket of Ubc9 while the aliphatic and acidic amino acids interact with surface residues on Ubc9 (Bernier-Villamor et al. 2002). Thus, recognition of consensus-site motifs appears to only be possible if they are part of an extended loop (as with RanGAP1); in an unstructured area such as with the transcription factor ETS-1 (Macauley et al. 2006); or in an N- or C-terminal region, as with SUMO2/3 (Tatham et al. 2001).

Two additional extended sumoylation motifs have also recently been identified. The first is the phosphorylation-dependent sumoylation motif (PDSM), which consists of the conventional sumoylation motif, followed by a phosphorylated serine residue and then a proline residue ($\Psi KXEXXpSP$). Examples which utilize the PDSM include heat shock factor-1 (HSF1), Smad nuclear interacting protein-1 (SNIP1), MEF2 and several others (Hietakangas et al. 2006). However, there are also examples where phosphorylation reduces sumoylation of a given target and the mechanism by which this occurs is not clear (Johnson 2004). A second extended motif is the negatively charged amino acid-dependent sumoylation motif (NDSM) which is characterized by the presence of a negative charge next to the basic SUMO consensus site (Yang et al. 2006).

It should be noted that not all sumoylated lysines lie within classical consensus sequences: reported non-consensus sequences include: TKET, TKED, AKCP, VKYP, VKFP and both lysines with the sequence GKVEKVD. There are many known sumoylated proteins that do not contain the $\Psi KX(D/E)$ consensus sequence and others that are still sumoylated when all lysines within their consensus sequences are substituted to arginine (Johnson 2004).
1.2.5. Substrates and Biological Functions of Sumoylation

Sumoylation of target proteins has been described to have a myriad of effects: indeed, there is no simple way to predict what the functional consequence of sumoylation of a given target protein will be. *In vivo*, sumoylation may influence any aspect of a target protein, including stability, localization or activity. Ultimately, at the molecular level, sumoylation modifies protein surfaces, thereby influencing inter- or intramolecular interactions. Conceptually, changes in protein-protein interactions may be due to masking of existing surfaces or introduction of new surfaces, such as those present on SUMO or at the interfaces between SUMO and the target protein. In some cases, SUMO-modification has even been reported to induce conformational changes upon the target protein, which may even result from non-covalent interactions with SUMO (Baba et al. 2005). While the vast majority of sumoylated proteins are nuclear, a growing number of non-nuclear proteins and even foreign proteins (such as viral proteins) have been identified as targets. A recent review states that more than 120 SUMO substrates are now known (Zhao 2007). These can be categorized into general groupings: transcriptional regulators; nuclear body-associated proteins; DNA recombination/replication/repair proteins; chromosome assembly and segregation proteins; cytoplasmic and transmembrane proteins; and viral proteins.

The sumoylation profile changes considerably during the cell cycle and given the robust sumoylation of cytoplasmic septins in *S. cerevisiae*, mention of cytoplasmic sumoylation targets is of particular interest. SUMO1 is implicated in mitochondrial dynamics because its overexpression or the depletion of SENP5 (which in this case can act upon SUMO1) both result in mitochondrial fragmentation, implying that sumoylation plays a role in mitochondrial fission/fusion (Harder et al. 2004; Zunino et al. 2007). Protein tyrosine phosphatase-1B (PTP1B), an endoplasmic reticulum membrane protein has also been demonstrated to be sumoylated, although the physiological implications of this are unclear (Dadke et al. 2007). Sumoylation of several plasma membrane proteins has also been implicated in the regulation of their respective channel/transport activities. Examples include potassium leak channels (Rajan et al. 2005), voltage-gated potassium channels (Benson et al. 2007), glutamate receptors (Tang et al. 2005), and kainate receptors (Martin et al. 2007a). In addition, sumoylation has been implicated in various cytoplasmic signaling capacities in neurons, where brain-specific Gαi G-protein subunit GTPase-activating proteins RGSZ1 and RGSZ2 have been shown to be sumoylated (Rodriguez-Munoz et al. 2007); focal adhesion kinase (Kadare et al. 2003), axin (Rui
et al. 2002); and others. In neurons, sumoylation also seems to play a critical role in mRNA trafficking by regulating the ability of La to bind either dynein or kinesin (van Niekerk et al. 2007).

There is a growing number of examples where SUMO antagonizes other modifications at specific lysine residues, such as ubiquitination and acetylation (Geiss-Friedlander and Melchior 2007; Zhao 2007). When sumoylation occurs on the same lysine used for ubiquitin-mediated degradation, this has been shown to exert a protective measure, thereby saving a given target from proteolysis under certain cellular conditions. Examples of such targets include IκBα (Desterro et al. 1998), Smad4 (Lin et al. 2003), Huntingtin (Steffan et al. 2004), PCNA (Ulrich et al. 2005), RAD52 (Sacher et al. 2006), phosducin (Klenk et al. 2006) and Mdm2 (Carter et al. 2007; Lee et al. 2006), among others. Sumoylation can also antagonize acetylation and this has been demonstrated to occur during morphogenesis of neuronal dendritic claws (Shalizi et al. 2006). Several transcription factors have now been shown to be cross-regulated by sumoylation and acetylation/deacetylation (Zhao 2007). As such, sumoylation is emerging as an important extranuclear regulator in neuronal function and has been implicated in most major neuropathologies (Martin et al. 2007b).

1.2.6. Non-covalent Interactions with SUMO

An increasing body of literature supports the notion of physiologically-relevant outcomes resulting from non-covalent interaction with sumoylation components. For example, the GTPase dynamin, which functions in endocytosis, has been described to interact with SUMO1, Ubc9 and PIAS1, thereby inhibiting its ability to oligomerize (Mishra et al. 2004). Interestingly, the E3 ubiquitin ligase parkin has also been demonstrated to interact with SUMO, thereby promoting its own nuclear transport, autoubiquitination and degradation (Um and Chung 2006). Recently the SIM (SUMO-interaction motif) has been defined which is characterized by a short sequence of hydrophobic amino acids flanked in acidic and/or serine residues (Hannich et al. 2005; Minty et al. 2000; Song et al. 2004). This motif is present in many SUMO-modified substrates, as well as sumoylation enzymes such as Uba2, and PIAS E3 ligases and RanBP2 (Reverter and Lima 2005; Song et al. 2004).
1.2.7. *S. cerevisiae* Septin Sumoylation

While characterizing sumoylation in *S. cerevisiae*, Johnson and Blobel discovered that several of the *S. cerevisiae* septins become sumoylated during mitosis, with conjugates appearing shortly before anaphase and disappearing abruptly at cytokinesis (Johnson and Blobel 1999). Intriguingly, only septin filaments on the mother side of the *S. cerevisiae* bud-neck were sumoylated. Four major SUMO attachment sites were identified using site-directed mutagenesis: one in Cdc3p, one in Cdc11p and two in Shs1p. All were found near the termini of these septins, within the consensus (I/L/V)KX(D/E), consistent with the established sumoylation consensus sequence at the time. Mutational analysis suggested that sumoylation was critical for disassembly of septin ring structures following cytokinesis. Consistent with others’ observations, Johnson and Blobel only observed modification of a very small proportion of the total cellular quantities of these septins. This required trapping cells at the G2/M spindle checkpoint with nocodazole (trapping division with the septin hourglass intact), lysing directly in chaotropic agents (6M guanidine) and subsequent purification from unmodified septins. They also used N-ethylmaleimide in their buffers, which reportedly inhibits the very active SENPs, which are released from their various cellular compartments upon cell lysis.

An additional study by Johnson and Gupta (Johnson and Gupta 2001) demonstrated that a barely detectable portion of Siz1p (which is predominantly nuclear) was preferentially localized to the mother side of the septin ring during cytokinesis and was responsible for directing sumoylation of the septins. This was the first reported case of PIAS proteins functioning as E3 ligases for SUMO. This localization was dependent upon phosphorylation of Siz1p, although *in vitro* assays suggested that this phosphorylation had no effect on the ability of Siz1p to promote septin sumoylation. Further, individual elimination of the SIZ1 gene (but not SIZ2) resulted in the loss of most septin sumoylation. Simultaneous elimination of both SIZ genes resulted in a slowed cell cycle and various cell division defects. Curiously, a proportion of cells underwent cytokinesis at wildtype rates, making interpretation of this result difficult. Nonetheless, Siz proteins differ in their target specificity, although it seems that they may exhibit some functional redundancy. Simultaneously, two additional studies were published, which also supported the functional characterization of PIAS proteins as SUMO E3 ligases for *S. cerevisiae* septins (Kahyo et al. 2001; Takahashi et al. 2001a).
It should be noted that the studies by Johnson and colleagues did not take into account the effect that several simultaneous point mutants may have had on the stability of the septin ring, independent of sumoylation. In addition, one sumoylation site was found within the G4 sequence of the nucleotide binding region of Cdc3p and the effect of abrogation of sumoylation at this site could not be assessed due to the generation of a synthetic lethal phenotype upon mutagenesis of this lysine to arginine. This may account for why these researchers were unable to eliminate all sumoylation at the mother-bud neck in their point mutant septin background. In these studies, they also assessed whether or not abrogation of sumoylation had any effect on septin turnover during the cell cycle, concluding that septin levels remain relatively consistent throughout the cell cycle, consistent with recent results presented by M. McMurray at the 2007 septin meeting in Monta Verità, Switzerland (Gladfelter and Montagna 2007). While inconclusive with regard to the physiological relevance of septin sumoylation, these studies established that the \textit{S. cerevisiae} septins are robustly sumoylated during a specific stage in the cell cycle, clearly defined Siz1p and Siz2p as SUMO E3 ligases and provided clear evidence of differential localization and modification of the mother versus daughter septin rings, supporting the notion that the overall septin structure possesses some form of polarity. The work by Johnson and colleagues leaves the reader with the exciting notion that sumoylation may be regulating septin filament dynamics or some other aspect of septin function, opening the door to further studies in \textit{S. cerevisiae} and mammals.
1.3. Spermatogenesis and Organization of the Seminiferous Epithelium

1.3.1. Organization of the Seminiferous Epithelium

Organized of the Testis

The testis is the site of male germ cell production. As such, extensive cell proliferation and division occurs in this tissue. Interestingly, the role of septins in the testis has not been thoroughly examined.

The testis consists of two major compartments: the interstitial (or intertubular) compartment and the seminiferous tubule compartment. The interstitial compartment contains the blood and lymphatic vessels, as well as androgen-producing Leydig cells. Macrophages are also present in the interstitial compartment, and in some species they account for up to 25% of the cells in this compartment, although their function here is unknown. The seminiferous tubules are highly convoluted and travel predominantly in the long axis of the testis. Thus, a transverse histological section through the long axis of most species can be used to visualize cross-sectioned tubules. The seminiferous tubule compartment is bounded by the lymphatic endothelium and the myoid cells and acellular elements which together form the boundary tissue or limiting membrane of the tubule. There are basement membranes between the lymphatic endothelial cells, the myoid cells and the cells within the tubule. The myoid cells are contractile and are the major motive force for sperm along the seminiferous tubules. Along with the basal lamina, the myoid cells provide the structural surface for Sertoli cells and basal compartment cells of the seminiferous tubule.
Figure 7. Anatomy of the Testis.

Organization of seminiferous tubules within the testis. Seminiferous tubules run predominantly in the direction of the long axis of the testis (left; blue). A typical cross section shows germ cells at several different stages of development. Successively more mature germ cells are found closer to the lumen (light purple), with the exception of elongating spermatids (grey), which are pulled down into deep crypts in the Sertoli cells (not shown), appearing interspersed between germ cells of earlier-stages of development.
The Sertoli Cell

Sertoli cells are somatic cells: during puberty, they stop dividing and are thought to remain at a relatively stable population throughout the lifespan of the animal. Sertoli cells are elaborately equipped to support spermatogenesis; they interact with and control the various phases of germ cell development, forming and breaking tight junctions as they pass the developing germ cells progressively luminal. Sertoli cells are attached to various other cells and acellular elements. They are attached to the basal lamina via hemidesmosomes; to each other by desmosomes, gap junctions and tight junctions (blood-testis barrier) and to germ cells via highly specialized structures which include: desmosome-like junctions, gap junctions, ectoplasmic specializations and tubulobulbar complexes. Sertoli cells further function to create two permanent (basal and adluminal) and one transient (intermediate) compartment within the seminiferous epithelium. Basal compartment cells, which include spermatogonia and spermatocytes, have relatively free access to nutrients traveling through the lymphatic and vascular systems.

General Organization of the Seminiferous Tubule

The general organization of cells within the seminiferous tubules is depicted in Figure 8. Spermatogenesis can be divided up into three phases based on functional considerations: 1) the proliferative phase (spermatogonia; not shown in Figure 8), in which cells undergo rapid and successive division; 2) the meiotic phase (spermatocytes), in which genetic material is recombined and segregated; and 3) the differentiation or spermiogenic phase (spermatids), in which spermatids transform into the familiar elongated mature sperm cells. Detailed staging of spermatogenesis in rat is presented by Russell and colleagues and forms the basis of this introduction (Russell et al. 1990).

In all species, male germ cell numbers are vastly increased early in spermatogenesis. The spermatogonial cell population undergoes numerous mitoses building up its own population by 1000-fold, followed by a 4-fold expansion during meiosis. In the rat, a single germ cell clone can give rise to 4,096 interconnected spermatids. While spermatogonial proliferation is complicated, the end result is the generation of many differentiated spermatogonia connected by intercellular bridges: this connectivity is believed to promote their synchronous development. Spermatogonia reside basally within the seminiferous tubule, generally showing one flattened side along the basal lamina and a rounded surface in contact with the Sertoli cell.
Differentiated spermatogonia are called spermatocytes once prophase of the first meiotic division can be distinguished. Prophase lasts for approximately three weeks in the rat and is characterized by a gradual morphological transition from one phase of prophase to another; the size of the cells and their nuclei also gradually increases, as well as the perinuclear Golgi apparatus. Nuclear changes have been used to subdivide this process into several stages: preleptotene, leptotene, zygotene, pachytene and diplotene. The latter stage is the culmination of this long prophase; the remainder of the cell division process is rapidly completed, followed by a second rapid meiotic division. Spermatocytes are generally round, with condensed chromatin and within the seminiferous epithelium, they are found mid-way between the basal lamina and the lumen.

Spermiogenesis is characterized by the spectacular transformation of round spermatids into mature spermatozoa. Once nuclear reshaping begins, spermatids are easy to differentiate, although early stage spermatids (so-called “round spermatids”) can be difficult to discern from late stage spermatocytes. Elongation takes approximately 3 weeks in the rat, so spermatids are also abundant in seminiferous tubule cross-sections. Elongate spermatids (spermatids in the process of elongating into mature spermatozoa) occupy deep crypts within Sertoli cells and are found at various positions in the seminiferous epithelium. These often appear in cross sections to be between the other germ cells.
Myoid cells and acellular elements make up the basement membrane. Sertoli cells (orange) stretch the entire thickness of the epithelium, forming tight junctions (TJ; red) with one another, as well as specialized junctions with germ cells, such as the ectoplasmic specialization (ES; red). Successively differentiated germ cells are found approaching the lumen: meiotic spermatocytes are located nearest the basement membrane and are characterized by large nuclei with condensed chromatin. Spermatids in the early stages of elongation can be identified by the presence of an extending acrosomal vesicle (A; blue/black); an enlarged Golgi apparatus (G; green) is observed in both of these cell types. Elongating spermatids are often observed interspersed between germ cells of earlier stages of differentiation as they are pulled into Sertoli cell crypts before release (spermiation). Nuclei are denoted by “N”.

Figure 8. Organization of the Seminiferous Epithelium.
1.3.2. **Spermiogenesis and Spermatozoon Structure**

Spermiogenesis begins with the development of a flagellum: centrioles move from a perinuclear localization to the plasma membrane, where an axoneme (classical 9+2 microtubule arrangement) grows from one centriole. As the axoneme extends, drawing the plasma membrane out around it, the centrioles move back to the nuclear envelope and implant into a specialized indentation, called the implantation fossa. The flagellum then gradually moves to be directly opposite the growing acrosome, such that the acrosomal and flagellar poles of the nucleus are established (Figure 9).

While the flagellum is forming, the acrosome forms via secretion from the Golgi. The acrosome is observed initially as a large perinuclear vesicle, but then flattens onto the surface of the nucleus. The acrosome has been likened to a huge lysosome and contains enzymes and cofactors essential for degradation of the zona pellucida (outer covering) of the ovum (female gamete), such that fertilization is possible. After the acrosome is formed, the Golgi remains swollen and moves distally away from the nucleus and into the tail region of the cell. Gradually, the spermatid elongates, drawing cytoplasm out along its tail, while the nucleus condenses and re-shapes into an ovoid and then sickle-shaped structure, with the tightly packed acrosome at its tip. Accessory components are added to the flagellum to form its middle, principle and end pieces. Mitochondria are recruited from the cytoplasm into a helical pattern around the middle piece of the flagellum, while the annulus begins to move down the axoneme. Outer dense fibres form both in the midpiece and the principle piece, while a fibrous sheath forms distal to the mitochondrial sheath. Although the mature flagellum imparts motility to the cell, capability for motility is developed during post-testicular epididymal maturation. Further, sperm are not vigorously motile until they are placed in the female reproductive tract. A schematic of a mature sperm cell is shown in Figure 10.
Figure 9. Spermiogenesis.

Elongation of the axoneme begins with movement of the centrioles to the plasma membrane and their subsequent implantation onto the nuclear envelope at the implantation fossa (A, B). During
these early steps, an accumulation of electron dense material is observed at the regions of the plasma membrane with high negative curvature, in the immediate vicinity of the growing axoneme. As the acrosomal vesicle (blue) flattens onto the surface of the nucleus, the chromatoid body (red) appears and travels along the nuclear envelope towards the forming annulus (black), where it reportedly deposits some material (C, D). At this point, mitochondria (orange) align along the axoneme as the annulus travels down it stopping mid-way, while the Golgi (green) moves away from the nucleus, towards the end of the axoneme, in the residual cytoplasm (E). The fibrous sheath is assembled along the region posterior to the mitochondrial sheath and anchors at/near the annulus. The majority of the cytoplasm is removed, leaving only cytoplasmic droplets, which are removed during epididymal maturation (F).
The principal piece is characterized by a sheath of mitochondria covering the central axoneme, the latter of which consists of a 9+2 microtubule arrangement encased in a highly stable protein core (outer dense fibres). In the principal piece, the axoneme is also encased in a fibrous sheath, adding additional mechanical stability to the axoneme. The end piece is characterized by the presence of only the axonemal microtubules.
1.3.3. **Conserved Roles for Septins in the Testis**

SEPT2 and SEPT9 are both highly expressed in testis (Figure 3). A role for septins in the testis was first described in *D. melanogaster*, where septins were demonstrated to localize to male ring canals (Carmena et al. 1998). These stable, cytoskeletal structures are located on the inner surface of membrane invaginations between interconnected germ cells. Much like the structure which forms at the mother-bud neck in *S. cerevisiae*, or the cleavage furrow during mammalian cell division, this cytoskeletal structure restricts the opening between cells, while allowing for the sharing of cytoplasm between germ cells. Interconnected mammalian germ cells initially appear to undergo a process identical to somatic cell cytokinesis. However, the midbody and central spindle break down via an unknown mechanism (without the requirement for abscission of cells), leaving a stable intercellular bridge between germ cells. Similar to *D. melanogaster*, septins have also been localized to a similar intercellular bridge structure in mammals (Greenbaum et al. 2007). In somatic cells, SEPT9 has been described to localize to the mitotic spindle, a localization unique so far only to this septin (Surka et al. 2002). However, in the mammalian testis, SEPT9 has been observed in direct co-localization with SEPT2 and SEPT7, along the cortex of intercellular bridges, reminiscent of where SEPT2 is observed during mammalian cytokinesis (Surka et al. 2002). Similar to their conserved role in somatic cytokinesis, septins evidently play an ill-defined, yet conserved role in establishment and/or maintenance of intercellular bridges between germ cells.

1.3.4. **The Annulus and SEPT4**

**Annulus and Chromatoid Body**

Recently, a subset of septins was also demonstrated to be essential for the formation of the annulus structure in spermatozoa (Ihara et al. 2005; Kissel et al. 2005). The annulus is a proteinaceous membrane-associated ring, surrounding the axoneme, which localizes to the region between the middle and principle pieces in mature spermatozoa (see Figures 9 and 10). EM studies first identified a proteinaceous structure associated with the inner leaflet of the plasma membrane and this was predicted to represent the nascent annulus. The first origins of this structure were observed to accumulate very early in spermiogenesis, surrounding the base of the extending axoneme, on the regions of the inner leaflet of the plasma membrane with a high
negative curve, although the identity of the accumulation is unknown (see Figure 9). This structure is then thought to travel down the axoneme, to its final destination, as mitochondria are aligned along the principal piece (Fawcett et al. 1970; Fawcett and Phillips 1969). These early electron microscopy studies suggest that another structure, the so-called chromatoid body, briefly associates with the early annulus, prior to its movement down the axoneme, contributing electron-dense material to the structure. The chromatoid body is unique to male germ cells and is first observed as a fibrous/granular structure in the interstices of mitochondria clusters in meiotic spermatocytes. After meiosis, in round spermatids, the chromatoid body coalesces into one highly visible, perinuclear structure that is believed to function as an intracellular focal domain for organization and control of RNA processing (Kotaja and Sassone-Corsi 2007). Once coalesced into a single structure, the chromatoid body remains in close association with the nuclear envelope: its close association with the nuclear pores has been suggested to allow for direct exchange of materials with contents of the nucleus. It is then that it moves along the nuclear envelope to the base of the axoneme, where it comes into close association with the annulus and then gradually diminishes in size while traveling down the axoneme with the annulus, ultimately disappearing altogether.

The chromatoid body is often observed in close association with the Golgi immediately after it coalesces into one structure, very early in spermiogenesis. However, as the chromatoid body moves around the nuclear envelope towards the forming annulus, the Golgi moves out into the tail cytoplasm, where it remains, enlarged, until late in spermiogenesis when it is removed with the excess cytoplasm (Figure 9).

**Disruption of the Sept4 Gene in Mice**

Very recently, the targeted disruption of the Sept4 gene in mice identified septins as crucial components of the annulus (Ihara et al. 2005; Kissel et al. 2005). In knockout mice, testicular morphology was normal, but the annulus was completely absent from mature spermatozoa, as determined by both electron microscopy and immunofluorescence.

The mechanical stability of the axoneme was severely compromised and sperm tails were bent back on themselves up to 180°. The fibrous sheath appeared to form normally, but was no longer anchored at/near the annulus, contributing to this mechanical instability. One group (Kissel et al. 2005) observed substantial alterations in mitochondrial morphology, consistent with a role for
the SEPT4 isoform ARTS from this organelle (Larisch et al. 2000). They noted that while spermatid elongation proceeded normally and mitochondria lined up along the axoneme, the cytoplasmic droplets of the head and neck region were not removed. Notably, caspase activity has been demonstrated as essential for removal of these cytoplasmic droplets (Arama et al. 2003), prompting the authors suggest a consistency with the previously observed role for SEPT4 in apoptosis via interaction with the caspase XIAP (Larisch et al. 2000). The other group (Ihara et al. 2005) determined that SEPT1, SEPT6 and SEPT7 were also components of the annulus and that in the absence of SEPT4, these septins were completely delocalized along the entire length of the sperm tail. They also observed that while generation of ATP was not impaired in knockout animals, its use was. This led them to the hypothesis that the inner dynein arms which bind to microtubules and drive flagellum motility were impaired in their ability to function. In addition they observed stalling of kinesins near the narrow band of membrane marking the absent annulus, indicating defects in intraflagellar transport proteins required to move cargo distally towards the end of sperm tails, consistent with the inability of sperm to use ATP.

These studies both suggest that the annulus is not required for elongation of spermatids, but rather, for the later stages of spermiogenesis, as the fibrous sheath is forming and must anchor in the region of the annulus. They suggest that the annulus is required for the overall mechanical stability of mature spermatozoa. Unfortunately, neither of these research groups examined the initial formation of the annulus in any detail, with regard to its previously-described deposition on the plasma membrane very early in spermiogenesis and its interaction with the chromatoid body. As such, details of the formation of the annulus, especially with respect to when septins localize to it during its formation, remain elusive.
1.4. Hypotheses

The identification of septin binding partners, through various interaction screens, will allow for subsequent characterization of these interactions, furthering the understanding of septin function in mammals.

Specific Hypothesis 1:

Septins interact with each other and other proteins. These interactions are likely to regulate the function of septins.

Specific Hypothesis 2:

Septins exhibit tissue-specific expression and may exhibit specific sub-cellular distributions within those tissues, which may in turn control cellular function in a given tissue.

Aims:

1. To use the yeast two-hybrid system to conduct an in-depth examination of septin:septin interactions.

2. To conduct septin interaction screens to identify candidate septin interactor proteins for subsequent characterization.

3. To conduct a thorough characterization of the localization of septins in the testis.
2. MATERIALS AND METHODS

2.1. Nomenclature

Both rodent and human septins were used in this work. For simplicity, septin gene and gene product names are presented in the form consistent with the current rules of naming for human septins (Macara et al. 2002). Thus, all gene names are presented in lower case, italics with the first letter capitalized (for example, Sept4) and all protein names are presented in capitals, not italicized (for example SEPT4).

2.2. DNA Manipulation and Cloning

2.2.1. Yeast Two-Hybrid: Full-length Septins

The following cDNAs were isolated from the rat brain cDNA library (Clontech) screen using SEPT5 as the bait and in some cases PCR-amplified, with the primers listed, for transfer into the pEG202 plasmid: Sept6 (GI 109512511: 5’cagatgtcagagatggcagcggcccgatata3’ and 5’ccatgtctcgagtttaattcttctttt3’); Sept11 (GI 109500352: 5’ccagcccttcttgagtgtgagatg3’ and 5’cagttggaattcttactttttctcatctgt3’) and rat Sept12_v2 (GI 66911992; shuttled directly using EcoRI and XhoI). The following sequences were PCR-cloned, from existing Trimble lab constructs, into yeast expression vectors using EcoRI and/or XhoI and constructs were confirmed by sequencing: human Sept1 (GI 15082493: 5’attagaattcatggacaaggagtactggg3’ and 5’atatcctcgagcaggggccgtcactgtgct3’); human Sept2 (GI 56550108: 5’aaagaattcatgtctgaagcccaact3’ and 5’aaactcgagtcacagctggtggtcccaacg3’); human Sept4 (GI 17986250: 5’gctatactcgagatggccgcccgtctcttgagtgg3’ and 5’ggctcagctcgatagttactttcatctctgt3’); human Sept7 (GI 19684120: ggtacactcgagatggcactagggacctgtg3’ and 5’gctctcagtctgtaaaagatctctccttttt3’); human Sept8 (GI 1503987: 5’atagatctcgagatggagagggccggatctca3’ and 5’attattctcgatccgacacggtttg3’); human Sept9 (GI 34782782: 5’atagattctgagatggagggccgcagctca3’ and 5’attattctcgagatctcctgtcgagcttg3’); human Sept10 (GI 7688656: 5’atagattctgagatggagggccgcagctca3’ and 5’attattctcgagatctcctgtcgcggctg3’); human Sept12_v1 (GI 91984167:
5’atatgaattcatggacccctgagcgcctcccc3’ and 5’gcgcctcgagtcagaactcatcatcagaatcgtc3’). Rat Sept3 (GI 9507084) was subcloned from an existing vector using XhoI; wildtype and S58N rat Sept5 (Beites et al. 1999) were subcloned from existing vectors using EcoRI and XhoI. Human Sept14 (GI 153252197) was isolated in the laboratory of Dr. Elizabeth Petty (University of Michigan) in a yeast two-hybrid screen of a testis library using SEPT9 as the bait and provided to me in pCR-XL-TOPO; I subcloned it directly from this vector using EcoRI.

