Characterization of a Novel Interaction Between Septins and the Adenomatous Polyposis Coli Tumor Suppressor.

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Department of Biochemistry
University of Toronto

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Abstract

Septins are evolutionarily conserved proteins with roles in chromosome congression and segregation, cytokinesis and microtubule destabilization. Septins form homo- and hetero-oligomeric complexes, which are thought to act as dynamic scaffolds. We identified SEPT2/9/11/10 as novel interacting partners of adenomatous polyposis coli (APC), a bona fide tumor suppressor. Since septins and APC have similar roles and knockdown phenotypes, I sought to determine if they work together to perform their cellular functions. I showed that APC co-immunoprecipitates with endogenous septins in colon cancer cell lines. Using siRNA, I found that SEPT2 and APC may function within the same pathway to regulate DNA congression and segregation. Co-depleting SEPT9 with APC slightly alleviates the chromosome congression and segregation defects caused by siAPC alone. siSEPT9 increased abscission times, which was rescued by co-depleting APC. Future studies should elucidate the significance of the rescue data obtained upon APC and SEPT9 co-depletion and APC’s interactions with SEPT10/11.
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<td>binder of Rho GTPases</td>
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<td>colorectal cancer</td>
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<td>CRIB</td>
<td>Cdc42, Rac interactive binding</td>
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EB-1  end binding protein 1
ECL  enhanced chemiluminescence
EDTA  ethylene diamine tetraacetic acid
F-actin  filamentous actin
FAP  familial adenomatous polyposis
FBS  fetal bovine serum
G1  gap 1
GAP  GTPase activating protein
GAPDH  glyceraldehyde 3-phosphate dehydrogenase
GDP  guanine diphosphate
GFP  green fluorescent protein
GSK3β  glycogen synthase kinase-3β
GTP  guanine triphosphate
GTPase  guanine triphosphatase
h  hour
hAPC  human adenomatous polyposis coli
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP  horseradish peroxidase
hSept  human septin
IgG  immunoglobulin G
IF  immunofluorescence
IP  immunoprecipitation
IQGAP1  calmodulin binding, GTPase activating protein 1
kDa  kilodalton
KAP3  kinesin associated protein 3
LB  lysogeny broth
LEF-1  lymphoid enhancer-binding factor-1
Lipo 2000  lipofectamine 2000
LOH  loss of heterozygosity
M1-M3  middle 1-3
mA  milliamp
MAP  microtubule associated protein
MCR  mutation cluster region
MLL  mixed lineage leukemia
mm  millimeter
mM  millimole
MT  microtubule
MW  molecular weight
NaCl  sodium chloride
NES  nuclear export signal
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</tr>
<tr>
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<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<td>sodium dodecyl sulfate – polyacrylamide gel electrophoresis</td>
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<td>shRNA</td>
<td>small hairpin ribonucleic acid</td>
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<td>siRNA</td>
<td>small interfering ribonucleic acid</td>
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</tr>
<tr>
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<td>super optimal culture</td>
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<td>V</td>
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Chapter 1
Introduction

1.1 The Septins

The septins were initially discovered through a genetic screen for temperature-sensitive mutants that interfered with cell cycle progression in budding yeast (Hartwell et al. 1973). Under lax temperature conditions, the cells underwent normal cell division through budding. However, at the restrictive temperature, the cells partitioned nuclear material equally between the two buds (called karyokinesis) but failed to undergo cleavage furrow ingression, leading to the formation of multinucleated cells. This study identified four cell division cycle (Cdc) mutants: Cdc3, Cdc10, Cdc11 and Cdc12. To date, three additional septins have been identified in Saccharomyces cerevisiae: Sep7, which also plays a role in cytokinesis and Spr3 and Spr28, sporulation-specific septins (Mino et al. 1998; Fares et al. 1996; De Virgilio et al. 1996). Septins have now been identified in all mammals and fungi, but not in plants (Leipe et al. 2002).

1.1.1 Septins in yeast

In yeast, it was demonstrated that septins assemble as 10 nm rings found at the mother-bud neck, which form a collar or hourglass shape during cytokinesis (Figure 1) (Ford & Pringle 1991). Septin complex formation is a pre-requisite for assembling the yeast septin ring at the bud neck, and this event is regulated by the guanine triphosphatase (GTPase) Cdc42p (Versele & Thorner 2004; Caviston et al. 2003). Septin rings are thought to perform a number of functions ranging from acting as diffusions barriers throughout cytokinesis to acting as scaffolds for recruitment of proteins such as actin and myosin (Bi 2001).

During yeast division, the septins form a ring in late G1 prior to the emergence of a visible bud neck. After materialization of the bud, the ring extends into the aforementioned
hourglass shape and persists throughout division (Cid et al. 2001). During cytokinesis, the hourglass splits into two rings that each associate with the mother and daughter cells. These rings persist until formation of a new ring in the next round of division (Cid et al. 2001).

Figure 1: Septins localize to the mother-bud neck during yeast cytokinesis. Panel A: The septins polymerize into 10 nm rings at the mother-bud neck during cytokinesis in yeast cells. Black arrows indicate septin rings, which form a collar or hourglass-like shape at the bud neck. Scale bar represents 0.1 μm. Adapted from © Rockefeller University Press, 1976. Byers and Goetsch, J. Cell Biol. 69:717-721. Panel B: Fluorescent micrograph of Saccharomyces cerevisiae with GFP-tagged Sep7. White arrow indicates septin rings formed during cytokinesis. Sep7 is labeled in green, and the outline of the cell is shown in red. Scale bar represents 10 μm. Adapted from the Philippsen lab (2006).

1.1.2 Septin function in metazoans

There is a diverse number of septins found in eukaryotes: Caenorhabditis elegans have been found to express only two septins, Drosophila melanogaster express five septins and
mammals express at least 13 septins (Weirich et al. 2008). In humans, 14 septins have been discovered and named SEPT1 to SEPT14.

1.1.2.1 The human septin genes

Human septins are implicated in processes including DNA congression and segregation, microtubule and actin dynamics and cytokinesis. In sharp contrast to yeast septins, which function exclusively at the mother-bud neck during cytokinesis, mammalian septins localize throughout the cytoplasm, cytoskeleton and plasma membrane (Cao et al. 2009). The 14 human septin genes contain a central GTP-binding domain, which is flanked by a polybasic (PB) region and a septin unique element (SUE) (Figure 2, Panel A) (Spiliotis & Nelson 2006). While the function of the SUE remains to be elucidated, the PB domain has been found to interact with phosphoinositides (Zhang et al. 1999). In some cases, septins may also possess a C-terminal coiled coil region and/or an N-terminal proline-rich motif used in oligomerization with other septins and proteins (Sheffield et al. 2003; Nagata et al. 2004).

Based on amino acid similarities, mammalian septins have been placed in four groups designated by single family members: the SEPT2, SEPT3, SEPT6 and SEPT7 groups (Figure 2, Panel B) (Kartmann & Roth 2001; Kinoshita 2003; Hall et al. 2005). The SEPT2 group comprises SEPT1, SEPT2, SEPT4 and SEPT5. Also, members of the SEPT2 group contain the variable N-terminal proline-rich and C-terminal coiled coil domains, though the latter is truncated in comparison to septins from groups 6 and 7 (Spiliotis & Nelson 2006). The SEPT3 group encompasses SEPT3, SEPT9 and SEPT12; this group does not contain the variable C-terminal coiled coil region. The SEPT6 group comprises SEPT6, SEPT8, SEPT10, SEPT11 and SEPT14, which contain all the essential and variable domains. Lastly, the SEPT7 group includes SEPT7 and SEPT13, and they lack the proline-rich, N-terminal extension (Spiliotis & Nelson 2006).
Figure 2: Schematic representation of the general structure of septins and their classifications based on amino acid similarity. Panel A: All septins contain a central, GTP-binding domain flanked by a polybasic (PB) region and a septin unique element (SUE). The N-terminal proline-rich and C-terminal α-helical coiled-coil regions are variable. Panel B: Septins are classified into four groups: SEPT2 (SEPT2, SEPT1, SEPT4 and SEPT5), SEPT3 (SEPT3, SEPT9 and SEPT12), SEPT6 (SEPT6, SEPT8, SEPT10, SEPT11 and SEPT14) and SEPT7 (SEPT7 and SEPT13). The SEPT2 group contains a shorter C-terminal coiled-coil region than the SEPT6 and SEPT7 groups, whereas the SEPT3 group lacks the C-terminal coiled-coil domain altogether. Septins belonging to the SEPT6 group contain the major domains as well as the variable proline-rich and coiled-coil domains. The SEPT7 group lacks the proline-rich domain. Adapted with permission from Spiliotis and Nelson (2006), Journal of Cell Science: jcs.biologists.org.
1.1.3 The septin filaments

1.1.3.1 Septin complex formation

It has been suggested that septins function based on their abilities to polymerize into hetero- and homo-oligomeric complexes and highly ordered microfilaments (Versele & Thorner 2004). These filaments are thought to be composed of two or more different septins. This is consistent with studies from budding yeast, where the septin ring at the mother-bud neck was found to contain Cdc3, Cdc10, Cdc11, Cdc12 and Sep7 (Versele & Thorner 2004). Similarly, septin filaments are found in mammals, and the best characterized is the SEPT2/6/7 complex, which will be discussed later (Figure 3) (Sirajuddin et al. 2007). Furthermore, the SEPT7/11/9 complex was previously described in rat embryonic fibroblast cells, and the SEPT3/5/7 complex was found in rat brain (Hanai et al. 2004; Lukoyanova et al. 2008). In addition, SEPT7 is thought to be unique and the glue that holds together members of the other groups (Spiliotis & Nelson 2006). Consistent with this, SEPT7 siRNA-mediated knockdown has been shown to cause depletion of other septins despite the fact that the siRNA targets SEPT7 specifically (Kremer et al. 2005).

In mammals, new evidence is emerging that septin complexes contain one septin from each group. In addition, it has been suggested that septin members in the same group can substitute for one another within a complex. For instance, SEPT6 in the SEPT2/6/7 complex can be replaced with SEPT8, SEPT10 or SEPT11 (Kinoshita 2003; Hall et al. 2005). While it has been speculated that SEPT13 may substitute for SEPT7 in all the complexes, the fact that the SEPT13 mRNA transcript is not translated into a functional polypeptide casts doubt on this possibility (Hall et al. 2005; Cao et al. 2007; Cao et al. 2009).
The recent crystallization of the human SEPT2/6/7 complex provided some insight into how individual septin subunits interact with each other (Sirajuddin et al. 2007). First, the crystal structure showed that the septin complex was apolar and made up of a dimer of heterotrimers (Figure 3). Second, the structure showed that two interaction interfaces are employed in interactions between individual septin subunits: the G interface, involving the GTP-binding domain, and the NC interface, which requires interaction between the N- and C- termini (Figure 3). Third, the crystal structure showed that the SEPT2/SEPT2 dimer and SEPT6/SEPT7 interact at the NC interface, whereas SEPT2/SEPT6 interact at the G interface. The former observation...
is inconsistent with the lone SEPT2 dimer that assembles in the absence of the other septins. This dimer was found to interact at the G interface for reasons which are currently not understood (Sirajuddin et al. 2007). Fourth, SEPT2 and SEPT7 each bind one molecule of GDP, whereas SEPT6 binds GTP (Figure 3).

Despite these fruitful findings, the SEPT2/6/7 complex was crystallized in the absence of the coiled coil regions, which have been previously shown to be important for septin interaction with members of different groups (Low & Macara 2006). This, coupled with previous suggestions that one member from each septin group is needed to form a complex, may render the SEPT2/6/7 crystal structure unphysiological in mammalian systems. Intriguingly, new experimental data from our lab, where the coiled coil regions were kept intact, demonstrated that SEPT9 — a septin from the missing group — interacts at both ends of the SEPT2/6/7 complex (Kim, unpublished).

1.1.3.2 Regulation of septin complex formation

1.1.3.2.1 The role of guanine nucleotides

With the exception of yeast Cdc3 and Cdc11 and fly Sep2, animal and yeast septins are able to bind and hydrolyze GTP (Field et al. 1996; Mendoza et al. 2002; Versele & Thorner 2004; Sheffield et al. 2003). Specifically, it has been demonstrated that GTP-binding by competent yeast septins is required for formation of the septin collar during cytokinesis (Nagaraj et al. 2008), and mutation of residues within the GTPase can alter assembly, appearance, localization and/or functionality of the septin filaments (Kinoshita et al. 1997; Casamayor & Snyder 2003; Vega & Hsu 2003; Hanai et al. 2004; Robertson et al. 2004; Ding et al. 2008). As previously mentioned, bound guanine nucleotides are associated with the SEPT2/6/7 complex (Figure 3) (Sirajuddin et al. 2007).
Recent studies have shown that rates of nucleotide exchange and hydrolysis by septin filaments are slow in vitro, that the turnover of GTP within septin complexes is slow in vivo, and that the septin filaments have no in vitro GTPase activity (Frazier et al. 1998; Kinoshita et al. 2002; Sheffield et al. 2003; Vrabioiu et al. 2004; Huang et al. 2006). In sharp contrast to these studies, fly septin complexes were found to contain a GDP:GTP ratio of 2.6:1, suggesting that nucleotide exchange or hydrolysis occurred during filament polymerization (Field et al. 1996). However, another study demonstrated yeast septin complexes do not undergo significant events of nucleotide exchange or hydrolysis in vivo (Vrabioiu et al. 2004). The investigators of this study concluded that nucleotide hydrolysis is necessary to stabilize septin complexes but is dispensable for septin function.

Furthermore, it was discovered that GDP or GTP binding facilitated conformational changes in the SEPT2 dimer, allowing the GTP-bound protein to form a bent complex (Sirajuddin et al. 2009). Threonine 78 is a conserved amino acid within the SEPT7 and SEPT2 groups and is necessary for GTP binding and hydrolysis (Sirajuddin et al. 2009). Interestingly, this residue is missing from the SEPT6 group, leading Wittinghofers’s group (2009) to suggest this as the reason why SEPT6 is bound to GTP, and not to GDP, in the crystal structure (Sirajuddin et al. 2009).

1.1.3.2.2 Other regulators of septin filament polymerization

The first evidence for Cdc42p involvement in septin filament organization came from yeast, where it was found to be required for the entire process of collar formation (Caviston et al. 2003; Gladfelter et al. 2002; Smith et al. 2002). Cdc42p, a product of the CDC42 gene, is a small, Rho-family GTPase which is required for polarized growth in yeast (Park & Bi 2007).
Mutations in Cdc42p hamper localizations of both actin and septins to the yeast bud site (Gladfelter et al. 2001; Jeong et al. 2001).

In mammals, Cdc42 has been shown to play dual roles in facilitating organization of actin and septin filaments (Caviston et al. 2003; Gladfelter et al. 2002; Smith et al. 2002). Specifically, the Cdc42 effector protein, Borg3, binds directly to the SEPT2/6/7 complex using its CRIB (for Cdc42, Rac interactive binding) domain (Joberty et al. 2001). Borg3 overexpression can destabilize robust septin filaments into fibrillar structures, and this phenotype is resolved by expressing constitutively active Cdc42, which uncouples the SEPT-Borg3 interaction (Joberty et al. 2001).

IQGAP1, another Cdc42 effector protein, was shown to play a role in septin filament organization. IQGAP1 was named for its homology to GTPase activating proteins (GAPs) and its IQ (calmodulin binding) motifs (Rittmeyer et al. 2008). More importantly, IQGAP1 was found to bind directly to SEPT2, and uncoupling this interaction by expressing IQGAP1’s SEPT2-binding region resulted in formation of thick, elaborate and randomly distributed SEPT2 filaments throughout the cell, whereas, in control cells, SEPT2 maintained a juxtanuclear and plasma membrane localization with only few SEPT2 filaments present in some cells. Additionally, IQGAP1 depletion interfered with SEPT2 localization to the plasma membrane (Rittmeyer et al. 2008).

Finally, anillin, a member of the conserved family of pleckstrin homology domain proteins, is implicated in septin localization and function (Kinoshita et al. 2002; Field et al. 2005). Disruption of the SEPT-anillin interaction through expression of the septin-interacting region of anillin alters septin organization and actin bundling (Kinoshita et al. 2002). More
strikingly, mutations in anillin abolish septin recruitment to the cleavage furrow and also affect furrow ingression rates (Field et al. 2005).