2.2.2. Yeast Two-Hybrid: Septin Truncation Constructs

Full-length and truncation mutants for rat Sept5 were constructed by PCR amplification from existing Trimble lab constructs, using the following specific primers:

- 5’aaagaattcatgagcacaggccgtgcctgta3’ and 5’aaactcgagtcactggtcctgcattgcgtg3’ (full length);
- 5’aaagaattcatgagcacaggccgtgcctgta3’ and 5’aaactcgagacagtgcggcctggctg3’ (aa1-292);
- 5’aaagaattcatgagcacaggccgtgcctgta3’ and 5’aaactcgagacagtgcggcctggctg3’ (aa1-235);
- 5’aaactcgagtcactggtcctgcattgcgtg3’ and 5’aaagaattgctacactgcaccagggg3’ (aa28-368);
- 5’aaactcgagtcactggtcctgcattgcgtg3’ and 5’aaagaattgctacactgcaccagggg3’ (aa48-368).

Similarly, for rat Sept12_v2 was PCR-amplified from pJG4-5-Sept12 isolated in the SEPT5 yeast two-hybrid screen using the following primers: 5’aaacacgaattcatggatgagaggcgaacacc3’ and 5’aaacacgaattcatggatgagaggcgaacacc3’ (full length) and 5’aaacacgaattcatggatgagaggcgaacacc3’ and 5’aaacacgaattcatggatgagaggcgaacacc3’ (aa1-324);

5’aaacacgaattcatggatgagaggcgaacacc3’ and 5’aaacacgaattcatggatgagaggcgaacacc3’ (aa1-282); 5’aaacacgaattcatggatgagaggcgaacacc3’ and 5’aaacacgaattcatggatgagaggcgaacacc3’ (aa1-235); 5’aaacacgaattcatggatgagaggcgaacacc3’ and 5’aaacacgaattcatggatgagaggcgaacacc3’ (aa31-356); 5’aaacacgaattcatggatgagaggcgaacacc3’ and 5’aaacacgaattcatggatgagaggcgaacacc3’ (aa50-356); 5’aaacacgaattcatggatgagaggcgaacacc3’ and 5’aaacacgaattcatggatgagaggcgaacacc3’ (aa228-356). Resultant PCR products were directionally cloned into the yeast two-hybrid vectors pEG-202 and pJG4-5, respectively, using EcoRI and XhoI. The S58N point mutant of Sept5 was described elsewhere (Beites et al. 1999) and was PCR-amplified using primers for full-length amplification described above.
2.2.3. Yeast Two-Hybrid: PIAS and Ubc9

Full-length rat PIAS1 cDNA was obtained in the SEPT5 yeast two-hybrid screen. cDNAs were obtained as noted and PCR-amplified with the primers listed. Rat PIAS3 (KChAP) cDNA: Dr. Barbra Wible (Wible et al. 1998) 5’aatggaatcatgtggatgtgtccggt3’ and 5’agttaaggaacctgactgacgctat3’; human PIASy cDNA: Dr. Fletcher White (Sturm et al. 2000) 5’gttagaattcatggcggcggacagtgt3’ and 5’gaccacgcccggacagtgtcggata3’; rat PIASxα: Dr. Jorma Palvimo (Moilanen et al. 1999) 5’ttaagaatcatgcggatattcag3’ and 5’atatctcgagtcactgtgcacagtat3’; human Ubc9: Dr. Ronald Hay (Desterro et al. 1997) 5’taaatagaatcggcggactgacgctat3’ and 5’atagacgctagtatgagcagcagcaactat3’. All PCR products were cloned directionally into pJG4-5 with EcoRI and XhoI.

2.2.4. Bacterial Expression

PIASxα (rat) bacterial expression vectors were provided by Dr. Jorma Palvimo (University of Helsinki) and included: pGEX-4T3-PIASxα and pGEX6P1-Δ347-418PIASxα. Dr. Ronald Hay (University of Dundee) provided pGEX-2T-Ubc9 (human). pGEX-KG-Sept5 (rat) was generated previously in the lab (Beites et al. 1999). pGEX-UlpI was a gift from Dr. Mark Hochstrasser (Yale University). Empty cloning vector pGEX-KG was used to express free GST.

2.2.5. Baculovirus-Mediated Expression

For baculovirus expression, SEPT2 was provided by Dr. YiWei Huang, in the Trimble lab (Huang et al. 2006). Human Sept12_v1 and rat Sept12_v2 were subcloned from pJG4-5, directly into pFastBacHTA (Invitrogen, Mississauga, ON, Canada) using EcoRI and XhoI.

2.2.6. Mammalian Expression

GFP-tagged Constructs:

Rat Sept12_v2 cDNA was subcloned from pJG4-5 into a modified version of pEGFPC1 (Clontech) using EcoRI and XhoI; pEGFPC1 was linearized by cleavage with BglII and BamHI followed by insertion of the following annealed oligonucleotides:
5’gatctgaattccggtcagctgcagg3’ and 5’gatecctgagtcgcgccgaattca3’. Similarly, pEFGPC1-PIAS1 was generated by subcloning of rat PIAS1 cDNA (isolated in SEPT5 yeast two-hybrid screen) into the above vector. PIASxα was directly subcloned from pJG4-5 into the above vector to create pEGFPC1-PIASxα. pEGFPN1-Sept5 was provided by Dr. XiaoRong Peng (Trimble lab). pEGFPC1-Sept14 was constructed by subcloning from pCR-XL-TOPO using EcoRI and XhoI.

**Myc-tagged Constructs:**

pcDNA-myc-Sept5 was created using pGEX-KG-Sept5 as a template for PCR amplification using the oligonucleotides 5’ccggaattcagctgcagg3’ and 5’ccgctcgagatggtcctgcatttgctgctt3’. The resultant PCR product was cloned using the EcoRI and XhoI. pcDNA3.1-Myc-Sept4 was provided by Ms. Hong Xie (Trimble lab). Myc-tagged Ubc9 (human) was constructed by digesting the pGEX-2T-Ubc9 (from Dr. Ronald Hay) with BamH1 and EcoR1 and directly ligating into Myc tagged pcDNA3.1 (provided by Mr. Richard Collins, Trimble lab). pFLAG-CMV2-Ubc9 (mouse) was provided by Dr. Jorma Palvimo (University of Helsinki).

**HA-tagged Constructs:**

HA-Ubc9 (human) was constructed by digesting the pGEX-2T-Ubc9 (from Dr. Ronald Hay) with BamH1 and EcoR1 and directly ligating into HA-tagged pcDNA3.1 (provided by Mr. Richard Collins, Trimble lab). pcDNA3-HA-SUMO 1/2/3 (human) were provided by Dr. Ronald Hay (University of Dundee).

**Flag-tagged Constructs:**

pcDNA3.1-Flag-Sept5 (rat) was provided by Dr. XiaoRong Peng (Trimble lab); Flag-tagged truncations of Sept5 were created by replacing wildtype Sept5 with Sept5 truncations previously cloned into pJG4-5; Sept1 was sub-cloned directly into the pcDNA3.1-Flag vector from pJG4-5, using EcoRI and XhoI. Mouse pCMVJ-PIAS3 was a gift from Dr. Ke Shuai (University of California, Los Angeles).

*Sept9* cDNA was subcloned into a pcDNA-Flag vector, analogous to that described elsewhere (Surka et al, 2002).
His\textsubscript{6} and His\textsubscript{6}-Myc-tagged SUMOs

For expression of His\textsubscript{6}-tagged SUMOs, pcDNA3.1 (-) was modified by annealing and phosphorylating two oligos (5’ctagctatcagcacccattgcacccaccaccacccag3’ and 5’gatcctggtgatgtgtgtgtgatgcacgacctggtgatcagtagat3’). Annealing these oligos created a 5’ NheI and a 3’ BamHI site and an internal ClaI site and the annealed oligo was ligated into the NheI and BamHI sites in pcDNA3.1(-). Insertion was checked by digesting the construct with ClaI and sequencing. The sequence for the active form of SUMO1 (terminal amino acids: glycine-glycine) was amplified by PCR using pGEX-2T-SUMO1 (Ronald Hay, University of Dundee) as a template and primers, one containing a BamHI site and the other a KpnI site (5’aatggatccatgtctgacccagggacccagaaacc3’ and 5’atatggtacctcctccgctgctgctg3’). The PCR product was digested with these enzymes and cloned into the same sites in the His\textsubscript{6}-modified pcDNA 3.1 (-) described above. The sequences for the active forms of SUMO2 and 3 were amplified by PCR using pGEX SUMO2 and 3 (Ronald Hay, University of Dundee) as templates and primers containing BamHI sites and EcoRI sites (SUMO2 primers: 5’atatggatccatgtccgaggagaagcccaaggag3’ and 5’atatggtacctcctccgctgctgctg3’). The PCR products were digested with these enzymes and cloned into the same sites in the His\textsubscript{6}-modified pcDNA3.1 (-) described above. To make the Myc-tagged variation of the His\textsubscript{6}-tagged SUMO constructs an oligo containing the Myc sequence with a 5’ BamHI site and 3’ BglII site and an internal HindIII site (5’gatccatggacgagacgatcgcagcagggacccagta3’ and 5’gatctagcttcaggtctcctcctcgatcagcagctctcctcatg3’) was cloned into the BamHI site following the His\textsubscript{6} tag. Insertion was confirmed by cutting with HindIII and direction was checked by sequencing.

2.2.7. Point Mutation of Sept12

Mutagenesis was done according to Stratagene’s Quickchange\textsuperscript{TM} Site-Directed Mutagenesis Kit (Stratagene) with some modifications. Each mutagenesis reaction was run with and without the primers (as a negative control) in a total volume of 50μl. Two μl of each reaction was removed before adding 2μl DpnI after which the reaction was incubated for 2 hours at 37°C. Competent
DH5α were transformed with 10μl of each mutagenesis reaction and 2μl of each reaction before the DpnI digest, as a positive control. Two colonies from each mutagenesis plate were miniprepped and sequenced in both directions to confirm the mutagenesis and check that no additional mutations were introduced. A rat SEPT12_S61N mutant was created, changing serine-61 to asparagine, using following primers: 5’agtgggetaggcaagaccaccatggtgaacat3’ and 5’aatgtgttcaccatggtttcttgectagccacct3’ and pJG4-5-Sept12 (rat) was used as the template.

2.2.8. Point Mutation of Sept5

Several point mutants of pcDNA3.1-Flag-Sept5 were created in order to change several predicted SUMO-acceptor lysine residues into alanines. These mutants were created as described above using the following primers: K40R: 5’gcaccgcaagtctcaggaagaggtttgacttc3’ and 5’gaagtcgaaccttctctgacttgcgtggc3’; K180R: 5’gaaggctgtcagcagagggtaaatgcttc3’ and 5’ggaacgatgttcacacctctctgagcgccttc3’; K189R: 5’gttcacctctctgagcgccttc3’ and 5’gaacccagcagcgtcgtgtcagcgccttc3’; K347R: 5’gaagctcatcaggatgagggatgaagagctgaggc3’ and 5’gcctcagctcctctcatcctctgtgacctt3’.

2.2.9. Point Mutation of SUMO2

A mutation was introduced into the His6-myc-tagged SUMO2, as described above. This changes the lysine at position 11 to arginine using the following oligos: 5’gcccaaggagggttgaggacagaatgaccac3’ and 5’ggctcattgctcctcatacctctgagcgc3’.

2.2.10. Transformation of Escherichia Coli Competent Cells

For routine transformation, 1ng of circular DNA or 50-100ng of ligated plasmid DNA (usually about 10μl of the ligation mix) was added to 100μl of E.coli competent cells and incubated on ice for 20 minutes. Following a brief heat shock at 42°C for 2 minutes and incubation on ice for 15 minutes, the E.coli/DNA mix was then incubated with 1 mL of LB at 37°C for 1 hr. The cells were then spun at 14 000xg for 30sec and resuspended in 100μl of LB supplemented with 100μg/ml of ampicillin (Sigma) or 50μg/ml of kanamycin (Sigma). Routinely, both 10% and
90% of the culture was plated onto LB/agar plates with the appropriate antibiotic and incubated overnight at 37°C.

### 2.3. Yeast Two-Hybrid Studies

#### 2.3.1. Yeast Two-Hybrid Screen

An adult rat brain cDNA library constructed in the cloning vector pJG4-5 (Origene) was screened separately using Sept5-LexA and LexA-Sept12, expressed from the pNLexA and pEG202 plasmids, respectively. The yeast strain EGY48 was used for library screens. With the exception of yeast strain RFY206 (Clontech), all other plasmids and yeast strain EGY48 were kindly provided by the lab of Roger Brent (Finley and Brent 1995). All yeast two-hybrid methods were performed as previously described (Finley and Brent 1995; Gietz and Schiestl 1991; Gietz et al. 1995) and interactions were assessed by ability of diploid yeast to grow on selective media and to activate the LacZ reporter. Isolated plasmids were sequenced with the following primers: 5’ccagcctcttgctgagtggagatg3’ and 5’gacaagccgacaaccttgattgca3’ (Finley and Brent 1995).

#### 2.3.2. Septin:Septin Pairwise Crosses

Each mammalian septin cDNA was subcloned into bait (pEG202) and prey (pJG4-5) vectors. Each bait vector was transformed separately into yeast strain RFY206 (ura3 his3 trp1 leu2 MATa) along with pSH18-34 LacZ reporter (ura3) and selected on Glu ura⁻ his⁻ plates. Prey vectors were transformed into yeast strain EGY48 (ura3 his3 trp1 leu2 MATα) and selected on Glu trp⁻ plates. Each RFY206 septin transformant was streaked out in parallel horizontal lines on Glu ura⁻ his⁻ plates and each EGY48 septin transformant was streaked out on Glu trp⁻ plates in parallel vertical lines. Using the same replica velvet, the RFY206 and EGY48 derivatives were printed such that the streaks from both plates were perpendicular to one another. The print of both strains was lifted from the velvet onto a YPD plate and incubated overnight at 30°C. Replicas were made from the YPD plate onto Gal ura⁻ his⁻ trp⁻ X-gal plates and incubated
overnight at 30°C. Septin:septin interactions were identified by the formation of blue colonies at the intersection between the two yeast strains.

2.3.3. **Septin:PIAS/Ubc9 Pairwise Crosses**

Each mammalian PIAS cDNA was subcloned into prey (pJG4-5) vectors. Each prey vector was co-transformed into yeast strain EGY48 (ura3 his3 trp1 leu2 MATα) along with pSH18-34 LacZ reporter (ura3) and pEG202-Sept5. Transformants were selected on Glu ura’ his’ trip’ plates. Colonies were subsequently restreaked into patches onto Gal ura’ his’ trp’ X-gal plates and incubated at 30°C. Septin:PIAS interactions were identified by the degree of blue colour after 2-3 days.

2.4. **Recombinant Protein Expression**

2.4.1. **Expression and Purification of GST-fusion Constructs in E. Coli**

To express recombinant SEPT5, an *E.coli* culture harbouring the expression plasmid was inoculated into 3ml of Luria Bertani (LB) broth containing 100 μg/ml of ampicillin for approximately 8hrs at 37°C in a shaking incubator. The 3 ml was then added to 50ml of LB broth, and incubated overnight. The next day the culture was diluted into 500ml of LB for 1hr or until the OD₆₀₀ reached 0.8 to 1.0. At this time, 0.1mM of isopropyl-β-D-thiogalactopyranoside (IPTG) was added and the culture was switched to a 30°C bacterial shaker and incubated for an additional 6hrs. The cells were then spun at 6,000 x g, 4°C for 10 minutes, the supernatant decanted and the pelleted cells were resuspended in 5-7ml of cold resuspension buffer (PBS-T (PBS-T = 150mM NaCl, 12.5mM NaH₂PO₄, 90mM Na₂HPO₄, 0.05% tween-20), supplemented with 0.1% β-mercaptoethanol, and the following protease inhibitors: 1μg/ml pepstatin, 1μg/ml leupeptin, 2mM benzamidine, 1mM PMSF). Using a French Pressure Cell Press (Spectronic Instruments) the bacterial cells were lysed at 20,000psi at a rate of about 30 drops per minute. Successful bacterial lysis was often accompanied by a darkening of the suspension. To increase the yield of soluble protein, the lysis was further incubated with 1% triton X-100 for 30 minutes.
at 4°C followed by a spin at 15,000 rpm in a JA-17 rotor (Beckman) for 15 minutes to remove cellular debris and insoluble fusion proteins.

To purify the protein from bacterial lysates, glutathione agarose beads (Sigma, G4510) were pre-swollen in water, washed twice in PBS-T and diluted to a 50% slurry. The beads were then incubated with the pre-cleared lysate for 2hrs at 4°C, with constant rotation and then washed five times with 10ml of ice cold PBS-T. The volume was readjusted to achieve a 50% bead slurry. Protease inhibitors (1mM PMSF, 1µg/ml leupeptin and pepstatin, 2mM benzamidine) were added if the SEPT5 fusion protein was to be left on the beads for subsequent assays.

To remove the GST carrier from the fusion proteins, the beads were washed twice in thrombin cleavage buffer (TCB; 20mM HEPES-KOH pH 7.8, 150mM KCl, 2.5 mM CaCl₂, 0.5% tween-20 and freshly added 0.1% β-mercaptoethanol) and incubated with 500μl of TCB with the addition of 5 units of thrombin (Sigma, T7513). Although efficient cleavage occurred after 10min at room temperature, some cleaved protein remained bound to the beads, possibly through aggregation of the protein at high concentration. The thrombin was then inactivated by the addition of 1 mM (4-(2-aminoethyl)-benzenesulfonylflouride HCl) (AEBSF) and the aforementioned protease inhibitors were also added. Incubation of this protein with thrombin lead to the production of two fragments, a predominant 45 kDa species reflecting the full length SEPT5 and a minor lower band possibly due to degradation of the protein from some protease sensitive site. For most studies the protein was used immediately as overnight incubation at 4°C resulted in loss of its GTP binding capability (C.L. Beites, personal communication).

In spite of its low solubility, a typical yield of 1.5-2.5mg/ml of protein was eluted from the glutathione beads per 500ml starting culture. In our experience, many unlysed bacterial pellets or pre-cleared lysates of other GST fusion proteins can be frozen at -80°C with purification continued at a later date, but several attempts at recovery of GST-SEPT5 after freezing (in the presence or absence of glycerol, or within the bacteria after induction) have been unsuccessful. Most of the protein remained in insoluble aggregates and the little that did bind to the glutathione beads could no longer bind GTP (C.L. Beites, personal communication).
Protein isolates were quantified and assessed for purity by Coomassie-stained SDS-PAGE, running varying amounts of purified BSA alongside. Quantification was estimated based on band intensity relative to BSA standards.

All other GST-fusions used in this thesis were grown and expressed as for the GST-SEPT5 except that the cultures were kept at 37°C, for 4hrs, during induction with IPTG. Incubation with glutathione beads, washes and elutions were carried out as above.

**2.4.2. Expression and Purification of His₆-fusion Constructs in Insect Cells**

The N-terminal His₆-tagged proteins were overproduced in SF21 insect cells as recommended by the manufacturer (Invitrogen). Cells infected for 4 days with baculovirus were disrupted by sonication, and the lysate was centrifuged at 10 000 g for 10 min to pellet debris. The supernatant was purified on NTA agarose (Qiagen). The proteins were washed twice with the equilibration buffer (40mM Tris, 200mM NaCl, 20% glycerol, 0.4mM PMSF, 1mg/mL of leupeptin and pepstatin, 8mM imidazole, pH 8.0), and subsequently eluted by the same buffer but containing 150mM imidazole.

**2.4.3. Phosphatidylinositol phosphate (PIP) Strip Binding**

Recombinant protein (from baculovirus expression) was quantified using BioRad Reagent (BioRad) and purity was verified by SDS-PAGE. PIP strips (100pmol/spot; Echelon Biosciences) were probed with recombinant proteins, as per product directions. An anti-His₆ antibody and the secondary antibody and detection system described below for Western Blotting were used for detection.

**2.5. Nucleotide Extraction and Exchange**

Septins were overexpressed and purified using the baculovirus system (Invitrogen) and SF21 insect cells, as previously described (Huang et al. 2006; She et al. 2004). Protein was acid-denatured and nucleotide was resolved by reverse-phase high pressure liquid chromatography (R-HPLC) as described (Huang et al. 2006; She et al. 2004). ³H-GDP exchange assays were also
conducted as previously described (Huang et al. 2006; She et al. 2004). Briefly, non-radioactive recombinant proteins were incubated for increasing time periods in buffer containing $^3$H-GDP, protein was collected on nitrocellulose by vacuum and radioactivity was counted.

2.6. Northern Blotting

Testes were pulverized under liquid nitrogen and 100mg frozen, powdered tissue homogenate was weighed directly into tissue lysis reagent. The RNeasy Mini Kit $^{TM}$ (Qiagen) was used to isolate total RNA from all tissues except brain, brainstem, epididymis and testis; the RNeasy Lipid Tissue Mini Kit $^{TM}$ (Qiagen) was used for the latter four tissues. Northern blotting was conducted as per standard methodology. Briefly, RNA was quantified using UV absorbance (260nm) and 20μg/lane RNA was resolved by agarose gel electrophoresis and transferred to Nytran membrane by capillary action. Rat Sept12_v2 cDNA was gel-purified using the QIAquick Gel Extraction Kit (Qiagen) and 100ng was heat-denatured and labeled with dCTP-P$^{32}$ using the High Prime Kit (Roche, Indianapolis, IN, USA) and hybridized to the membrane. Signal was detected by phosphorimaging. RNA ladder (BioCan Scientific) and ribosomal RNA were visualized with ethidium bromide and Nytran membrane was stained prior to exposure with methylene blue to mark the ladder and rRNA and to verify adequate and equal transfer of RNA.

2.7. RT-PCR

Conducted as previously described (Yenugu et al. 2006), using the Qiagen OneStep RT-PCR kit (Qiagen). Briefly, 1μg total RNA (isolated as above) was used with primers specific to β-actin or Sept12, respectively. Sept12 primers were designed to amplify the first 700bp of the open reading frame, allowing assessment of presence/absence of exon4. β-actin primers: 5’cgtgggccgcctagccacca3’ and 5’ttgccctagggttcaggggg3’; Sept12 primers: 5’atggagtgagggcgaacacc3’ and 5’tcagaggagtctgtcattgtc3’. 100pb ladder was purchased from Fermentas.
2.8. Antibodies

2.8.1. Generation of anti-SEPT12 Antibodies

The immunogenic peptide matching the sequence of the C-terminal 15 amino acids of rat SEPT12 (CPEKVRKRSKDPRDDE) was synthesized with an N-terminal cysteine residue and purified by HPLC (Hospital for Sick Children). It was then sent to Antibodies Incorporated (Davis, CA) where it was coupled to keyhole limpet hemocyanin and used to immunize rabbits. The above peptide was reduced using Reduce-Imm Immobilized reductant Column™ (Pierce) and then covalently linked to SulfoLink™ (Pierce) beads as per product specifications. To purify antibodies from serum, 5mL of serum was diluted into 45mL of phosphate-buffered saline (PBS) and incubated, rotating, overnight at 4°C. After degassing, the solution was poured into a fritted column and drained. A succession of washes was conducted with 50mL volumes of degassed buffers: A. PBS; B. 2M NaCl and 0.1M sodium phosphate, pH 7.0; C. 0.1M sodium borate, pH 9.1; D. PBS, pH 4.5. Antibodies were eluted with 20mM glycine and 200mM NaCl, pH 2.5 in 0.8mL fractions, which dripped directly into neutralizing buffer (0.4mL of 0.1M Tris, pH 8.5). Fractions containing the highest amount of antibody were determined by measuring absorbance at 280nm. After brief dialysis in PBS to adjust pH to neutrality using Slide-A-Lyzer cassettes™ (molecular weight cut-off 10kD, volume 0.5mL (Pierce)), fractions were diluted into glycerol to a final glycerol concentration of 50% and stored at -20°C.

2.8.2. Previously-generated Septin Antibodies

Rabbit polyclonal antibodies specific to SEPT2, SEPT4 and SEPT9, respectively were generated in the Trimble lab and are described elsewhere (Ahuja et al. 2006; Surka et al. 2002).

2.8.3. Commercial Antibodies

The following rabbit polyclonal antibodies specific to the antigens listed were used: LexA (Invitrogen #46-0710, 1:10,000); Myc (Santa Cruz #A-14; 1:2,000); Flag (Sigma #F7425; 1:250); SUMO2/3 (AbCam #ab3742-100; 1:250). The following mouse monoclonal antibodies specific to the antigens listed were used: Myc (Santa Cruz #sc-40, 1:1,000); Green Fluorescent
Protein (GFP) (Santa Cruz #sc-9996; 1:500); Flag (Sigma #M2; 1:5,000); Poly-histidine (Covance #MMS-156, 1:1,000); GM130 (BD Signal Transduction #610823; 1:250); hemagglutinin (HA)-Horse Radish Peroxidase (HRP) (Roche #12082700; 1:5,000); HA (Covance #MMS101R; 1:1,000). Goat and/or Donkey anti-rabbit/anti-mouse-HRP conjugates were used as secondaries in Western blotting (BioRad; 1:5,000). Similarly, goat and/or donkey fluorescent secondary antibodies were used for immunofluorescence (Jackson ImmunoResearch Labs, 1:1,000).

2.9. Western Blotting

With the exception of developmental and multi-tissue blots, primary antibodies and quantities loaded are noted in figure legends. For the former, tissues were purchased and/or procured as previously described, homogenized by mortar and pestle under liquid nitrogen and immersed directly into 2x SDS loading buffer. GFP-SEPT12 stable CHO cells growing at approximately 50% confluency were scraped and lysed directly in 2x SDS loading buffer for the GFP-SEPT12 control. Protein concentration was determined relative to a BSA standard curve by spotting equal sample volumes (2μL) on filter paper, staining with coomassie blue and quantification of signal intensity using ImageJ and Microsoft Excel software. 20μg quantities of protein per sample were loaded on gels with protein standards (BioRad). Samples were resolved by SDS-PAGE and transferred to polyvinyl difluoride (PVDF) (Millipore) and Western blotting was performed using appropriate secondary antibody-HRP conjugates and standard protocol. Signal was detected by ECL Western blotting Detection Reagent (GE Healthcare) and exposure to autoradiography film.

2.10. Cell Culture and Transfection

2.10.1. Transient Transfection

Chinese Hamster Ovary (CHO) cells were routinely transfected with 1μg of each plasmid DNA, using FuGene6™ transfection reagent as per product specifications (Roche). Cells were maintained in alpha-MEM supplemented with 10% FBS, 0.2 IU/mL penicillin and 0.2μg/mL
streptomycin. Transiently transfected cells were fixed and stained for imaging or lysed for sumoylation assays approximately 20-24 hours post-transfection.