1.1.4 Septins in the cytoskeleton

1.1.4.1 Septins and the actin cytoskeleton

Actin stress fibers co-localize with septins, and polymerization of each is dependent on the other. In other words, septin depletion causes loss of actin stress fibers, and actin disruption results in loss of septin filaments (Figures 4 and 5) (Kinoshita et al. 2002; Schmidt & Nichols 2004). In mammals, SEPT2 has also been found to localize to cortical actin filaments at the cleavage furrow (Kinoshita et al. 1997). Interestingly, fluorescent recovery after photobleaching experiments clearly showed that the SEPT2-actin association is highly dynamic as SEPT2 exchanged in and out of septin filaments and also in and out of actin-dependent SEPT2 polymers (Schmidt & Nichols 2004). The investigators of this study suggest that these dynamic behaviors may be necessary for assigning different cellular functions to SEPT2.
Figure 4: Septin filaments localize to actin stress fibers, and this localization is dependent on the actin cytoskeleton. Panel A: SEPT2 (green) was found to co-localize (yellow) with actin stained with phalloidin (red) in NIH3T3 cells in interphase. Panel B: After a half hour treatment with cytochalasin D to depolymerise actin, septins are observed in rings and not in filaments. White arrow indicates septin ring. Scale bars represent 1 μm. Adapted from Kinoshita (2002).

Figure 5: Maintenance of actin cytoskeleton integrity is dependent on septin filaments. Panel A: NRK cells transfected with SEPT2 siRNA. Areas indicated with o’s and white arrows show decreased SEPT2 (red) levels in cells. Panel B: NRK cells expressing SEPT2 siRNA were stained for actin (green). Areas indicated by o’s and white arrows show decreased actin bundles, concomitant with a decrease in SEPT2 levels. Scale bars represent 20 μm. Adapted from Schmidt and Nichols (2004).
1.1.4.2 **Septins bind to and destabilize microtubules**

Several independent studies have shown that septins have microtubule-dependent functions. Specifically, fruit fly septins purified using a chromatographic column packed with F-actin were able to rebind microtubules in vitro (Sisson et al. 2000). Additionally, SEPT9 was shown to co-localize and co-purify with microtubules in interphase and mitotic cells (Figures 6 and 7) (Nagata et al. 2003; Surka et al. 2002). Nelson’s group also showed in 2005 that SEPT2 and SEPT6 form a network of fibrillar structures at the midplane of epithelial cells during cell division.

Additionally, it was determined that the SEPT2/6/7 complex binds directly to MAP4, microtubule-associated protein 4, and inhibits MAP4-dependent microtubule bundling in vitro (Kremer et al. 2005). Furthermore, Kremer and colleagues demonstrated that septin depletion increases microtubule stability, though it was difficult to ascertain the septin(s) specifically involved in microtubule destabilization because they depleted SEPT7, which also depletes other septins (Kremer et al. 2005). However, recent work from our lab showed that transfection of a SEPT9-specific siRNA into mammalian cells increased microtubule stability (Surka, unpublished). Due to the fact that septins can bind to both microtubules and actin, it has been suggested that they participate in coordination of both cytoskeletons through interactions, at least in part, with the Cdc42 signaling pathway. Consistent with this, septins associate with Cdc42 effector proteins anillin, Borg3 and IQGAP1, as previously mentioned (Joberty et al. 2001; Kinoshita et al. 2002; Field et al. 2005; Rittmeyer et al. 2008).
Figure 6: Septins co-localize with microtubule networks. **Top Panel:** SEPT9 (green) was found to co-localize (yellow) with microtubule networks (red) in HMEC cells in interphase. **Bottom Panel:** Enlarged images of the boxed areas of the top panel are shown. Scale bars represent 20 μm. Adapted from the Journal of Biological Chemistry. Nagata, et al. Filament Formation of MSF-A, a Mammalian Septin, in Human Mammary Epithelial Cells Depends on Interactions with Microtubules. JBC. 2003; 16: 18538-18543 © the American Society for Biochemistry and Molecular Biology.

1.1.5 Septins in mitosis

In metazoans, septins were found to localize to the cleavage furrow, midbody, plasma membrane and the actin and tubulin cytoskeletons (Figure 7) (Kinoshita 2006; Spiliotis & Nelson 2006). In fruit flies, septins are found at the contractile apparatus during mitosis, and their depletions result in formation of binucleated cells (Neufeld & Rubin 1994). Similarly, C.
elegans septins localize to the cleavage furrow, and mutations in them cause postembryonic mitotic defects (Nguyen et al. 2000).

It has been abundantly demonstrated that septins are required for mammalian cytokinesis (Surka et al. 2002). Though much of the investigation of septin function in cell division has focused on SEPT2 and SEPT9, new evidence is emerging for SEPT6 and SEPT11 functions in mitosis. SEPT2 depletion was shown to cause formation of binucleated cells and defects in chromosome congression and segregation (Kinoshita et al. 1997; Spiliotis et al. 2005). During mitosis, lagging chromosomes result in unequal partitioning of genetic material into the two daughter cells due to SEPT2 removal, giving rise to aneuploidy (Spiliotis et al. 2005). Similarly, new work from our lab has shown that SEPT6 depletion does not affect cell division and that SEPT11 depletion causes binucleation and spindle mispositioning in HeLa cells (Estey, under review; Estey, personal communication).
Figure 7: Mammalian septins localize to the cleavage furrow, midbody, plasma membrane and the actin and tubulin cytoskeletons. **Top Panel (A, B):** SEPT2 localizes to the midbody and the cell cortex in MDCK cells. Blue arrow in A indicates midbody during cytokinesis. White and red arrows in B show SEPT2 localization at the cell cortex and plasma membrane, respectively (see C). C: SEPT2 (red) localizes to the plasma membrane (green) in interphase MDCK cells. D: SEPT2 co-localizes (blue; arrow heads) with microtubule networks and actin (red; arrows) in interphase MDCK cells. Scale bars represent 10 µm. Adapted with permission from Spiliotis and Nelson (2006), Journal of Cell Science: jcs.biologists.org. **Bottom Panel:** In HeLa cells, SEPT2 (green) localizes to the cleavage furrow during late anaphase but eventually decorates the midbody during early to late telophase. SEPT9 (red) is found at the distal ends of cells during metaphase to early telophase (filled arrows) and localizes to tubulin at the midbody bridge in late telophase (open arrows). Red arrow points to the cleavage furrow in late anaphase and to the midbody during early and late telophase. Scale bars represent 10 µm. Adapted from © ASCB; Surka and colleagues (2002) (http://www.molbiolcell.org.myaccess.library.utoronto.ca).
Furthermore, SEPT9 knockdown, similar to those of SEPT2 and SEPT11, results in formation of binucleated cells. However, in addition to this, SEPT9-depleted cells presented a more striking phenotype: they contained a significant amount of daughter cells joined by midbody bridges during cytokinesis (Estey, under review). The midbody bridge is made up of membrane and a very dense array of microtubule networks which must be severed in order to physically separate the two daughter cells in a process dubbed abscission (Gromley et al. 2005). SEPT9 is believed to recruit the exocyst component, Sec8, to the midbody during late cytokinesis (Estey, under review). The exocyst complex facilitates delivery of vesicles to the midbody bridge, where they fuse with each other and with the plasma membrane to break the midbody bridge (Fielding et al. 2005; Gromley et al. 2005).

1.1.6 Septin pathobiology: their involvement in cancer

Due to the fact that septins are involved in diverse cellular processes, it is not surprising that their deregulation is linked to a number of diseases, particularly cancers. It has been recently demonstrated that the expression patterns of human septin genes are quite complex and that alterations in these expression levels are seen in cancers.

1.1.6.1 Septins are MLL fusion partners

The first evidence for septin involvement in cancer came from leukemia patients, where the N-terminus of the mixed lineage leukemia (MLL) locus on chromosome 11 was found to be fused, in frame, to almost the entire open reading frame of the C-terminus of the SEPT9 gene (Osaka et al. 1999). It was subsequently discovered that MLL was also fused to the SEPT2, SEPT5, SEPT6 and SEPT11 loci to produce chimeric fusion proteins (Cerveira et al. 2006; Taki et al. 1999; Ono et al. 2002; Kojima et al. 2004). Though over 50 MLL fusion partners have been identified to date, five septins are fused to MLL, making this gene family the most frequently involved (by 10%) in MLL-related acute myeloid leukemia (Cerveira et al. 2006).
MLL chimeric fusion proteins are implicated in aberrations in MLL function and are associated with poor prognosis in leukemia (Cerveira et al. 2006).

It is generally believed that candidate proteins must possess either a transactivation or oligomerization domain in order to be competent for MLL fusion. Though SEPT9 lacks the coiled coil region used for oligomerization, it has been suggested that its N-terminal proline-rich region may be used to form oligomers (Nagata et al. 2004; Cerveira et al. 2006). Taken together, oligomerization of MLL-fused septins may promote leukaemogenesis by altering activity of the MLL protein, possibly through recruitment of transcriptional activators (Cerveira et al. 2006). Consistent with this, oligomerization of MLL-SEPT6 is important for in vitro immortalization of hematopoietic progenitors (Ono et al. 2005).

1.1.6.2 SEPT9 and cancer

In comparison to all other septins, SEPT9 has received the most attention in the field of cancer research. In addition to the MLL fusion, SEPT9 was linked to cancer by the Pedersen group, who found that the SEPT9 locus was a common retroviral integration site in mouse T-cell lymphomas (Sørensen et al. 2000). Furthermore, the SEPT9 locus on chromosome 17 was found to be a frequent site for allelic imbalance in sporadic breast and ovarian cancers (Kalikin et al. 2000; Russell et al. 2000). Additionally, depending on the tumor, SEPT9 expression levels are either upregulated or downregulated (Scott et al. 2005; Bennett et al. 2008).

Septin genes are known for their high degree of alternative splicing, and SEPT9 alone possesses at least five N-terminal isoforms (named SEPT9_v1 to SEPT9_v5) (McDade et al. 2007). Interestingly, there is increasing evidence that SEPT9 isoforms are not functionally equivalent. More specifically, the _v4 transcript has been found to be expressed in normal tissues, whereas the _v4* transcript is the predominant form in tumors. The _v4 and _v4* transcripts encode for the same polypeptide but differ at their extreme 5’ untranslated regions and in their translational efficiencies (McDade et al. 2007). Furthermore, recent studies have
demonstrated that expression of the \_v1 transcript transforms breast epithelial cells, whereas the \_v3 transcript reverses transformation (Gonzalez et al. 2007). Additionally, the \_v1 gene product promotes cell proliferation by stabilizing c-Jun-N-terminal kinase and angiogenesis by preventing degradation of hypoxia-inducible factor 1 (Gonzalez et al. 2009; Amir et al. 2009; Amir et al. 2010). More interestingly, \_v2 promoter methylation has been reported in colorectal cancers and is an effective biomarker for early detection of this disease (Grützmann et al. 2008).

1.1.6.3 Other septins in cancer

It has been demonstrated by several groups that SEPT4 may be involved in cancers. While SEPT4 undergoes alternative splicing to yield four transcripts, the Bradeion and ARTS transcripts are the best characterized (Paavola et al. 1999; Zieger et al. 2000). It was shown that downregulation of these transcripts inhibits tumorigenesis (Tanaka et al. 2002). Furthermore, two other laboratories showed that the ARTS transcript could promote apoptosis (Larisch et al. 2000; Gottfried et al. 2004). Consistent with these studies, Elhasid and colleagues (2004) reported that SEPT4 may act as a tumour suppressor because its expression is lost in most cases of childhood acute lymphoblastic leukaemia.

Furthermore, SEPT2 depletion has been implicated in genomic instability as it induces accumulation of lagging chromosomes and binucleated cells, suggesting that SEPT2 deregulation may be involved in tumorigenesis (Kinoshita et al. 1997; Spiliotis et al. 2005). In agreement with these findings, others have reported upregulation of SEPT2 expression levels in human hepatoma carcinoma cells (Yu et al. 2009).
### 1.2 The adenomatous polyposis coli (APC)

APC is a 2843 residue, 310 kDa gene product of the APC gene, which is ubiquitously expressed in all normal tissues and organs (Kojima et al. 2004). APC is a complex, multi-domain protein mutated in many cases of colorectal cancers (CRCs) (Figure 8). In fact, it is well documented that APC germline mutations in the colonic epithelium is an early event in familial adenomatous polyposis (FAP) coli, an autosomal dominantly inherited disease with a predisposition to CRCs (Powell et al. 1992). This is largely due to APC’s crucial function as a negative regulator of the Wnt pathway, which will be discussed later.
Figure 8: **Diagrammatic representation of the structure of full-length APC containing 2843 amino acids.** Embedded within the N-terminus of APC are armadillo repeats (with an internal NLS), two NESs and three 15 amino acid repeats used for β-catenin binding. Immediately following this is the middle region, which contains the mutation cluster region and three 20 amino acid repeats used for enhanced β-catenin binding. The C-terminus contains interactions that are lost upon APC truncation: the remaining four 20 amino acid repeats, the last three NESs, axin binding (SAMP repeats), two NLSs, basic region (for binding microtubules and actin) and the end binding protein-1 (EB-1) binding domain. Adapted from A novel function of adenomatous polyposis coli (APC) in regulating DNA repair, 271, Jaiswal and Narayan, Cancer Letters, 272-280., © (2008), with permission from Elsevier.

Despite the fact that APC is best known for its intricate function in the Wnt pathway, its cytoskeletal functions have received more attention recently and have also been suggested to contribute to tumorigenesis when deregulated (Rusan & Peifer 2008). Specifically, APC has roles in microtubule dynamics in mitosis (Nakamura et al. 2001), chromosome congression and segregation (Green et al. 2005; Kaplan et al. 2001), cell migration through interaction with actin (Watanabe et al. 2004), and it shuttles in and out of the nucleus using its nuclear localization and export signals (Jaiswal & Narayan 2008). Upon mutation of the APC gene, the C-terminal
region of the protein is truncated, resulting in the uncoupling of interactions with many proteins and cellular structures (Figure 8).

1.2.1  **APC’s roles in Wnt signaling**

1.2.1.1 **Gut tissue homeostasis**

Towards the end of the human alimentary canal lies the small and large intestines (or colon), which are lined by a single layer of epithelial cells. These epithelial cells are made up of invaginations known as crypts, which are surrounded by villus protrusions at their orifices (only in the small intestine) (Figure 9). The crypts are the proliferative part of the intestine, they are monoclonal, and they are maintained by multipotent stem cells (Polakis 2000; Pinto & Clevers 2005).

These stem cells give rise to transit amplifying cells, which then proliferate rapidly into non-proliferating daughter cells, that, in turn, differentiate into four epithelial cell groups: paneth, enterocyte, enteroendocrine and goblet (Pinto & Clevers 2005). Differentiation is finalized when the latter three cells migrate upwards to the top of the villi (in small intestine) or to the surface of the epithelium (in the colon). Here, they undergo apoptosis and are shed into the intestinal lumen (Hall et al. 1994). At the same time, paneth cells migrate downwards, ultimately residing at the base of the crypt. The entire intestinal epithelium is exfoliated every 5 days in humans, the amount of time required for cells to travel from the base of the crypt to the top of the villi or to the surface of the epithelium (Wright & Irwin 1982). This continuous renewal of the intestinal mucosa ensures homeostasis to optimize absorptive functions (Pinto & Clevers 2005).
As previously mentioned, the gut epithelium establishes a balance between stem cell proliferation, migration, differentiation, and apoptosis, and Wnt factors play a crucial role in
maintaining this balance. CRCs are initiated when these processes are perturbed, resulting in excessive proliferation and aberrations in cell migration (Pinto & Clevers 2005). CRCs begin when stem cells at the base of the crypt transform into adenomas, which can eventually develop into malignancies called adenocarcinomas. Approximately 15% of CRCs occur as a result of germline mutations in the APC gene to cause FAP, and 85% of CRC cases occur sporadically (Lynch & de la Chapelle 2003). Somatic mutations in the APC gene are associated with up to 85% of these sporadic CRCs, and heterozygous, activating mutations in the β-catenin gene are found in approximately 10% of the remaining cases of sporadic CRCs (Lynch & de la Chapelle 2003).

1.2.1.2 Truncation of APC in the colonic epithelium

APC is a classic tumor suppressor gene due to the fact that both alleles must be inactivated for loss of function. The APC gene is first truncated due to generation of a premature stop codon as a result of nonsense or frame-shift mutations (Figure 10). The mechanism underlying the lack of APC can be a second truncating mutation, or more typically, a loss-of-heterozygosity (LOH) mutation (Albuquerque et al. 2002).