2.10.2. Establishment of Stably Transfected CHO cells

The CHO stable cell lines were established by transient transfection of pEGFP-Sept12 or pcDNA3.1-His6Myc-SUMO1/2/3, respectively (as above), followed by dilution and plating in 96-well dishes and subsequent maintenance in media with 1.5mg/mL G418 sulfate. This allowed for selection of individual clones which were subsequently expanded and maintained in larger culture volumes.

2.11. Animal Work

2.11.1. Animal Handling

Adult Wistar rats were purchased from Charles Rivers Laboratories and all animal work was done in accordance with humane and ethical treatment of animals, as approved by the Hospital for Sick Children Animal Care Committee.

2.11.2. Sperm and Tissue Procurement

Spermatozoa were isolated from freshly dissected epididymis and vas deferens tissue by mincing tissue and placing it into warmed culture media (Wier and Rumberger 1995). For the testis developmental blots, testes from one animal per age timepoint were dissected from freshly sacrificed Wistar rats and snap-frozen in liquid nitrogen; all other rat tissues were purchased from Harlan Laboratories.

2.11.3. Tissue Sectioning

Freshly isolated testes were snap-frozen in mounting/sectioning media, in an isopentane/dry ice bath. An in-house cryostat was used to generate 7μm cryosections (Department of Pathology, Hospital for Sick Children) which were subsequently used for immunofluorescence studies,
using the standard fixation, immunostaining and imaging techniques described herein for cell lines.

2.12. Immunostaining and Image Acquisition

In all cases, actin staining was visualized with rhodamine-conjugated phallloidin (Molecular Probes) as per product specifications and DNA was visualized with bisbenzimide (Sigma-Aldrich) at a concentration of 5μg/mL for 10 minutes. All fluorescent secondary antibodies were purchased from Jackson ImmunoResearch Labs and used as previously described (Surka et al. 2002). Stably transfected CHO cells were either grown directly on glass coverslips (interphase) or dividing cells were enriched for by shakeoff and plated onto poly-D-lysine coated coverslips and allowed to adhere for 20min. Stable cells and all transient transfections in the mammalian sumoylation study were fixed with 3% paraformaldehyde in PBS, processed and imaged with epifluorescence, as previously described (Surka et al. 2002). Isolated spermatozoa were placed onto poly-D-lysine coated coverslips, allowed to adhere on ice for approximately 60 minutes and fixed as above. 0.1% triton X-100 in blocking solution (3% milk powder in PBS) was applied to permeabilize for 10min. Mitochondria were labeled with MitoTracker Orange™ (Invitrogen) prior to fixation and according to product specifications. For all remaining transfections, CHO cells were grown and prepared as stable cells, except 0.1% triton X-100 in blocking solution was used to permeabilize for 2 minutes; Tissue sections were permeabilized and blocked similarly. All remaining imaging was done using a Quorum Spinning Disk Confocal Microscope; a Leica DMIRE2 inverted fluorescence microscope equipped with a Hamamatsu Back-Thinned EM-CCD camera and spinning disk confocal scan head, equipped with 4 separate diode-pumped solid state laser lines (Spectral Applied Research: 405nm, 491nm, 561nm, 652nm), 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 and all image analysis was done using this system. Representative optical slices (captured at a Z-spacing of 0.2μm) are shown.
2.13. Sumoylation Assay

2.13.1. NTA Precipitation

20 hours after transfection, CHO cells were lysed by scraping directly into 1 ml lysis buffer, pH 8.0 (100 mM NaH₂PO₄, 10mM Tris, 6M guanidine hydrochloride, 0.1% sarkosyl and 20mM imidazole). After incubation for 10min, room temperature, the cells were passaged through a 27G needle three times followed by sonication (3 x 30sec bursts). Insoluble material was pelleted at 10 000 rpm, for 5 min and 1% of the starting material was kept as input. 25μl of Ni-NTA beads was first washed in lysis buffer and then incubated end over end with 90% of the lysate for 30min, at room temperature. The beads were then washed twice with washing buffer (pH 8.0) containing 100mM NaH₂PO₄, 10mM Tris, 8M Urea, 0.1% sarkosyl and 20mM imidazole followed by washing with the same buffer at pH 6.3. After three washes with ice-cold PBS, SDS sample buffer (40μl) was added to the purified material on the beads for subsequent SDS-PAGE. 10% of the starting material was subjected to trichloroacetic acid precipitation and used as a whole cell extract. The proteins were analyzed by Western blotting using the appropriate antibodies, as described.

2.13.2. Ulp1 Cleavage of NTA Precipitates

CHO cells triply transfected with Flag-Sept5, Flag-Ubc9 and His₆-SUMO2 were purified on NTA beads as described above. After a final wash with PBS, the beads were incubated either with 50μl of PBS as a control or 50μl of purified recombinant Ulp1 for 5 minutes at 37°C. The beads were then spun down at 5200 rpm, for 2 minutes and the supernatant and beads were boiled in 2x SDS loading buffer, separated by SDS-PAGE and transferred to PVDF.

2.14. Immunoprecipitations

2.14.1. SEPT9:SEPT14

60mm dishes of CHO cells (approx 80% confluency) were cotransfected for 24 hours with Flag-Sept9 and GFP-Sept14, using Fugene transfection reagent (Roche). Upon lysis in 40mM Tris,
pH 8.0, 100mM NaCl, 1% triton X-100 and standard protease inhibitors (1mM PMSF, 1µg/ml leupeptin and pepstatin, 2mM benzamidine), lysates were incubated with rotation for 1 hour at 4°C and clarified by high-speed centrifugation. 2µg Anti-Flag M2 monoclonal antibodies (Sigma) were used for immunoprecipitation; controls were conducted with purified mouse IgG. Following addition of antibodies, proteinG-agarose was added (Invitrogen) and samples were rotated for several more hours at 4°C. Following extensive washing in the above buffer, followed by two washes in PBS, samples were resolved by SDS-PAGE and transferred to PVDF (Millipore) and Western blotting was performed using standard protocol.

2.14.2. **SEPT9 from Testis Lysate**

Immunoprecipitation was performed as described above, except 20µg of anti-SEPT9 antibody was used; this antibody is described elsewhere (Surka et al. 2002). Rabbit IgG was used as the control (first lane). Briefly, 1 rat testicle (Harlan Laboratories) was homogenized using a mortar and pestle, under liquid nitrogen; then in 40mM Tris, pH 8.0, 100mM NaCl, 1% triton X-100 and standard protease inhibitors (1mM PMSF, 1µg/ml leupeptin and pepstatin, 2mM benzamidine) in a dounce mechanical homogenizer. After rotation for 1 hour at 4°C, followed by clarification by high-speed centrifugation, antibodies were added and rotation was continued. After approximately 4 hours, proteinA-agarose beads were added (Invitrogen) and immunoprecipitation was conducted overnight. Following extensive washing in the above buffer, followed by two washes in PBS, immunoprecipitated proteins were resolved by SDS-PAGE, stained with coomassie, and bands of interest were excised. After standard trypsin digestion, bands were subjected to MALDI-TOF. Unique peptide hits of greater than 95% accuracy were compared against public databases allowing for identification of corresponding proteins. Mass spectrometry and database searching was conducted by the Hospital for Sick Children Advanced Protein Technology Centre.
2.15. Bioinformatic Analyses

2.15.1. Generation of Phylogenetic Trees
The minimal conserved septin amino acid sequence (G1 sequence to end of SUE) was entered into ClustalW at the Kyoto Bioinformatics Institute server (http://align.genome.jp) using default settings.

2.15.2. Assigning Identity to Yeast Two-hybrid Clones
Insert-containing prey plasmids isolated from yeast two-hybrid screens were sequenced with the primers specific to both the 5’ and 3’ ends of the insert, listed previously. Automated sequencing was done in-house, at the Hospital for Sick Children’s “The Centre for Applied Genomics”. National Center for Biotechnology Information (NCBI) databases were searched using the Basic Local Alignment and Search Tool (BLAST) algorithm to identify the closest matching sequence.

2.15.3. Identification of SEPT14
BLAST analysis using the NCBI server was used to determine the sequence of the rat Sept14 cDNA, using human Sept14 cDNA, human Sept14 gene structure and expressed sequence tag (EST) clones as templates. To identify SEPT14 by mass spectrometry, this protein sequence was inputted into the database of known rat protein sequences that was used to blast all peptide sequences obtained from mass spectrometry. Alignments were performed with ClustalW and BLAST (NCBI).

2.15.4. Assigning Identity to SEPT9 Co-immunoprecipitates
Unique peptide hits of greater than 95% accuracy were compared against the current NCBI rat protein database (24,198 sequences) allowing for identification of corresponding proteins. Data was further analyzed using the freely available program Scaffold Viewer (www.proteomeviewer.com/scaffold/scaffold_viewer.htm).
3. SEPT5 YEAST TWO-HYBRID INTERACTIONS

Cloning for the septin:septin crosses was conducted with the help of summer students Mathew Estey and Min Lin. I conducted all remaining work with yeast, including the yeast two-hybrid screen. I am grateful to the laboratory of Dr. Roger Brent for provision of all yeast two-hybrid vectors and yeast strains. This work is not published elsewhere.

3.1. Rationale

The first portion of this chapter makes use of the yeast two-hybrid system to examine septin-septin interactions. I then use this system to map SEPT5:SEPT5 binding domains. At the time I began this study, most literature suggested that the septins interacted directly with one another to form a unit complex composed of two copies of each of the septins present (Field et al. 1996; Frazier et al. 1998; Hsu et al. 1998). This unit complex was then competent for self-polymerization into higher order filamentous structures. However, the specific ordering and structure within this unit complex was not known, nor was it clear how specific septins bound to themselves and to one another. This outstanding question was my motivation for creating a series of SEPT5 truncation mutants in order to map at least one septin:septin binding interface.

Septin complexes had also been isolated from mammals, but it remained elusive whether or not this was similar in structure to the S. cerevisiae complex (Beites et al. 1999; Frazier et al. 1998; Oegema et al. 1998). While septins have been compared across species, there are not clear mammalian orthologues of the S. cerevisiae septins. Thus, additional questions arise, such as:

1. Is there specific ordering within mammalian septin complexes?

2. Are there septins that are strictly conserved and required in all septin complexes?

3. How do septin complexes differ between organisms/tissues/cell types?

4. Is there redundancy within the mammalian septin family that has been introduced by the expansion of this gene family in mammals, allowing for compensation in the absence of a given septin; is this particular to any specific septins?
It was my goal to use the yeast two-hybrid system to provide the foundation work for such answers by completing a comprehensive pairwise interaction map of all known mammalian septins.

In the second portion of this chapter, I conduct a yeast two-hybrid screen to identify SEPT5 binding partners. At the onset of this project, previous work from our lab had identified SEPT5 as an interaction partner of the essential conserved neuronal SNARE protein, syntaxin-1 (Beites et al. 1999). It was further shown that SEPT5 is highly enriched in post-mitotic neurons and that it may somehow work in concert with the SNARE complex to regulate synaptic vesicle secretory events during neurotransmission, which requires syntaxin-1. This was a very exciting finding at the time, and SEPT5 represented one of the most extensively characterized septins of the brain-enriched septins. However, it remained unclear how SEPT5 was functioning on a molecular level and exactly what its role was in neurons. Thus, I began my studies with yeast two-hybrid screening of an adult rat brain cDNA library in order to identify additional SEPT5 interaction partners to glean further insight into the molecular role of SEPT5 in neurons.

### 3.2. Results: Septin:Septin Interactions Using the Yeast Two-Hybrid System

#### 3.2.1. Mapping the Potential Mammalian Septin:Septin Interactome in Yeast

As a starting point in determining septin association hierarchy, I cloned all the mammalian septins that were known at the time into yeast two-hybrid vectors and conducted pairwise crosses. Many interactions were observed and this is shown in Figure 11. In most cases, a given pair of septins either does or does not interact in both orientations. Thus, Figure 11 is bi-fold symmetric about a central axis (hatched line), as one would expect if protein tags are not altering the ability of proteins to interact. Interactions which do not exhibit this bi-fold symmetry are marked with the letters a-f. Notably, SEPT5 is capable of self-interaction.
Constructs listed to the left were expressed as LexA fusions from pEG202. Constructs listed across the top were expressed from the pJG4-5 vector. In most cases, similar results were found in both orientations; paired anomalies are marked with colour-coordinated letters a-f. Results were generated by expression of respective constructs in haploid yeast strains EGY-48 (pJG4-5) and RFY-206 (pEG-202) and their successive mating on YPD and replica plating onto selective media with X-gal. Degree of blue colour is indicative of the strength of interaction. Expression of all constructs was verified with Western blots using either anti-HA antibodies (pJG4-5) or anti-LexA antibodies (pEG-202) (not shown).

Figure 11. Pairwise Interaction of Mammalian Septins 1-11.
3.2.2. Mapping Septin:Septin Interaction Domains in Yeast

In this study, I examined the binary homomeric SEPT5:SEPT5 interaction. To do this, I created a series of truncation mutants and expressed them as fusion constructs in the yeast two-hybrid system. I sequentially truncated from the 5’-end of the cDNA, progressively removing first the sequence encoding the unique N-terminal extension; poly-basic (lipid-binding) domain, right up to the start of the G1 sequence; followed by the entire N-terminus, from just downstream from the G4 sequence. From the 3’-end of Sept5, I removed coding sequence for the unique C-terminal extension (including the predicted coiled-coil) and then the majority of the C-terminal half, downstream from the G4 sequence within the nucleotide-binding domain. The binary interactions and associated schematics of the truncations are shown in Figure 12.

Interestingly, full length wildtype (WT) SEPT5 homodimerized strongly, whereas the S58N point mutant which cannot bind nucleotide (Beites et al. 1999) bound very weakly to WT SEPT5 and not at all to itself. Only truncation of the very N-terminal 27 amino acids preserved self-association, although this was only observed in one orientation: when LexA-(Δ1-27)SEPT5 and B35-WTSEPT5 were expressed in yeast. This result is somewhat unclear, but suggests that the protein tags may influence the interaction by creating stearic hindrance. Nonetheless, the minimal sequence required for SEPT5:SEPT5 self-association is all but a very small portion at the N-terminus, which is alternately spliced in different SEPT5 variants (Yagi et al. 1998; Zieger et al. 2000). These results are shown in Figure 12.
Figure 12. Assessment of SEPT5 Mutant Self-Interaction.

(A) Schematic of SEPT5 truncations assessed. (B) Pairwise interaction grid: constructs listed to the left were expressed as LexA fusions from pEG202. Constructs listed across the top were expressed from the pJG4-5 vector. Results were generated by expression of respective constructs in haploid yeast strains EGY-48 (pJG4-5) and RFY-206 (pEG-202) and their successive mating on YPD and replica plating onto selective media with X-gal. Degree of blue colour is indicative of the strength of interaction. Expression of all constructs was verified with Western blots using either anti-HA antibodies (pJG4-5) or anti-LexA antibodies (pEG-202) (not shown).
3.3. Results: SEPT5 Yeast Two-Hybrid Screen

*Sept5* was cloned into the LexA yeast two-hybrid vector pNLexA for screening using the LexA system. Unlike the Gal4-based yeast two-hybrid systems, the LexA system allows for use of the pNLexA vector where bait proteins are fused at their C-terminus to the N-terminus of the transcriptional activator’s DNA-binding domain, thereby exposing a free N-terminus of the bait protein during screening (Toby and Golemis 2001). This was of particular interest in the screening of septins, as most septins (including SEPT5) contain a predicted C-terminal coiled-coil sequence and I was concerned that this would bias the screen towards the identification of non-specific coiled-coil interactions. A saturating rat brain cDNA library screen produced several unique clones; all of these were verified by back-transformation into the original bait-containing yeast strain.

Prior to screening against a library of interest, it is crucial to verify that the bait protein is expressed by the yeast and that it is (1) able to enter the yeast nucleus and bind to appropriate operator sequences and (2) not able to autoactivate the reporter genes. Using any of the available yeast two-hybrid systems, it is easy to test for expression of the bait construct using Western blotting with antibodies specific either to the tag added to the bait or with antibodies specific to the bait itself. By expressing the bait construct in the yeast in the absence of the prey construct, or library, it is also simple to confirm that the bait construct does not autoactivate the reporter genes. An additional advantage to the LexA system is that it allows one to verify that the bait construct is able to enter the nucleus and bind to operator sequences, using a repression assay (outlined in Figure 13).
Bait construct is expressed, enters the yeast nucleus, and does not cause autoactivation of reporter genes. (A) Western blot of yeast lysate, blotted with anti-SEPT5 monoclonal antibodies (SP20), showing specific expression of the Sept5-LexA fusion construct. (B) Yeast haploid strain EGY-48 was co-transformed with LacZ reporter plasmid pSH18-34 and either pNLexA-Sept5, pSH17-4 or pRHFM1, respectively to ensure that the bait construct could not activate reporter genes on its own. This is clearly shown in the leftmost panel of (B), by the lack of blue colour. pSH17-4 expresses the LexA-Gal4 activation domain and acts as a positive control, while pRHFM1 expresses only LexA and acts as a negative control. (C) pJK101 is transformed alone or with either pNLexA-Sept5 or pRHFM1, respectively, in order to assess ability of the bait construct to enter the yeast nucleus and bind LexA operator sequences. In the absence of repression (pJK101 alone, rightmost panel of (C)), LacZ gene transcription is active, producing robust blue colour; as LexA-fusion constructs enter the nucleus and bind the LexA operators, transcription is repressed (pNLexA-Sept5 and pRHFM1).
Upon confirmation of expression of the bait fusion construct and confirmation that it is capable of entering the nucleus and binding to LexA operators, I screened an adult rat brain library (Origene). This library was created containing inserts ranging from 0.4 – 4kb. The inserts were directionally cloned using 5’-EcoRI and 3’-XhoI. A saturating screen was then performed, where transformants were initially plated on minimal glucose medium lacking histidine, uracil and tryptophan (to select for the transformed plasmids). Colonies were then scraped, resuspended and titred and plated onto galactose medium lacking histidine, uracil and tryptophan and also lacking leucine, to select for transformants able to activate reporter leucine biosynthesis genes. This latter media also contained 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) to enable selection of transformants capable of simultaneously activating expression of the other reporter gene, LacZ. Positive clones were picked during the first four days of the screen and restreaked on selective media to confirm activation of both reporters. In total, 113 positive colonies were isolated. Library plasmids were then isolated from the yeast using standard glass bead and phenol:chloroform lysis. Plasmids were electroporated into bacterial strain KC8, which is unable to synthesize tryptophan. By growing transformants on media lacking tryptophan, with the addition of ampicillin, I was able isolate adequate quantities of respective clone bait plasmids for sequencing at the Hospital for Sick Children (Toronto) DNA sequencing facility. Sequences were then compared against databases using the BLAST algorithm (NCBI) to assign identities (Table 3), yielding 12 novel cDNAs. All plasmids were back-transformed into yeast to confirm interaction.
### Table 3. SEPT5 Yeast-Two Hybrid Results.

cDNA sequences corresponding to unique SEPT5 binary interactors identified in yeast two-hybrid screening of an adult rat brain cDNA library. Left-most column (A) lists the septin interactors; the remaining columns (B) pertain to the non-septin interactors.

<table>
<thead>
<tr>
<th>Septin</th>
<th>Non-septin</th>
<th>Match [organism/score]</th>
<th>NCBI Accession No.</th>
<th>Full-length cDNA (bp)</th>
<th>Screen (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sept2</td>
<td></td>
<td>PIAS1 [Rn/100%]</td>
<td>NM_001101829</td>
<td>1956</td>
<td>1-1956</td>
</tr>
<tr>
<td>Sept5</td>
<td></td>
<td>PIASα [Rn/100%]</td>
<td>BC078775.1</td>
<td>1719</td>
<td>1309-1719</td>
</tr>
<tr>
<td>Sept6</td>
<td></td>
<td>VPS53 [Rn/100%]</td>
<td>NM_001101813</td>
<td>2499</td>
<td>1575-2355</td>
</tr>
<tr>
<td>Sept10</td>
<td></td>
<td>SGIP1 [Rn/100%]</td>
<td>XM_001058167</td>
<td>2424</td>
<td>496-2424</td>
</tr>
<tr>
<td>Sept11</td>
<td></td>
<td>Hypothetical (BTB) [Rn/100%]</td>
<td>XM_001079342</td>
<td>5970</td>
<td>3178-5970</td>
</tr>
<tr>
<td>*Sept12</td>
<td></td>
<td>PSB4 [Mm/92%]</td>
<td>BC008241</td>
<td>795</td>
<td>736-795</td>
</tr>
</tbody>
</table>

*Unique septin cDNA at the time of screening; henceforth termed Sept12, consistent with adopted septin nomenclature and the first mention of Sept12 in the literature (Kinoshita 2003).
3.3.1. **Septin:Septin Interactions**

Several full-length septin cDNAs were isolated in the screen and are listed in Table 3. With the exception of Sept12, they all represent previously known sequences and at least initial characterizations have now been published for all of them.

3.3.2. **Identification of a Novel Septin cDNA**

Through detailed database searching and sequence comparison, I determined that the uncharacterized septin sequence (Sept12) is indeed novel and is conserved in mammals. Panel A of Figure 14 depicts the dendrogram resulting from the alignment of the amino acid sequence of the highly conserved portion of septins, containing the GTP-binding domain followed by the SUE. This alignment suggests that the new septin is most closely related to SEPT3 and SEPT9 and that all three of these septins likely originated from a common ancestor gene. The SUE is defined as the region of high sequence identity, across all septins, following the G4 domain. It is approximately 110 amino acids in length, is specific to septins, and speculated to contribute to a conserved property of septins, which remains enigmatic. For this comparison, I have defined the end of the SUE as the amino acid sequence LYE (SEPT1), HFE (SEPT9) or HYE (remaining septins). Alignments were performed using ClustalW2, on the European Bioinformatics server (EBI). SEPT12 is not predicted to contain a C-terminal coiled-coil sequence, when analyzed using the COILS algorithm, which is readily available on-line (Lupas et al. 1991). It does, however contain the other septin sequence elements, as shown in Figure 14, panel B.
Figure 14. Properties of SEPT12.

Phylogenetic tree generated by ClustalW2 alignment of SEPT1-SEPT12 shows that SEPT12 is unique but contains conserved septin primary sequence elements. (A) Human protein sequences from the start of the G1 domain to the end of the Septin Unique Element (SUE) were aligned, as in Figure 1. Branching is assumed to represent the degree of phylogeny where branch lengths are proportional to the inferred evolutionary change. By sequence comparison alone, SEPT12 is most closely related to SEPT3 and SEPT9. (B) SEPT12 contains the sequence elements/motifs conserved in septins: polybasic domain (PB; blue), the G1, G3 and G4 sequences of the GTP-binding domain (red) and the SUE (yellow).
3.3.3. Septin:Non-Septin Interactions

PIAS1 and PIASxα (PIAS2/ARIP3) are members of the SUMO E3 ligase family. The full-length PIAS1 cDNA and the 385 nucleotides of PIASx corresponding to the 3’ end of the cDNA of the alpha isoform were isolated in this screen, suggesting that mammalian septins may be subject to modification by sumoylation, as has been observed in *S. cerevisiae* (Johnson and Blobel 1999; Johnson and Gupta 2001). VPS53 is a member of the recently characterized tripartite VPS complex, which has been shown to regulate Golgi to endoplasmic reticulum retro-trafficking (Conibear and Stevens 2000; Liewen et al. 2005). Similarly, SGIP1 has recently been shown to play a role in endocytic events (Uezu et al. 2007). The hypothetical BTB-containing protein contains a “Bric-a-brac/Tramtrack/Broad Complex” (BTB) domain; a domain established to determine a unique three-dimensional fold with a large interaction surface which facilitates a versatility of protein-protein interactions (Perez-Torrado et al. 2006). SEPT5 interacted with the C-terminal half of this protein; the coding sequence for the clone isolated began just downstream of the BTB domain but did not include this domain. The final interactor is the one of the proteasome beta subunits, which is intriguing given the connection between SEPT5 and parkin, but it is often obtained as a false positive in yeast two-hybrid screening.

3.4. Discussion

Some anomalies were observed in this pairwise interaction study and are likely attributable to steric hindrance introduced by protein tags. A frustrating limitation of the yeast two-hybrid system is that it is not possible to discern whether or not homodimerization of bait/prey septins is occurring prior to assessing pairwise interaction, although the recent septin unit complex crystal suggests that this is not likely to occur (Sirajuddin et al. 2007). Nonetheless, it is not clear from this study whether or not heteromeric interactions are true 1:1 interactions, or whether they require respective prior homodimerization. In addition, this study does not take into account the many splice forms of the different septins and how this might alter septin:septin interaction specificity. However, the crystal structure of the core mammalian septin complex suggests that septins of the same sub-family as SEPT2 (SEPT1, SEPT4 and SEPT5) should self-associate and this was observed for all members except SEPT1. In addition, it suggests that SEPT6 sub-family members should not self-associate and this was observed for all members (SEPT6, SEPT8,
SEPT10 and SEPT11), consistent with the current model (Sirajuddin et al. 2007). Unfortunately, the model does not account for the sub-family containing SEPT3, SEPT9 and SEPT12, making it difficult to predict binary interactions.

The results of the septin:septin interactome study expand substantially upon the known septin interactions (Joo et al. 2005) and contain additional observations, which may be of use in explaining other results, particularly when more information is available with regard to specific expression patterns and septins which co-isolate in specific complexes. It serves as a starting point for the consideration of whether or not members of a given septin subfamily might substitute for one another in the unit complex. For example, although SEPT4 and SEPT5 are almost identical, SEPT4 and SEPT7 interact, whereas SEPT5 and SEPT7 do not. SEPT4 has not been observed in primary hippocampal neurons, whereas SEPT5 is highly expressed in these cells (Beites et al. 1999; Peng et al. 2002; Tsang 2007). This would suggest that if the core septin complex contains SEPT2/6/7, then SEPT4 would directly associate with the core complex via SEPT7 and SEPT5 likely interacts via another septin. An understanding of hierarchy in septin:septin interactions is particularly important for understanding the types of complexes that may be forming due to tissue and cell-type specific expression and to understand more complex issues, such as the observed developmental compensation which has sometimes been reported upon septin knockout and which may or may not be associated with an obvious phenotype (Ihara et al. 2005; Kissel et al. 2005; Ono et al. 2005; Peng et al. 2002).

It is not surprising to observe that homodimerization of SEPT5 required almost the entire full-length protein. Although studies in *S. cerevisiae* have suggested C-terminal coiled-coil regions are capable of mediating septin-septin interaction, they also suggest N-terminal regions of at least some septins are involved in mediating septin:septin interactions (Versele et al. 2004; Versele and Thorner 2005). Detailed studies are lacking for septins which are not predicted to contain coiled-coil sequences, although homodimerization has been observed (Versele et al. 2004). Overall, my results are consistent with the current model for septin unit complex formation (Sirajuddin et al. 2007; Versele et al. 2004; Versele and Thorner 2005).

Several full-length septins were identified as SEPT5-interactors in the yeast two-hybrid screen, as expected. Several early studies in *S. cerevisiae, D. melanogaster* and mammals demonstrated that septins copurified as a complex (Fares et al. 1996; Hsu et al. 1998) and that across
organisms, this complex contains approximately two copies of each respective septin protein (Field et al. 1996; Frazier et al. 1998). Other studies demonstrated endogenous septin:septin interaction as well as the ability of septins to form exogenous filaments, but did not predict such specifics of the unit septin complex (Beites et al. 1999; Frazier et al. 1998; Oegema et al. 1998).

At this point, many independent studies have reported interactions between several of the mammalian septins and several are listed in the recent review by Joo and colleagues (Joo et al. 2005). More recent studies have examined the nature of septin:septin interaction in great detail, both in *S. cerevisiae* (Versele et al. 2004) and in the recent publication of the crystal structure of the mammalian Sept2/6/7 complex (Sirajuddin et al. 2007).

Additional support for the isolation of these specific septins comes from their previous characterizations: SEPT2 is expressed in all organs, including in the brain (Xie et al. 1999), where it was previously found associated with SEPT5 (Beites et al. 1999). Both SEPT5 and SEPT6 are also known to be highly expressed in brain (Beites et al. 1999; Kinoshita et al. 2000). SEPT10 was first cloned from a dendritic cell library and then was shown by Northern blotting to be broadly expressed. SEPT11 was recently isolated in a complex of brain septins, and was subsequently shown by Northern blotting to be widely expressed (Hanai et al. 2004). The expression profiling studies of all the septins using microarrays has confirmed their expression in brain (Hall et al. 2005). Further, Western blotting, reveals that all are abundantly expressed in brain (Figure 3), which supports the identification of multiple septins as SEPT5 binding partners in this screen.

Notably, a novel septin cDNA was isolated in this screen and encodes for a 356 amino acid protein, which contains all of the conserved sequence elements of the septin protein family. This novel septin is most similar to SEPT3 and SEPT9 and is predicted not to contain a C-terminal coiled-coil sequence. A thorough analysis of the tissue expression of the novel septin and the biochemical properties of its protein product is presented in Chapter 5 and it is hereafter referred to as *Sept12*/SEPT12, consistent with current nomenclature (Kinoshita 2003).

Several intriguing non-septin candidate partner proteins for SEPT5 were also identified in this screen. PIAS proteins contain a critical Really Interesting New Gene (RING) domain, which allows them to interact with both the SUMO-bound E2 enzyme Ubc9 as well as the substrate, thereby generating an isopeptide link between SUMO and a specific lysine residue in the target
protein. Based on the conservation of critical cysteine residues within the RING domains across both ubiquitin ligase and PIAS protein family members, these ligases are believed to function similarly (Schmidt and Muller 2003). Cell cycle-dependent sumoylation of *S. cerevisiae* septins was described just prior to the completion of my screening, in work by Erica Johnson (Johnson and Blobel 1999; Johnson and Gupta 2001) and its further examination in the context of SEPT5 is presented in the following chapter. Further support for the interaction between SEPT5 and RING-containing PIAS proteins came from the recently reported interaction between the RING-type E3 ubiquitin ligase parkin and SEPT5 (Zhang et al. 2000).

The remaining SEPT5-interactors were not studied further due to limited information and/or resources available at the time of completion of the screen. These interactions may be specific to SEPT5, a subset of septins, or may be conserved across all the septins. They represent exciting new candidate interactions for studying the cellular role of septins.

VPS53 is a member of the tripartite vacuolar protein sorting (VPS) complex composed of VPS52/53/54. At the time of screening, VPS orthologues had not been identified in mammals. In *S. cerevisiae*, it is known that this complex is required for trafficking from the late Golgi compartment (Conibear and Stevens 2000). It has recently been revealed that a similar complex exists in mammals and appears to function in an analogous fashion, regulating transport back to the endoplasmic reticulum (Liewen et al. 2005). It should also be mentioned that Golgi-localization of septins has been observed during neurite outgrowth both in the Trimble lab (unpublished observations) and elsewhere (Hsu et al. 1998).

SGIP1 has also only recently been characterized (Uezu et al. 2007). This protein appears to play an integral role in endocytosis through its binding to both AP2 clathrin adaptors and phosphoinositides. Work by the Robinson lab in Australia demonstrated an enrichment of SEPT3 in synaptosome preparations from brain, consistent with presence of endocytic proteins, such as synaptophysin and dynamin (Xue et al. 2004). This research suggests that SEPT3 may function in endocytosis at nerve terminals through association with endocytic machinery.

The cDNA encoding large BTB domain-containing hypothetical protein, for which bona fide orthologues (ESTs) exist in other mammals, was also isolated. Nothing is known of the function of this protein, and a limited number of sequences are available in databases. The BTB-only proteins Skp1 and ElonginC are long-established adaptor molecules in E3 ubiquitin ligase
complexes. These two proteins form complexes with Cul1 and F-box proteins or Cul2 and SOCS-box proteins, respectively, to form the specificity-conferring E3 ubiquitin ligase. BTB-containing proteins are emerging in the literature as common, wide-ranging regulators through their combination with the above proteins to form active E3 ubiquitin ligases. It is tempting to speculate that this protein may participate in the regulated turnover of septins or that septins complexes may function as a scaffold for the ubiquitination of substrates under strict spatio-temporal regulation, such as during the cell cycle. Interaction of SEPT5 with E3 ubiquitin ligase parkin has already been shown to promote degradation of SEPT5. Mutations in the parkin gene result in a recessive form of early-onset Parkinson Disease, in which accumulation of septins is observed in affected neurons. Overexpression of SEPT5 also shows a similar phenotype (Choi et al. 2003). These studies suggest a critical importance in maintaining the appropriate cellular balance between synthesis and degradation of septins for healthy neuron function, highlighting the importance of the specific interaction between E3 ubiquitin ligases and septins.

Finally, the proteasome beta subunit 4 (PSB4) is an intriguing interaction partner, considering the established links between SEPT5 and parkin, as well as the previously described BTB-containing SEPT5 interactor. The core of the proteasome is a barrel-like macromolecule made up of four stacked heptamer rings. The two central (inner) rings are made up of 7 beta subunits each and effect the enzymatic proteolysis of substrates. The outer rings are made up of 7 alpha subunits each and play a role in recognition and guiding of substrates towards the proteolytic centre (Dong et al. 2003; Son et al. 2005). While again, it is tempting to suspect a link between SEPT5 and proteasome-mediated hydrolysis, there exists no evidence that septins are part of the extensively studied and well-conserved core proteasome or the proteasome substrate recognition process (Wolf and Hilt 2004). Further, proteasome subunits are commonly found false positives when using the LexA-based yeast two-hybrid system to detect binary protein interactions (Wolf and Hilt 2004).
4. MAMMALIAN SEPTIN SUMOYLATION

I worked in close collaboration with Dr. Cresina Beites and Dr. Carol Froese to study mammalian septin sumoylation. Data presented in Figures 17, 18, 19, 24, 25 and 26 was generated by Dr. Crestina Beites. Data presented in Figure 27 was generated by Dr. Carol Froese. This work is not published elsewhere.

4.1. Rationale

At the time I completed my screen for SEPT5-interacting proteins, PIAS-dependent sumoylation of \textit{S. cerevisiae} septins had only recently been described. These early studies suggested that septin sumoylation promoted the breakdown of higher order septin filaments following cytokinesis (Johnson and Blobel 1999; Johnson and Gupta 2001). There were no reports of mammalian septin sumoylation. As such, examination of septin sumoylation was a promising next step for my studies.

It seemed reasonable that because sumoylation had remained undetected for so long due to the low proportion of a given target protein that is sumoylated at a given time, the transient nature of this process and the high activity of the isopeptidases that remove SUMO from target proteins (Johnson 2004) the mammalian septins would likely be sumoylated in a cell-cycle dependent manner, like the \textit{S. cerevisiae} septins. Further rationalization of this pursuit was provided by the observation that in \textit{D. melanogaster}, septins interacted directly with the E1 (Uba2 subunit) and the E2 (Ubc9), respectively, and exhibited a convincing degree of co-localization, despite the authors’ inability to observe endogenous sumoylation (Shih et al. 2002).

The suggestion that sumoylation might play an essential role in septin filament breakdown or priming for breakdown was a particularly exciting observation for the septin field at the time I started this work because virtually nothing was known about the regulation of filament disassembly. As such, sumoylation immediately rose to a high priority of study in a diversity of septin-dependent cellular contexts, in a variety of commonly studied organisms.
Upon identification of PIASxα and PIAS1 in yeast two-hybrid screens using SEPT5 as the bait, I reasoned that septin sumoylation was likely to be conserved across eukaryotes and that septins would also be sumoylated in mammalian cells. The conservation of this interaction from *S. cerevisiae* to mammals is striking and many of the mammalian septins, including SEPT5, contain classical sumoylation consensus sequences. As such, I set out to confirm the interaction between SEPT5 and the enzymes of the sumoylation modification pathway and attempted to demonstrate mammalian septin sumoylation with the eventual goal of determining its physiological relevance.

### 4.2. Results

#### 4.2.1. Confirmation of Septin-Sumoylation Enzyme Interaction

To address interaction with components of the sumoylation machinery, I obtained cDNAs for all PIAS proteins, as well as Ubc9, and cloned them into the yeast two-hybrid prey vector to assay for interaction with SEPT5. Controls were conducted, as in Figure 13 (not shown). When yeast was retransformed with SEPT5 and the original interactors: the C-terminal fragment of PIASxα and full-length PIAS1, the interaction was confirmed (Figure 15). In addition, SEPT5 interacted weakly with PIAS3 and strongly with Ubc9. This initial finding suggests a conserved binary interaction between septins at two different levels of the SUMO pathway: the E2 and the E3. The conservation of this interaction across eukaryotes suggests that septins in higher eukaryotes may also be sumoylated during cell division or other cellular processes.

As an additional means to confirm the interaction between SEPT5 and sumoylation enzymes, I expressed and purified recombinant proteins from *E. coli* for direct binding studies. I conducted binary binding assays using glutathione-S-transferase (GST) tagged variants of SEPT5, Ubc9 and both wildtype PIASxα and a ΔRING mutant of PIASxα which has been shown to be incapable of binding to Ubc9 (Kahyo et al. 2001); see Figure 16 for a schematic of PIASxα.
Figure 15. SEPT5 Directly Interacts with Sumoylation E2 and E3s.

Yeast strain EGY-48 was co-transformed with the LacZ reporter plasmid (pSH18-34) and vectors expressing LexA-SEPT5 bait fusion construct (pEG-202) and respective vectors encoding B42-PIAS/Ubc9 prey fusion constructs (pJG4-5). Degree of blue colour correlates with the strength of interaction.
Recombinant GST-SEPT5 was produced in bacteria and the GST tag was cleaved off with thrombin. 0.3nanomoles of free, unbound SEPT5 was then incubated with 0.3nanomoles of GST fusion proteins on glutathione beads, as noted above. In the final lane, 1 part soluble Ubc9 was added to the binding mixture, greatly enhancing the interaction between SEPT5 and PIASxα. Following extensive washing, all protein isolated on glutathione beads was resolved by SDS-PAGE and immunoblotted for SEPT5 with the SP20 monoclonal antibody.
SEPT5 bound directly to both variants of PIASxα, as well as Ubc9. SEPT5 binding to mutant PIASxα implies that this interaction is not dependent upon the region of PIASxα which is required for binding to Ubc9, supporting the potential for a trimeric complex to form. This assay does not, however, identify the region(s) of SEPT5 responsible for binding to PIASxα and Ubc9; as such it is not possible to speculate whether their binding is expected to be simultaneous or mutually exclusive.

Figure 16 also corroborates the finding that SEPT5 binds directly to Ubc9. The binding of SEPT5 to PIASxα appears to be greatly enhanced by addition of Ubc9 (last lane). This supports the postulation that PIASxα may form a trimeric complex with both SEPT5 and Ubc9, and that this may be a more stable complex than the respective binary interactions, as is suggested in similar work by Kahyo et al (Kahyo et al. 2001).

Thus, I have demonstrated by two independent means that binary interactions can occur between SEPT5 and two levels of the sumoylation pathway: the E2 (Ubc9) and the E3 (PIAS family members). In addition, in vitro binding assays also showed a direct interaction between SEPT5 and SUMO1 (not shown). Together, these findings strongly support the evolutionary conservation of an interaction between septins and the sumoylation pathway.

4.2.2. Development of a Sumoylation Assay for SEPT5 in CHO cells

Our initial varied approaches to observe supportive evidence of SEPT5 sumoylation were taken directly from successful literature examples and included: in vitro translation and subsequent sumoylation with recombinant proteins (Buschmann et al. 2001; Johnson and Gupta 2001); transfection and immunoprecipitation (Buschmann et al. 2001); endogenous staining (Kirsh et al. 2002); and cell synchrony (Johnson and Gupta 2001). Unfortunately, none of these methods were successful in our hands (data not shown).

After reading that another research group was only able to observe sumoylation of their target protein only upon transfection of His6-tagged SUMO into cells, followed by cell lysis in chaotropic salt conditions (which instantaneously deactivates highly active SUMO isopeptidases) and subsequent purification of sumoylated species by nickel affinity chromatography (from
isopeptidases and unmodified substrate), we designed our assay in order to follow a similar approach (Müller et al. 2000).

All sumoylation assays in this thesis were conducted using transient overexpression of Sept5 and components of the mammalian sumoylation pathway in CHO cells, followed by direct lysis into a 6M guanidine buffer. Cells for each individual assay condition were grown in 60mm dishes to approximately 50-80% confluency prior to transfection. Post-transfection, cells were maintained for approximately 20 hours prior to lysis. The CHO cell line was selected because it is a well characterized and actively-dividing mammalian cell line which produces endogenous SEPT5. In interphase CHO cells, SEPT5 localizes to stress fibres, as has been observed for other septins (Joberty et al. 2001; Kinoshita et al. 1997; Surka et al. 2002; Xie et al. 1999). During cytokinesis, as with other septins, SEPT5 becomes incorporated into the prominent cortical septin structure found in close apposition to the actomyosin cleavage apparatus (Kinoshita et al. 1997; Surka et al. 2002; Xie et al. 1999). Initial sumoylation assays were conducted using only His6-tagged SUMO1 and SUMO2, for subsequent isolation on nickel-nitrilotriacetic acid (NTA) beads. For the remaining assays, a Myc tag was inserted between the His6 tag and the SUMO start codon, allowing for immunoblotting using clean and well-characterized anti-Myc antibodies following purification on NTA beads. With the exception of Figure 17, all assays employed this second purification step on NTA beads.

We reasoned that testing only SUMO1 and SUMO2 was adequate because SUMO2 and SUMO3 differ by only four amino acids and phylogenetic analysis indicates that the SUMO3 gene is derived from the SUMO2 gene (Su and Li 2002). We also used mature SUMO isoforms, eliminating the need for SENP-dependent proteolytic activation. In addition, early studies suggested substantial differences between the cellular roles of SUMO1 and SUMO2/3 modification, but not between modification by SUMO2 and SUMO3 (Saitoh and Hinchey 2000; Tatham et al. 2001). Early studies also reported direct interaction between the sumoylation E2, Ubc9, and a large number of proteins (Yeh et al. 2000). There were several studies demonstrating that Ubc9 was both necessary and sufficient to direct substrate sumoylation because it is capable of directly interacting with substrates and effecting sumoylation at lysines embedded in the standard sumoylation consensus sequence, ΨKX(D/E) (Bernier-Villamor et al. 2002; Desterro et al. 1998; Eloranta and Hurst 2002; Okuma et al. 1999; Sampson et al. 2001; Tatham et al. 2001). As such, we did not include PIAS proteins in our initial sumoylation
assays. In addition, low-level septin sumoylation was still observed in a SIZ1/SIZ2 deletion strain, suggesting the E3 is not absolutely necessary for sumoylation of septins because *S. cerevisiae* has only two PIAS-type SUMO E3 ligases.

The results of one of the most promising early experiments, suggesting sumoylation of SEPT5, are depicted in Figure 17. In this assay, *Sept5* was overexpressed along with SUMO1 or SUMO2 with or without Ubc9 in each case. When lysates were resolved by SDS-PAGE, transferred to PVDF and immunoblotted for SEPT5, the presence of a very faint higher molecular weight band was detected only in the lane where both Ubc9 and SUMO2 were overexpressed with *Sept5* (final lane, asterisked). This is precisely what we would expect to see in the case of SUMO-modified SEPT5, because since its original observation (Lee et al. 1998), covalent modification by SUMO has been demonstrated to consistently shift the mobility of modified protein by approximately 20kDa (per SUMO) relative to its unmodified form. Therefore, this result suggested to us that only a very small proportion of the overexpressed SEPT5 was modified and that isolation of sumoylated species away from the bulk of expressed SEPT5 would be an essential step in further experiments.
Figure 17. Overexpression of SUMO2, Ubc9 and SEPT5: Sumoylation.

CHO cells were transfected with plasmids expressing the fusion constructs indicated above. 1% of total cell lysates were separated by SDS-PAGE and Western blotted with anti-SEPT5 antibodies (top panel) or with anti-Flag antibodies (bottom panel) to detect Ubc9. Asterisk denotes possible SUMO-modified SEPT5. Conducted by Dr. Crestina Beites.
To confirm that this upper band was SUMO-modified SEPT5, His<sub>e</sub>-SUMO modified proteins were isolated from the lysate, using NTA beads as described above, and the entire isolate was resolved by SDS-PAGE and Western blots were conducted. As Figure 18 shows, modification of SEPT5 by SUMO2 was much more robust than by SUMO1. Also, the addition of Ubc9 greatly enhanced this modification (lane 6) consistent with its observed role in direct recognition and subsequent modification of lysine residues embedded in accessible sumoylation sequences in target proteins. In Figure 18, when lanes 5 and 6 were stripped and re-probed with antibodies specific to SUMO2/3 (right-hand panel), the banding pattern clearly matches that observed when previously blotted for SEPT5 (left-hand panel).
Figure 18. **SEPT5 is Covalently Modified by SUMO2.**

CHO cells transfected with vectors expressing the fusion constructs noted were lysed directly in chaotrope salt and SUMO-conjugates were purified on NTA beads. The isolated proteins were then fractionated by SDS-PAGE and Western blotted and probed with SP20 antibodies against SEPT5 (left panel). In the right-hand panel, the last two lanes of the left-hand panel (lanes 5 and 6) were stripped and re-probed with anti-SUMO2/3 antibodies. The asterisk denotes either other substrates purified by the His6-SUMO2 column or free His6-SUMO2. Conducted by Dr. Crestina Beites.
4.2.3. Verification that SEPT5 is Sumoylated

To exclude the possibility that SEPT5 was aggregating to the beads in an aberrantly migrating guanidine and SDS-resistant aggregate, the NTA precipitates were incubated with purified recombinant *S. cerevisiae* isopeptidase Ulp1p or with PBS as a control. Ulp1p is capable of cleaving both *S. cerevisiae* SUMO (Smt3p) and mammalian SUMO1 from its conjugates, although there was no literature at the time to suggest that Ulp1p could process or cleave mammalian SUMO2 conjugates (Yeh et al. 2000). However, incubation of the NTA purified precipitates with Ulp1p but not PBS reveals a distinct shift in the pattern of SUMO-SEPT5 conjugates (Figure 19, lanes PBS vs Ulp1) with the predominant band running just below 47kDa, consistent with the size of unmodified form of SEPT5.

Although it was expected that the unmodified version of SEPT5 would be found in the supernatant fraction and not in the bead fraction since it is no longer bound to the NTA column via the His6-SUMO2, aggregation or polymerization of SEPT5 has likely occurred, resulting in the detection of unmodified SEPT5 in the bead fraction. This atypical aggregation of SEPT5 can be explained by the lack of the denaturing detergent sarkosyl in the incubation buffer. Typically, 0.1% sarkosyl is added to the buffers used to lyse and wash the Ni-NTA bound material. However, since this particular experiment necessitated more native conditions that would not denature the enzyme Ulp1p, the detergent was omitted. Nevertheless, the shift in mobility of SEPT5 by SDS-PAGE confirms that the higher molecular weight septin is indeed bound to the NTA beads via a covalent SUMO2 interaction.
Figure 19. SUMO2 is Cleaved from SEPT5 by the SENP Ulp1p.

NTA-purified complexes from CHO cells expressing His$_6$-SUMO2, Flag-SEPT5 and Flag-Ubc9 were incubated with either PBS or Ulp1p for 5 minutes at 37°C. The beads were then spun at 5200 x gravity and the resulting supernatants (S) as well as the beads (B) were analyzed by Western blotting with anti-SEPT5 antibodies (SP20). 1% of the total cell lysates (Input lane) was loaded to determine the mobility of the unmodified form of SEPT5. Conducted by Dr. Crestina Beites.
4.2.4. Examination of Potential Sumoylation Sites in SEPT5

Site-directed mutagenesis is frequently employed to as a means to verify sites of endogenous post-translational modification. Conservative mutation of potential lysines embedded in target sumoylation sequences to arginines has been successfully used to determine physiologically-relevant sumoylation sites in many well-characterized sumoylation targets, such as RanGAP and PML (Kamitani et al. 1998; Lee et al. 1998). Therefore, we began by sequentially mutating all lysines to arginines in consensus sequences resembling sumoylation consensus sequences in SEPT5.

A schematic of SEPT5 is shown in panel A of Figure 20 and the corresponding primary amino acid sequence in panel B. Consensus sequences are underlined and potential target lysines are asterisked; these represent four of the 31 total lysines within the rat SEPT5 amino acid sequence. Notably, the first three putative sumoylation sites are located in or very near to conserved septin sequence elements: the polybasic (bold) and G4 (boxed) sequences, respectively. The last lysine is located in the predicted C-terminal coiled-coil sequence (bold, italics), just downstream from a consecutive series of proline residues, which may allow the very C-terminus of SEPT5 to adopt a unique structure. Panel C shows the results of the sumoylation assay: it is clear from this experiment that each of the point mutants is modified to the same degree as wildtype SEPT5 (lane 2), suggesting either that the physiologically-relevant lysine has not been mutated, or that several lysines are modified simultaneously. Curiously, mutation of lysine-40 resulted in an overall upward shift of mobility in SEPT5 by SDS-PAGE. In addition, this mutant form of the SEPT5 is also hyper-sumoylated, suggesting that this mutant may be in less tightly folded (non-native) conformation, making it more competent for sumoylation. In the final lane of Figure 20, panel C, a mutant of SUMO2 (K11R) was tested which has been reported to be unable to form poly-SUMO chains because the lysine at position eleven has been changed to arginine (Tatham et al. 2001). However, the ladder-like pattern observed (lower panel) suggests either that SEPT5 is being modified at multiple lysines, or that SUMO2 is forming chains by some means other than through lysine-11.
Figure 20. SEPT5 Point Mutants are Still Sumoylated.

Schematic of SEPT5 (A) and the corresponding primary amino acid sequence (B). Consensus sequences are underlined and potential target lysines are asterisked; the polybasic sequence is in bold and the elements of the GTP-binding domain are boxed; the predicted coiled-coil is in bold italics, respectively. (C) vectors expressing the above-noted fusion constructs were transfected into CHO cells and standard sumoylation assays were conducted; specific point mutants are denoted above each lane. Western blots of inputs (upper) and NTA-purified SUMO conjugates (lower) probed with anti-SEPT5 antibodies (SP20) demonstrate that each of the point mutants is modified to the same degree as wildtype SEPT5 (lane 2). In addition, a SUMO2 K11R mutant, reported incapable of forming poly-SUMO chains, still modifies SEPT5 multiple times.
In attempt to further clarify the relevant SEPT5 *in vivo* sumoylation site(s), I created truncations of SEPT5 for use in sumoylation assays. Schematics are shown in panel A of Figure 21 and results in panel B. Interestingly, the extreme C-terminus of SEPT5 is heavily sumoylated, despite the fact that this region of SEPT5 has only one lysine embedded in a consensus sumoylation sequence. At least one lysine in the N-terminus of SEPT5 is also SUMO-modified, as both truncations lacking increasing portions of the C-terminus appear capable of mono-sumoylation.
Figure 21. C-terminus of SEPT5 is Robustly Sumoylated.

Schematics of the truncations are shown in panel A. (B) Vectors expressing the above-noted fusion constructs were transfected into CHO cells and standard sumoylation assays were conducted; the region of SEPT5 (amino acids) expressed is noted above each lane. Western blots of inputs (B, left) and NTA-purified SUMO conjugates (B, right) probed with anti-Flag antibodies (Sigma) are shown. A single modification was observed in the C-terminal truncations of the protein (lanes 1 and 2), whereas robust multi-sumoylation was observed when just the C-terminal 127 amino acids was expressed. This portion of SEPT5 contains a stretch of proline residues, followed by the predicted coiled-coil sequence.
Figure 21 suggests that the very C-terminus of SEPT5 is capable of robust modification by SUMO2. Assuming that SUMO2 is not forming chains, as SUMO2 K11R gave similar results (not shown), multiple modification sites must exist within the very C-terminus of SEPT5, at non-consensus lysine residues. However, there are only four lysine residues present in the very C-terminus of SEPT5. In addition, when overexpressed in CHO cells, these SEPT5 truncations were frequently observed to coalesce into punctate structures in close approximation to the nuclear envelope (not shown), which suggests the possibility of an overexpression artifact due to proximity of sumoylation enzymes localized to the nuclear pore complex and within the nucleus.

Collectively, the mutation and truncation analysis suggest that SEPT5 is capable of undergoing sumoylation at multiple lysines; likely at non-consensus lysines; but these studies do not clarify the degree of sumoylation or identify the physiologically-relevant residues. Indeed, the possibility of overexpression artifacts makes interpretation of the observed results complicated. To further address SEPT5 sumoylation in a more selective environment, I turned my attention back to full-length wildtype SEPT5 and the PIAS proteins which I originally identified as SEPT5-interactors.

**4.2.5. Effect of PIAS Proteins on Sumoylation of SEPT5**

Our initial studies (Figures 17-21) support the hypothesis that Ubc9 is sufficient to promote the sumoylation of SEPT5, preferentially by SUMO2. However, at the time of this work, PIAS proteins were being described to enhance the sumoylation of several substrates at specific physiologically-relevant lysine residues. Such substrates included: p53 (Kahyo et al. 2001); the androgen receptor, c-jun and PIAS proteins themselves (Kotaja et al. 2002); and of course the *S. cerevisiae* septins (Johnson and Gupta 2001). As such, it was my expectation that inclusion of PIAS proteins in sumoylation assays would enhance SEPT5 sumoylation, possibly even allowing me to then identify the physiologically-relevant sumoylated lysine(s). I used PIASxα and PIAS1 in standard sumoylation assays to address this, because these were the two PIAS family members originally identified in the SEPT5 yeast two-hybrid screen and because out of the four PIAS proteins, these two interacted most strongly with SEPT5 in a binary fashion (Figures 15 and 16).

In Figures 22 and 23, the dependence of SEPT5 sumoylation on PIASxα and PIAS1, respectively, is tested. SEPT5 is expressed alone as a negative control (lane 1). SEPT5 was then
co-transfected with Ubc9 and each of the respective SUMO proteins (lanes 2-4), generating results similar to those observed previously (Figure 18). In lanes 5-7, SEPT5 is expressed with PIA$\alpha$/PIAS1 and each of the respective SUMOs, but in the absence of exogenous Ubc9. This resulted in very poor sumoylation; substantially less than with Ubc9 alone. In fact, it is comparable to the level of sumoylation observed when SEPT5 is coexpressed with only SUMO, in the absence of excess sumoylation enzymes (not shown). In the final lanes (8-10), Ubc9 and the respective PIAS proteins are included in the assay, but this only resulted in restoration of sumoylation to the level observed with Ubc9 alone. In addition, the proportion of sumoylated PIAS increased substantially.