The site of truncation within the APC protein influences development of CRCs. Germline mutations within the MCR (from amino acids 1263 to 1587) of the APC gene are directly correlated with greater severity in the polyposis phenotype (Albuquerque et al. 2002; Ficari et al. 2000; Polakis 2000). Patients with mutations outside the MCR, closer to the C-terminal region, exhibit milder phenotypes. For instance, animals with truncating mutations in the APC protein at amino acid 1638 do not develop CRCs and survive through adulthood (Smits et al. 1999). Collectively, these data suggest that a truncating mutation is important for
deregulation of APC’s nuclear shuttling abilities and loss of interaction with β-catenin and the cytoskeleton, resulting in aberrant function and tumorigenesis (Fodde & Brabletz 2007).

**Figure 10:** The most frequent truncations of the APC gene observed in CRCs. The full-length, 2843 amino acid protein is truncated at amino acids 1309, 1338 or 1462 due to formation of a premature stop codon within the MCR.

### 1.2.1.3 APC mediates degradation of cytoplasmic β-catenin

Wnts are signaling molecules that play pivotal roles in normal development and are involved in three pathways: Wnt/calcium, planar cell polarity and canonical signaling (Veeman et al. 2003; Katoh 2005; Logan & Nusse 2004; Pinto & Clevers 2005). The canonical pathway (henceforth called the Wnt signaling pathway) is the best characterized due to its vast implications in embryonic development and adult tissue homeostasis. As previously mentioned,
Wnt signaling is an integral part of gut stem cell maintenance and leads to CRCs when deregulated (Logan & Nusse 2004).

The primary feature of this pathway is degradation of the β-catenin oncoprotein by the so-called “destruction complex”, which assembles in the cytoplasm in the absence of Wnt stimulation (Figure 11). The destruction complex consists of two scaffolding proteins, axin and APC, and two kinases, GSK3β (glycogen synthase kinase-3β) and CKIα (casein kinase 1α). Briefly, APC targets cytoplasmic β-catenin to the destruction complex with the help of axin, where it is marked for proteolytic degradation through phosphorylation by GSK3β and CKIα (Liu et al. 2002). APC binds to β-catenin through its 15 amino acid repeats, and the avidity of this interaction is enhanced by binding via the 20 amino acid repeats, which are activated through APC phosphorylation by GSK3β (Rubinfeld et al. 1996; Ha et al. 2004).
Figure 11: The generally accepted model of the canonical Wnt signaling pathway. Left: In the absence of Wnt stimulation, the destruction complex assembles in the cytoplasm to facilitate phosphorylation and degradation of β-catenin. β-catenin cannot enter the nucleus to initiate transcription of genes involved in proliferation. Right: When Wnt binds to its receptor or in the absence of APC, the destruction complex is destabilized and cannot phosphorylate or mediate β-catenin degradation. β-catenin accumulates and translocates into the nucleus, where it interacts with LEF-1/TCF to initiate transcription of genes (such as c-myc and cyclin d1) involved in cell proliferation. Adapted from A Wnt-Wnt Situation, 4, He, Developmental Cell, 791-797., © (2003), with permission from Elsevier.
Conversely, in the presence of Wnt stimulation in normal cells or in the absence of APC in cancer cells, Dishevelled (Dvl), a cytoplasmic scaffold protein, is activated through a poorly understood mechanism. Dvl facilitates disassembly of the destruction complex by mislocalizing axin to the plasma membrane and displacing GSK3β from the destruction complex (Cliffe et al. 2003; Lee et al. 2003; Schwarz-Romond et al. 2007). Ultimately, β-catenin is not phosphorylated or degraded, and it translocates into the nucleus and binds to lymphoid enhancer-binding factor-1/T-cell factor (LEF-1/TCF) to initiate transcription of genes involved in cell proliferation (Behrens et al. 1996; Li et al. 2005; Gan et al. 2008). Constitutive activation of the Wnt pathway initiates tumorigenesis through two mutually exclusive mechanisms: APC truncating mutations or β-catenin activating mutations (Pinto & Clevers 2005). Both mutations allow β-catenin to evade phosphorylation and degradation (Sparks et al. 1998).

1.2.1.4 **Truncated APC can bind to β-catenin**

1.2.1.4.1 **The intricate balance between APC truncation and β-catenin degradation**

It has been recently demonstrated that many truncated forms of APC can associate with β-catenin, albeit less effectively in comparison to the full length protein (Schneikert et al. 2007). It is of note, however, that this binding is insufficient to target β-catenin for degradation as mutant APC is unable to bind axin. Nonetheless, the fact that some binding occurs between truncated APC and β-catenin suggests that redundancies exist within the APC gene to allow the truncated product to perform some of its cellular functions, as complete deletion of this gene is extremely rare and probably detrimental to cells (Näthke 2004). Also, it is possible that truncated APC itself plays a crucial role in tumor progression. This idea was supported recently by studies which showed that depletion of truncated APC in colon cancer cell lines hampered proliferation and initiated apoptosis (Schneikert & Behrens 2006; Brocardo et al. 2008).
Collectively, this suggests that, while cancers benefit from altering APC function, it should be done moderately to maximize the benefits. This “just-right” signaling model is thought to optimize the impairment of APC function such that the right amount of β-catenin accumulates in the nucleus to foster the right amount of intestinal tumorigenesis (Albuquerque et al. 2002). Indeed, prolific amounts of nuclear β-catenin have been shown to lead to apoptosis, effectively countering tumorigenesis (Kim et al. 2000).

1.2.1.4.2 APC sequesters β-catenin in the nucleus

Embedded within the APC protein are three nuclear localization signals (NLSs) and five nuclear export signals (NESs) (Henderson & Fagotto 2002). The most essential NLS appears to be the one within the armadillo domain as truncated APC lacking the other two NLSs is able to enter the nucleus (Smits et al. 1999; Galea et al. 2001). Truncated APC binds weakly to nuclear β-catenin and is able to sequester it within the nucleus, preventing transcriptional activation of its target genes (Neufeld et al. 2000). This sequestration is powered by the fact that the APC- and LEF-1/TCF-binding sites on β-catenin overlap, allowing APC to displace β-catenin from LEF-1/TCF (Orsulic et al. 1999). Conversely, wildtype APC binds to nuclear β-catenin and transports it to the cytoplasm (via its NESs) for degradation.

1.2.2 APC’s Wnt-independent functions – Its role in the cytoskeleton

Though many consequences of APC loss are explained by its function in the Wnt signaling pathway, additional observations in tissues and animal models lacking APC indicated that APC has Wnt-independent functions (Oshima et al. 1997; Sansom et al. 2004). This is consistent with the presence of many domains within the APC protein that interact with cellular structures and proteins not involved in the Wnt pathway (Figure 12). Also, while APC truncation and β-catenin mutation are mutually exclusive and thought to be functionally
equivalent, tumors with β-catenin activating mutations are not as invasive and do not form adenomas as large as those observed in full length APC-deficient tumors (Samowitz et al. 1999). This suggests that other factors besides Wnt deregulation contribute to tumorigenesis upon APC truncation. More specifically, human adenomas with truncated APC experience aberrant migration by moving towards the crypt base instead of upwards towards the top of the crypt, where they should undergo apoptosis (Oshima et al. 1997; Sansom et al. 2004). These data suggest that APC truncation alters the direction of cell migration in the intestinal mucosa. Since cytoskeletal elements play roles in migration of epithelial cells, scientists sought to ascertain the connections between APC and the cytoskeleton in order to fully elucidate its role as a tumor suppressor.

![Cartoon representation of the Wnt-independent interacting partners of APC.](image)

**Figure 12: Cartoon representation of the Wnt-independent interacting partners of APC.** Embedded within the APC armadillo repeats is a nuclear localization signal (NLS) and binding sites for IQGAP1 and KAP3. Two more NLSs are present in the middle to C-terminal regions of APC. The basic region of APC interacts with microtubules (MTs) and F-actin. Lastly, APC interacts with microtubule-associated protein, EB-1, at plus ends. Adapted from A novel function of adenomatous polyposis coli (APC) in regulating DNA repair, 271, Jaiswal and Narayan, Cancer Letters, 272-280., © (2008), with permission from Elsevier.
1.2.2.1 APC and the tubulin cytoskeleton

1.2.2.1.1 APC binds to tubulin

Many studies have shed a great deal of light on a previously unappreciated function of APC: its ability to bind the tubulin cytoskeleton. One of these studies demonstrated that a 200-amino acid stretch within the C-terminal basic domain of APC not only interacts directly with microtubules but also induces microtubule bundling in vitro (Munemitsu et al. 1994). Additionally, endogenous APC in epithelial cells localizes to microtubule ends in fixed samples, specifically in areas of active migration (Näthke et al. 1996). APC was later found to travel along microtubule cables towards their plus ends and also to stabilize microtubules (Figure 13) (Mimori-Kiyosue et al. 2000; Mimori-Kiyosue et al. 2000; Zumbrunn et al. 2001).

**Figure 13: APC stabilizes microtubule networks in vivo. Left:** Distribution of tubulin in PTK2 cells transfected with wildtype APC. Red arrow shows robust microtubule networks. **Right:** PTK2 cells transiently transfected with GFP-APC\(\Delta\)MT (lacking residues 2168-2451) experienced unstable microtubule networks. Red arrow shows reduced microtubule networks. Adapted from Zumbrunn and colleagues (2001).
1.2.2.1.2 APC binds to microtubule-associated proteins and clusters at membrane protrusions

Interestingly, APC can also interact with microtubules in a manner independent of the basic domain and dependent on two microtubule-associated proteins: EB-1 and kinesin-associated-protein 3 (KAP3). APC mutants lacking the basic domain bind indirectly to microtubules through association with EB-1, via the EB-1 binding domain immediately following the basic domain (Su et al. 1995). Interestingly, APC-2 and APC-L, homologs of APC that lack the basic domain, interact with EB-3, a relative of EB-1, suggesting that this interaction is conserved and may be an alternate mechanism for facilitating APC interaction with microtubules (Nakagawa et al. 2000).

A second mechanism of mediating APC interaction with microtubules is through KAP3. Briefly, Jimbo and colleagues (2002) demonstrated that APC associates with microtubule-dependent motor proteins KIF3A-KIF3B through direct interaction with KAP3. Uncoupling the KAP3-APC interaction hampered APC cluster accumulation at the ends of microtubules, which was concomitant with a decrease in cell migration (Jimbo et al. 2002). However, more work is needed to determine whether APC clusters at microtubule plus ends are transported by the KIF3A-KIF3B-KAP3 complex or if APC is recruited to microtubule plus ends that already contain the KIF3A-KIF3B-KAP3 complex (Jimbo et al. 2002). Consistent with the former possibility, previous studies showed that APC movement to microtubule plus ends was abrogated upon treatment with ATP poisons (Mimori-Kiyosue et al. 2000). Lastly, though APC’s N-terminal armadillo repeats are sufficient for interacting with KAP3, truncated APC (lacking only the C-terminus) is unable to cluster at microtubules ends in colon cancer cell lines despite the presence of the KAP3-binding site. These data suggest that other proteins which
interact with the C-terminus of APC may be required, not for facilitating the interaction, but for proper functioning of the APC-KAP3 complex (Jimbo et al. 2002).

1.2.2.1.3 Overview of APC function in mitosis

In interphase, APC localizes to relatively few microtubules and is enriched at only a small number of clusters near the cell periphery (Mimori-Kiyosue et al. 2000b; Dikovskaya et al. 2001a). Conversely, during mitosis, APC localizes to microtubules and co-operates with EB-1 at the cortical attachment site of astral MTs, where both proteins position and anchor the mitotic spindle (Tirnauer & Bierer 2000). APC and EB-1 are also important for high fidelity chromosome congression and segregation (Draviam et al. 2006; Zhang et al. 2007). Furthermore, two independent studies demonstrated that APC loss causes excessive spindle rotation and failed spindle anchorage (Draviam et al. 2006; Caldwell et al. 2007). In fact, these APC-depleted cells experienced mitotic slippage as they exited mitosis prior to forming a cytokinetic furrow, leading to formation of cells with more than one nucleus (Figure 14) (Caldwell et al. 2007). Notably, the function of the APC-EB-1 interaction is evolutionarily conserved, as their yeast homologs, Bim1 and Kar9, respectively, are required for migration and positioning of the nucleus to facilitate equal partitioning of genetic material between the mother and the bud (Beach et al. 2000; Bloom 2000). Mammalian and yeast APC and EB-1 have also been shown to localize to the centrosomes during mitosis and also co-purify with centrosome components (Louie et al. 2004). Lastly, similar to yeast and mammalian septins, Kar9 depletion has been reported to cause formation of binucleated cells (Miller & Rose 1998).
Figure 14: Formation of tetraploid cells in the absence of APC due to spindle rotation and premature mitotic exit. Upon APC truncation, the mitotic apparatus is unable to anchor the mitotic spindle, and it undergoes a series of rotations. During rotation, the cell experiences mitotic slippage before formation of the cytokinetic furrow, generating binucleated cells. Adapted from © Rusan and Peifer; 2008, Journal of Cell Biology. 181:719-726.

Stable microtubules are required for formation of the mitotic spindles which facilitate equal division of DNA during mitosis. Interestingly, APC has been shown to facilitate proper kinetochore capture by microtubules and to stabilize this interaction (Figure 15) (Fodde et al. 2001; Kaplan et al. 2001; Rusan & Peifer 2008). Consistent with this observation, APC truncation induces formation of lagging chromosomes by interfering with chromosome congression and segregation, ultimately giving rise to aneuploidy (Figure 16) (Kaplan et al. 2001; Zhang et al. 2007).
Figure 15: APC localizes at kinetochores to facilitate microtubule capture. Panel A: In mitotic PTK2 cells, endogenous APC (red) localizes to kinetochore proteins, marked with CREST (green), where it clusters at microtubule plus ends (magenta). DNA is shown in blue, and APC is indicated by white arrow. Scale bar represents 5 µm. Panel B: Enlarged ten-fold in comparison to panel A, and APC is marked by white arrow. Adapted from Dikovskaya and colleagues (2001).
Figure 16: Loss of APC induces genomic instability caused by formation of lagging chromosomes. Panel A: In the presence of wildtype APC, the microtubule (MT)-kinetochore interaction is stable, allowing equal portioning of chromosomes in anaphase and progression to euploidy. Panel B: In the absence of APC, the MT-kinetochore interactions are unequal or weak, giving rise to lagging chromosomes. Lagging chromosomes are not pulled apart symmetrically due to microtubule destabilization, giving rise to aneuploidy. Purple arrow points to lagging chromosome in daughter cell. Adapted from © Rusan and Peifer; 2008. Originally published in Journal of Cell Biology, 181:719-726.
Since APC truncation is a very early event in CRCs and since CRCs exhibit genomic instability, APC was proposed to play a role in maintaining genomic stability (Rusan & Peifer 2008). This assertion is supported by the fact that APC depletion causes formation of binucleated cells and lagging chromosomes, which are hallmarks of genomic instability (Figures 14 and 16) (Caldwell et al. 2007; Rusan & Peifer 2008). Lastly, APC has been found to localize to the midbody bridge during mitosis, though the functional significance of this localization is currently unclear (Munemitsu et al. 1994; Hanson & Miller 2005).

1.2.2.2 APC and the actin cytoskeleton

1.2.2.2.1 APC interacts with F-actin and actin stress fibers

Moseley and colleagues (2007) unambiguously showed that the C-terminal, basic region of APC associates directly with F-actin, bundles actin filaments and co-localizes with stress fibers. Further, they found that microtubules and F-actin competed for APC binding, and that EB-1 expression promotes the former interaction (Moseley et al. 2007). Others have also found that APC localizes to actin-rich junctions in the gut epithelium (Langford et al. 2006; Langford et al. 2006). Under certain concentrations, Moseley and colleagues also determined that APC could crosslink actin and microtubules.

Interestingly, other labs have demonstrated that the APC-tubulin interaction is the dominant cytoskeletal association in mammalian cells. However, when the tubulin cytoskeleton is disrupted, APC clusters are released from microtubule ends and associate with the actin cytoskeleton (Mimori-Kiyosue et al. 2000). The APC-cytoskeleton interaction that is favored may depend on the presence of microtubule-binding domains and APC’s interaction with other proteins. For instance, APC-2/L or E-APC, homologs of APC which lack microtubule-binding domains, may favor the actin cytoskeleton (Dikovskaya et al. 2001). While the studies
summarized here demonstrated a crucial link between APC and the two major cytoskeletal elements, it was Kaibuchi’s group who elucidated the physiological relevance of these interactions by linking APC to IQGAP1.