I next questioned the potential role of SEPT5 as a platform for the sumoylation of PIAS proteins themselves. While sumoylation of PIAS proteins has been observed, its potential regulatory role has not been examined (Kotaja et al. 2002). However, when sumoylation assays were conducted, as above, examining the sumoylation status of PIA$\alpha$/PIAS1 in the presence and absence of exogenous SEPT5, there was no difference in the proportion of sumoylated PIAS (not shown).
Figure 22. PIASxα does not Enhance SEPT5 Sumoylation.

Vectors expressing the above-noted fusion constructs (top left) were transfected into CHO cells and standard sumoylation assays were conducted. Immunoblots of inputs (0.5%) and NTA isolates were probed with antibodies against SEPT5 (SP20), Ubc9 (anti-FLAG), PIASxα (anti-GFP) and/or SUMO1/2/3 (anti-Myc). In lanes 2-4, the inclusion of Ubc9 and any of the SUMOs was sufficient to drive robust sumoylation of SEPT5. Lanes 5-7 demonstrate that PIASxα alone did not appreciably enhance sumoylation of SEPT5. Inclusion of both Ubc9 and PIASxα restored SEPT5 sumoylation to that observed with Ubc9 alone (lanes 2-4) and only drove enhancement of PIASxα sumoylation; particularly with SUMO2 and SUMO3 (lanes 8-10).
Figure 23. PIAS1 does not Enhance SEPT5 Sumoylation.

Vectors expressing the above-noted fusion constructs (top left) were transfected into CHO cells and standard sumoylation assays were conducted. Immunoblots of inputs (0.5%) and NTA isolates were probed with antibodies against SEPT5 (SP20), Ubc9 (anti-FLAG), PIAS1 (anti-GFP) and/or SUMO1/2/3 (anti-Myc). In lanes 2-4, the inclusion of Ubc9 and any of the SUMOs was sufficient to drive robust sumoylation of SEPT5. Lanes 5-7 demonstrate that PIAS1 alone did not appreciably enhance sumoylation of SEPT5. Inclusion of both Ubc9 and PIAS1 restored SEPT5 sumoylation to that observed with Ubc9 alone (lanes 2-4) and only drove enhancement of PIAS1 sumoylation; particularly with SUMO2 (lanes 8-10).
Inclusion of PIAS proteins in these sumoylation assays did not enhance SEPT5 sumoylation. Thus, we were not able to further clarify the results of previous assays, in which Ubc9 alone was used to drive sumoylation, as it had been our expectation that inclusion of PIAS proteins in sumoylation assays would promote sumoylation. In an attempt to move forward from encouraging, yet unclear results of sumoylation assays, we began co-localization experiments with the goal of confirming cellular co-localization of SEPT5 with sumoylation components, as further evidence of a relationship between septins and the sumoylation pathway.

4.2.6. Localization of SUMO1/2 with respect to SEPT5 in CHO cells

When low levels of GFP-SEPT5 are produced in CHO cells, the expression pattern during interphase matches that which is observed for endogenous SEPT5: filamentous localization along actin-based stress fibres. Initial co-localization experiments were limited to interphase cells due to the marked absence of transfected cells undergoing division at the time of fixation and imaging. GFP-Sept5 was co-expressed with each of the SUMOs, respectively. As shown in Figure 24, co-localization was never observed between GFP-SEPT5 and SUMO1 (A-C), consistent with previous observations (Saitoh and Hinchey 2000; Su and Li 2002). Overexpressed SUMO1 always remained predominantly nuclear and we could not detect any effect on cytoplasmic filament formation by SEPT5. SUMO2 (D-F) and SUMO3 (not shown) were also observed to be nuclear, consistent with literature reports (Saitoh and Hinchey 2000; Su and Li 2002). However, in some cases, we observed direct co-localization of SEPT5 and SUMO2/3 at the cell periphery, in what appeared to be juxtamembranous regions (G-I). SEPT5 never appeared filamentous in such cases, suggesting that interaction with SUMO2/3 or modification by SUMO2/3 may be affecting filament formation.
Figure 24. SEPT5 and SUMO2/3 Co-localize

CHO cells were transfected with vectors expressing near-endogenous levels of GFP-SEPT5 (green) and either HA-SUMO1 (A-C; red) or HA-SUMO2 (D-I; red). Cells were transfected for 20 hours, fixed with paraformaldehyde and immunostained with primary antibodies against HA, followed by fluorescent secondary antibodies (Jackson Labs). Representative epifluorescent images are shown. SUMO1 was never observed outside the nucleus (A-C). SUMO2 was observed both exclusively localized to the nucleus (D-F) and also in the cytoplasm (G-I). When SUMO2 was cytoplasmic, septin filaments were notably absent, with co-localization appearing at the cortical regions of the cell. Similar results were observed for SUMO3. Conducted by Dr. Crestina Beites.
4.2.7. Localization of Ubc9 with respect to SEPT5 in CHO cells

Ubc9 was also been reported to be predominantly nuclear (Saitoh et al. 2002; Zhang et al. 2002). Much like the SUMOs, our observations were consistent with this (Figure 25, A-C). However, we also observed that Ubc9 co-localized with SEPT5 in the cortical periphery of some transfected cells, as with SUMO2/3 (Figure 25, D-F). As seen above with SUMO2, in cells exhibiting co-localization, SEPT5 was not observed to form filaments, again suggesting that either interaction with Ubc9 or sumoylation may be responsible for altering the SEPT5 interfaces responsible for filament formation.
Figure 25. SEPT5 and Ubc9 Co-localize.

CHO cells were transfected with vectors expressing near-endogenous levels of myc-SEPT5 (green) and HA-Ubc9 (red). Cells were transfected for 20 hours, fixed with parafomaldehyde and immunostained with epitope-specific primary antibodies, followed by fluorescent secondary antibodies. Representative epifluorescent images are shown. Ubc9 was observed both exclusively localized to the nucleus (A-C) and also in the cytoplasm (D-F). When Ubc9 was cytoplasmic, septin filaments were notably absent, with co-localization appearing at the cortical regions of the cell. Conducted by Dr. Crestina Beites.
4.2.8. Co-localization of PIAS3 with Endogenous SEPT5

PIAS proteins have been described as predominantly nuclear, although at the time of this work, there were at least two literature examples of their interaction with cytoplasmic proteins. PIAS3 was described to interact with voltage-dependent potassium channel subunits (Kuryshev et al. 2000; Kuryshev et al. 2001; Wible et al. 1998) and Siz1p with the S. cerevisiae septins (Johnson and Blobel 1999). Even in these studies, co-localization was either not observed or barely detectable.

In order to further examine the PIAS:SEPT5 interaction and its potential relevance, both proteins were overexpressed in CHO cells. In this experiment (Figure 26), overexpression with a 1:1 ratio of Sept5:PIAS3 expression plasmids resulted in the expected expression patterns: SEPT5 in the cytoplasm; diffuse and/or filamentous, depending on the expression level, and PIAS3 contained in the nucleus. However, when the ratio of Sept5 expression plasmids transfected was increased to 3-fold higher than PIAS3, the PIAS3 protein produced appeared to be drawn from the nucleus. Interestingly, this did not result in co-localization: both proteins, respectively, appeared to be forming non-specific aggregates. While this result is difficult to interpret, it does suggest that an interaction of some sort occurs which shifts the balance of PIAS3 from the nucleus to the cytoplasm. Like the yeast two-hybrid results (Figure 15), these results do support the conservation of a sequence element within the PIAS proteins that is responsible for septin binding. This particular finding also supports the notion that PIAS proteins may be predominantly nuclear, but that they are capable leaving the nucleus and likely cycle between the nuclear and cytoplasmic compartments. This, in turn, would allow for interactions with cytoplasmic proteins and possibly their modification by SUMO.
CHO cells were transfected with vectors expressing GFP-SEPT5 (green) and Flag-PIAS3 (red). Cells were transfected for 20 hours, fixed with parafomaldehyde and immunostained with epitope-specific primary antibodies, followed by fluorescent secondary antibodies. Representative epifluorescent images are shown. When expressed in a 1:1 ratio (1µg each plasmid), GFP-SEPT5 displayed typical cytoplasmic diffuse localization and Flag-PIAS3 displayed characteristic nuclear localization (A-C). However, when the amount of GFP-SEPT5 plasmid was increased to 3-fold greater than Flag-PIAS3 (3µg and 1µg, respectively), Flag-PIAS3 was no longer exclusively nuclear and also appeared as granular puncta in the cytoplasm. Conducted by Dr. Crestina Beites.
From the work presented thus far in this chapter, it is still unclear if there is a physiological relevance to the interaction of SEPT5 with the sumoylation machinery and the possible sumoylation of SEPT5 \textit{in vivo}. The only implication of functional relevance is the loss of septin filaments during overexpression of excess sumoylation pathway components. However, this may be artifactually forced to occur due to the interactions between SEPT5 and components of the sumoylation machinery. These results do suggest the possibility that septin filament formation may be regulated either by sumoylation or by non-covalent interaction with components of the sumoylation pathway. In this case, sumoylation of septins during cytokinesis may regulate filament dynamics, as they have been suggested to do in \textit{S. cerevisiae} (Johnson and Gupta 2001). Generation of stably-transfected His\textsubscript{6}MycSUMO cell lines allowed us to examine the localization of SUMO in relation to septins during the stages of cell division.

\textbf{4.2.9. \textit{SUMOs do not Co-localize with Endogenous SEPT2 during Cytokinesis}}

To more conclusively determine if the sumoylation pathway is linked to the septins during cytokinesis, as occurs in \textit{S. cerevisiae}, we generated His\textsubscript{6}Myc-SUMO stable CHO cell lines. This powerful tool allowed us to image SUMO alongside endogenous septins during all stages of the cell cycle. Conducting mitotic shake-offs, followed by plating onto poly-lysine coated coverslips allowed for enrichment of cells undergoing division which were then immediately fixed with paraformaldehyde for immunofluorescence and imaging. Representative images of SUMO1 (A-C) and SUMO2 (D-F) stables are shown in Figure 27. SUMO3 is not shown, as its localization matched SUMO2. SUMO1/2 staining is in red and SEPT2, which has identical localization to SEPT5 in CHO cells, is shown in green. These experiments clearly demonstrate that SUMO does not appreciably co-localize with septins at the cleavage furrow during cytokinesis, as occurs in \textit{S. cerevisiae}, in turn suggesting that septin sumoylation does not occur to an appreciable degree during mammalian cell division. The distribution pattern observed for the SUMOs matches that which has been recently published, with the development of the SUMO field and the on-going characterization of sumoylation (Ayaydin and Dasso 2004).

Further evidence that septins are not sumoylated in standard cell lines such as CHO is provided by our inability to isolate endogenously sumoylated septins from these stable cell lines (not shown). In these experiments sumoylated species were isolated on NTA beads from both
asynchronously dividing and synchronized cell populations. While septin sumoylation is not likely occurring during cell division, it conceivably plays some role in the regulation of septin dynamics in other highly specialized cellular processes involving septins.
Figure 27. Septins and SUMOs do not Co-localize During Cytokinesis.

Mitotic shake-offs were conducted on CHO cells stably expressing near-endogenous levels of His$_6$Myc-SUMO1/2/3. Cells were fixed with paraformaldehyde and immunostained with epitope-specific primary antibodies, followed by fluorescent secondary antibodies. Representative epifluorescent images are shown. SEPT2 (green) localized to the cleavage furrow during cytokinesis, matching previous literature publications. His$_6$MycSUMO1 (red; panels A-C) exhibited punctate staining during early stages of mitosis, then relocalized to the nuclear envelope during cytokinesis, never overlapping with the localization of SEPT2. His$_6$MycSUMO2 remained diffuse, throughout the cell during all stages of cytokinesis, not ever showing any appreciable overlap in localization with SEPT2. Similar results were obtained for His$_6$MycSUMO3 (not shown). Conducted by Dr. Carol Froese.
4.3. Discussion

Septins are a family of GTP-binding proteins that are conserved throughout evolution of eukaryotes, from *S. cerevisiae* to mammals. In the various organisms in which they have been studied, septins have been suggested to: participate in cell cycle checkpoint; assist in the orientation of the spindle polar body; act as a diffusion barrier; regulate membrane traffic; and serve as a molecular scaffold to concentrate essential signaling molecules.

Septins are essential for cytokinesis in *S. cerevisiae* and mammals, even though their respective division processes are very distinct. They form a filamentous, hour glass-shaped array in close apposition to the cytoplasmic face of the inner leaflet of the cell membrane. The details of filament formation and breakdown remain elusive, although it is known that the master regulator Cdc42 plays a role in establishing the cytokinetic septin ring (Caviston et al. 2003; Gladfelter et al. 2002), that phosphorylation plays a role in filament fluidity (Dobbelaere et al. 2003) and that filaments are likely assembled from a “unit complex” likely consisting of two copies of each of several individual septins (Sirajuddin et al. 2007; Versele and Thorner 2005). Studies in *S. cerevisiae* have demonstrated that the higher order septin structure becomes robustly modified by SUMO during cytokinesis, and abrogation of sumoylation by point mutation originally suggested that this sumoylation might regulate filament breakdown, following cytokinesis (Johnson and Blobel 1999). The bulk of the sumoylation was found to be directed by the SUMO E3 ligase, Siz1p, which is an orthologue of the mammalian PIAS SUMO E3 ligases (Johnson and Gupta 2001).

Our initial studies demonstrated that the interaction between SEPT5 and components of the sumoylation machinery is conserved from *S. cerevisiae* through to mammals. SEPT5 interacts directly with several members of the PIAS family, including PIASxα, PIAS1 and PIAS3. SEPT5 did not interact with PIASy and this may be because PIASy lacks the C-terminal S/T sequence, which is highly conserved in the other PIAS family members, including *S. cerevisiae* Siz1p (Sharrocks 2006). This is supported by the original finding that SEPT5 interacted specifically with the C-terminal 136 amino acids of PIASxα, which contains this sequence element. SEPT5 also interacted directly with the sumoylation E2, Ubc9, initially suggesting to us that Ubc9 may direct septin sumoylation in the absence of an E3. *S. cerevisiae* septins were still sumoylated in a SIZ1/SIZ2 deletion strain background, further supporting this notion. In
vitro binding assays corroborate the interactions we observed using the yeast two-hybrid system, further suggesting that the most stable interaction is a tripartite interaction between SEPT5, the E2 and the E3. If PIAS proteins function in sumoylation in a manner analogous to RING-type ubiquitin ligases, the formation of a tripartite complex is to be expected, as has been proposed by many groups. SEPT5 bound equally well to a mutant of PIASα lacking the Ubc9-binding domain, suggesting that PIASxα and SEPT5 bind to Ubc9 using interfaces distinct from those required for their interaction with one another.

Sumoylation of SEPT5 was achieved in asynchronously growing CHO cells, under conditions of overexpression of SEPT5, Ubc9 and SUMO1/2/3. The most robust sumoylation was observed using SUMO2. Sumoylated SEPT5 constituted only a very small fraction of the total expressed SEPT5, although this is consistent with what is seen with other sumoylated proteins (Johnson 2004). This is also consistent with the observation that SUMO2 and SUMO3 constitute the largest pool of “free” unconjugated SUMO in the cell and that SUMO1 is believed to be mostly conjugated (Saitoh and Hinchey 2000) because unconjugated SUMO2 would then be abundant in the cell for sumoylation of SEPT5.

Efforts to map the SEPT5 sumoylation site by both point mutation and truncation analysis were not conclusive, although we did not exhaustively mutate each of the 31 lysines (and combinations thereof) found in the SEPT5 primary amino acid sequence. Our studies suggest either that SEPT5 is sumoylated at multiple C-terminal non-consensus lysines and/or that SUMO2 chains are forming at one or more sites, through non-consensus lysines in SUMO2. Recent literature suggests that both of these scenarios are possible. State-of-the-art mass spectrometry was recently used on SUMO-modified isolates to demonstrate that although the preferred site for chain formation by SUMO2 and SUMO3 is the lysine at position eleven, they can both form chains using at least one alternate lysine (Matic et al. 2008). In addition, several substrates have now been identified which are SUMO-modified at lysines which are embedded in non-consensus sequences (Geiss-Friedlander and Melchior 2007).

It was our expectation that by including PIAS proteins in sumoylation assays, we would introduce the appropriate specificity to identify the physiologically-relevant sumoylation sites in SEPT5, as Johnson and Gupta were able to do with the *S. cerevisiae* septins (Johnson and Gupta
However, sumoylation was not even enhanced by PIAS proteins relative to background levels, making this impossible to discern with our assay system.

The results of our co-localization studies further support the conserved interaction between septins and the sumoylation machinery, but strongly suggest that septin sumoylation does not occur during cytokinesis. The absence of a concentration of any of the SUMO isoforms at the cleavage furrow, where septins concentrate, has also now been verified by other research groups (Ayaydin and Dasso 2004). There exists no evidence of cell cycle-dependent mammalian septin sumoylation in standard cell lines, despite the publication of several broad-spectrum proteomic approaches to identifying sumoylation targets (Ganesan et al. 2007; Gocke et al. 2005; Zhao et al. 2004). One would expect to see this even given even a low proportion of dividing cells growing asynchronously in culture, as this has been demonstrated for septins in similar proteomic studies using *S. cerevisiae* (Panse et al. 2004; Sacher et al. 2005; Wykoff and O'Shea 2005).

The sumoylation pathway has been proven essential in a variety of organisms studied to date (Johnson 2004). In *S. cerevisiae*, elimination of Ubc9 results in defects in chromosome condensation and segregation (Seufert et al. 1995). In Ubc9 knockout mice, death occurs early in embryogenesis. On the cellular level, elimination of Ubc9 has been reported to result in major chromosome condensation and segregation defects (Nacerddine et al. 2005). These defects precede the timing of robust *S. cerevisiae* septin sumoylation and Ubc9 has not been observed to concentrate at the septin ring during cytokinesis. A minute portion of the *S. cerevisiae* E3 Siz1p leaves the nucleus and localizes to the septin ring during cytokinesis (Johnson and Gupta 2001; Makhnevych et al. 2007), but mammalian PIAS proteins have not been observed at the cleavage furrow with septins; there are no published reports of this and our attempts to visualize PIAS proteins during cytokinesis were unsuccessful. Furthermore, our attempts to generate PIAS stable cell lines resulted in cells with grossly aberrant nuclear morphology, which could not be maintained in culture. Our subsequent attempts to generate PIASxα-specific antibodies were unsuccessful, and availability of other anti-PIAS antibodies was limited at the time of these studies.

Septins have been localized to mammalian kinetochores, implicating their involvement in chromosome congression and segregation. However, this work is limited to only two studies and
has not been explored thoroughly (Spiliotis et al. 2005; Zhu et al. 2008). SUMO-modification of many essential kinetochore-localized proteins has been reported in mammals and in most cases, their modification is essential to maintain the integrity of the kinetochore (Dasso 2008; Zhao 2007). Very recently, localization of the essential kinetochore protein CENP-E was shown to depend on its ability to bind poly-SUMO2/3 chains (Zhang et al. 2008). This same protein was shown by Spiliotis and colleagues to localize to kinetochores in a septin-dependent manner. As such, it is very tempting to speculate evolutionarily-maintained interdependence between septins and sumoylation in mammalian cell division, but in a structure such as the kinetochores, which is distinct from the more prominent septin hourglass structure at the cleavage furrow and prior to its formation.

It is unlikely that septins function in analogously at *S. cerevisiae* kinetochores because mutating individual septin genes results in a cytokinesis defect, which occurs after nuclear division (Hartwell 1971). Furthermore, no clear CENP-E orthologues exist in *S. cerevisiae* (Hildebrandt and Hoyt 2000). As such, if there is any interplay between septins and the sumoylation pathway at kinetochores, it is likely an expanded/evolved/modified role of septins specific to mammalian cell division.

Publication of the *S. cerevisiae* septin sumoylation story provided a convincing first line of evidence that septin sumoylation was likely to play a critical role in septin filament disassembly. However, since its publication, sumoylation has not been demonstrated to fulfill this role for septin filaments in any other cellular context. There were no experiments in the original study to control for the possible extraneous effects introduced by so many simultaneous point mutations. As such, it is now speculated that replacement of several endogenous *S. cerevisiae* septins with mutants harbouring several simultaneous point mutations may have been responsible for alteration of filament dynamics thereby causing a delay in filament disassembly following cytokinesis. Furthermore, the SIZ1/SIZ2 mutant *S. cerevisiae* strain showed slowed progression through cell division, but did not exhibit an obvious cytokinesis defect; nor was septin ring disassembly altered.

The work presented in this chapter provides an excellent starting point for the future study of the interaction between the septins and the sumoylation pathway. There are many additional theories to explain the conservation of this interaction throughout evolution, which are suggested
from current literature examples. Such studies may prove fruitful, but were not reasonable pursuits within the time confines of the project presented herein. For example, septins may be sumoylated in response to cellular stress, as a means to antagonize another lysine-based post-translational modification, or as a means to regulate transcriptional activity they may possess. It is also quite possible that septins never become sumoylated under endogenous conditions in mammalian cells, despite the varied processes in which both constituents are involved. It might be the interaction between septins and the sumoylation pathway components that regulates septin function. Alternatively, septins may function as a scaffold to recruit sumoylation pathway components and septin-interacting SUMO substrates.

Conjugation of SUMO2/3 to cellular proteins has been reported to increase substantially upon exposure to cellular stressors, such as electrophilic and oxidative agents (Manza et al. 2004). This is likely a protective mechanism employed by the cell to avoid major catastrophe, ultimately resulting in apoptosis. However, we were unable to detect any changes in septin sumoylation under such conditions (not shown). Sumoylation has also been reported to antagonize ubiquitination by competing for the same lysine in IkBα and several other substrates (Geiss-Friedlander and Melchior 2007; Muller et al. 2000; Zhao 2007), although we did not test for this.

The majority of sumoylation is believed to occur in the nucleus and to regulate gene expression through modification of transcription factors. However, septins are almost exclusively cytoplasmic. There have been recent reports of certain septins localizing to the nucleus. At the primary amino acid sequence level, SEPT4 is almost identical to SEPT5. There is a variant of SEPT4 which is released from mitochondria upon initiation of apoptosis, where it has been reported to activate caspases, further initiating apoptosis (Larisch et al. 2000). In addition, SEPT10 has been reported to localize to both the nuclear and cytoplasmic compartments upon its transfection into mammalian cells, although literature concerning SEPT10 is very limited (Sui et al. 2003). SEPT9_v1 has been localized to the nucleus and was demonstrated to effect the regulation HIF1α expression (Amir et al. 2006). As such, the interrelationship between septins and sumoylation may be limited to nuclear regulation of gene expression, exclusive of cytoplasmic septin functions.
It is also quite possible that mammalian septins are never sumoylated, but that their conserved interaction with components of the sumoylation machinery (exclusive of their modification) is of importance in the regulation of some aspect of septin function, such as filament dynamics. Many sumoylated proteins contain a SIM (SUMO-interacting motif) in their primary amino acid sequence, which is believed to promote sumoylation (Geiss-Friedlander and Melchior 2007; Kerscher 2007). Due to limited information at this time, it is not clear whether or not the septins have a conserved SIM. The GTPase dynamin has recently been shown to interact with both Ubc9 and SUMO1 and this interaction inhibits its oligomerization, although the dynamin monomer is reported not to contain a putative SIM (Mishra et al. 2004). It is noteworthy that we also detected direct interaction between SEPT5 and SUMO1 in a preliminary in vitro translation/binding study, but this was not pursued further (not shown). Intriguingly, a relationship between sumoylation and septin localization to stress fibres is suggested by our data. However, the details of this relationship remain elusive and difficult to discern because of the small proportion of sumoylated septin relative to total expressed septin and the inability to map a sumoylation site on SEPT5.

Work on Candida albicans from the Konopka lab has suggested that in this organism, septins may direct the sumoylation of other proteins with which they interact (Martin and Konopka 2004). In this study, septins were not found to be sumoylated, even during nocodozole arrest, which traps cells in division when the prominent septin hourglass is formed. However, SUMO immunoreactivity was localized to bud necks and hyphal septae, demonstrating co-localization of sumoylated proteins with the septins. In a blind approach to identify candidate sumoylated interactors, we were able to demonstrate sumoylation of several syntaxins, as well as the rho kinase ROCK in our overexpression assay. However, we were not able to demonstrate endogenous sumoylation or any physiological consequence thereof. A more thorough assay directed at a septin-specific function would be of great interest in addressing the possibility that septins might act as a sumoylation scaffold in mammals.

The PIAS proteins used in this study were originally isolated from a brain library, despite their varied prominent tissue expression. Sept5/SEPT5 is expressed almost exclusively in brain and substantial evidence suggests that SEPT5 functions in the regulated secretion of neurotransmitters (Beites et al. 2005; Beites et al. 2001; Beites et al. 1999). Recent literature provides convincing evidence that SEPT5 is also found in neurons at the base of dendritic spines,
where it is part of a septin complex forming a collar-like structure at the base of the spine in apposition to the inner plasma membrane leaflet (Tada et al. 2007; Xie et al. 2007). In these studies, knockdown of SEPT7 resulted in the loss of this septin structure and alteration of dendritic spine morphology. It has been postulated that this neuronal septin complex may function analogously to the septin collar formed during *S. cerevisiae* cell division by functioning as both a diffusion barrier and a scaffold. Dendritic spines are distinct compartments and their capacity to constantly restructure is crucial for synaptic plasticity (learning and memory). As such, they are responsive to a variety signaling cues resulting in their remodeling. Another recent study has shown that despite exceptionally low levels of expression in brain (Moilanen et al. 1999), PIASx promotes dendrite formation in the cerebellar cortex by promoting the sumoylation of phosphorylated nuclear transcription factor MEF2A (Shalizi et al. 2007). As such, it is enticing to speculate that there may be some link between septin sumoylation status and PIASx: perhaps in the regulation of dendritic spine morphology. In *S. cerevisiae*, the septin collar has been reported to function as a signaling scaffold triggering mitotic exit and perhaps an analogous function might be attributable to the septins during dendritic spine remodeling in neurons.

Future studies should be directed at providing conclusive evidence of a biological effect of interaction between septins and the sumoylation machinery. This may or may not include the sumoylation of septins and should start with work suggested in the above discussion. In addition, the sumoylation inhibitor protein Gam1 has recently been described (Boggio and Chiocca 2005; Colombo et al. 2002). Through inhibition of the sumoylation E1, this viral protein halts all sumoylation and actually causes the elimination of sumoylation E1 and E2 enzymes from the cell. Gam1 promises to be a powerful tool in the pursuit of function functional consequences of protein target sumoylation.
5. CHARACTERIZATION OF SEPT12

The majority of this work was completed independently and appears as a first author publication in the journal *Cell Motility and the Cytoskeleton* (October 2007, 64(10): 794-807). Data presented in Figure 33 was generated collaboratively with Dr. Carol Froese. Data presented in Figure 34 was generated collaboratively with Mr. Dénis Reynaud. Figure 35 was generated with the help of Mr. Nolan Beise. Data presented in Figure 52 was generated by Mr. Mathew Estey. Testes perfusion fixation (for generation of Figures 40-43) was performed by Mr. Marvin Estrada in the Hospital for Sick Children Animal Facility (Toronto). I am extremely grateful to Dr. Rachel Johnson for generation of the C-terminal peptide used for antibody production. In addition, Dr. Mimi Lam from the animal facility at the Hospital for Sick Children taught me how to procure viable sperm. The immuno-localization of SEPT12 in tissue sections and the yeast two-hybrid screen and interaction results are not published anywhere other than this thesis.