1.2.2.2 APC is involved in polarized cell migration

The Kaibuchi group showed that IQGAP1 interacts directly (in an actin-dependent manner) with the armadillo repeats of APC, and more importantly, this interaction was shown to be involved in cell polarization and migration (Watanabe et al. 2004). It was also determined that depletion of APC or IQGAP1 hampered re-orientation of the microtubule organizing centre during wound healing and recruitment of actin to leading edges, necessities for directional migration. Furthermore, APC loss inhibited formation of the actin meshwork and recruitment of IQGAP1 to the leading edges of migrating cells, and vice versa (Watanabe et al. 2004). Altogether, Kaibuchi’s group demonstrated that, during migration, IQGAP1 cross-links actin filaments and recruits APC, which, in turn, stabilizes microtubules directly and/or indirectly to promote formation of the stable actin meshwork needed for migration at leading edges (Watanabe et al. 2004). Lastly, it is noteworthy that, similar to the KAP3-APC interaction, truncated APC can still bind to IQGAP1, but this interaction is insufficient to promote IQGAP1 accumulation at leading edges or for polarized cell migration (Watanabe et al. 2004).

1.3 Rationale and Hypothesis

The mass spectrometry data obtained in the Angers lab identified SEPT2, 9, 10 and 11 as part of an APC interactome. Due to the large body of literature indicating several interesting overlaps in septin and APC functions, we decided to further investigate this proteomic data. APC and septins localize to similar cellular structures such as the midbody, actin and microtubule networks (Moseley et al. 2007; Surka et al. 2002; Rittmeyer et al. 2008; Watanabe et al. 2004; Spiliotis & Nelson 2006). Interestingly, APC and septins have opposing roles in
microtubule stability, where APC stabilizes microtubules networks and SEPT9 destabilizes them (Kremer et al. 2005; Zumbrunn et al. 2001).

Additionally, independent depletion of septins and APC in both yeast and mammalian systems are associated with phenotypes including chromosome congression and segregation defects and binucleation, giving rise to genomic instability (Hartwell et al. 1973; Surka et al. 2002; Draviam et al. 2006; Spiliotis et al. 2005; Caldwell et al. 2007; Miller & Rose 1998). During the preparation of this thesis, the Liakopoulos lab published a paper showing that the APC yeast homologue, Kar9, is degraded during mitosis to optimize spindle positioning and anchorage and that this degradation is abrogated in yeast septin mutants (Kammerer et al. 2010). Based on these similarities and the mass spectrometry data, we decided to further explore this interaction. To this end, we proposed two hypotheses:

1. Septins interact endogenously with APC

2. This interaction is involved in genomic instability.

To investigate these hypotheses, we performed immunoprecipitation and co-localization studies using endogenous proteins from ARPE-19, HCT116 and SW480 cells. HCT116 and SW480 cell lines are colon cancer cell lines, where the former harbors full-length APC with a β-catenin phosphorylation-resistant mutant and the latter expresses truncated APC with wildtype β-catenin. Once the interactions were verified, we completed single and double siRNA-mediated depletions of septins and APC in ARPE-19 cells in order to elucidate the functional relevance of these associations. ARPE-19 cells were chosen due to their good karyotype.
Chapter 2
Materials and Methods

2.1 **Mass Spectrometry**

Endogenous APC was immunoprecipitated in Dr. Stephane Angers’s laboratory (Leslie Dan Faculty of Pharmacy, University of Toronto, Ontario, Canada), and the associated proteins were subjected to tandem mass spectrometry. APC was immunoprecipitated from HCT116 cells using the C-APC Ab (see section 2.4).

2.2 **cDNA expression constructs**

A cDNA construct expressing a truncated form of human (h) APC (pEGFP-C3-APC-1309) was obtained from Dr. Kathleen Goss (University of Chicago, Illinois, USA). cDNA constructs expressing other truncations of hAPC (pEGFP-C1-APC-M1, pEGFP-C1-APC-M2 and pEGFP-C1-APC-CT) were kind gifts from Dr. Kozo Kaibuchi (Nagoya University, Aichi, Japan). Two cDNA constructs (pcDNA3.1-FLAG-SEPT9_v1 and pEGFP-SEPT9_v1) expressing the longest isoform of hSEPT9 were obtained from Dr. Carol Froese (Trimble Laboratory, Toronto, Canada).

2.3 **Reagents**

Octyl-β-D-glucopyranoside (OGP) and nonidet P-40 (NP-40) were obtained from BioShop (Ontario, Canada), whereas Triton X-100 (TX-100) was obtained from Sigma Aldrich (Missouri, USA). Unless otherwise stated, all reagents used for polymerase chain reaction (PCR), including the PCR purification kit, were purchased from Bio Basic Incorporations (Ontario, Canada). All materials used for digestion and ligations were obtained from New
England Biolabs (NEB, Massachusetts, USA) unless otherwise specified. All double stranded, small interfering RNAs (ds siRNAs) were from Dharmaco (Colorado, USA) and synthesized with dTdT overhangs at each 3' terminus. Protein (Prot) A and ProtG sepharose beads were obtained from Sigma Aldrich and GE Healthcare Life Sciences (New Jersey, USA), respectively. All immunoglobulin (IgG) sera were obtained from Sigma Aldrich. Lipofectamine (Lipo) 2000 and fluorescent secondary Abs were bought from Invitrogen (California, USA). Secondary HRP-conjugated Abs were purchased from Cedarlane (Ontario, Canada). The NuPAGE antioxidant was obtained from Invitrogen. Lastly, β-mercaptoethanol, bisbenzimide stain and DAKO were obtained from BioShop, Sigma Aldrich, and Invitrogen, respectively.

2.4 Antibodies

Three antibodies (Abs) against hAPC were used in our studies: rabbit C-APC (A300-981A) from Bethyl (Texas, USA), mouse APC-ALi 12-28 from Abcam (Massachusetts, USA) and mouse APC-Ab-7 from Calbiochem (California, USA). 10C10, the only mouse monoclonal septin Ab used in our studies, was previously generated in our laboratory. All other septin Abs used in our studies are rabbit polyclonal Abs and were also lab-generated. All septin Abs were used for immunoprecipitations and Western blots as required. A mouse monoclonal FLAG Ab was used for both immunoprecipitation and Western blot experiments (Sigma Aldrich). A rabbit polyclonal Ab was used to immunoprecipitate GFP-tagged proteins (Invitrogen), and a monoclonal version (Santa Cruz, California, USA) of the Ab was used for Western blotting. A mouse monoclonal Ab directed against GAPDH was obtained from Millipore (Massachusetts, USA) and used for Western blotting. A mouse monoclonal α-tubulin Ab was obtained from Sigma Aldrich and used for Western blotting and immunofluorescence.
2.5 Cell culture and transfection

HEK293 and ARPE-19 cells were obtained from American Type Culture Collection (ATCC, Virginia, USA). SW480 and HCT116 cells were kindly provided by Dr. Stephane Angers (Leslie Dan Faculty of Pharmacy, University of Toronto, Ontario, Canada). HEK293, SW480 and HCT116 cells were maintained in DMEM supplemented with 10% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin and 2mM L-glutamine (Wisent, Quebec, Canada). ARPE-19 cells were maintained in DMEM/F-12 supplemented with 10% FBS, L-glutamine (365.10 mg/L), 15mM HEPES and 3.151 g/L glucose (Lonza, Maryland, USA). All cells were kept at 37°C under 5% CO₂.

SW480, HEK293 and ARPE-19 cells were transfected using Lipo 2000 according to the manufacturer’s instructions. Briefly, Lipo 2000 was incubated for 5 min at room temperature (RT) in the desired volume of serum-free DMEM. Then, the vector or siRNA to be transfected (already added to an equal volume of serum-free DMEM) was added to the Lipo 2000-DMEM mixture and allowed to incubate for 20 min at RT before being added drop-wise to cells. The nucleic acid-Lipo 2000 mixture was removed five hours (h) later and replaced with fresh media.

2.6 PCR-cloning of FLAG-APC-NT

2.6.1 PCR

pcDNA3.1-FLAG-APC-NT was constructed using pEGFP-APC-1309 as a template with 5’-GACCCCGGAATTTCATGGCTGCAGCATTATGATCAGTGG-3’ as the forward primer and 5’-CTTATCGTGCAGACTGCTGCTATTCTTCGTGCTGCTC-3’ as the reverse primer. For the forward and reverse primers, EcoRI and Sall sites are underlined, respectively. To make a 100 μL PCR reaction mixture, 5 μL each of the template and both forward and reverse primers
were added to 72 µL of autoclaved distilled water (dH₂O). This was followed by addition of 2 µL dNTPs, 10 µL TSG plus 10X reaction buffer and, finally, 1 µL TSG plus DNA polymerase. PCR was performed using the MJ Research PTC-200 Peltier Thermal Cycler according to the manufacturer’s instructions (GMI Incorporations, Minnesota, USA). Briefly, the template was unwound at 95ºC for 1 min, annealed to primers at 55 ºC for 0.5 min and extended at 72 ºC for 1 min for a total of 30 cycles. The PCR product was purified using a PCR purification kit and eluted in 50 µL of elution buffer (EB).

2.6.2 Digestion

Both the PCR product and the empty FLAG vector were digested in a 50 µL mixture at 37 ºC for 1 h. Briefly, 1 µL each of SalI and EcoRI were added to a mixture containing 2 µL purified PCR product or empty FLAG vector, 34 µL of autoclaved dH₂O, 5 µL of 10X BSA and 5 µL of NEBuffer 3. Digestion products were purified again using a PCR purification kit and eluted in 50 µL of EB.

2.6.3 Ligation

Digested, PCR-purified NT was used in a 3 molar excess when compared to the digested, PCR-purified FLAG empty vector. Ligation was performed in a 20 µL mixture by adding appropriate amounts of NT and/or FLAG empty vector (from section 2.6.2) to autoclaved dH₂O, 2 µL T4 DNA ligase 10X buffer and, finally, 1 µL T4 DNA ligase. FLAG-NT and FLAG-control ligations were allowed to proceed for 15 min at RT, followed by transformation into DH5α. For transformation, 50 µL competent DH5α were added to each 20 µL mixture and incubated on ice for 10 min. Samples were heat-shocked at 42ºC for 1 min, which was followed by another 10 min incubation on ice. 1 mL of SOC was added to the DH5α-
ligation mixture, and this was followed by a 2 h rescue at 37 °C with shaking. The samples were centrifuged at 400 x g for 5 min and plated on LB-agar (supplemented with 0.1 mg/mL AMP) overnight in a 37°C incubator. Colonies were picked the following day and grown in LB containing 0.1 mg/mL AMP overnight in a 37°C shaker. Plasmids were isolated from several colonies using the GeneJET Plasmid Miniprep Kit (Fermentas Life Sciences, Ontario, Canada), and those containing the inserts (confirmed by digestion with EcoRI and SalI) were sequenced completely (ACTG Corporations, Ontario, Canada).

2.7 Investigation of the SEPT-APC association in vivo

2.7.1 Immunoprecipitations (IP)

2.7.1.1 Immunoprecipitations of endogenous proteins from HCT116 cell lysates

HCT116 cells from a confluent dish were harvested via scraping, centrifuged at 130 x g for 10 min and washed with cold PBS. Cells pelleted after suspension in PBS were treated with 1 ml of cold IP buffer (20 mM Tris, pH 7.4, 100mM NaCl, 2 mM EDTA and 1% NP-40) supplemented with (1:25 of a 10 mL IP buffer solution containing) a mini protease inhibitor tablet (Roche, Mannheim, Germany). In addition, the cells were lysed using a 25G needle, and the lysate was rotated end-to-end at 4°C for 15 min. During this incubation, 20 μL of a ProtG was blocked with 3% BSA for the next 2.5 h at 4°C with end-to-end rotation. The lysate was then centrifuged at 14,000 x g for 15 min at 4°C, and about 1.0 mg of the supernatant was pre-cleared for 45 min using 20 μL of a 50:50 slurry of ProtG sepharose beads. The beads were discarded and the supernatant was incubated with 2.5 μg of 10C10 Ab (to immunoprecipitate endogenous SEPT9) or 2.5 μg of mouse IgG for 1h at 4°C with end-to-end rotation. After this incubation, the BSA-blocked beads were washed three times with IP buffer and then incubated with the lysate-Ab mixture overnight at 4°C on a rotator. The next morning, the beads were
washed four times with IP buffer, and all samples were suspended in 20 μL of SDS-PAGE loading dye and 2 μL β-mercaptoethanol. The immunoprecipitation samples were then boiled for 10 min for Western blot analyses.

To investigate the reciprocal interaction, another experiment was performed exactly as described above, except endogenous APC was immunoprecipitated using 4 μg of the C-APC Ab, and ProtA sepharose beads were used instead of ProtG beads.

2.7.1.2 Immunoprecipitations of epitope-tagged proteins from HEK293 cell lysates

HEK293 cells were transfected with either GFP-APC-N and FLAG-SEPT9_v1, GFP-APC-M1 and FLAG-SEPT9_v1, GFP-APC-M2 and FLAG-SEPT9_v1, GFP-APC-CT and FLAG-SEPT9_v1 or GFP-SEPT9_v1 and FLAG-APC-NT. Transfection was completed as previously described in section 2.5. Briefly, each co-transfection was performed by mixing 20 μL of Lipo 2000 (in 1 mL serum-free DMEM) with 4 μg of each plasmid (also in 1 mL serum-free DMEM). After sufficient incubation, the Lipo 2000-DNA mixture was added drop-wise to a 70% confluent 10 cm dish of cells and later replaced with fresh media. Two days after transfection, cells were collected through scraping and lysed as mentioned in section 2.7.1.1 except 1% NP-40 was replaced with 1% OGP. Other changes include pre-clearing 500 μg of supernatant with a combined 20 μL amount of 50:50 slurries of both ProtA and G sepharose beads. The beads were discarded and the supernatant was incubated with 1.5 μg of FLAG Ab, 1.5 μg of mouse IgG, 1.5 μg GFP or 1.5 μg rabbit IgG for 1 h at 4°C with end-to-end rotation. Again, all beads were blocked with 3% BSA and washed before incubation with the lysate-Ab mixture overnight. All subsequent steps were completed as described in section 2.7.1.1.
2.7.1.3 Immunoprecipitations of endogenous proteins from SW480 cell lysates

A confluent dish of SW480 cells, with a subset of cells kept aside for immunofluorescence analysis (see section 2.7.2), was harvested and lysed with OGP as described in sections 2.7.1.1 and 2.7.1.2. In addition, the supernatant was pre-cleared using 20 μL of a 50:50 slurry of ProtG sepharose beads. The beads were discarded and the supernatant was incubated with 1.5 μg of 10C10 Ab (to immunoprecipitate endogenous SEPT9) or 1.5 μg of mouse IgG for 1h at 4°C with end-to-end rotation. All subsequent steps were completed as described in sections 2.7.1.1 and 2.7.1.2.

2.7.2 Immunofluorescence – Part I: Co-localization studies of septins and APC

Cells from sections 2.7.1.3 were set aside for immunofluorescence. Briefly, SW480 cells were grown on 25 mm coverslips overnight. At RT, cells were fixed for 20 min with 3% formalin in PBS, inactivated using 25 mM ammonium chloride and 25 mM glycine in PBS for 15 min and permeabilized using 0.2% TX-100 in PBS for 10 min. Coverslips were blocked in 3% BSA in PBS overnight at 4°C and stained using primary (1°) and secondary (2°) Abs the following morning. Briefly, 1° Abs used for staining were diluted in 80 μL of PBS and incubated on parafilm for 1.5 h at RT and then washed three times in PBS. Ab dilutions are as follows: APC-Ab-7 1:70, SEPT2 Ab 1:500, SEPT9 Ab 1:100 and SEPT11 Ab 1:500. Coverslips were then incubated with either DαM Alexa 488 (A488) or DαR Cyanine 3 (Cy 3) fluorescent 2° Abs to detect 1° Abs against APC and septins, respectively. All 2° Abs were used at dilutions of 1:1000 in 100 μL PBS on parafilm and incubated for 1h at RT. Coverslips were then incubated with bisbenzimide stain (DAPI; 1:10,000) in PBS for 5 min and subsequently washed four times with PBS. Coverslips were finally mounted onto glass slides with DAKO.
The cells were viewed and imaged under the 63X oil immersion objective on a Leica DM IRB Quorum Spinning Disk Confocal Microscope equipped with a Hamamatsu 9100-12 EM-CCD camera and Improvision Volocity 5 Image Acquisition Software (Leica, Wetzlar, Germany). For Z stack acquisition, 3 µm slices of cells were obtained. To detect the A488, Cy3 and DAPI signals, 491 nm, 561 nm and 405 nm lasers were used, respectively. Images were co-localized using Volocity 5 and exported as TIFF files.