5.1. Rationale

At the time I discovered the Sept12 cDNA, very little was known about the extent of the mammalian septin gene family. Mammalian septins SEPT2 and SEPT9 were among the first to be described and essential roles in cytokinesis were demonstrated (Kinoshita et al. 1997; Surka et al. 2002). At this time many new septins were being discovered and the known number of mammalian septins was constantly growing.

Interestingly, mammalian septins are expressed at high levels in post-mitotic tissue and have been implicated in many cellular roles in addition to cytokinesis, including neurotransmission (Beites et al. 1999; Kinoshita et al. 2000). While some septins were shown to be ubiquitously expressed (Kinoshita et al. 1997), others, such as SEPT5 exhibited a high degree of tissue specificity (Beites et al. 1999). As such, it was important to characterize the expression of this novel septin gene and attempt to determine its function in mammals.
5.2. Results

5.2.1. Isolation of Sept12 cDNA and Characterization of Gene

The cDNA encoding rat Sept12_v2 was isolated in a yeast two-hybrid screen using rat SEPT5 as the bait. In humans, the gene encoding Sept12 is located at chromosome 16p13.3, and covers approximately 10 kilobases (kb). Sept12_v2 consists of 9 exons spliced together to give a final coding sequence of 1,077 base pairs (Figure 28). ESTs in the NCBI database indicate that two mature mRNA transcripts exist in mammals, resulting from differential splicing of exon 4 (Fig.1a-c). Sept12 is likely conserved amongst all mammals, as sequences from many species exist in the NCBI database; including human, monkey, mouse, rat, and cow. The two major isoforms of SEPT12 have been named version 1 (v1) and version 2 (v2), with v1 corresponding to the shorter isoform, contrary to the accepted septin nomenclature (Macara et al. 2002). Sept12 transcripts have been isolated from several tissues, but the majority of transcripts are from testis.

The primary amino acid sequence of SEPT12 (Figure 28, B and C) contains the consensus GTP-binding and polybasic domains, as well as the septin unique element. While the absence of exon 4 may confer unique properties, v1 still shows significant Ras sequence identity and contains all the necessary primary sequence elements for guanine nucleotide binding and hydrolysis. A more surprising observation, however, is that the G4 sequence is ARAD with arginine replacing the lysine of the consensus (XKXD) found in the majority of GTPases (Bourne et al. 1991) and all other septins. As previously described, SEPT12 is predicted to lack a C-terminal coiled-coil sequence and shows highest sequence identity to SEPT3 and SEPT9 (Russell and Hall 2005).
Figure 28. Sept12 Gene and its Product.

(A) The genomic DNA sequence of Sept12 encompasses 10kb at chromosome 16p13.3.  (B) SEPT12 protein sequence: two mature forms exist, arising from differential splicing of exon 4. SEPT12 contains the septin consensus GTP-binding (red), polybasic (blue) and SUE (yellow) sequences. Of note, the G4 domain contains an arginine in place of the strictly conserved lysine which is present in all other septins.  (C) Complete amino acid sequence of human SEPT12_v2; G1, G3 and G4 sequences are boxed and exon 4 is underlined.
Yeast mating intersections: yeast is expressing rat SEPT12_v2 as bait and all other respective septins as prey. Intensity of blue colour indicates strength of interaction as correlated with degree of LacZ activation (blue colour). SEPT12 is capable of interacting with all septins except Sept10 and the other members of its sub-family, SEPT3 and SEPT9. In addition, mutation of a conserved serine to alanine in the G1 sequence of the nucleotide binding domain of SEPT5 (S58N) reduces pairwise binding. A comparable mutation in SEPT12 (r12S61N_v2) abrogates SEPT12 homodimerization. Identical results were obtained using SEPT12_v1 as the bait.
As septins are known to homodimerize and to directly interact with members of different subgroups to form unit complexes, I sought to determine relevant septin binding partners of SEPT12, both as confirmation that SEPT12 is a bona fide septin and to provide a starting point for the further study of SEPT12. As shown in Figure 29, SEPT12 can homodimerize and interact robustly with most mammalian septins, with the exception of the other members of its subgroup (SEPT3 and SEPT9) and SEPT10. The latter has been observed in the nucleus, where it may have a function unique from that described for other septins (Sui et al. 2003). In addition, this pairwise interaction appears to be dependent upon the ability of septins to coordinate a guanine nucleotide. Septin point mutants in the G1 sequence (GXXGXGKST), where serine is changed to asparagine, are unable to bind nucleotide (Beites et al. 1999). In SEPT12, the analogous mutation abrogates pairwise septin interaction.

I also examined the binary homomeric interaction between SEPT5 and SEPT12, respectively, or their binary heteromeric interaction. To do this, I created a series of SEPT5 truncation mutants and expressed them as fusion constructs in the yeast two-hybrid system, as outlined in Chapter 3. For SEPT12, I sequentially truncated from the 5’-end of the cDNA, progressively removing first the coding sequence for the unique N-terminal extensions; poly-basic (lipid-binding) domain, right up to the start of the G1 sequence; followed by the entire N-terminus, from just downstream from the G4 sequence. From the 3’-end of Sept12, I removed first the unique C-terminal extension; then a region of high homology to SEPT9, between the former region and the end of the SUE. I then removed a substantial piece of the SUE; followed by the entire C-terminal half of the protein. The binary interactions and associated schematics of the truncations are shown in Figures 30 and 31.

Figure 30 depicts the homodimerization study using SEPT12 truncation mutants. Similar to SEPT5 (Chapter 3), SEPT12 homodimerized strongly and this was dependent upon the presence of entire protein, with the exception of the C-terminal 32 amino acids. Upon examination of the interaction between SEPT5 and SEPT12 (Figure 31), I observed that for preservation of heteromeric interaction, SEPT5 and SEPT12 could only tolerate truncations of the same regions that were tolerated for preservation of their respective homomeric interaction. The interaction between the isolated C-terminal half of SEPT12 and the isolated N-terminal half of SEPT5 may represent an isolated binary interaction of the sort occurring at the NC-dimer interface, as the
recent crystal structure suggests is possible (Sirajuddin et al. 2007), not involving the nucleotide-binding regions of these septins.
Figure 30. SEPT12 Self-interaction.

(A) Schematic depicting the truncations assessed. (B) A pairwise interaction grid: constructs listed to the left were expressed as LexA fusions from pEG202; constructs listed across the top were expressed from the pJG4-5 vector. Results were generated by expression of respective constructs in haploid yeast strains EGY-48 (pJG4-5) and RFY-206 (pEG-202) and their successive mating on YPD and replica plating onto selective media with X-gal. Degree of blue colour is indicative of the strength of interaction. Expression of all constructs was verified with Western blots using either anti-HA antibodies (pJG4-5) or anti-LexA antibodies (pEG-202) (not shown).
Figure 31. SEPT5:SEPT12 Interaction.

SEPT5 constructs listed to the left were expressed as LexA fusions from pEG202. SEPT12 constructs listed across the top were expressed from the pJG4-5 vector; similar results were found in both orientations (data now shown). Results were generated by expression of respective constructs in haploid yeast strains EGY-48 (pJG4-5) and RFY-206 (pEG-202) and their successive mating on YPD and replica plating onto selective media with X-gal. Degree of blue colour is indicative of the strength of interaction. Expression of all constructs was verified with Western blots using either anti-HA antibodies (pJG4-5) or anti-LexA antibodies (pEG-202) (not shown). Schematics of SEPT5 truncations are shown in Figure 12.
5.2.2. **Generation of GFP-SEPT12 Stable CHO Cell Line**

As additional proof that SEPT12 is a *bona fide* septin, I generated a CHO stable cell line producing rat GFP-SEPT12_v2 to confirm a septin-like expression pattern in a cellular context (Figure 32). It is well established that septins localize to actin stress fibres in interphase cells and to the cleavage furrow during cytokinesis: colocalizing either with the actomyosin cleavage apparatus (Kinoshita et al. 1997) or the microtubule-based mitotic spindle (Surka et al. 2002). Unlike its subfamily member SEPT9, which localizes to microtubules in cytokinetic cells, stably expressed GFP-SEPT12 localizes to actin-based structures in both interphase and dividing cells (Figure 32), coincident with the known localization of septins to which it can bind, such as SEPT2 (Kinoshita et al. 1997; Surka et al. 2002).
Figure 32. SEPT12 Localizes to Actin-based Structures.

(A-C) Interphase cells: (A) SEPT12; (B) Filamentous actin; (C) merge. During interphase, GFP-SEPT12 (green) co-localizes with actin stress fibres (red) in an intermittent filamentous co-localization pattern, along the length of stress fibres (C). (D-H) Representative images of cells progressing through cytokinesis. SEPT12 (green) displays cortical localization and subsequent enrichment at the cleavage furrow, colocalizing with the actomyosin cleavage apparatus (actin shown in red). Epifluorescent images acquired on a Leica DM IRE2 inverted microscope, using OpenLab software. Actin visualized with rhodamine-phalloidin. Size bar = 15μm.
5.2.3. Phosphoinositide Binding to SEPT2 and SEPT12

To assay whether SEPT12 isoforms are capable of binding phosphoinositides, I incubated recombinant SEPT12 with membranes spotted with various purified lipids (PIP strips), as shown in Figure 33. Poly histidine-tagged SEPT12 was successfully obtained in very high purity, using a baculovirus-mediated expression system in SF21 insect cells (Figure 33, panel A). Both SEPT12 isoforms bound specifically to the same set of phosphoinositides, showing a preference for mono-phosphorylated phosphoinositides, consistent with SEPT2 and previously published findings for *S. cerevisiae* septins (Casamayor and Snyder 2003; Zhang et al. 2000). Interestingly, by this assay, SEPT2 and SEPT12 also interacted robustly with phosphatidylserine, which is known to be enriched on the inner leaflet of the plasma membrane of mammalian cells.
Figure 33. SEPT12 Binds Phosphoinositides and Phosphatidylserine.

(A) Coomassie stained SDS-PAGE of recombinant proteins (approx. 5μg/lane) purified from baculovirus-infected SF21 cells (Invitrogen). (B) Schematic of phospholipid loading, PIP strips (Echelon) containing 100pmol lipid/spot. Abbreviations: lysophosphatidic acid (LPA); lysophosphatidylcholine (LPC); phosphatidylinositol (PI); phosphatidylinositol-3-phosphate (PI(3)P); phosphatidylinositol-4-phosphate (PI(4)P); phosphatidylinositol-5-phosphate (PI(5)P); phosphatidylethanolamine (PE); phosphatidylcholine (PC); sphinosine-1-phosphate (S(1)P); phosphatidylinositol-3,4-diphosphate (PI(3,4)P$_2$); phosphatidylinositol-3,5-diphosphate (PI(3,5)P$_2$); phosphatidylinositol-4,5-diphosphate (PI(4,5)P$_2$); phosphatidylinositol-3,4,5-triphosphate (PI(3,4,5)P$_3$); phosphatic acid (PA); phosphatidylserine (PS); blank. (C) SEPT2 binding. (D) Rat SEPT12_v2. (E) Human SEPT12_v1. Images were obtained by incubation of recombinant protein with the membrane followed by incubation with anti-His$_6$ antibodies and detection with HRP-coupled secondary antibodies; resultant film exposures are shown.
5.2.4. Guanine Nucleotide Binding to SEPT12

As a means to further characterize the biochemical properties of SEPT12, I assayed for bound nucleotide and conducted nucleotide exchange assays, as previously described (Huang et al. 2006; She et al. 2004). Both SEPT12 isoforms purified bound to guanine nucleotides in a GDP:GTP ratio of approximately 2:1 (Figure 34, panel B). Site-directed mutagenesis was conducted to change the arginine in the G4 sequence (XKXD) to lysine and this was found to result in abrogation of nucleotide binding and substantially reduced ability to form filaments when overexpressed in mammalian cell lines (data not shown). In contrast to SEPT2, neither isoform of SEPT12 exchanged GDP to any measurable degree over a period of up to 3 hours (Figure 34, panel C), consistent with previous reports for most other septins (reviewed in Versele and Thorner, 2005).
Figure 34. SEPT12 Binds Guanine Nucleotides.

(A) R-HPLC elution profile of controls 500pmol GDP and 500pmol GTP. (B) Sample chromatograph of nucleotide extraction from human SEPT12_v1 (500pmol protein). (C) \(^3\)H-GDP exchange using a final concentration of 0.2µM of each respective protein. Human SEPT2 readily exchanges radioactive GDP in solution for non-radioactive GDP (with which it co-purifies), whereas human SEPT12_v1 does not exchange bound GDP. Bovine serum albumin (BSA) is used as the negative control. Recombinant septins were expressed in SF21 cells using the baculovirus system, as shown in Figure 33. Non-specific peaks in (A) and (B) are marked with asterisks.
5.2.5.  Tissue Expression of Sept12 by RT-PCR and Northern Blotting

I originally identified Sept12 in a yeast two-hybrid screen from a rat brain cDNA library using SEPT5. All septins described to date are expressed in the mammalian brain (Kinoshita et al. 2000; Tsang 2007); as such, I sought to confirm expression in brain and determine if Sept12 was expressed elsewhere. RT-PCR was conducted with primers specific to Sept12 in order to amplify the first 700bp, containing exon 4. RNA isolated from stably transfected CHO cells was used as a positive control (Figure 32). Of the tissues examined, the most robust signal was from testis (Figure 35, A). A smaller fragment corresponding to Sept12_v1 was observed upon overexposure of the blot (Figure 35, B), suggesting that the major Sept12 isoform in testis is _v2. At very high overexposure, a very faint band matching the size corresponding to Sept12_v2 in testis was observed in most tissues, similar to that reported by Hall et al. (2005). Northern blots confirmed RT-PCR results, showing a prominent band in only testis (Figure 35). Figure 35 (C) clearly shows a prominent band of the expected size (approx. 1,100 base pairs) and that the major location of Sept12 expression is testis. To determine the developmental expression profile of Sept12, mRNA was isolated from the testis of rats of different ages. In developing rat testis, expression of Sept12 increases steadily until sexual maturity (approx. 49 days post-partum), where it remains expressed at a relatively constant level (Figure 35, D).
Figure 35. *Sept12* RNA is Highly Enriched in Testis.

(A) RT-PCR was conducted on total RNA isolated from multiple rat tissues. *Sept12*-specific primers yielded either a 700-bp fragment (*Sept12* v2) or a 400pb fragment (*Sept12* v1). β-actin is amplified as a control. (B) Overexposure of testis lane (from (A)) demonstrates that
*Sept12_v1* (arrowhead) is present in very small amounts relative to *Sept12_v2*. (C) 20μg per lane total RNA isolated from multiple tissues was resolved by agarose gel electrophoresis, transferred to Nytran+ and probed with ^32^P-labelled *Sept12_v2* cDNA. (D) With sexual development, the levels of *Sept12* transcript increase in the testis, coincident with a role in sperm maturation. *Sept12* levels do not change appreciably after sexual maturity (approx. 49 days post-partum). Age of rats in days post-partum is indicated. 20μg total RNA isolated from stably transfected GFP-*Sept12* CHO cells is used as a positive control (lane 1 in both blots). Ethidium bromide staining of 28S RNA on the agarose gel prior to transfer, is used to verify equal loading (lower panels).
5.2.6. Generation of SEPT12-specific Antibodies in Rabbit

To confirm Sept12 expression analysis (Figure 35) and further explore the function of SEPT12 in the testis, I generated SEPT12-specific antibodies. To control for potential cross-reactivity, I confirmed that my antibody was able to detect only rat SEPT12 from a complete panel of all previously described mammalian septins (Figure 36, A and B). As a further control, I show that incubation of our antibody with the original immunization peptide prior to blotting abolishes the detection of recombinant and endogenous SEPT12 (Figure 36, C).
Figure 36. SEPT12-specific Antibodies.

All septins were expressed in yeast strain RFY-206 from pEG-202 as LexA fusions; whole lysate was resolved by SDS-PAGE, transferred to PVDF and blotted with anti-SEPT12 antibodies. (A) Approximately equal loading of each septin was verified by anti-LexA immunoblotting. (B) Probing of an identical blot with affinity-purified polyclonal antibodies against SEPT12
demonstrate specificity for rat SEPT12. (C) Western blot of stably transfected GFP-SEPT12 CHO cell line lysate, adult testis lysate and recombinant rat SEPT12 (left); SEPT12 antibody pre-incubated with immunization peptide completely abrogates any signal on an identical blot (right). Lower panel shows C-terminal SEPT12 peptide used for immunization as well as corresponding mouse and human sequences. Asterisk denotes species-specific peptide used for immunizing rabbits.
5.2.7. **Anti-SEPT12 Antibodies Detect SEPT12 in Testis and Epididymis**

Multi-tissue and developmental Western blots using affinity-purified anti-SEPT12 antibodies confirmed RNA work, showing that the protein is only highly expressed in the testis and epididymis (Figure 37), relative to other tissues examined, and that expression onset in the testis is consistent with mRNA expression (Figure 35). Epididymis consists mostly of transcriptionally quiescent mature sperm; as such, mRNA transcripts encoding components of mature sperm are not found in this tissue. Western blots also support RT-PCR findings that the larger SEPT12_v2, is the major splice variant expressed, as our antibodies routinely detected a single protein band of approximately 40kDa, significantly higher than the approximately 35kDa expected for SEPT12_v1. Overall, these results are highly suggestive that SEPT12 functions predominantly in spermatozoa.
Figure 37. SEPT12 Protein is Only Present in Testis.

(A) Tissue samples were homogenized, prepared in SDS loading buffer, resolved by SDS-PAGE (20μg total lysate/lane) and transferred to PVDF. The SEPT12 protein is detected exclusively in testis and epididymis, suggestive of a role for SEPT12 in mature sperm. (B) SEPT12 expression peaks during the latter stages of sexual maturity and remains constant into adulthood, consistent with a maintained role in spermatogenesis or a function in mature sperm. GFP-SEPT12 stable cell lysate is used as a positive control in both blots and age in days post-partum is indicated.
To test the hypothesis that SEPT12 is present in spermatozoa, I isolated mature spermatozoa from epididymis and vas deferens, stained them to mark the mitochondrial sheath, then fixed and stained them with anti-SEPT12 antibodies. As shown in Figure 38, A-C, these antibodies very specifically stain a punctate structure demarcating the end of the mitochondrial sheath, which is most certainly the annulus, as indicated by identical SEPT4 staining (Figure 38, E). As both septin antibodies were raised in rabbit, co-localization studies were not readily possible. Pre-incubation of SEPT12 antibodies with the original immunogen peptide prior to immunostaining completely blocked any signal (Figure 38, D). Figure 38 (F) shows a single spermatozoon at a lower magnification with SEPT12 staining showing the position of the annulus in relation to the head of the sperm and the end of the mitochondrial sheath. Figure 38 (G) clearly demonstrates that upon reorientation of the 3-D optical reconstruction, SEPT12 immunostaining reveals the ring-like structure of the annulus. This ring structure often appeared non-uniform in 3-D optical reconstructions, likely due to an artifact of fixation, staining and/or imaging.
Figure 38. SEPT12 is a Component of the Annulus.

(A-C) A mature spermatozoon labeled with SEPT12 ((A) green) and MitoTracker ((B) red); overlay is shown in (C). (A) A prominent structure demarcating the end of the mitochondrial sheath and characteristic of the annulus is detected with our SEPT12 antibodies; SEPT12 was absent from all other regions of spermatozoa. (D) Pre-incubation of SEPT12 antibodies with immunization peptide eliminated this SEPT12 signal. (E) Confirming this structure is the annulus, antibodies specific to SEPT4 (green) also detect an identical structure demarcating the end of the mitochondrial sheath (red). (F) Overlay of lower magnification of a mature sperm cell labeled with SEPT12 (green), MitoTracker (red) and nuclear stain (blue). (Gii) and (Giv) show a high magnification of the SEPT12 staining shown in (A); (Gi) and (Giii) show relative orientation of 3-D optical reconstruction. Note the hollow, ring-like structure characteristic of the annulus. Images acquired using a Quorum Spinning Disk Confocal Microscope. Representative 0.2µm sections are shown in (A-F). 3-D optical reconstructions of only the relevant region from A are shown in Gii, iv. (A-E): size bars = 15µm; (F): size bar = 25µm; (Gii), (Giv): size bar ~1µm.
5.2.9. **SEPT12 Co-localizes with other Annulus Septins when Overexpressed**

Due presumably to the insoluble cytoskeletal and membrane associated nature of SEPT12 in spermatozoa, I was unable to coimmunoprecipitate it with other septins that have been reported to constitute the annulus; namely SEPT1, 4, 6 and 7 (Ihara et al. 2005). Thus, in an attempt to confirm direct interaction of SEPT12 with annulus septins, I overexpressed SEPT12 in combination with these septins in a heterologous system (CHO cells). SEPT4 never formed filaments on its own when overexpressed and almost always appeared diffuse and cytoplasmic (Figure 39, A). SEPT12 was almost always filamentous in nature, forming short, linear filaments or cytoplasmic aggregates when overexpressed alone (Figure 39, B). However, upon co-expression of SEPT4 and SEPT12, the observed localization and organization of both proteins changed dramatically (Figure 39, C). Almost every cotransfected cell contained highly curved filaments incorporating both septins and supporting the *in vivo* interaction of these two septins. It is tempting to speculate that the SEPT4:SEPT12 interaction may impart a characteristic curvature allowing the very formation of the highly curved, closed-ring annulus structure; however, overexpression in CHO cells is far removed from the sperm tail. The specific importance of the SEPT4-SEPT12 interaction is emphasized by the observation that SEPT4 did not form filaments when co-overexpressed with the closest relatives of SEPT12, SEPT3 or SEPT9, respectively. Also, while SEPT1, 2, 6 and 7 co-localized with SEPT12 at least to some degree when co-overexpressed, they never formed filaments together (Figure 39, D and data not shown). During septin overexpression I did not see perturbation of the actin or tubulin cytoskeleton networks and thus speculate the structures seen are predominantly septin-based.
Figure 39. SEPT12 forms Filaments with SEPT4.

(A) The distribution of Myc-SEPT4 is consistently diffuse and cytoplasmic, lacking any evidence of ability to form filaments. (B) GFP-SEPT12 regularly forms cytoplasmic filaments of varying length and thickness, as well as structures that appear to be cytoplasmic aggregates. (C) When Myc-SEPT4 (red) and GFP-SEPT12 (green) are co-overexpressed, a highly reproducible, characteristic expression pattern is observed in virtually all cotransfected cells: both septins co-localize completely in long, highly curved cytoplasmic filaments, often wrapping around the nucleus many times. (D) Flag-SEPT1 (red) and GFP-SEPT12 (green) can interact when co-overexpressed, but never in filaments. Images acquired as in Figure 38; bar = 15 μm.
5.2.10. In Testis, SEPT12 Likely Originates from the Golgi

Having localized SEPT12 to the annulus in mature spermatozoa with highly specific antibodies, the most logical next direction was to examine the distribution of SEPT12 in tissue sections. The *D. melanogaster* septin pnut has been reported to localize to intercellular bridges between germ cells, where anillin and actin are both found (Hime et al. 1996). SEPT2, SEPT7 and SEPT9 have recently been observed to transiently localize to a similar structure in mammals (Greenbaum et al. 2007), although these particular septins have not been reported at the annulus. Virtually nothing is known of how the annulus forms, as all initial studies were done using electron microscopy, assessing morphology of various structures in the testis. While formation of the annulus is now known to be dependent upon SEPT4, it is still not known what all of the molecular constituents of it are or how it forms, prior to its first known localization as a ring-shaped structure on the plasma membrane, at the base of elongating sperm heads (Fawcett et al. 1970; Fawcett and Phillips 1969).

In my experiments, perfusion-fixed tissue was cryosectioned to 7μm thickness and placed onto microscope slides. Standard solubilization and immunochemistry (as used for cell lines) was used to label SEPT12 and cis-Golgi matrix protein, GM130. As shown in Figure 40, the Golgi marker GM130 reveals large, punctate structures in apposition to nuclei of round germ cells (pre-elongation); GM130 staining directly overlaps with SEPT12 staining. During this stage of male germ cell differentiation, the Golgi swells substantially, re-orientates towards the nucleus and directs vesicles to the forming acrosomal vesicle, which is located in association with the nuclear envelope (Russell et al. 1990). This finding is suggestive that SEPT12 either plays an earlier role in germ cell differentiation, or that SEPT12 and possibly other annulus septins, are processed through the Golgi prior to their arrival at the base of elongating sperm heads.
Figure 40. **SEPT12 is Golgi-localized in Seminiferous Epithelium.**

(A) GM130 staining (green) reveals a punctate pattern at pre-elongation stages of differentiation in most seminiferous tubule cross sections (green), as is known from the literature. L refers to the lumen of the seminiferous tubule; the dotted line is the divide between adjacent seminiferous tubules; arrows point to representative regions of overlapped staining. (B) Co-staining of SEPT12 (red) reveals a similar staining pattern and substantial overlap is shown in the overlay. (C). Testes were perfusion-fixed and stained using standard immunohistochemistry techniques and imaged using spinning disc confocal microscopy. Bar = 50μm.
Upon closer examination of stained tissue sections, I observed that SEPT12 and Golgi staining are highly co-localized in what are likely late spermatocytes or early spermatids (Figure 40). This co-localization persists during spermiogenesis into the intial portion of elongation, when the Golgi moves into the distal cytoplasm, out in the tail of the elongating spermatids (Figure 41). However, a divergence into two separate entities occurs during spermiation: this is somewhat evident from Figure 41, but becomes more clear at a higher magnification, shown in Figure 42.

Arrows on Figure 42 (1-3) mark robust co-localization of SEPT12 with the Golgi, appearance of divergence of SEPT12 from the Golgi, and finally, the presence of a distinct annulus at the base of elongating sperm heads. An even closer magnification of what appear to be representative stages is shown in Figure 43, where a high degree of co-localization is shown in panel A, through to almost complete divergence in panel E.
Figure 41. SEPT12 Localizes to the Distal Cytoplasm of Elongating Spermatids.

(A,B) Two representative low magnification of seminiferous tubule cross sections show that SEPT12 (green) and GM130 staining (red) are still co-localized during the elongation stages of differentiation, when the Golgi moves away from the nucleus and into the cytoplasm. L refers to the lumen of the seminiferous tubule; the dotted line is the divide between adjacent seminiferous tubules. Testes were perfusion-fixed and stained using standard immunochemistry techniques and imaged using spinning disc confocal microscopy; bar = 50μm.
Figure 42. SEPT12 and Golgi Markers Diverge During Elongation of Spermatids.