2.8 Western blots

2.8.1 Detection of septins, truncated APC and GAPDH

Samples for Western blot analyses were resolved on 10% polyacrylamide gels and transferred onto PVDF membranes at 200 mA for over 2 h. The membranes were blocked overnight in 5% milk-PBST at 4°C on a shaker and probed with 1° and 2° Abs the next day for 1 h each. The following Abs were used for Western blots: mouse FLAG 1:7500, rabbit SEPT2 Ab 1:1000, rabbit SEPT9 Ab 1:1000, rabbit SEPT11 Ab 1:5000, mouse GAPDH 1:100,000 and mouse GFP 1:500. 2° HRP-conjugated Abs against the mouse and rabbit 1° Abs were used at dilutions of 1:5000. After washing, blots were treated with Western Lightning Plus ECL reagent (PerkinElmer, Massachusetts, USA) and exposed to autoradiography film.

2.8.2 Detection of full-length APC and alpha-tubulin

To resolve full-length APC, samples for Western blot analysis were run on 3-8% Tris-Acetate Gels (Invitrogen). Lysate and buffer preparations, resolution on gel and protein transfer onto PVDF were completed according to the manufacturer’s instructions. Briefly, the gels were run at a minimum voltage of 150 V, and proteins were transferred onto PVDF overnight at 10 V. During gel resolution and transfer, appropriate amounts of NuPAGE antioxidant were added to
the buffers according to the manufacturer’s instructions. The next day, the PVDF was blocked in 5% milk-TBST for 7 h and was incubated with the APC-ALi Ab diluted 1:1,000 overnight at 4°C on a shaker. The lower MW area of the blot (around 60 kDa) was incubated with the α-tubulin Ab in 3% BSA (1:5,000) at 4°C overnight with constant shaking. The next day, the blots were washed for 1 h, and 2° Abs and application of ECL proceeded as described in section 2.8.1.

2.9 Double stranded, small interfering RNAs (ds siRNAs)

We used a ds siRNA sequence which has been previously shown to effectively knockdown hAPC (5’-AGGGGCAGCAACTGATGAAAA-3’) (Brocardo et al. 2005; Brocardo et al. 2008). As control, we used siRNA directed against rodent (r) SEPT9, which does not recognize the human counterpart (5’-GCAGCGACCATGAGTATCA-3’). For the knockdown studies, I obtained working aliquots (see below) of siRNAs against hSEPT 2, 9 and 11 and rSEPT9 from Dr. Caterina Di Ciano-Oliveira (Trimble Laboratory, Toronto, Canada). siRNAs against hSEPT2, 9, and 11 are, respectively, listed as follows: 5’-GAATATTGTGCCTGTCATTG-3’, 5’-GCACGATATTGAGGAGAAA-3’ and 5’-CAAGAGGAATTGAAGATTAAA-3’.

All ds siRNAs were dissolved in DEPC-treated water to a concentration of 40 pmol/µL and stored at -80°C in working aliquots. ARPE-19 cells grown to 60% confluence in 6-well plates were transfected with siRNA using Lipo 2000 as described in section 2.5. Briefly, 3 µL of Lipo 2000 (in 400 µL of serum-free DMEM) were added to 360 pmol ds siRNA, which was already dissolved in 400 µL of serum-free DMEM. After allowing sufficient incubation of the Lipo 2000-siRNA mixture, the 800 µL solution was added to one well of a 6-well plate. The cells were subsequently washed and re-plated as required for Western blots,
immunofluorescence and time-lapse microscopy. With the exception of time-lapse microscopy which began approximately 60 h post-transfection, all other analyses were performed 72 h after knockdown.

Each knockdown condition was completed in one well of a 6-well plate. For single knockdowns, 360 pmol of one type of ds siRNA was used. For double knockdowns, 360 pmol of each siRNA was combined to make 720 pmol. Briefly, eight knockdowns were prepared: siCntrl, siAPC, siSEPT2, siSEPT9, siSEPT11, siAPC/SEPT2, siAPC/SEPT9 and siAPC/SEPT11.

3.0 **Quantification of mitotic defects in ARPE-19 cells**

3.0.1 **Immunofluorescence – Part II: Analyses of mitotic defects in ARPE-19 cells**

3.0.1.1 **Scoring for cytokinetic and abscission defects in fixed ARPE-19 cells**

ARPE-19 re-plated from the siRNA studies were grown on coverslips and rinsed with warm PBS. Cell fixation and staining were performed as previously explained in section 2.7.2 except only tubulin and DAPI were stained here. For three independent experiments, 200 cells were counted for each knockdown. For every 200 cells counted, the percentages of interphase cells with greater than one nucleus and mitotic daughter cells joined by midbodies were documented. Counts were performed using the 63X oil immersion objective on a Leica DMIRE2 Inverted Fluorescence Microscope (Improvision, California, USA), and images were captured using a Confocal Microscope as in section 2.7.2.
3.0.1.2 **Scoring for lagging chromosomes in ARPE-19 fixed-cell preparations**

ARPE-19 re-plated from the siRNA studies were grown to 70% confluence in a 6-well plate such that one well represented one knockdown condition. 55 h post-transfection, 50 ng/mL of nocodazole were added to the cells. Sixteen hours later, the nocodazole-treated cells were washed off using a 1 mL pipette and allowed to attach on poly-D-lysine-coated coverslips for 5 min. Coverslips were incubated with a solution of poly-D-lysine (Sigma Aldrich) for at least 1 h at RT with constant agitation, and the coverslips were washed thoroughly with PBS after coating. After attachment to coated coverslips, the cells were released from nocodazole treatment by washing three times with DMEM. Nocodazole release proceeded for 1 h, and the cells were prepared for staining as previously described in section 2.7.2 except only DNA was stained here. For three independent experiments, 200 cells were counted for each knockdown. For every 200 cells counted, the percentage of cells in telophase with lagging chromosomes was documented. Cell counts and image acquisitions were performed as described in sections 2.7.2 and 3.0.1.1.

3.0.2 **Time-lapse microscopy in ARPE-19 cells**

ARPE-19 cells treated with single or dual siRNAs were imaged in HPMI (RPMI powder (Gibco/Invitrogen) supplemented with 15mM NaCl and 20mM HEPES, pH 7.4) at 37°C on a Nikon TE2000 Inverted Microscope adapted with a Solent environmental chamber. A Nikon Plan-Fluor 40X/0.6NA objective was employed, and DIC images were acquired using a Hamamatsu camera every 3 min. Volocity 5 was used for image acquisition, analysis and manipulation. The time from anaphase onset to midbody abscission was determined for each cell that started division within 12 h of imaging. Imaging was initiated approximately 60 h after
siRNA transfection. Cells that did not unambiguously complete abscission were excluded from the analyses.

3.0.3 **Statistics**

Statistical significance between the different conditions in the siRNA studies was determined by performing one-way Analysis of Variance (ANOVA) using Instat Software (version 3.05, GraphPad) (Ottawa General Hospital, Ontario, Canada) with a 95% confidence interval. In the event that ANOVA allowed rejection of the null hypothesis, the Student-Keuls Newman multiple comparison test was employed to determine where significance existed.
Chapter 3
Results

3.1  A septin complex comprising of SEPT2, 9, 10 and 11 is a novel interacting partner of APC in HCT116 cells

To identify novel interacting partners of APC, the Angers lab immunoprecipitated endogenous APC from HCT116 colon cancer cell lines, and the sample was analyzed using tandem mass spectrometry (Figure 17). Though many signals were identified, as a septin lab, we focused on APC’s putative interaction with the septin complex. The septin complex identified in the screen comprised SEPT2, 9, 10 and 11 (Figure 17). SEPT9 produced the greatest number of peptides, followed by septin 2, 10 and, finally, 11. Specifically, SEPT9 produced 26 peptides, and septins 2, 10, and 11 produced 15, 7, and 5 peptides, respectively. Interestingly, SEPT2 and 9 produced more peptides than axin-2 and β-catenin, which are bona fide APC-binding partners. These findings raised the possibility that septins may be physiological interacting partners of APC.
Figure 17: Septins 2, 9, 10 and 11 are novel APC-interacting proteins in HCT116 cells. Mass spectrometry data from the Angers lab suggests that a septin complex comprised SEPT2, 9, 10 and 11 is a putative interacting partner of APC. Other known APC-binding partners, axin-2 and β-catenin, were also identified in the screen. SEPT2 and 9 produced greater numbers of peptides than axin-2 and β-catenin. Curved-edged rectangles indicate known APC-interacting partners, whereas normal rectangles show septins as novel APC-interacting partners.
3.2 Full-length APC and septins interact endogenously in HCT116 cells

To further investigate the mass spectrometry data obtained by the Angers lab, I immunoprecipitated the endogenous septin 2/9/10/11 complex and APC from HCT116 cell lysates. The septin complex and APC were immunoprecipitated from 1 mg of cell lysates, and the samples were resolved on Western blots (Figure 18). To bring down the septin complex, SEPT9 was immunoprecipitated using our lab-generated 10C10 Ab, and APC was immunoprecipitated using the C-APC commercial Ab.

Figure 18: Full-length APC and septins interact endogenously in HCT116 cells. Immunoprecipitations of the endogenous SEPT2/9/10/11 complex and APC from HCT116 cell lysates. Panel A: APC co-immunoprecipitates endogenously with the septin complex but not with mouse IgG alone (3rd lane versus 2nd lane). Red arrow indicates APC enrichment in SEPT9 immunoprecipitation. Panel B: Immunoprecipitation of endogenous APC (but not mouse IgG) pulls down the septin 2/9/10/11 complex, which was confirmed by detecting SEPT9 on Western blots (3rd lane versus 2nd lane). Green arrow indicates SEPT9 enrichment in APC immunoprecipitation. APC was immunoprecipitated and detected on Western blots using the C-APC Ab, while SEPT9 was immunoprecipitated with 10C10 and probed on Western blots with the lab-generated, SEPT9 rabbit Ab. Western blots were resolved with 10% input. APC is not visible in the input lanes due to C-APC Ab limitations (see discussion).
As control, rabbit IgG and mouse IgG were added to the lysates in the absence of C-APC and 10C10, respectively. Mouse IgG and 10C10 were captured using ProtG beads, while rabbit IgG and C-APC were captured using ProtA beads. The Western blots were probed for APC and SEPT9 to monitor their enrichment in the immunoprecipitations. Although the APC protein is difficult to immunoprecipitate and resolve on Western blots due to its large size, it is, nonetheless, evident that APC is enriched in the SEPT9 immunoprecipitation in comparison to mouse IgG alone (Figure 18, Panel A). In the reciprocal experiment, the septin complex co-immunoprecipitates with APC but not with rabbit IgG alone (Figure 18, Panel B). Taken together, these data demonstrate that septins and full-length APC are endogenous interacting partners.

3.3 The N-terminus of APC (1-1309) interacts with septins in HEK293 cells

To determine which region of APC is responsible for interacting with septins, I tested different truncations of full-length APC for the ability to associate with septins. To this end, I performed immunoprecipitation experiments with epitope-tagged proteins. Briefly, HEK293 cells were co-transfected with either GFP-APC-N and FLAG-SEPT9, GFP-APC-M3 and FLAG-SEPT9 or GFP-APC-CT and FLAG-SEPT9. In all cases, the GFP epitope was immunoprecipitated from 0.5 mg lysates using the GFP rabbit Ab, which was subsequently detected on Western blots with the GFP and FLAG mouse Abs (Figure 19). Under these conditions, only GFP-APC-N was found to interact with FLAG-SEPT9, suggesting that the interaction may be confined to the N-terminus of APC (Figure 19, Panel A versus Panels B and C).
Figure 19: The N-terminus of APC (1-1309) interacts with septins in HEK293 cells while the C-terminus and middle regions are insufficient for the interaction. Immunoprecipitation of GFP-APC-N, GFP-APC-M3 or GFP-APC-CT co-expressed with FLAG-SEPT9 from HEK293 cell lysates. In all cases, GFP was immunoprecipitated using the GFP rabbit Ab and the Western blots were probed with both the GFP and FLAG mouse Abs. Panel A: FLAG-SEPT9 was detected in the GFP-APC-N immunoprecipitation but not in the rabbit IgG control (3rd lane versus 2nd lane). Green arrow shows that FLAG-SEPT9 is enriched in GFP-APC-N immunoprecipitation. Panels B and C: GFP-APC-M3 and GFP-APC-CT do not interact with FLAG-SEPT9. Red arrows show that FLAG-SEPT9 is not found in GFP-APC-M3 and GFP-APC-CT immunoprecipitations. All Western blots were resolved with 10% input.
Once I determined that GFP-N-APC could interact with septins, I sought to determine if the interaction could be detected in the reciprocal experiment. I co-transfected HEK293 cells once again with GFP-APC-N and FLAG-SEPT9. Here, I immunoprecipitated both the GFP and FLAG epitopes from 0.5 mg lysates using the GFP and FLAG Abs, respectively. The samples were resolved on Western blots for detection of the GFP and FLAG tags. I found that FLAG-SEPT9 could co-immunoprecipitate with GFP-APC-N and vice versa (Figure 20).

![Western Blot Image]

**Figure 20:** The N-terminus of APC (1-1309) and septins maintain a reciprocal interaction in HEK293 cells. Immunoprecipitation of GFP-APC-N and FLAG-SEPT9 co-expressed in HEK293 cell lysates. GFP was immunoprecipitated using the GFP rabbit Ab and the Western blots were probed with the GFP mouse Ab. The FLAG epitope was immunoprecipitated and detected on Western blots using the FLAG mouse Ab. **Left:** GFP-APC-N, but not the rabbit IgG control, can interact with FLAG-SEPT9 (5th lane versus 4th lane). Red arrow indicates that FLAG-SEPT9 is found in the GFP-N immunoprecipitation. Film was exposed for 1 sec. **Right:** GFP-APC-N can be detected in the FLAG-SEPT9 immunoprecipitation but not in the mouse IgG control (3rd lane versus 2nd lane). Green arrow indicates that GFP-APC-N is found in the FLAG-SEPT9 immunoprecipitation. Film was exposed for 4 sec. All Western blots were resolved with 5% input.
3.4 The N-terminus of APC (1-1309) is both necessary and sufficient to interact with septins in HEK293 cells

After verifying the interaction between septins and APC-N, I sought to determine which region of APC-N was responsible for the interaction. To this end, I co-transfected HEK293 cells with FLAG-SEPT9 and GFP-APC-N (as positive control) or FLAG- or GFP-SEPT9 with three APC-N truncations: FLAG-APC-NT, GFP-APC-M1 and GFP-APC-M2. Under these conditions, I found that the entire N-terminal region of APC was required to interact with septins as further truncations of GFP-APC-N abrogated the interaction (Figure 21).
Figure 21: The N-terminus of APC (1-1309) is both necessary and sufficient to interact with septins in HEK293 cells. Immunoprecipitation of FLAG-APC-NT or GFP-APC-M1 from HEK293 cell lysates when co-expressed with GFP-SEPT9 and FLAG-SEPT9, respectively. GFP-APC-M2 was also co-transfected with FLAG-SEPT9. As positive control, FLAG-SEPT9 was co-expressed with GFP-APC-N. For the APC-M1 and APC-M2 experiments, GFP was immunoprecipitated using the GFP rabbit Ab, and the Western blots were probed with GFP and FLAG mouse Abs. Western blots were resolved with 10% input. For the APC-NT and APC-N experiments, FLAG was immunoprecipitated and detected on Western blots using the FLAG mouse Ab. 

**Panels A-D:** GFP-APC-N can interact with FLAG-SEPT9 (Panel D, 3rd lane versus 2nd lane) but FLAG-APC-NT, GFP-APC-M1 or GFP-APC-M2 are insufficient for the interaction. Red arrows indicate immunoprecipitations of GFP- or FLAG-APC truncations where FLAG- or GFP-SEPT9 are not found, whereas the green arrow indicates enrichment of GFP-APC-N in the FLAG-SEPT9 immunoprecipitation.
3.5 The N-terminus of APC (1-1338) interacts with septins endogenously in SW480 cells

3.5.1 The N-terminus of APC (1-1338) co-immunoprecipitates endogenously with septins in SW480 cells

Though I have presented a strong body of evidence suggesting that APC-N interacts with septins, these experiments were performed in overexpressed systems. Because overexpression of genes of interest could result in co-immunoprecipitation of proteins due to increased protein amounts, it is necessary to investigate interactions with endogenous protein levels. To do this, I performed endogenous immunoprecipitations of the septin complex in SW480 cell lysates, a colon cancer cell line harboring a 1-1338 N-terminal truncation of APC. To this end, I immunoprecipitated SEPT9 from 1 mg of SW480 cell lysates and detected both endogenous SEPT9 and APC on Western blots (Figure 22). SEPT9 was immunoprecipitated using the 10C10 mouse Ab and detected on Western blots using our SEPT9 rabbit Ab, whereas APC was detected using the APC-ALi mouse Ab. Under these conditions, I found that truncated APC interacts with septins endogenously (Figure 22).
Figure 22: The N-terminus of APC (1-1338) interacts with septins endogenously in SW480 cells. Immunoprecipitations of endogenous SEPT9 from SW480 cell lysates. SEPT9 was immunoprecipitated using 10C10, and the Western blots were probed with both SEPT9 and APC-Ali Abs to detect SEPT9 and APC, respectively. APC can be detected in the septin complex immunoprecipitation but not in the mouse IgG control (3rd lane versus 2nd lane). Red arrow indicates that endogenous APC co-immunoprecipitates with SEPT9. Western blots were resolved with 5% input.