(A) GM130 staining (green) reveals large punctate structures in association with round nuclei in most seminiferous tubule cross sections, characteristic of pre-elongation stages of differentiation, as is known from the literature. L refers to the lumen of the seminiferous tubule; R and E refer to “round” and “elongated”, respectively. (B) Co-staining of SEPT12 (red) and overlay, (C). Arrows point to (1) regions of substantial overlap; (2) regions where SEPT12 staining appears to become distinct from GM130 staining and (3) SEPT12 staining in characteristic annulus pattern, with no associated GM130 staining. Testes were perfusion-fixed and stained using standard immunochemistry techniques and imaged using spinning disc confocal microscopy. Bar = 20μm.
Figure 43. Divergence of SEPT12 Staining from Golgi, Magnified.

(A-E) GM130 staining (green) and SEPT12 staining (red) reveals initial co-localization in large perinuclear punctate structures and what appears to be subsequent separation of these structures into distinct entities during spermatid elongation (as suggested by Figures 40-42). Testes were perfusion-fixed and stained using standard immunochemistry techniques and imaged using spinning disc confocal microscopy. Bar = 1μm.
These results suggest that the annulus assembles into its final ring structure from association with the Golgi. Unfortunately, our attempts to observe other annulus-localized septins in testis sections failed due to a very low signal-to-noise ratio, using available septin antibodies and this method for visualization. Interestingly, despite testis-specific expression, SEPT12 is not observed at intercellular bridges between germ cells, as has been observed for other septins. I verified the presence of such bridges by staining for anillin (not shown).

5.2.11. SEPT12 Yeast Two-Hybrid Screen

Upon completing an initial characterization of the expression of Sept12 and its protein product, I next sought to further determine relevant molecular interactions SEPT12 may have during annulus formation and its stabilization at the end of the mitochondrial sheath. A natural follow-up was to conduct a yeast two-hybrid screen to identify candidate interactors; this was completed with the help of two summer students, Min Lin and Mathew Estey. An adult rat brain cDNA library was screened because it was in my possession from the previous screen using SEPT5. It was my hope that physiologically-relevant interaction partners would still be revealed, as many genes are known to be expressed in a bimodal distribution between brain and testis.
Table 4. SEPT12 Yeast-two Hybrid Results.

cDNA sequences corresponding to unique SEPT12 binary interactions identified in yeast two-hybrid screening of an adult rat brain cDNA library. Left-most column (A) lists the septin interactors; the remaining columns (B) pertain to the non-septin interactors.

<table>
<thead>
<tr>
<th>Septin</th>
<th>Non-septin</th>
<th>Match [organism/score]</th>
<th>NCBI Accession No.</th>
<th>Full-length cDNA (bp)</th>
<th>Screen (bp)</th>
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<tr>
<td>Sept4</td>
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<td>1293</td>
<td>1-1293</td>
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<tr>
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<td>MAGI-2 [Rn/98%]</td>
<td>AF130819</td>
<td>3345</td>
<td>2731-3345</td>
</tr>
<tr>
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<td></td>
<td>MAGI-1 [Rn/100%]</td>
<td>NM_001030045</td>
<td>3768</td>
<td>3124-3768</td>
</tr>
<tr>
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<td></td>
<td>PIN1 [Rn/100%]</td>
<td>NM_001106701</td>
<td>498</td>
<td>1-498</td>
</tr>
<tr>
<td>Sept11</td>
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<td>NM_031682</td>
<td>786</td>
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<td></td>
<td></td>
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<td></td>
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<td>NM_001012212</td>
<td>1140</td>
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</table>
The screen identified several interactors, listed in Table 4. Several septins were identified, as expected by my previous screen using SEPT5 (Table 3). The majority of clones were identified to be SEPT4, in strong support of a relevant binary interaction between SEPT12 and SEPT4; this supports their interaction in the annulus, and possibly in a septin-based structure in the brain.

Several intriguing non-septin cDNAs were also identified and fall into two main categories: structural proteins and enzymatic/modifier proteins. Tektin2 is of great interest, as it is a key structural protein in the sperm tail (Tanaka et al. 2004). Tektins are insoluble, filamentous proteins that were originally identified in the flagella of sea urchin sperm (Chang and Piperno 1987; Pirner and Linck 1994). Two members of the MAGUKs (Membrane Associated Guanylate Kinase) with Inverted domain structure (MAGI) family were also identified. Members of this well-characterized protein scaffold family have recently been revealed to play a critical role in neuronal synapse assembly and maintenance (Iida et al. 2004). Protein-Interactor of NIMA-1 (PIN1) is a peptidyl prolyl isomerase which effects cis/trans isomerization about prolines of the protein backbone of its targets in response to phosphorylation of N-terminally adjacent phosphorylated serine or threonine residues. It has since been demonstrated to play a critical role in the cell cycle in mammals (Lu et al. 1996) as well as many other processes, including cell growth regulation, genotoxic and stress response, immune response, germ cell development and neuronal differentiation and survival. 17β-hydroxysterol dehydrogenase-10 (HSD17B10) is a mitochondrial metabolic enzyme, which is constitutively expressed in the liver, brain and gonads (He et al. 2001). Its mutation has been linked to progressive neurodegeneration and mental retardation (Yang et al. 2005). The MOZ, Ybf2/Sas3, Sas2, and Tip60 (MYST) family of proteins catalyzes histone acetylation and may link septins to tubulin acetylation, as has been previously implicated in the literature (Kremer et al. 2005). The final non-septin SEPT12-interacting protein identified in the screen was calreticulin3, an endoplasmic reticulum resident chaperone protein, which is not likely to interact with septins endogenously.

These protein interactors were transformed back into yeast and interactions were re-assessed using the yeast two-hybrid system to verify interactions. Notably, the interactions were all validated. None of the interactions were maintained with a dominant negative point mutant of SEPT12, suggesting that native-structure, or possibly even self-association of SEPT12 is required for these interactions.
When *Sept12* was truncated sequentially from both ends, only removal of the very C-terminus allowed for the interaction to be maintained, as shown in Figure 44. This is consistent with the regions required for SEPT12:SEPT12 self-association (Figure 12).
Figure 44. Interaction with SEPT12 Requires all but its Extreme C-terminus.

Constructs listed to the left were expressed as LexA fusions from pEG202. Constructs listed across the top were expressed from the pJG4-5 vector. Results were generated by expression of respective constructs in haploid yeast strains EGY-48 (pJG4-5) and RFY-206 (pEG-202) and their successive mating on YPD and replica plating onto selective media with X-gal. Degree of blue colour is indicative of the strength of interaction. Expression of all constructs was verified with Western blots using either anti-HA antibodies (pJG4-5) or anti-LexA antibodies (pEG-202) (not shown). Schematics of SEPT12 truncations are shown in Figure 12.
5.3. Discussion

Sept12 is a novel septin, encoded on human chromosome 16. Differential splicing of exon 4 occurs, giving rise to two Sept12 isoforms, although the longer isoform is much more abundant in the NCBI EST database. My RT-PCR and Western studies suggest Sept12_v2/SEPT12_v2 is the major isoform produced in the rat. It is particularly noteworthy that Sept12_v1 lacks exon 4, as this exon encodes a conserved septin primary amino acid sequence element and no septin isoforms lacking this sequence have been observed and/or described to date. Both SEPT12 isoforms bind to all the same septin family members in a pairwise manner, as well as to each other. Comparison of septin primary amino acid sequences to Ras family members suggests the additional sequence added to SEPT12 by exon 4 may not be necessary for proper folding of the nucleotide binding and hydrolysis domain. Indeed, like SEPT12_v2, SEPT12_v1 is capable of binding guanine nucleotides and does not exchange GDP any more rapidly than SEPT12_v2. While the lack of exon 4 may not inhibit proper folding of this domain, it may impart some function upon SEPT12_v1 unique from other septins. As such, SEPT12_v1 may have unique cellular localization, protein binding partners, or influence on the formation and/or regulation of higher order septin structures. Further studies will be required to address this.

The observation that SEPT12 can interact with almost all other septins is surprising because SEPT3 and SEPT9 do not exhibit such broad interaction capability (see Figure 11). All three sub-family members interact with only SEPT8 and SEPT11, suggesting that if they are capable of functional redundancy this would be limited to complexes containing one or both of these septins. Interestingly, SEPT12 contains very short N- and C-terminal extensions, which may account for its ability to self-associate and form filaments when overexpressed alone, consistent with that observed for SEPT2 (Huang et al. 2006).

Overall, results of the truncation interaction study suggest that SEPT5 and SEPT12 must be almost full-length in order to adopt their native conformations and interact with one another. This suggests that their interaction is dependent upon the correct folding of the core septin domain and that it is not mediated by N- or C-terminal extensions. While the septin:septin interaction model based on the crystal structure offers an explanation for this sort of interaction, it does not account for septins of the sub-family to which SEPT12 belongs. As such, it is
tempting to speculate that the SEPT5:SEPT12 interaction interface is one of those depicted in the current model (Sirajuddin et al. 2007).

Consistent with this notion, the generation of CHO cells stably expressing GFP-SEPT12 showed that SEPT12 co-localizes with other septins at the actomyosin cleavage apparatus in the cleavage furrow, during cytokinesis, unlike SEPT9, which has been localized to the mitotic spindle during this process (Surka et al. 2002). This suggests that SEPT9 and SEPT12 interact preferentially with different cellular structures and that these two septins are not likely to exhibit functionally redundant roles.

In vitro lipid binding assays were consistent with previous reports for septins and point to the recruitment of septins to membrane domains where active membrane remodeling events are occurring coincident with the generation of phosphoinositides. Phosphoinositides are known to play key roles in a variety of cellular processes, including vesicle trafficking and actin remodeling and have recently shown to be essential for cytokinesis and flagellar biosynthesis (Field et al. 2005; Wei et al. 2008). SEPT2 and SEPT12 both also bound robustly to the inner leaflet phospholipid, phosphatidylserine, highly suggestive that septins may have a general affinity for the inner surface of the plasma membrane through this interaction.

Biochemical characterization of the nucleotide binding and exchange properties were consistent between SEPT12 isoforms and with most findings reported to date. Like most septins, SEPT12 was purified from eukaryotic cells bound to guanine nucleotides, in a 2GDP:1GTP ratio. Surprisingly, this required the arginine residue found in the G4 sequence of SEPT12, which is present as a conserved lysine in all other known septins. Thus, other elements of the SEPT12 primary amino acid sequence must specifically select for and stabilize arginine in this position, while maintaining conserved septin nucleotide binding properties. I was unable to exchange nucleotide in SEPT12, suggesting that rather than a dynamic signaling role, the binding of nucleotide is more likely to play a structural role in proper septin folding, as has been suggested previously (Vrabioiu et al. 2004).

Surprisingly, Sept12 transcripts and SEPT12 protein were produced predominantly in testis, as verified with RT-PCR, Northern and Western blotting. While mRNAs encoding Sept12 have been isolated from many tissues in a diversity of organisms, including brain, I speculate that the SEPT12 protein is expressed in a small subset of cells in these tissues, where it may still have
some prominent role. Alternatively, it may also be ubiquitously present in extremely low copy number, as has been shown for ODF2, which was originally characterized as a testis-specific protein (Hoyer-Fender et al. 1998; Nakagawa et al. 2001).

In the rat, post-natal testis development involves a series of well-characterized phases of germ cell proliferation culminating in sexual maturity at approximately 49 days after birth (Slaughter et al. 1987). While low transcript and protein levels are detectable very early in development, the onset of robust Sept12/SEPT12 expression is coincident with the first cycle of development of spermatocytes into spermatids, which occurs from approximately days 29-49, post-partum. During this stage of spermatogenesis, sperm cells undergo substantial morphological changes, each acquiring a long flagellum and the distinctive structure of a mature sperm cell. Mitochondria also become densely packed and aligned along the middle portion of the tail and must be partitioned from the principle piece of the tail (see Figure 10).

Coincident with a role in spermatozoa, I clearly observe SEPT12 in a ring-shaped structure demarcating the end of the mitochondrial sheath in mature sperm. This staining is highly specific and indicates that SEPT12 must also be a component of the annulus, as has been reported for SEPT1, 4, 6 and 7, even though based on unshown immunostaining results, SEPT12 was previously suggested to be absent from this structure (Ihara et al. 2005). Additional support for this argument comes from pairwise septin interaction studies using the yeast two-hybrid system and overexpression in CHO cells. I observed that SEPT12 interacted with all previously reported septin components of the annulus and formed unique, highly curved filaments upon interaction with integral annulus component, SEPT4, in CHO cells.

Spermatogenesis is extremely complex and takes approximately 7-8 weeks in the adult rat. Detailed descriptions of the maturation process classify spermatogenesis into stages based on the grouping of germ cell types at particular phases of development in cross-sectioned seminiferous tubules. Again, I refer the reader to a thorough introduction to and morphological characterization of this process in rat, which is presented in work by Russell and colleagues and forms the basis for staging in all literature pertaining to rat spermatogenesis (Russell et al. 1990).

During the meiotic phase of spermatogenesis, the Golgi apparatus sits in direct apposition to the nucleus and swells up to 2-3μm in diameter: it is a defining feature of these cells (Suarez-Quian et al. 1991). At the beginning of spermiogenesis, the acrosomal vesicle begins to form in direct
apposition to the nuclear envelope, by directed secretion from the Golgi. However, after the formation of the acrosome, the Golgi remains enlarged during most of the remaining stages of spermiogenesis and the reason for this has not been identified. Upon examination of SEPT12 distribution in testis sections, I observed direct co-localization of SEPT12 with Golgi marker GM130 during the pre-elongation stages (most likely meiotic or round spermatid stages), consistent with observations for other Golgi-localized proteins in male germ cells (Matsuda et al. 2004). This appears to be followed by segregation of the SEPT12-containing structure from the Golgi and its subsequent relocalization to the reported first origins of the annulus proper at the base of sperm heads (Fawcett et al. 1970; Fawcett and Phillips 1969), consistent with the change in morphology of germ cells from a round to elongated form. This suggests that annulus septins are sequestered in the Golgi until the formation of the annulus at the base of the sperm head is directed by the appropriate signaling cue. In at least one case, it has been suggested that the Golgi functions as a storage compartment until later stages of spermiogenesis, at which time large vesicles containing specific proteins become distinct from the Golgi and are targeted to the appropriate locations in the cell, prior to removal of residual cytoplasm and the completion of spermiogenesis (Cesario et al. 1995). A very recent study even suggests ubiquitin-dependent sorting is involved in this process (Morokuma et al. 2007). In addition, disruption of the Golga3 gene, whose gene product has been shown to localize to the Golgi, results in the loss of elongating germ cells, although the reason for this is somewhat obscure (Matsuda et al. 2004; Matsukuma et al. 1999). It is well established that the *S. cerevisiae* septins are specifically recruited to PI(4)P-rich domains in the plasma membrane during cytokinesis (Casamayor and Snyder 2003). Interestingly, in mammalian cells, PI(4)P is specific to the Golgi and this lipid is responsible for localization of some proteins to the Golgi (Wang et al. 2007).

Using the marker anillin, I did not observe any SEPT12 localization to intercellular germ cell bridges, where septins and other components of the cell division machinery have been observed previously (Greenbaum et al. 2007). This suggests that the formation of the annulus is distinct from the role of septins in germ cell bridges.

Unfortunately, high-resolution morphology studies were limited by tissue fixation conditions, and this did not allow me to thoroughly examine SEPT12 distribution in an accurate stage-dependent manner. My antibody is rat-specific, requiring perfusion fixation in order to preserve detailed morphology for accurate staging, due to the size of the organ. Further, lipid-rich testis...
requires Bouin’s fixative for preservation of detailed morphology. Unfortunately, the SEPT12 epitope was not accessible under any conditions other than upon fixation with paraformaldehyde or methanol, when cryosectioning either before or after fixation. Following Bouin’s fixation, a variety of antigen retrieval techniques proved unsuccessful. As such, it was not possible to accurately stage specifically when SEPT12 begins to accumulate in association with the Golgi and specifically when the pre-annulus SEPT12-containing structure becomes a distinct entity from the Golgi. Nonetheless, the results of the localization of SEPT12 in tissue sections presents the exciting evidence that the annulus originates from the Golgi and that this “pre-annulus” likely coalesces onto a specific patch of plasma membrane at the base of elongating sperm heads, allowing for the formation of the annulus proper. This is a substantial expansion upon the work of Fawcett, demonstrating clear origins of the annulus from a secretory organelle. This finding is consistent with some previous observations: Golgi localization of septins was first reported in PC-12 cells (Hsu et al. 1998) and has been reported again recently in epithelial cells (Spiliotis et al. 2008). The Trimble lab has routinely observed septin co-localization with Golgi markers in early-stage differentiating primary neurons (C.W. Tsang, personal communication). Further, this clearly eliminates the possibility that the origin of the septins in the annulus is from either the chromatoid body or another source. It will be of utmost interest to more thoroughly map the early stages of annulus formation in testis and identify the signaling cues responsible, possibly allowing for the identification of conserved processes during directed protein targeting in a variety of processes involving septins.

The results of the yeast two-hybrid study suggest several very interesting potential in vivo SEPT12 interaction partners, including several structural proteins and enzymatic/modifier proteins:

1. Tektins are particularly noteworthy because they share several properties of septins: they are 40-60kDa, coiled-coil-containing proteins that form filaments. Tektins have been characterized in a wide variety of organisms: they are conserved, essential components of axonemes across phyla in diverse types of motile/cilia-containing cells: such as flagellated algae, mammalian sperm flagella and ciliated tracheal epithelia. In mammals, all of the five known tektins are most highly expressed in testis, where they display varying localizations along the length of sperm flagella (Iguchi et al. 2002; Iida et al. 2006; Murayama et al. 2008; Roy et al. 2004; Wolkowicz et al. 2002; Xu et al. 2001). In sperm flagella, tektins play a major role in stabilizing the
structure of the axoneme and disruption of both the Tekt2 and Tekt4 genes, respectively, results in major axonemal defects and motility defects (Roy et al. 2007; Tanaka et al. 2004) reminiscent of the phenotype seen in the Sept4 knockout mouse. Tekt2, which was identified in my screen, has been shown to localize to the principal piece in sperm (Iguchi et al. 2002; Wolkowicz et al. 2002). This suggests a means of localization or anchoring of the annulus via septin interactions with other structural proteins in the sperm flagellum and identification of this interaction provides an excellent starting point for such studies. Fascinatingly, Tekt2 was recently shown to localize to the mitotic spindle in dividing mammalian cells, where it is essential for spindle integrity (Durcan et al. 2008). This suggests that tektins have important roles outside of the testis and suggests the likelihood of a conserved septin:tektin interaction during cytokinesis.

2. PIN1 was originally identified as an interactor of the mitotic kinase NIMA in Aspergillus nidulans (Lu et al. 1996). It has since been shown to be essential for mammalian mitosis, where it interacts with a myriad of phosphorylated proteins and effects isomerization about prolines preceded by a phosphorylated serine/threonine (Lu et al. 1996). Many PIN1:substrate interactions take place during mitosis (Shen et al. 1998); indeed, PIN1 is very highly expressed in testis, where germ cells are constantly being produced. PIN1 is also highly expressed in neurons, where it was first described to participate in the regulation of Tau and has been implicated in the pathology of Alzheimer disease (Lu et al. 1999). In testis, PIN1 has been demonstrated to play a critical role in the generation of spermatogonia from primordial germ cells. PIN1 knockout mice exhibit age-dependent degeneration of spermatogenic cells leading to a complete loss of germ cells by 14 months of age (Liou et al. 2002). The septins are known to become phosphorylated both in the nervous system and during mitosis, but the detailed consequences of phosphorylation remain elusive. An understanding of the regulation of septins and their interactions within higher order septin structures is also very poorly understood. Septin filament status has been observed to change between a static and fluid state throughout mitosis and can be correlated to both septin phosphorylation state and major structural reorganization of septin filaments (Dobbelaere et al. 2003; Vrabioiu et al. 2004). As such, the interaction of PIN1 with septins is of particular interest because it is conceivable that the action of PIN1 may impart structural changes by isomerization of septins, but only once they are phosphorylated at specific residues in response to other cellular signaling cues. Further support of a conserved interaction between septins and PIN1 comes from synthetic lethal screens in S.
cerevisiae, PIN1 orthologue, ESS1, has been shown to interact genetically with *S. cerevisiae* septin CDC12 (Davierwala et al. 2005). PIN1 has essential roles in the cell cycle prior to formation of the septin hourglass structure; as such, it is conceivable that an essential role for PIN1 in regulation of septins has remained undetected thus far. In a study identifying phosphorylated proteins in mitotically-arrested mammalian cells, SEPT9 was observed to be phosphorylated at a PIN1 consensus site (Beausoleil et al. 2006). Mathew Estey, now a graduate student in the lab, has compiled compelling evidence that phospho-dependent interaction of PIN1 with SEPT9 is critical during cell division (M.P. Estey, personal communication). PIN1 is likely to represent a critical, conserved septin-interactor, which plays a fundamental role in septin regulation in a diversity of cellular processes.

3. MAGI family members are transmembrane proteins characterized by the presence of an N-terminal guanylate kinase domain, which functions not as a kinase, but in protein-protein interaction. They also have multiple tandem PDZ (PSD-95-Discs-Large/ZO-1) protein-protein interaction motifs extending from the kinase domain to the C-terminus (Montgomery et al. 2004). SEPT12 has a putative C-terminal PDZ-binding domain and it interacted with the most C-terminal of the 5 consecutive PDZ domains in MAGI1/2, suggesting either a structural/stabilizing role, or perhaps participation in temporally regulated targeting upon exposure of the C-terminal PDZ domain. Critical roles for septins in neurite outgrowth, synapse assembly and mature synapse functioning has long been implied and a detailed study of this interaction in a neuronal context could be very informative (Beites et al. 1999; Caltagarone et al. 1998; Hsu et al. 1998; Kinoshita et al. 2000; Vega and Hsu 2003; Walikonis et al. 2000; Xue et al. 2004).

4. HSD17B10 is a mitochondrial metabolic enzyme which is essential for the metabolism of isoleucine and branched chain fatty acids (Yang et al. 2005). Its mutation causes a spectrum of clinical conditions from mild mental retardation to progressive infantile neurodegeneration. It has also been linked to Alzheimer disease because of its affinity for amyloid-β peptide. Although the significance of an interaction with septins remains unclear, there are increasing reports of alternative localizations and functions for proteins originally described to have classical functions, such as enzymes and channels. One such example is the testis anion transporter, whose family members have been shown to transport chloride. This protein has
recently been localized to the annulus, where it was demonstrated to be essential for sperm tail differentiation and motility (Toure et al. 2007).

5. MYST2 is a histone acetyltransferase which is also expressed most highly in testis (Sharma et al. 2000). A previous study found that septins bind directly to microtubule associated protein-4 (MAP4) and somehow destabilize microtubules, perhaps by sequestration of cytoplasmic MAP4 pools. Correspondingly, upon septin knockdown, an increase in tubulin acetylation was observed (Kremer et al. 2005). This is particularly exciting, given that the enzymes responsible for tubulin acetylation remain unidentified, but that a histone deacetylase has been shown to remove acetyl groups from microtubules (Westermann and Weber 2003). My isolation of MYST2 suggests that under resting conditions, septins may also function to regulate tubulin acetylation and that MYST2 may be the acetyltransferase responsible. It is also possible that septins are acetylated themselves, although this has not been described to date and the functional consequence of this remains speculative.

Due to its specific high expression in testis, relative to other tissues, Sept12/SEPT12 is unique among the septins described to date and may represent a novel target for fertility treatment without the need to disrupt other key processes in the body. This study also suggests an answer to a long-standing question in the field of spermatogenesis: how the annulus originates prior to its deposition on the plasma membrane at the base of elongating sperm heads. My co-localization studies strongly suggest the annulus forms via targeted secretion of a “pre-annulus” vesicle from the Golgi, which then localizes to the membrane invaginations near the base of the elongating sperm head. Yeast two-hybrid studies provide an additional solid starting point for further study of the specific role of SEPT12 in the formation and maintenance of the integrity of the highly conserved and essential mammalian sperm tail annulus. Additional binding partners may be involved in broader septin regulation. Recent evidence reveals the absence of Sept12 expression in testis of infertile men. In these studies, significantly reduced or absent Sept12 expression was matched to both maturation arrest and Sertoli cell-only forms of sterility (Lin et al. 2006), both of which result in the absence of mature spermatozoa being produced by the seminiferous epithelium. This study supports my finding that Sept12/SEPT12 is highly produced in germ cells and is a component of the annulus, a structure specific to mature spermatozoa and essential for their mobility.
6. OTHER SEPTINS IN THE TESTIS

This work was conducted in collaboration with the lab of Dr. Elizabeth Petty (University of Michigan) and is published as a co-first author paper in the journal *Mammalian Genome* (November, 2007; 18(11):796-807). I conducted all work presented in this chapter, although it does not all appear in the published paper; additional information pertaining to Sept14/SEPT14 generated in the Petty lab can be found in the published paper. Testes perfusion fixation (Figures 45-48) was performed by Mr. Marvin Estrada in the Hospital for Sick Children Animal Facility (Toronto). Mass spectrometry represented in Figure 49 was conducted by Ms. Kelly Hogue at the Hospital for Sick Children Advanced Protein Technology Centre (Toronto). The immunolocalization of SEPT2 and SEPT9 has not been published anywhere other than this thesis.

6.1. Rationale

Virtually nothing is known of septin expression and function in the testis, and only with the very recent publication that SEPT4 is crucial for annulus formation (Ihara et al. 2005; Kissel et al. 2005) has there been attention drawn to septin function in the testis. While conducting the initial characterization of Sept12/SEPT12, I was able to examine the localization of additional septins for which the Trimble lab possesses excellent antibodies, namely SEPT2 and SEPT9 because they are also highly expressed in testis (see Figure 3). In addition, I was able to use anti-SEPT9 antibodies to conduct immunoprecipitation experiments in order identify proteins that are capable of interaction with SEPT9 in testis lysate.

6.2. Results

6.2.1. Localization of SEPT2 and SEPT9 in Testis Sections

While conducting immunohistochemical analysis of SEPT12 on testis sections, I also immunostained sections with antibodies against SEPT2 and SEPT9. Fascinatingly, both of these antibodies localized predominantly to a structure unique from that observed with anti-SEPT12 antibodies
(Figure 45), and distinct from intercellular bridges, where these particular septins have been previously described to localize (Greenbaum et al. 2007). Initial experiments demonstrated that SEPT2 and SEPT9 staining were indistinguishable; as such, only representative anti-SEPT9 immunostaining is shown. A high degree of co-localization is observed between SEPT2/9 and actin, in structures reminiscent of Sertoli cell apical ectoplasmic specializations (AES), which surround invaginations holding elongating sperm heads. These highly specialized structures are characterized by the presence of bundles of hexagonal actin filaments, endoplasmic reticulum, tubulin, and a variety of adherens junction, adaptor and scaffolding proteins (Lee and Cheng 2004b). The AES surrounds elongating spermatids during most of the elongation process, until it develops into the tubulobulbar complex on the concave side of the elongating spermatid head, just a few days before release of the mature spermatid into the lumen of the seminiferous tubule (Lee and Cheng 2004b). At this point, the AES is believed to be internalized and trafficked to the basal ES (BES), which is believed to contribute to adherence of Sertoli cells to the basement membrane (Guttman et al. 2004).