3.5.2 The N-terminus of APC (1-1338) co-localizes with septins endogenously in SW480 cells

To determine whether the interaction between septins and APC observed in the immunoprecipitation experiments also existed in intact cells, I fixed SW480 cells and stained for SEPT9 and APC. SEPT9 was stained using our lab-generated, rabbit polyclonal Ab, whereas APC was stained using the commercial mouse monoclonal Ab-7. Endogenous APC was detected using the fluorescent A488 2° Ab, and endogenous SEPT9 was detected using the fluorescent DøR Cy3 2° Ab. After acquisition of the images on a Confocal Microscope, they
were merged using the Volocity 5 software to assess co-localization, and the images were exported as TIFF files (Figure 23).

![Co-localization of endogenous APC (1-1338) with SEPT9 in SW480 cells.](image)

**Figure 23:** Co-localization of endogenous APC (1-1338) with SEPT9 in SW480 cells. SW480 cells were fixed and stained with our lab-generated SEPT9 Ab and commercial APC-Ab-7 to detect SEPT9 (red) and APC (green), respectively. Scale bars represent 17 µm. Images were merged using the Volocity 5 Software on the Quorum Spinning Disk Confocal Microscope. **Top-Right:** APC and SEPT9 co-localize (yellow) on filamentous structures present at the cell membrane. White arrow indicates co-localization at the cell periphery. **Bottom-Right:** APC and SEPT9 co-localize at the midbody during cytokinesis. Blue arrow indicates co-localization at the midbody.
When SW480 cells were fixed and stained with Abs against SEPT9 and APC, two filament-related co-localization patterns were observed: co-localization was observed at the plasma membrane in interphase and at the midbody bridge during cytokinesis (Figure 23). Moreover, the interaction data presented so far has been with SEPT9 under the assumption that the other septins were also interacting with APC. To verify this, I fixed and stained SW480 cells with Abs directed against endogenous SEPT2 (Figure 24) or SEPT11 (Figure 25). SEPT10 was excluded from this and further experiments because of the lack of a reliable Ab. Indeed, SEPT2 and SEPT11 exhibited comparable co-localization patterns with APC as seen with SEPT9. The interaction was, once again, confined to filamentous structures at the cell membrane and to the midbody during cytokinesis. These co-localization data provide further support for the endogenous interaction between septins and APC and suggest that SEPT9 is a reliable method of detecting the septin complex.
Figure 24: Co-localization of endogenous APC (1-1338) with SEPT2 in SW480 cells. SW480 cells were fixed and stained with our lab-generated SEPT2 Ab and commercial APC-Ab-7 to detect SEPT2 (red) and APC (green), respectively. Scale bars represent 17 µm. Images were merged using Volocity 5 Software on the Quorum Spinning Disk Confocal Microscope. **Top-Right:** APC and SEPT2 co-localize (yellow) on filamentous structures present at the cell membrane. White arrow indicates co-localization at the cell periphery. **Bottom-Right:** APC and SEPT2 co-localize at the midbody during cytokinesis. Blue arrow indicates co-localization at the midbody.
Figure 25: Co-localization of endogenous APC (1-1338) with SEPT11 in SW480 cells. SW480 cells were fixed and stained with our lab-generated SEPT11 Ab and commercial APC-Ab-7 to detect SEPT11 (red) and APC (green), respectively. Scale bars represent 17 µm. Images were merged using Volocity 5 Software on the Quorum Spinning Disk Confocal Microscope. **Top-Right:** APC and SEPT11 co-localize (yellow) on filamentous structures present at the cell membrane. White arrow indicates co-localization at the cell periphery. **Bottom-Right:** APC and SEPT11 co-localize at the midbody during cytokinesis. Blue arrow indicates co-localization at the midbody.

I also examined other stages of cell division to determine when septins and APC interact. Interestingly, co-localization was not observed during prophase and metaphase (Figure 26),
suggesting that the interaction may occur exclusively at sites where cytoskeletal filaments are available. Cells in anaphase and early telophase were not captured as they were difficult to enrich.

Figure 26: Endogenous septins and APC (1-1338) do not co-localize in SW480 cells in prophase and metaphase. SW480 cells were grown on coverslips, fixed and stained with the SEPT9 Ab and APC-Ab-7. SEPT9 (red) and APC (green) were detected using DαM and A488, respectively. Images were merged using the Volocity 5 Software on the Quorum Spinning Disk Confocal Microscope. Top Panel: Localization of SEPT9 and APC in SW480 cell in prophase. DNA is shown in blue. White arrow points to cell in prophase. Scale bars represent 16 µm. Bottom Panel: Localization of SEPT9 and APC in SW480 cell in metaphase. Scale bars represent 17 µm.
3.6 siRNA-mediated depletions of septins and APC in ARPE-19 cells

To determine the physiological relevance of the interaction between septins and APC, I investigated the consequences of depleting these proteins. Specifically, I used siRNA sequences known to efficiently knockdown human APC and septins (Figure 27). Since independent depletions of septins and APC have been linked to multinucleation and chromosome congression/segregation and abscission defects, I sought to investigate the severity of these phenotypes upon APC and septin co-depletions. Also, since there is currently no known role for APC at the midbody, I also sought to investigate if it is involved in abscission. For the knockdown studies, I utilized ARPE-19 cells, primary retinal epithelial cells.

Figure 27: siRNA-mediated depletions of septins and APC in ARPE-19 cells. Three days after siRNA transfection, a confluent well of a 6-well plate was lysed for each condition. Lysates were resolved on Western blots and probed using antibodies against APC, SEPT2, SEPT9 or SEPT11. GAPDH and α-tubulin were also detected on Western blots as loading controls. Panel A: APC is effectively depleted from ARPE-19 cell lysates in all single and double knockdowns when compared to control. Panels B-D: Septins 2, 9 or 11 were efficiently depleted from ARPE-19 cell lysates in all single and double knockdowns when compared to controls.
To ensure that ARPE-19 cells are appropriate for the knockdown studies, I fixed and stained them for endogenous septins and APC to ensure the presence of the interaction. As an example, co-localization between SEPT9 and APC is shown in Figure 28. Similar to SW480 cells, appreciable co-localization was observed between APC and SEPT9 at the midbody during cytokinesis. However, APC only partially co-localized with septins in interphase cells (Figure 29). Specifically, co-localization was seen only between some APC puncta and dense, cytoplasmic septin filaments.

![Fig 28](image)

**Figure 28: Endogenous septins and APC co-localize at the midbody in ARPE-19 cells during cytokinesis.** ARPE-19 cells were grown on coverslips, fixed and stained with our SEPT9 Ab and APC-Ab-7. SEPT9 (red) and APC (green) were detected using DαM and A488, respectively. White arrow indicates co-localization (yellow) at the midbody. Scale bars represent 17 µm. Images were merged using the Volocity 5 Software on the Quorum Spinning Disk Confocal Microscope.
3.6.1 Investigation of chromosome segregation defects in fixed ARPE-19 cells

To investigate chromosome segregation defects in the different knockdowns, I synchronized ARPE-19 cells with nocodazole and released them into telophase (Figure 30). Cells expressing the following siRNAs were fixed and stained for DAPI: control, APC, SEPT2, SEPT9, SEPT11, APC/SEPT2, APC/SEPT9 or APC/SEPT11. The cells were counted using an Inverted Fluorescence Microscope, and the percentage of cells with lagging chromosomes was determined for each knockdown condition.

Figure 29: Endogenous septins and APC co-localize partially during interphase in ARPE-19 cells. ARPE-19 cells were grown on coverslips, fixed and stained with the SEPT9 Ab and APC-ALi. SEPT9 (red) and APC (green) were detected using DαM and A488, respectively. White arrow indicates area of septin filament co-localization (yellow) with some APC puncta. Scale bars represent 16 µm. Images were merged using the Volocity 5 Software on the Quorum Spinning Disk Confocal Microscope.
Figure 30: Distinguishing complete chromosome segregation from lagging chromosomes in siRNA-transfected ARPE-19 cells. ARPE-19 cells were grown on coverslips, fixed and stained with DAPI to detect DNA (blue). Scale bars represent 17 μm. Images were captured using a Confocal Microscope. **Left:** High fidelity chromosome segregation. **Right:** Abnormal chromosome segregation.

3.6.1.1 **SEPT2 and APC may be involved in chromosome congression and segregation in ARPE-19 cells**

Since other labs have independently implicated APC and SEPT2 in chromosome segregation, I sought to investigate the consequences of knocking down these proteins in ARPE-19 cells. More importantly, I wanted to determine if co-depleting APC and SEPT2 would yield a phenotype additive of the two single knockdowns. Consistent with previous studies, I determined that knocking down APC or SEPT2 increased the incidence of lagging
chromosomes to 28% and 25%, respectively, in comparison to 13% in the control cells (Figure 31). While an approximate two-fold increase was observed upon removal of SEPT2 or APC, the differences were not statistically significant according to the one-way ANOVA. Furthermore, the incidence of lagging chromosomes upon co-depleting APC with SEPT2, though still different (27%) from control, was not significantly different from singly depleting APC or SEPT2, suggesting that these two proteins may function within the same pathway to regulate chromosome segregation. Since these studies were limited to the examination of lagging chromosomes in telophase, I cannot rule out the possibility that they do not include remnants of chromosome congression defects, especially since both APC and SEPT2 are also involved in this process. As a result, the lagging chromosome data presented henceforth will be attributed to defects in both DNA congression and segregation.

3.6.1.2 Co-depletion of SEPT9 with APC rescues siAPC-mediated chromosome congression and segregation defects

Knocking down SEPT9 did not have a significant effect on chromosome congression and segregation (18%), which is not surprising as SEPT9 has not been previously implicated in these processes. However, co-depleting SEPT9 with APC slightly alleviated the siAPC-mediated chromosome congression and segregation defects, suggesting that siSEPT9 may compensate somewhat for the lack of APC (Figure 31). Specifically, co-depleting SEPT9 with APC reduced the incidence of lagging chromosomes from 28% to 17.5% in comparison to siAPC alone. Though this was not statistically significant according to the one-way ANOVA, SEPT9 depletion conferred a 60% decrease in lagging chromosomes when compared to siAPC alone.
Figure 31: In ARPE-19 cells, SEPT2 and APC may function together to regulate chromosome congression and segregation while SEPT9 reduces the incidence of lagging chromosomes when co-depleted with APC. Error bars, s.e.m. (n = 3; 200 cells from each independent experiment). None of these values were statistically significant using one-way ANOVA (p=0.06221). As significance was lost immediately after performing the ANOVA, the multiple comparison tests were omitted.

3.6.1.3 Depletion of SEPT11 or its co-depletion with APC does not affect chromosome congression and segregation

Under the conditions investigated, I found no statistically significant differences in the incidence of lagging chromosomes in cells transfected with siSEPT11 (12.3%) or siCtrl (13%).
Furthermore, co-depleting APC with SEPT11, though still different from control, did not affect siAPC’s ability to impair chromosome segregation (28%) (Figure 31). These data suggest that, in my hands, SEPT11 does not play a role in chromosome congression and/or segregation independently or with APC.

3.6.2 **Investigation of cytokinetic and abscission defects in fixed ARPE-19 cells**

Since septin and APC depletions have been independently shown to cause multinucleation, I sought to investigate the consequences of co-depleting septins with APC. Furthermore, while SEPT9 has been shown to localize to the midbody and regulate abscission, there is currently no role reported for APC at the midbody. For the knockdowns, ARPE-19 cells expressing all the siRNAs of interest were plated on coverslips, fixed and stained with α-tubulin and DAPI (Figure 32). The cells were counted using an inverted fluorescence microscope, and the percentages of cells with more than one nucleus and persistent midbody bridge attachments were determined (Figure 33).
Figure 32: Representative images of siRNA-transfected ARPE-19 cells with unresolved cytokinesis in comparison to control cells with normal cytokinetic events. ARPE-19 cells were grown on coverslips, fixed and stained with DAPI (blue) and α-tubulin (green). Scale bars represent 17 µm. Images were acquired on a Confocal Microscope. **Top-Left:** Mononucleated cell after normal cytokinesis. **Top-Right:** White arrow points to binucleated cell with unresolved cytokinesis. **Bottom-Left:** Two normal daughter cells after a successful abscission event. **Bottom-Right:** Red arrow points to two daughter cells with failed or delayed abscission characterized by persistent midbody bridge attachment.
Figure 33: Quantification of fixed ARPE-19 cells with multiple nuclei and persistent midbody bridge attachments upon depletion of septins and APC. White bars represent the percentage of cells with multiple nuclei (>1), and black bars represent the percentage of cells attached by midbodies. Error bars, s.e.m. (n = 3; 200 cells from each independent experiment. None of these values were statistically significant using one-way ANOVA (p>0.05).

Despite the fact that multinucleation has been previously described by other groups upon septin or APC depletion, I did not observe a statistically significant increase in this defect for any of the knockdown conditions (Figure 33). In fact, the most striking multinucleation defect I observed was for the SEPT11 knockdown, where the percentage of cells increased from 1.12%
to 4.33% (Figure 33). For midbody bridge attachment, the highest defect was observed for SEPT9 co-depletion with APC, where the percentage of cells increased from 1.83% to 3.67% (Figure 33). Though these values were obtained from three independent experiments, the high variability and their low occurrence rates (less than 5%) rendered the aforementioned increases statistically insignificant (p>0.05, ANOVA). These discrepancies may be due to differences in the methods and cell lines utilized in these studies in comparison to published literature.

3.6.3 Investigation of abscission defects in live ARPE-19 cells

To investigate the effect of knocking down septins and APC on abscission, I followed ARPE-19 cells using time-lapse microscopy. Cells were scrutinized based on the time it took, from anaphase onset, to complete abscission (Figure 37).

3.6.3.1 Depletion of SEPT2, SEPT11 or their corresponding co-depletions with APC does not cause abscission defects

Consistent with previous findings from our lab, SEPT2 or SEPT11 knockdown did not affect average abscission times (Table 1). Specifically, SEPT2 depletion resulted in an average abscission time of 1.91 h, and SEPT11 depletion resulted in an average abscission time of 1.58 h (Table 1). The average abscission time after SEPT11 depletion is comparable to that of APC depletion (1.97 h) and control (2.30 h). Furthermore, co-depletion of SEPT2 or SEPT11 with APC did not affect mean abscission times (2.30 h and 2.17 h, respectively) (Table 1).

Furthermore, I determined that, within 4 h of anaphase onset, 91.3% of cells transfected with control siRNA successfully abscised. This number is very comparable to the percentage of SEPT2 (94.7%)- or SEPT11 (100%)-siRNA transfected cells that completed abscission within 4 h of anaphase onset (Figures 34 and 35). Similarly, 100% of cells transfected with siRNA
against APC completed abscission within this 4 h window (Figures 34 to 36). Consistent with the aforementioned data, co-depletion of SEPT2 or SEPT11 with APC allowed, respectively, 90% and 92.6% of siRNA-transfected cells to complete abscission within 4 h of anaphase onset. These numbers were not significantly different from one another or from the control (p>0.05, Table 2), suggesting that SEPT11 and SEPT2 may not be involved in abscission.

**Table 1:** Mean abscission times found for live ARPE-19 cells expressing single or double APC and/or septin siRNAs.

<table>
<thead>
<tr>
<th>siRNA transfected</th>
<th>Mean abscission times (± h)</th>
<th>Number of cells counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.30 ± 0.10</td>
<td>23</td>
</tr>
<tr>
<td>APC</td>
<td>1.97 ± 0.03</td>
<td>19</td>
</tr>
<tr>
<td>SEPT2</td>
<td>1.91 ± 0.06</td>
<td>19</td>
</tr>
<tr>
<td>SEPT9</td>
<td>4.42 ± 0.12</td>
<td>21</td>
</tr>
<tr>
<td>SEPT11</td>
<td>1.58 ± 0.02</td>
<td>21</td>
</tr>
<tr>
<td>APC/SEPT2</td>
<td>2.30 ± 0.06</td>
<td>23</td>
</tr>
<tr>
<td>APC/SEPT9</td>
<td>2.07 ± 0.04</td>
<td>19</td>
</tr>
<tr>
<td>APC/SEPT11</td>
<td>2.17 ± 0.05</td>
<td>27</td>
</tr>
</tbody>
</table>

3.6.3.2 **Co-depletion of SEPT9 with APC rescues siSEPT9-mediated abscission defects**

Since the functional significance of APC’s localization to the midbody is currently unclear, I sought to determine if it is involved in abscission. The average abscission time for cells transfected with siAPC was found to be 1.97 h, in comparison to control siRNA-
transfected cells with an average abscission time of 2.30 h (Table 1). Consistent with previous work from our lab, I found that SEPT9 depletion significantly delays abscission times (4.42 h) when compared to control cells (Table 1). Intriguingly, APC co-depletion with SEPT9 significantly decreased average abscission times (2.07 h), suggesting that APC loss compensates for SEPT9 loss.

Furthermore, while nearly all cells transfected with control or APC siRNA abscised successfully within 4 h of anaphase onset, only 52.4% of SEPT9 siRNA-transfected cells abscised in the same period of time (Figure 36). In addition, when siAPC was co-transfected with siSEPT9, 100% of the cells abscised within 4 h (Figure 36), and this result was statistically significant (p<0.001; Table 2). In fact, the percentage of cells expressing the double APC/SEPT9 siRNAs that completed abscission was statistically indistinguishable (p>0.05, Table 2) from cells expressing control siRNA, suggesting that APC removal from SEPT9-deficient cells was sufficient to restore normal abscission times. Furthermore, the percentage of cells that completed abscission when depleted of SEPT9 was significantly different when compared to all other single septin knockdowns (p<0.001; Table 2). This suggests that, among the septins investigated in the present study, SEPT9 plays a unique role in abscission.
Figure 34: Single or double APC and SEPT2 depletions do not cause abscission defects in live ARPE-19 cells. Dividing ARPE-19 cells transfected with Cntrl, APC, SEPT2 or APC/SEPT2 siRNA were monitored by time-lapse microscopy. The time from anaphase onset to midbody abscission was determined for each cell. The following number of cells were counted for each knockdown: n = 23 cells for Cntrl, n = 19 cells for APC, n = 19 cells for SEPT2 and n = 23 for APC/SEPT2. When compared to each other, none of these curves were found to be significantly different using one-way ANOVA (p>0.05).
Figure 35: Single or double APC and SEPT11 depletions do not cause abscission defects in live ARPE-19 cells. Dividing ARPE-19 cells transfected with Cntrl, APC, SEPT11 or APC/SEPT11 siRNA were monitored by time-lapse microscopy. The time from anaphase onset to midbody abscission was determined for each cell. The following number of cells were counted for each knockdown: n = 23 cells for Cntrl, n = 19 cells for APC, n = 21 cells for SEPT11 and n = 27 for APC/SEPT11. When compared to each other, none of these curves were found to be significantly different using one-way ANOVA (p>0.05).
Figure 36: Co-depletion of APC with SEPT9 rescues siSEPT9-mediated abscission defects in live ARPE-19 cells. Dividing ARPE-19 cells transfected with Cntrl, APC, SEPT9 or APC/SEPT9 siRNA were monitored by time-lapse microscopy. The time from anaphase onset to midbody abscission was determined for each cell. The following number of cells were counted for each knockdown: n = 23 cells for Cntrl, n = 19 cells for APC, n = 21 for SEPT9 and n = 19 for APC/SEPT9. According to one-way ANOVA, there are statistically significant differences between some of these siRNA conditions (p<0.001).
Table 2: Summary of relevant p values computed by one-way ANOVA for abscission times in live ARPE-19 cells expressing single or double APC and/or septin siRNAs.

<table>
<thead>
<tr>
<th>siRNAs compared</th>
<th>p value and statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEPT9 vs. SEPT11</td>
<td>p&lt;0.001, significant</td>
</tr>
<tr>
<td>SEPT9 vs. SEPT2</td>
<td>p&lt;0.001, significant</td>
</tr>
<tr>
<td>SEPT9 vs. APC</td>
<td>p&lt;0.001, significant</td>
</tr>
<tr>
<td>SEPT9 vs. Control</td>
<td>p&lt;0.001, significant</td>
</tr>
<tr>
<td>SEPT9 vs. APC/SEPT9</td>
<td>p&lt;0.001, significant</td>
</tr>
<tr>
<td>APC vs. APC/SEPT9</td>
<td>p&gt;0.05, not significant</td>
</tr>
<tr>
<td>Control vs. APC/SEPT9</td>
<td>p&gt;0.05, not significant</td>
</tr>
<tr>
<td>Control vs. APC</td>
<td>p&gt;0.05, not significant</td>
</tr>
<tr>
<td>Control vs. SEPT2</td>
<td>p&gt;0.05, not significant</td>
</tr>
<tr>
<td>APC vs. APC/SEPT2</td>
<td>p&gt;0.05, not significant</td>
</tr>
<tr>
<td>SEPT2 vs. APC/SEPT2</td>
<td>p&gt;0.05, not significant</td>
</tr>
<tr>
<td>Control vs. SEPT11</td>
<td>p&gt;0.05, not significant</td>
</tr>
<tr>
<td>SEPT11 vs. APC/SEPT11</td>
<td>p&gt;0.05, not significant</td>
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<td>APC vs. APC/SEPT11</td>
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Figure 37: Restoration of normal abscission times in live ARPE-19 cells upon co-depletion of APC and SEPT9. Dividing ARPE-19 cells transfected with Cntrl, APC, SEPT9 or APC/SEPT9 siRNA were monitored by time-lapse microscopy. The time from anaphase onset (00:00) to midbody abscission was determined for each cell. A Nikon Plan-Fluor 40X/0.6NA objective was employed, and DIC images were acquired using a Hamamatsu camera every three min. Midbody bridges are indicated by white arrows, and abscission events are indicated with black arrows. Scale bars represent 100 µm. Panel A: An example of ARPE-19 cell expressing control siRNA abscised in 1:51 h. Panel B: ARPE-19 cell expressing APC siRNA abscised in 1:18 h. Panel C: ARPE-19 cell expressing siRNA against SEPT9 took 4:39 h to complete abscission. Panel D: An example of ARPE-19 cell expressing combined siRNAs directed against SEPT9 and APC abscised in 1:54 h.
Chapter 4
Discussion and Concluding remarks

4.1 Discussion

We initially obtained mass spectrometry data from the Angers lab showing a putative interaction between the septin 2/9/10/11 complex and the APC tumor suppressor protein. Previous studies have demonstrated that septins and APC localize to similar cellular structures including actin stress fibers, microtubules and midbodies (Moseley et al. 2007; Surka et al. 2002; Rittmeyer et al. 2008; Watanabe et al. 2004; Spiliotis & Nelson 2006; Schmidt & Nichols 2004; Hanson and Miller 2005; Moseley et al. 2007). As well, both SEPT2 and APC are involved in chromosome congression and segregation, and their suppressions cause formation of lagging chromosomes (Kaplan et al. 2001; Spiliotis et al. 2005). Furthermore, septins and APC have been implicated in cytokinesis, and their depletions in yeast and mammalian systems cause formation of binucleated cells (Hartwell et al. 1973; Surka et al. 2002; Draviam et al. 2006; Spiliotis et al. 2005; Caldwell et al. 2007; Miller & Rose 1998). Furthermore, it has been recently demonstrated that APC levels are tightly regulated during yeast cell division to facilitate proper spindle positioning, and this regulation is aberrant in the absence of septins (Kammerer et al. 2010). Lastly, SEPT9 depletion has been shown to increase microtubule stability, whereas APC suppression by siRNA has been shown to decrease microtubule stability (Kremer et al. 2005; Zumbrunn et al. 2001).

Due to these well documented similarities in septin and APC functions, we sought to determine if they work together to perform their cellular roles. To this end, I verified that septins and full-length APC interact endogenously in HCT116 colon cancer cell lines (Figure 18). It is noteworthy that, due to APC’s abnormally large size (>310 kDa), the bands seen on the Western blots are somewhat smeared possibly due to poor migration through the gels. Another important
point is that the APC antibody used here could only detect the protein from enriched sources such as immunoprecipitations. In other words, the antibody could not detect APC from lysates, explaining why APC could not be observed in the input lanes even when 10% of the immunoprecipitations were loaded on the gels. Nonetheless, these data clearly indicate that APC interacts endogenously with septins.

To determine which region of APC was responsible for the interaction, I performed immunoprecipitations using three APC truncations in HEK293 cells: GFP-APC-N (1-1309), GFP-APC-M3 (1211-2075) and GFP-APC-CT (2076-2483). I determined that GFP-APC-N could interact with FLAG-SEPT9, whereas the middle region (1211-2075) and C-terminus (2076-2483) were insufficient for the interaction (Figures 19 and 20). These results were surprising because I initially imagined that, since APC and septins have overlapping cytoskeleton-related functions, they would interact near or at the actin/microtubule binding domain located at the C-terminus of APC. Moreover, the results of my data indicate that APC interacts with septins through its N-terminus, the region maintained in cancers. As mentioned in the introduction, truncated APC can perform most of the functions executed by full-length APC, albeit less effectively. Consistent with the indispensability of APC as a crucial regulator of epithelial cells, the entire protein is rarely deleted in cancers because this is not beneficial for tumor progression. In fact, removal of truncated APC from colon cancer cell lines reversed oncogenic phenotypes and initiated apoptosis (Schneikert & Behrens 2006; Brocardo et al. 2008). Therefore, these data present an important and potentially physiologically significant interaction between septins and the N-terminus of APC, which has been shown to be necessary for tumorigenesis.

To narrow down the binding site of septins within the N-terminus of APC, further truncations were made and used for immunoprecipitations in HEK293 cells. In my hands, APC-
NT (1-220), APC-M1 (221-958) and APC-M2 (959-1338) were unable to interact with septins (Figure 21). In the same experiment, septins maintained interaction with the APC-N construct, suggesting that the entire N-terminus (1-1309) of APC may be required to interact with septins. Since I used APC-N truncations which have been previously found to interact with other proteins, it is unlikely that these constructs encountered problems folding properly (Kawasaki et al. 2003; Watanabe et al. 2004; Jimbo et al. 2002). Alternatively, it is conceivable that the regions where the truncations were made are also needed for the interaction. To address this possibility, future experiments will be aimed at making many different, overlapping APC-N truncations and testing them for the ability to interact with septins.

Though I have presented strong evidence for the interaction between septins and the N-terminus of APC, these experiments were performed with overexpressed proteins. In order to interrogate this interaction in a more physiological system, I utilized SW480 cells, a colon cancer cell line expressing a 1-1338 N-terminal truncation of APC. Consistent with the overexpression data, I determined that truncated APC was present in SEPT9 immunoprecipitations, suggesting that this interaction exists endogenously (Figure 22).

To further verify the interaction between APC and septins, I stained SW480 cells with antibodies against SEPT9 and APC. Co-localization was observed at the cell membrane in interphase and at the midbody during cytokinesis (Figure 23). Furthermore, the interaction data discussed so far has been limited to SEPT9, under the assumption that the other septins are also interacting with APC. To verify that this was the case, SW480 cells were fixed and stained with antibodies against APC and SEPT2 or SEPT11. Similar to the SEPT9 co-localization data, APC and SEPT2 or SEPT11 co-localized appreciably at the cell membrane in interphase cells and at the midbody during mitosis (Figures 24 and 25). Collectively, these co-localization data present
further evidence that septins interact endogenously with APC and also suggest that SEPT9 is a reliable method of detecting the septin complex.

Interestingly, co-localization was not observed during prophase or metaphase (Figure 26), suggesting that cytoskeletal filaments may be required for the interaction. This is consistent with the fact that co-localization was only observed at filament-rich areas such as the cell membrane and the midbody. Truncated APC has been shown to associate with microtubules; however, its association with actin has not been reported to date (Jimbo et al. 2002; Green & Kaplan 2003). This, coupled with the fact that microtubules are the predominant filaments at the midbody during cytokinesis (where appreciable co-localization was observed), suggests that the SEPT-APC interaction may be occurring on microtubules. To examine this possibility, it will be necessary to depolymerize microtubules using a short treatment with nocodazole.

Since I did not observe enough SW480 cells in anaphase and early telophase, I was unable to unambiguously assign the status of co-localization during these stages of mitosis. Since APC is typically localized to the centrosome and septins to the cell cortex and cleavage furrow during anaphase, it is unlikely that they form an interaction complex at this stage (Louie et al. 2004; Spiliotis & Nelson 2006). In telophase, however, APC transfers to microtubules at the cleavage furrow, where it may, presumably, be free to interact with septins (Liakopoulos et al. 2003). To ascertain if septins and APC interact during these two stages of mitosis, it will be necessary to synchronize cells using mitotic poisons, such as thymidine and/or nocodazole, and then perform immunoprecipitation and co-localization experiments.

To determine the functional relevance of the SEPT-APC association, I uncoupled the interactions using siRNA-mediated depletions of these proteins in ARPE-19 cells, primary retinal epithelial cells. Since septin and APC depletions have been tied to phenotypes resulting
in genomic instability, we decided to use a primary cell line with normal karyotype to reduce background and make the data obtained relevant to untransformed cells. To perform knockdowns, siRNAs previously shown to effectively knockdown septins and APC were utilized. Three days after the transfections, I scored fixed cells for binucleation, midbody bridge attachment and lagging chromosomes (Figures 30 and 32). Furthermore, I followed ARPE-19 cells using time-lapse microscopy and determined average abscission times from anaphase onset (Figure 37 and Table 1). In addition, lysates from these experiments were resolved on Western blots to determine efficiency of the knockdowns after three days (Figure 27). All experiments were performed independently three times, and on average, above 80% knockdown was achieved for all conditions (Figure 27).

To ensure that ARPE-19 cells are appropriate for the knockdown studies, I fixed and stained them for antibodies against SEPT9 and APC (Figures 28 and 29). Unlike SW480 cells, which displayed appreciable co-localization in interphase and cytokinesis, ARPE-19 cells only showed robust co-localization at the mibody during cytokinesis (Figure 28). However, the septin and APC staining in both cell lines are somewhat different. In SW480 cells, the septins were punctuate and filamentous, but thicker filaments were often present at the cell membrane, the region where co-localization was observed (Figures 23 to 25). ARPE-19 cells, on the other hand, had an even distribution of septin filaments and puncta throughout the cell and did not have thicker filaments present at the cell membrane (Figure 29). Additionally, while the APC staining patterns in SW480 cells during interphase were punctuate throughout the cytoplasm and somewhat filamentous at the cell membrane, APC staining in ARPE-19 cells at the same stage was exclusively punctuate (Figures 23 to 25 versus Figure 29). Also, ARPE-19 cells sometimes contained thick septin filaments in the center of the cytoplasm, where some co-localization was observed with a few APC puncta (Figure 29). Interestingly, puncta of full-length APC have also
been observed in other cell lines, and this observation has been attributed to recruitment of the destruction complex by axin (Faux et al. 2008). Since ARPE-19 cells express full-length APC, the puncta observed here could be the APC-axin puncta, which have been previously reported (Faux et al. 2008). While the differences in the staining patterns observed for APC and septins in SW480 and ARPE-19 cells are probably due to cell type-specific and protein truncation reasons, it is, nonetheless, clear that APC and septins interact in ARPE-19 cells. This further supports our decision to use ARPE-19 cells for the knockdown studies.

Since both APC and SEPT2 have been shown to play roles in chromosome segregation, I sought to determine the outcome of co-depleting these proteins in ARPE-19 cells (Spiliotis et al. 2005; Zhang et al. 2007; Kaplan et al. 2001). SEPT9 and SEPT11 single and double knockdowns with APC were also included in this experiment. To this end, I determined that depleting SEPT2 or APC increased the incidence of cells with lagging chromosomes by approximately two-fold, though this was not statistically significant (one way ANOVA; p=0.06221) (Figure 31). However, significance could have been gained if a 90% confidence interval was used instead of the standard 95% value. However, this is usually concomitant with an increase in the likelihood of introducing Type I errors. As a result, it would be necessary to repeat this experiment multiple times in order to unambiguously determine whether or not to reject the null hypothesis. Co-depleting these proteins did not enhance this phenotype (Figure 31), suggesting that APC and SEPT2 may function within the same pathway and/or maybe even within the same complex to regulate DNA segregation.