Closer examination of testis sections further supports the direct co-localization between SEPT2/9 and actin, at various stages of spermatid elongation, when the AES is known to be present (Figure 46, 47). Further, some reports demonstrate connection of this structure to the basal surface of the Sertoli cell via actin-based structures (Mruk and Cheng 2004a; Mruk and Cheng 2004b) and this is supported by observations shown in Figure 48.
Figure 45. SEPT9 Co-localizes with Actin Structures in the Seminiferous Epithelium.

(A) SEPT9 antibody staining (red) reveals punctate staining at low magnification, surrounding clusters of elongating sperm heads (not shown). Similar staining is observed with rhodamine-phalloidin, which detects filamentous actin (B; green). (C) High degree of co-localization is observed near apical (1) and basal (2) surfaces. Testes were perfusion-fixed and stained using standard immunochemistry techniques and imaged using spinning disc confocal microscopy. “L” denotes lumen of seminiferous tubule. (D) Schematic of elongating spermatids embedded in Sertoli cells (red); note ectoplasmic specialization (black concentrations at tips of sperm heads); other germ cells are not shown for simplicity. Size bar = 50 μm.
Figure 46. SEPT9 and Actin Ensheath Heads of Elongating Spermatids.

(A, C) SEPT9 staining (red) reveals SEPT9-ensheathed elongating sperm heads (nuclei stained with Hoescht, shown in blue). Similar staining is observed with rhodamine-phalloidin, which detects filamentous actin (B, D; green). Testes were perfusion-fixed and stained using standard immunochemistry techniques, then imaged using spinning disc confocal microscopy. Arrows in (A) and (B) point to filamentous structures leading basally. Size bar = 20μm.
Figure 47. SEPT9 Localizes to the Tubulobulbar Complex.

SEPT9 antibodies (red) reveal a concentration of SEPT9 at what is likely the tubulobulbar complex, located on the concave side of sperm heads just prior to spermiation (nuclei stained with Hoescht, shown in blue). Testes were perfusion-fixed and stained using standard immunohistochemistry techniques and imaged using spinning disc confocal microscopy. Size bar = 20μm.
Figure 48. SEPT9 and Actin Structures are Associated with the Basal Compartment of Seminiferous Tubule.

(A) SEPT9 staining (red) reveals filamentous SEPT9 structures, attached to the basal membrane (nuclei stained with Hoescht, shown in blue). Similar staining is observed with rhodamine-phalloidin, which detects filamentous actin (B; green). (C) High degree of co-localization is observed in filamentous structures leading down to the basal lamina (arrows). Testes were perfusion-fixed and stained using standard immunochemistry techniques and imaged using spinning disc confocal microscopy. Size bar = 20μm.
The findings for SEPT2/9 demonstrate a novel, previously uncharacterized localization for septins in testis and suggest they play a novel role in ES-mediated sperm maturation and/or subsequent release of sperm from the seminiferous epithelium. Co-localization of SEPT2 and SEPT9 with actin was observed during all stages of spermatid elongation; as such septins appear to be associated with the AES, BES and the tubulobulbar complex. However, more precise staging must be conducted in order to produce a more thorough analysis of these observations. Better fixation and staining protocols will be required to assess this because, as with SEPT12, antigens were irretrievably masked by use of Bouin’s fixative. In this case, studies are not limited by rat-specific antibodies and work with mice testis (which to not require perfusion fixation) could be pursued in future studies.

6.2.2. Immunoprecipitation of SEPT9 from Testis Lysate

To further probe the role of SEPT2 and SEPT9 in testis, I conducted anti-SEPT9 immunoprecipitation from testis lysate, with the goal of identifying coimmunoprecipitating proteins by mass spectrometry. In these experiments, adult rat testes were ground to homogeneity under liquid nitrogen and subsequently homogenized in buffer containing protease inhibitors, using a Dounce tissue homogenizer. Following solubilization in triton X-100 and subsequent high-speed clarification of the lysate, immunoprecipitations were conducted using rabbit polyclonal antibodies specific to SEPT9 and rabbit immunoglobulin G (IgG) as a negative control. Total immunoprecipitates were resolved by SDS-PAGE. Coomassie-stained protein bands were excised and subjected to trypsin digest and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF). Peptide fragments were compared to current protein databases, generating identities of the major peptide fragments from each section of the original gel lane. This is shown in Figure 49.

Many septins were identified, including two major isoforms of SEPT9, _v1 and _v2, which both contain the antigenic sequence recognized by our antibody. SEPT2 was identified by substantial peptide sequence coverage, as expected from co-localization studies. SEPT7 was also identified by an abundance of peptide sequences, suggesting its localization in the SEPT2/9 structure. SEPT8, SEPT11 as well as a new member of this sub-family, SEPT14, were also identified. SEPT14 is further discussed in the following section. Actin and tubulin were also identified in
abundance, supporting the observed localization of SEPT2/9 to actin and tubulin-rich structures. Many other proteins were identified, although none of these have been reported to interact with septins or to localize to the ES; most of them are metabolic enzymes or heat shock proteins. An interesting candidate interactor is protein-L-isoaspartate methyltransferase (PIMT), which is implicated in protection of isoaspartate-containing proteins from oxidation in the brain (Zhu et al. 2006). However, the relevance of many of these candidate septin interaction partners is not immediately obvious and these proteins may represent non-specific interactors.
Figure 49. Many Proteins Co-isolate with SEPT9.

SEPT9 polyclonal antibodies were used to immunoprecipitate SEPT9 from triton X-100-solubilized testes tissue. Total immunoprecipitate was subjected to SDS-PAGE and stained with...
Coomassie blue (shown to the left). Protein bands were excised, trypsin-digested and subjected to MALDI-TOF mass spectrometry at the Hospital for Sick Children Mass Spectrometry Facility. Peptide sequences with probabilities greater than 95% were compared against the current rat protein data base (NCBI), identifying several unique bands. Proteins identified are listed with their accession numbers and the percentage of total sequence coverage obtained from peptide sequences identified by mass spectrometry. MALDI-TOF and database searching conducted by Kelly Hogue.
6.2.3. Preliminary Characterization of SEPT14

Through participation in a collaborative study with the laboratory of Dr. Elizabeth Petty (University of Michigan), I was able to identify SEPT14 (Peterson et al. 2007) as one of the proteins that co-immunoprecipitated with SEPT9 from testis lysate (listed in Figure 49). The rat SEPT14 sequence is shown in Figure 50 with the peptides identified by mass spectrometry highlighted in bold.

The Petty lab originally identified the Sept14 cDNA in a yeast two-hybrid screen for SEPT9-interactors using a human testis library. They found that the Sept14 gene is encoded over approximately 65kb on chromosome 7p11.2. SEPT14 is most similar to SEPT10, as depicted by a phylogenetic tree generated from the alignment of all the septins (Figure 1); a schematic of SEPT14 is shown in Figure 51.
Figure 50. Primary Amino Acid Sequence of Human SEPT14.

Peptides unique to SEPT14 and corresponding to 23% total sequence coverage were identified in the SEPT9 immunoprecipitate. Peptides identified are shown in bold and G1, G3 and G4 sequence elements of the GTP-binding domain are outlined with hatched boxes.

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1-MSAQYPFVSALTVVAVALKVRENINIRCTLTTLGFGECPLPTQLVNSKI-50
51-QKGFSPNILCVGETGIGKSLINTLFNTNLKEAKSSHYSKVLKVKTYE-100
101-LLERNIPKLTVKVQYGDQINKEASYQPVDQYLAQFEAYLQEELIKIK-150
151-RSPMDYHDSRIHVCLVPTGPHSLSDLITLMKSIDRRVNIPLLIAKAD-200
201-SLSKNDLQRFKNNIMSELSNGVHIYQFLADDATAQVNSSLNLYLLCLF-250
251-LQGLLPFAVVSMEEVKRGVRGRHRPGVLOGYVENENHCDFVCLL-300
301-LCTHMDLKDQTHQHYECYRSRQLQKLGSDFKGNNKPVSFQEMYEAKR-350
351-QEFHDCQREEEELKQTFMRVKEKELTFKDAEKELEDKPEHLKRIQQEE-400
401-ILKLEEBRRKLEEQIIDFYKMAASESAQAQCNMVKKDQDRKK-445
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**Figure 51. Primary Sequence Features of SEPT14.**

SEPT14 is a unique septin but contains conserved septin primary sequence elements: polybasic domain (PB; blue), the G1, G3 and G4 sequences of the GTP-binding domain (red) and the Septin Unique Element (SUE; yellow).
Like *Sept12*, *Sept14* transcripts were also found to be exclusive to testis, in the tissues examined. Although neither an exhaustive comparison of tissues nor developmental expression profiles were conducted (Peterson et al. 2007), the vast majority of *Sept14* EST clones are derived from testis libraries, supporting the observed testis-specificity of expression. With the exception of *Sept12/SEPT12*, all mammalian septins studied thus far have been found to be abundant in brain; some septins are expressed in a subset of other tissues and others are ubiquitously expressed. While *Sept14* transcripts were not observed in fetal brain, *Sept14* may exhibit an age-dependent expression onset in this tissue later in development, as is observed with SEPT5 protein levels (Peng et al. 2002). I was able to use the human cDNA sequence to exhaustively search EST databases, thereby identifying the rat *Sept14* counterpart. Unfortunately, my attempts to generate SEPT14-specific antibodies were unsuccessful and I was not able to confirm testis-specific localization of the protein or conduct immunochemical localization of SEPT14 in tissue sections.

SEPT14 was found to interact with several other septins using the yeast two-hybrid system (Figure 52), as previously observed for the other septins. Notably, it either interacted very weakly or did not interact at all with its other sub-family members; namely SEPT6, SEPT8, SEPT10, SEPT11 and itself.

When overexpressed at low levels in CHO cells, SEPT14 co-localized with SEPT9 along stress fibres, matching the endogenous interphase distribution of SEPT9 and the observed distribution for other septins. A representative image is shown in Figure 53. I was also able to co-immunoprecipitate overexpressed SEPT9 and SEPT14 from these cells, further supporting the finding that these two proteins are capable of interacting stably *in vivo* (Figure 54).
Figure 52. SEPT14 Interacts with Other Mammalian Septins.

*S. cerevisiae* was used to express SEPT14 as the bait and all other respective septins as prey, as previously described. Intensity of blue colour correlates with the strength of interaction as determined by degree of LacZ activation (conducted by Mathew Estey).
**Figure 53. SEPT9 and SEPT14 Co-localize in CHO cells.**

When transiently overexpressed in CHO cells, GFP-SEPT14 (A, green) and Flag-SEPT9 (B, red) co-localized along stress fibres (C). Nuclei stained with Hoescht (shown in blue). Standard fixation and immunostaining were conducted; images were acquired using spinning disc confocal microscopy, as previously described. Size bar = 2μm.
Figure 54. SEPT9 and SEPT14 Co-immunoprecipitate.

CHO cells were transiently transfected with plasmids expressing GFP-Sept14 and Flag-Sept9, lysed and subjected to immunoprecipitation with anti-Flag antibodies. Twenty percent of the immunoprecipitated material was electrophoresed beside 5% of the initial cell lysate. Lane 2 shows that GFP-SEPT14 is able to coimmunoprecipitate with Flag-SEPT9, whereas this is not observed with non-specific rabbit IgG (lane 3). Lane 4 shows the expression of Flag-SEPT9.
Collectively, these results identify another novel septin, which appears to be testis-specific. Immunoprecipitation of endogenous SEPT9 from testis tissue identified SEPT14 and suggests that SEPT14 works in tandem with SEPT9 in testis. This suggestion is also supported by yeast two-hybrid interaction, co-localization during overexpression in mammalian cells and coimmunoprecipitation following transfection of mammalian cells. Further generation of SEPT14-specific reagents will be critical in identifying the specific functions of SEPT14 in the testis.
6.3. Discussion

In this chapter, a novel and previously undescribed localization for septins in seminiferous epithelium is presented. In seminiferous tubule cross-sections, SEPT2 and SEPT9 were localized to the region surrounding the heads of elongating spermatids. Here, an essential and testis-specific hybrid anchoring junction/tight junction called the apical ectoplasmic specialization (AES) is known to reside beneath the plasma membrane of surrounding Sertoli cells, where specialized Sertoli cell invaginations hold elongating spermatids. I also observe concentrations of septins at a morphologically similar structure found along the inner membrane of the Sertoli cell at the basement membrane, called the basal ectoplasmic specialization (BES).

While ultrastructurally very similar to the AES, the BES is thought to function mainly in adhesion of Sertoli cells to the basement membrane. It has recently been demonstrated that internalization of the AES upon spermiation (the final release of mature sperm) results in recycling of many components to the BES (Guttman et al. 2004); the function and dynamics of this ES turnover is unclear at this point.

Like the BES, the AES consists predominantly of filamentous actin bundles, which are sandwiched between the Sertoli cell plasma membrane and flattened cisternae of that same cell’s endoplasmic reticulum. As such, this connective structure is based almost entirely in the Sertoli cell; tight junctions are not present and little is known about the integral membrane junctional molecules of the elongating spermatid. The AES is critical for maintaining close attachment between the elongating spermatid and the Sertoli cell. As such, the AES has been described to have five major roles: to maintain the attachment of the spermatid head to the Sertoli cell; to orient and position the spermatid head; to shape the spermatid head; to draw the spermatid head into Sertoli cell crypts and finally, to regulate spermiation.

At the ultrastructural level, the ES has been thoroughly studied for several decades, but relatively little has been known about the molecular architecture, let alone the mechanisms regulating its dynamics. The filamentous actin found at the AES adopts a unique organization in hexagonal bundles whose orientation does not support those of a contractile system (Vogl and Soucy 1985). In addition, many proteins found at adherens junctions or focal contact points in other epithelia are also found here (Mruk and Cheng 2004a; Mruk and Cheng 2004b).
The AES contains three multi-protein complexes that mediate interaction between Sertoli and germ cells: cadherin/catenin, nectin/afadin/ponsin and integrin/laminin. A vast number of signaling molecules have also been found at this structure, implying cross-talk and substantial signaling complexity in the regulation of this structure. For example, isoforms of the three classical mitogen-activated protein kinase (MAPK) subfamilies (extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK) and p38MAPK) are found at the AES (Wong and Cheng 2005). In addition, there is evidence of specific molecular signaling cascades participating in the regulation of AES dynamics: the integrin/pFAK/PI 3-kinase/p130Cas/c-Jun N-terminal kinase (JNK) pathway and the integrin/RhoB/ROCK/LINK/cofilin pathways (Lee and Cheng 2004a; Lee and Cheng 2004b).

In addition to signaling and restructuring of adhesion molecules, a structure called the tubulobulbar complex forms towards the very end of the spermatid elongation phase, just prior to spermiation. This structure forms on the concave side of the sickle-shaped sperm head and is characterized by tube-like projections of spermatid plasma membrane into corresponding invaginations in the Sertoli cell membrane. It has been postulated to function both in the regulation of the final stages of spermiation and in the uptake of cell-cell adhesion molecules. As such, a variety of endocytic molecules, such as dynamin, protein kinase C-alpha (PKCα), as well as endosomal and lysosomal markers LAMP1 and SGP1, respectively, have been localized to this structure (Guttman et al. 2004).

Immunoprecipitation from testis lysate and subsequent MALDI-TOF mass spectrometry revealed several septins co-purifying with SEPT9 from testis, including another novel septin, SEPT14. Isolation of SEPT2 supports my observed co-localization between SEPT2 and SEPT9 in testis sections. Isolation of SEPT8, SEPT11 and SEPT14 further supports the widely observed finding that septins are found in complexes with one another in vivo. Several candidate non-septin proteins also co-purified with SEPT9, most notably the AES-enriched proteins, actin and tubulin. The likelihood of in vivo interaction of many of the other candidates with SEPT9 is uncertain given that many of them have not been previously reported to concentrate at the AES. One candidate interactor, PIMT, is of particular interest. Normally, it is highly expressed in the brain; however PIMT-deficient mice die from epileptic seizure 4-10 weeks after birth, supporting the critical role of this protein in nervous system function (Yamamoto et al. 1998). Through study of knockout mice, PIMT has been demonstrated to play critical roles in many aspects of...
neuronal function, including: synaptic and neuronal organization, motor activity and general neurophysiology (Farrar et al. 2005a; Farrar et al. 2005b; Ikegaya et al. 2001; Kim et al. 1999; Yamamoto et al. 1998). PIMT has been demonstrated to act upon many substrates which have been associated with septins, including: CRMP2 (Takahashi et al. 2003), actin and tubulin and dynamin (Zhu et al. 2006). Interestingly, the arginine methyltransferase Hsl7p is recruited to the septin hourglass structure in *S. cerevisiae* where it functions critically to somehow effect the phosphorylation of Swe1p in the morphogenesis checkpoint (Versele and Thorner 2005). As such, interaction of mammalian septins with PIMT may represent evolutionary conservation of this scaffold function and further studies will be required to verify the interaction and probe its biological significance.

Many aspects of ES dynamics are very poorly understood. Thorough and detailed examination of the role of septins at the ES may provide a better understanding of ES dynamics while revealing additional, broader insights into septin function in a variety of cellular contexts which have been reported to involve or depend on septins. The AES has been compared to a focal adhesion because it contains many proteins in common with this structure (Mruk and Cheng 2004a; Mruk and Cheng 2004b). However, it could also be compared to the cytokinetic cleavage furrow: both structures are highly dynamic and meticulously temporally regulated by a myriad of cell signaling events. Indeed, many common essential molecular players have been reported at both locations, including filamentous actin and tubulin, the septins, PIP2, Cdc42 and ROCK. In *S. cerevisiae*, the septins are influenced early in the cell cycle by master regulator Cdc42, causing them to coalesce into a patch on the membrane at the presumptive bud site (Gladfelter et al. 2002). In mammals, septins are also known to co-localize and/or interact with a number of AES-enriched components, but predominantly in the context of cell division. For example, septins have been shown localize to actin-based structures and to bind directly to tubulin (Kinoshita et al. 1997; Nagata et al. 2003; Surka et al. 2002); they co-localize with ROCK, a kinase which is essential for cytokinesis (Joo et al. 2007); bind phosphoinositides (Steels et al. 2007; Xie et al. 1999); and directly bind to myosin (Joo et al. 2007). Septins in the AES may be functioning as a cytoskeletal linker between various components of this structure, or as a signaling scaffold; convincing evidence has suggested such roles for septins during cytokinesis (Castillon et al. 2003). In addition, phospho-specific antibodies have identified that the AES contains a substantial concentration of phosphorylated proteins, implying phosphorylation is an important
component of regulating AES dynamics (Lee and Cheng 2004a; Lee and Cheng 2004b). This is further suggestive that septin filament dynamics at the AES may be regulated by phosphorylation, as has been demonstrated for \textit{S. cerevisiae} septins during mitosis (Dobbelaere et al. 2003). Finally, the septins may also be functioning to regulate some aspect of tubulobulbar function, given their previously reported interaction with dynamin and participation in endocytic events in the brain (Xue et al. 2004).

It should also be noted that during spermatogenesis, developing germ cells are gradually transferred luminally. However, near the end of the elongation phase, they are actively transported adlumenally, towards the basal membrane of the Sertoli cell. It is not well understood how or why this occurs, but this process is dependent upon active transport along microtubules and microtubule-based motor proteins dynein and kinesin have been implicated (Vogl 1996; Vogl et al. 2000). As such, septins may also function to coordinate some aspect of this transport via interaction with microtubules or microtubule-binding proteins, as suggested by some previous studies in other cell types (Kremer et al. 2005; Spiliotis et al. 2008; Spiliotis et al. 2005).

Simple experiments such as identification and characterization of additional septin binding partners in testis, for example through pull-downs and yeast two-hybrid screens of testis-derived libraries will serve as an excellent starting point for further defining the role of AES-localized septins. Such studies may provide insight into septin function in a variety of contexts, including cell division, while providing a further understanding of the functions and dynamics of the AES. Highly specific experiments will be required to more thoroughly probe the role of septins in the AES because disruption of several septin genes, including \textit{Sept2}, \textit{Sept7} and \textit{Sept9}, respectively, has been reported informally at conferences to result in embryonic lethal phenotypes. This suggests essential roles for these septins outside the testis, subsequently making it difficult to delineate the specific requirements for each of these septins during spermatogenesis. Due to the lack of adequate cell culture or other model systems with which to study spermatogenesis, a conditional knockout approach shows great promise in revealing specific functions of septins in the AES. The study of septin function in the AES has great promise in contributing to the further understanding of AES function and may reveal exciting parallels between spermatogenesis and other cellular processes involving septins.
The characterization of *Sept14*/SEPT14 was conducted in collaboration with the laboratory of Dr. Elizabeth Petty (Peterson et al. 2007). In this study, it was found that *Sept14* transcripts are predominantly expressed in testis. Sequence analysis revealed that SEPT14 contains all the primary sequence elements of a septin. Direct binary interaction between SEPT14 and SEPT9 and other septins was confirmed using yeast two-hybrid analysis. Interestingly, like SEPT12, SEPT14 showed a preference for interacting with septins in sub-families other than its own. This is consistent with the recent EM studies and crystal structure, which show that discrete subcomplexes of septins are likely to form by ordered polymerization of monomers into a “unit” septin complex. In this model, homodimerization does not occur prior to unit complex formation and only SEPT2 (or potentially members of this sub-family) and SEPT7 (and/or possibly SEPT13) homodimerize, driving higher-order filament formation (Gladfelter and Montagna 2007; John et al. 2007; Sirajuddin et al. 2007). This would place SEPT14 in place of SEPT6 in the model, between a SEPT2 and a SEPT7 sub-family member, respectively. As such, speculated placement of SEPT14 in the model is consistent with its observed inability to homodimerize or bind other members of its sub-family. These findings also support the notion that septin-based filaments with different constituent components likely give rise to filaments with different properties, and hence, different functions. Co-localization and co-immunoprecipitation of tagged variants of SEPT9 and SEPT14 from a standard cell line provided further support that SEPT14 is capable of interacting with SEPT9. In addition, SEPT14 co-immunoprecipitated with SEPT9 from testis lysate; this supports the concept that SEPT9 and SEPT14 function together in a complex endogenously, *in vivo*. This further suggests that SEPT14 may be part of the predominant seminiferous epithelium structure containing SEPT2 and SEPT9, which appears to be localized to the AES of Sertoli cells. Unfortunately, my attempts to generate SEPT14-specific antibodies were unsuccessful, limiting my ability to assess SEPT14 localization with immunostaining of testis sections. More thorough analysis will be necessary to determine biochemical properties of SEPT14, such as its ability to bind phosphoinositides and nucleotides. SEPT14-specific tools (such as antibodies) will prove invaluable in furthering the understanding of the role of SEPT14 in testis. These exciting findings reveal yet another member of the mammalian septin family and suggest a specialized role for this septin in the testis, as I found with SEPT12. It will be of utmost interest to unravel the roles of the predominant septins in the testis, particularly septins such as SEPT12 and SEPT14, which are very highly enriched in testis.
7. MAJOR CONCLUSIONS AND PERSPECTIVE

7.1. Overview

The studies presented herein are an original body of work and serve to further the understanding of septin function by:

1. Identifying many septin binding partners, including two novel septin family members;

2. Clarifying the role of the conserved interaction of septins with the sumoylation post-translational modification;

3. Characterizing the expression and functions of the two new septin family members.

Due to the novel nature of this work, studies were limited by time and resource constraints and are representative of a thorough, yet preliminary examination of these topics. The following sections outline the major findings of the thesis and the proposed direction of future work.

7.1.1. Septin Interaction Partners

This thesis was initiated with the study of septin:septin interactions, thereby clarifying the potential for a given pair of septins to interact, while supporting the requirement for virtually full-length septins to allow for this interaction. Where applicable, these results were consistent with the recently published model for septin unit complex formation. Several candidate proteins were then identified as septin interaction partners using yeast two-hybrid screens and septin immunoprecipitations. Such interactors included: SUMO E3 ligases of the PIAS family, and two previously undescribed septins: SEPT12 and SEPT14. The study of mammalian septin sumoylation and characterization of the novel septins constitute the bulk of this thesis work. Many additional interactors were also identified, many of which have not been previously described to interact with septins. As such, this body of work has produced a variety of testable implications on septin function, in a variety of cellular contexts, based on potential interaction partners and suggested follow-up work is outlined in the respective chapter discussion sections.
Further study of the interaction of these proteins with septins promises further clarification of the function of septins in various cellular contexts, beyond the scope of this thesis.

7.1.2. Mammalian Septins and Sumoylation

In these studies, it was determined that the interaction of septins with various components of the sumoylation pathway (E2, E3 and SUMO1) is conserved from *S. cerevisiae* to mammals. Efforts to map the sumoylation site on SEPT5 were inconclusive and co-localization studies suggested that septins localizing to the prominent septin collar during cytokinesis are not sumoylated in mammals, as they are in *S. cerevisiae*. Future study of mammalian septin sumoylation should be directed either to other septin functions in cells, such as during the earlier stage of cell division when the kinetochores form and are active, or the possible regulation of gene transcription.

Sumoylation of septins may be independent of/indirectly related to specific septin functions; as such, focus should also be directed towards such processes as cellular stress response and antagonization of ubiquitination, which both involve the SUMO pathway. The study of these topics is particularly relevant given that septins are highly expressed in many cell types, such as neurons and Sertoli cells, which reach a static population early in life. In addition, a more thorough examination of the potential role of conserved interaction between septins and the sumoylation machinery may allow for identification of functional consequences which do not arise due to covalent modification of septins by SUMO.

7.1.3. SEPT2, SEPT9, SEPT12 and SEPT14 in the Testis

This work represents a substantial contribution to the understanding of septin function in mammalian testis. Only SEPT4 had been reported as an essential component of the mammalian sperm tail annulus, prior to this work. The existence and preliminary characterization of two novel and conserved mammalian septins, SEPT12 and SEPT14, was presented in this thesis. Both of these septins are highly expressed in testis and virtually absent from other tissues tested. SEPT12 is produced in mammalian germ cells and becomes a component of the mature sperm tail annulus. Its localization in testis tissue sections suggests that the annulus forms via sequestration and then secretion from or division of the Golgi. This entirely original finding is a substantial expansion on the characterization of annulus formation, dating back to 1970s electron
microscopy studies. Further, it provides clear evidence that septins become associated with the annulus after the initial accumulation of electron dense material on the plasma membrane occurs (when the axoneme is just starting to form). This further suggests some sort of signaling and docking mechanism must be required for localization of the septin-containing structure to the pre-annulus.

In addition, SEPT2 and SEPT9 were revealed at the ectoplasmic specialization in the other major seminiferous epithelium cell type, the Sertoli cell. The integrity of this structure is essential for proper germ cell maturation and release of mature sperm from the seminiferous epithelium. A detailed description of the localization of SEPT14 in the testis is lacking, but co-immunoprecipitation experiments suggest that it functions in a complex with SEPT9, SEPT2 and several other septins. Due to their high expression in testis, disruption of the Sept12 and Sept14 genes in mice are speculated to have a prominent testis phenotype, allowing for assessment of the requirement of these gene products. Further examination of the roles of SEPT2 and SEPT9 may reveal substantial insight into conserved functions of septins in a variety of cellular contexts: testis-specific knockouts will be critical for this work due to the embryonic lethal phenotype resulting from the disruption of these genes in mice.
8. REFERENCES


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