While others have shown more severe chromosome segregation defects in cells depleted of APC and SEPT2, the techniques they utilized were slightly different from those used in this study. First, this is the first time this defect has been investigated in a human primary line, suggesting that differences between my data and others are likely due to cell and tissue type-
specific reasons (Spiliotis et al. 2005; Zhang et al. 2007; Kaplan et al. 2001). Second, a number of these studies attributed some DAPI puncta observed between daughter cells during anaphase or telophase to chromosome missegregation. Since I only counted cells with significant amount of DNA caught in between the two daughter cells (see Figure 30 for a representative image), my data may be underestimated (Spiliotis et al. 2005; Zhang et al. 2007; Kaplan et al. 2001).

It is noteworthy that since APC and SEPT2 also play roles in chromosome congression, it is possible that the percentage of lagging chromosomes presented in this study includes lagging chromosomes from earlier defects in chromosome congression (Spiliotis et al. 2005; Zhang et al. 2007). To ascertain if SEPT2 and APC work together to perform these functions, it will be crucial to determine if they interact directly using in vitro assays. To do this, our lab can perform GST pull downs with SEPT2 and the N-terminus of APC to determine if they can interact directly. This will be followed by a knockdown rescue approach, where endogenous SEPT2 will be replaced with an siRNA-resistant mutant unable to bind APC. This approach has been used by Estey and colleagues (under review), and it would ensure specific and selective uncoupling of the SEPT2-APC interaction while keeping all other SEPT2-related interactions intact. The cell lines expressing the SEPT2 mutant will then be interrogated for chromosome miscongression and missegregation in comparison to the parental cell line expressing an empty vector control. These studies will help to determine if APC and SEPT2 function together to regulate chromosome congression and segregation.

Alternatively, if SEPT2 and APC do not bind directly to regulate chromosome congression and segregation, it is possible that they are scaffolded within a complex comprising of centromere associated protein E (CENP-E) and the spindle checkpoint kinase BubR1. Indeed, APC has been found to co-operate with BubR1 for high fidelity chromosome congression and segregation, and SEPT2 depletion interferes with chromosome segregation by mislocalizing
CENP-E (Zhang et al. 2007; Spiliotis et al. 2005). Interestingly, it has been suggested that CENP-E and BubR1 co-operate during cell division to allow spindle checkpoint activation and proper chromosome segregation (Chan et al. 1998; Yao et al. 2000).

SEPT11 depletion did not affect chromosome congression and segregation significantly, and co-depleting it with APC did not affect siAPC-mediated induction of lagging chromosomes (Figure 31), suggesting that SEPT11 may not be involved in this process. Interestingly, preliminary work from our lab and established work from other labs have reported spindle misalignment in the absence of SEPT11 or APC, resulting in aberrant or failed cleavage furrow specification (Estey, personal communication; Draviam et al. 2006; Caldwell et al. 2007). It would therefore be of interest to explore any potential links between SEPT11 and APC with respect to spindle positioning and anchorage. As mentioned previously, this can be investigated by determining if SEPT11 can bind directly to APC and then replacing the endogenous protein with a mutant lacking the APC binding site.

Though SEPT9 depletion did not affect DNA congression and segregation, co-depleting it with APC slightly rescued siAPC-mediated induction of lagging chromosomes, suggesting that SEPT9 loss somewhat compensated for APC loss (Figure 31). As previously mentioned, SEPT9 depletion increases microtubule stability, whereas APC depletion decreases microtubule stability (Zumbrunn et al. 2001; Kremer et al. 2005). Specifically, APC is thought to stabilize the microtubule networks attached to kinetochores to facilitate high fidelity DNA congression and segregation, thereby, allowing equal partitioning of genetic material between the two daughter cells (Fodde et al. 2001; Kaplan et al. 2001; Rusan & Peifer 2008). Taken together, it is possible that knocking down SEPT9 with APC counteracts the microtubule instability encountered upon APC loss, thereby, restoring proper chromosome segregation by increasing microtubule stability. Moreover, it will be necessary to clarify SEPT9’s role in chromosome
congression and segregation through regulation of microtubule stability by utilizing $\text{APC}^{\text{Min/+}}$ murine cells, which possess mitotic defects including lagging chromosomes (Caldwell et al. 2007). These cells can be transfected with SEPT9 siRNA to determine if this can restore normal chromosome congression/segregation and genomic stability.

Furthermore, APC and septin depletions have been previously found to cause binucleation. Additionally, APC and septins localize to the midbody, and while SEPT9 depletion has been found to delay abscission, there is currently no role identified for APC at the midbody (Estey, under review; Munemitsu et al. 1994; Hanson & Miller 2005). In order to investigate a possible link between septins and APC in cytokinesis and to investigate if APC plays a role in abscission, I co-depleted these proteins in ARPE-19 cells. Unfortunately, I found no statistically significant accumulation of binucleated cells or cells connected by midbody bridges in the fixed ARPE-19 cell preparations (Figure 33).

Although others have shown that APC and septin losses cause mutinucleation, these studies differ from ours in a number of ways (Caldwell, et al., 2007; Surka, et al., 2002). First, the mutinucleation defect may be cell-type dependent. To my knowledge, investigation of this phenotype in a primary human cell line using fixed cell preparations has not been previously described. Others utilized HEK293 and/or HeLa cells, which have some cancer-like properties and probably exhibit a high background for these defects. As a result, HEK293 and HeLa cells may not be the most physiological systems for investigating these phenotypes. Furthermore, others from our lab have been unable to detect a significant accumulation of multinucleated cells upon SEPT9 knockdown in fixed ARPE-19 cells.

Second, although the Caldwell group (2007) showed that APC loss causes mutinucleation, this study used approaches distinct from ours. As alluded to earlier, they used
HEK293 cells. Additionally, the authors of this study stably expressed a truncated form of APC (1-1450) in cells, and this approach is different from depleting the endogenous protein. Specifically, APC\textsuperscript{1-1450} is thought to act as a dominant negative by oligomerizing with full length APC, preventing association with its physiological binding partners, thereby, effectively depleting the pool of competent APC (Green et al. 2005; Caldwell et al. 2007). Since siRNA-mediated knockdowns are not complete — given the likelihood that the small amount of APC remaining may be sufficient to drive cytokinesis — it is quite conceivable that this knockdown phenotype would be less severe than directly abrogating APC function through oligomerization with its truncated counterpart (Green et al. 2005; Caldwell et al. 2007).

Last, when I followed division of ARPE-19 cells using time-lapse microscopy, I noted that they utilized a great deal of traction and migratory mechanisms. There is a growing body of literature suggesting that traction and migratory forces can be used to complete cytokinesis, suggesting that ARPE-19 cells may evade the defects caused by our siRNAs using one or both of these mechanisms (Fukui 1993; Niswonger & O'Halloran 1997; Burton & Taylor 1997). In brief, these data suggest that, under the conditions employed, ARPE-19 cells are not appropriate for quantification of multinucleation and abscission defects using fixed cell samples. Alternatively, I could have used an approach where ARPE-19 cells are synchronized and released such that control cells would have sufficient time to complete cytokinesis (for example, two and a half hours from anaphase onset; see abscission data). This measure would facilitate confident scoring of cytokinetic defects in the knockdown cells because control cells are expected to have completed cytokinesis and abscission by the threshold time-point, and cells expressing siRNAs against septins or APC would accumulate in late telophase due to the inability to complete cytokinesis.
As mentioned previously, we followed ARPE-19 cell division using time-lapse microscopy to quantify abscission defects. Depletion of SEPT2, SEPT11 or APC did not affect average abscission times (Table 1), which is consistent with previous observations by others in our laboratory. Strikingly, SEPT9 depletion increased abscission times about two-fold (Table 1). In fact, nearly all control siRNA-transfected cells abscised in 4 h, whereas only half of SEPT9 siRNA-transfected cells abscised in the same amount of time (Figures 36 and 37). According to my one-way ANOVA computations, this delay in abscission caused by SEPT9 removal was statistically significant (Table 2). Interestingly, when APC was co-depleted with SEPT9, normal abscission time was restored, suggesting that APC loss compensated for SEPT9 removal during abscission (Figures 36 and 37).

As previously mentioned, SEPT9 and APC play reciprocal roles in microtubule stability, whereby SEPT9 depletion increases microtubule stabilization and APC depletion increases microtubule destabilization (Zumbrunn et al. 2001; Kremer et al. 2005). Since the midbody bridge contains very dense microtubule networks, it has been proposed that microtubule destabilization is a pre-requisite for abscission (Barr & Gruneberg 2007). In fact, spastin, a microtubule-severing protein, is recruited to the midbody during cytokinesis to facilitate a microtubule disruption event required for abscission (Connell et al. 2009). Taken together, it seems that the most economical explanation for my rescue data is that co-depletion of APC with SEPT9 rescues siSEPT9-mediated abscission defects by destabilizing MTs, thereby, making them more susceptible to cleavage. This increased microtubule susceptibility to cleavage may translate into the elevated number of cells that underwent abscission, resulting in a decrease in mean abscission times. To address this possibility, it will be important to stain for β-phospho tubulin, a marker for destabilized MTs, in ARPE-19 cells expressing both APC and SEPT9 siRNAs to determine if it is enriched at the midbody.
Despite these explanations, it is still somewhat perplexing that the slight decrease in abscission times encountered upon APC loss alone was not statistically significant (Table 1 vs. Table 2). There are some plausible explanations for this. First, I previously mentioned that ARPE-19 cells utilize a great deal of migration and traction forces during division. To my knowledge, HeLa and U2OS cells are the only other mammalian cells which have been employed in quantifying abscission times using live imaging (Estey, under review; Kouranti et al. 2006). Recent work from our lab has demonstrated that the average abscission times for normal HeLa cells is 3.9 h, and work from others has shown that normal abscission times for U2OS cells is around 5.4 h (Estey, under review; Kouranti et al. 2006). Taken together, it is evident that ARPE-19 cells abscise markedly faster than HeLa and U2OS (and probably other) cells, thereby underestimating the global abscission times reported in this study. It would be interesting to determine the outcome of performing these knockdowns in HeLa cells and re-quantifying abscission times.

Second, while only vanishingly small amounts of APC were found in our knockdown cell lysates, it is possible that we imaged some cells lacking the siRNA, thereby, overestimating the resulting mean abscission times. This limitation can be circumvented by repeating our experiments and replacing the APC siRNA with an APC shRNA vector expressing GFP to mark transfected cells.

Third, recent studies have identified an APC mammalian isoform, APC-2, which not only has great sequence similarity to APC, but also has overlapping functions with APC. Specifically, APC-2 can mediate β-catenin degradation and stabilize microtubules (van Es et al. 1999; Shintani et al. 2009). Therefore, it is plausible that the abscission and lagging chromosome phenotypes were masked by redundancy due to APC-2. Since APC-2 protein levels have never been interrogated in ARPE-19 cells and since its expression levels may vary
based on cell and tissue types, it is conceivable that APC-2 may be highly expressed in ARPE-19 cells, counteracting the phenotypes caused by APC depletion. The fact that intestinal cells undergo transformation in the absence of APC suggests that APC-2 may not fully compensate for the lack of APC in this tissue type. As a result, it may be necessary to repeat these studies in NCM460, a non-cancerous colon epithelial cell line (Moyer et al. 1996).

Last, it is possible that the APC siRNA is not fully penetrant, preventing complete manifestation of the abscission (and possibly the other) phenotypes investigated in the present study. Alternatively, our lab could repeat the knockdown studies using multiple APC siRNAs, which has been done by others (Brocardo et al. 2008).

To further complicate matters, lagging chromosomes are sometimes observed in untransformed cells at the midbody bridge during cytokinesis, and this phenomenon has been shown to slow down abscission. Specifically, Aurora B kinase has been reported to delay abscission upon accumulation of lagging chromosomes at the midbody bridge (Steigemann et al. 2009). This is because abscission is the last stage of cell division and therefore the last opportunity to resolve cytokinetic defects and maintain genomic stability (Steigemann et al. 2009). With this in mind, it is surprising that APC depletion induced formation of lagging chromosomes but did not increase average abscission times. To account for this observation, it is possible that APC loss inhibits Aurora B kinase activity at the midbody.

Though there is currently no published link between these proteins, I will entertain this possibility with a reasonable explanation. Assuming that the consequences of APC depletion in ARPE-19 cells recapitulate APC truncation in the normal colonic epithelium, it is plausible that chromosome missegregation coupled with fast abscission is, in fact, the optimal physiological response in cells devoid of APC. Interestingly, a similar concept has been proposed by the
Kaplan group (2005), who suggested that multiple forms of mitotic infidelity may co-operate to enhance the selective pressures which promote tumorigenesis in the absence of APC. The functional significance of this suggestion is further discussed below. Nonetheless, the use of multiple siRNAs for a more robust phenotype and/or APC<sup>Min+/-</sup> mouse cell lines will be crucial to resolve the discrepancies relating to APC’s possible role at the midbody.

The fact that APC and SEPT9 losses compensate for each other was unexpected and rather peculiar (Figures 31, 36 and 37). However, this is not the first time this has been reported. In a 2005 study, it was demonstrated that septin co-depletion with MAP4, another microtubule-stabilizing protein (like APC), resolved all cytokinetic defects (Kremer et al. 2005). These investigators suggested that, in order to facilitate cell cycle progression, there exists a balance between microtubule stabilization and destabilization. When septins are depleted, microtubules are overly stabilized, inhibiting cell cycle progression and giving rise to mitotic defects. This excessive microtubule stabilization may be counteracted by co-depleting a microtubule-stabilizing protein, thereby, providing the optimal level of microtubule dynamics required for normal cell cycle progression and genomic stability (Kremer et al. 2005).

Moreover, the physiological advantage of APC’s and SEPT9’s opposing roles in microtubule stability remains to be elucidated. As previously mentioned, the N-terminal region of APC is maintained in cancers, and its expression is thought to contribute to optimal tumorigenesis (Albuquerque et al. 2002). Despite the fact that cancer cells exhibit genomic instability due to chromosome congression/segregation and cytokinetic defects, the right amount of normal chromosome congression/segregation and cytokinesis must still proceed in order to deliver any genetic material to the daughter cells. As well, cancer cells must keep excessive, abnormal proliferation to a moderate level in order to avoid apoptosis initiation (Kim et al. 2000). Further, colon cancers have been reported to contain high rates of APC truncating
mutations (15% germline and 85% sporadic) and SEPT9 promoter hypermethylation (58%) (Lynch & de la Chapelle 2003; Grützmann et al. 2008). With these facts in mind, it would be very interesting to investigate if colon cancers couple APC truncation to SEPT9 downregulation via promoter hypermethylation. If this is the case, it could explain the rescue data I obtained when APC and SEPT9 were co-depleted. In other words, my siRNA data may have recapitulated what happens in vivo, where colon cancer cells concurrently silence SEPT9 and truncate APC in order to maintain an optimal level of genomic instability. Intriguingly, research has shown that DNA methylation—much like APC truncation—is a very early event in cancer progression, suggesting that events which trigger aberrations in the SEPT9 and APC genes may occur around the same time (Bennett et al. 2008). Future studies should be aimed at determining if the frequency of APC truncating mutations is related to SEPT9 promoter hypermethylation in human CRC samples.

SEPT10 was excluded from our studies because we do not have a reliable antibody for it. However, its potential link to APC must be explored because it was also found in the mass spectrometry screen (Figure 17). The antibody limitation will be circumvented by cloning SEPT10 into an expression vector and expressing it stably in a suitable cell line. Interestingly, the only literature which has attempted to characterize SEPT10 showed that it is not highly expressed in the normal colonic epithelium but overexpressed in SW480 colon cancer cells (where APC is truncated) (Sui et al. 2003). Though preliminary, these findings are exciting as they suggest a potentially interesting role for SEPT10 in intestinal tumorigenesis. Also, since SEPT10 remains one of the most poorly understood septins, investigation of its link with APC may be necessary to realize its function. As done in the present study, the APC-SEPT10 interaction will be explored using co-localization, immunoprecipitation and knockdown studies by employing the stable expression system mentioned above. More importantly, in order to
determine the function of SEPT10 in tumorigenesis, NCM460 cells will be transfected with a SEPT10 expression vector and scrutinized for development of oncogenic phenotypes. These cancer assays are well documented: briefly, NCM460 cells transfected with SEPT10 will be examined for increased cell motility and invasion, binucleation and aneuploidy, increased proliferation and decreased apoptosis (Gonzalez et al. 2007).

It is interesting that I obtained diverse phenotypes upon co-depletion of APC with different septins. Recent work from our lab has demonstrated that, although they were initially thought to function as part of a complex, individual septins may have distinct functions (Estey, under review), suggesting that the APC-SEPT2/9/10/11 complex may be dynamic. In other words, it is possible that there are instances in the cell where APC interacts with the septin complex and others where it interacts with individual septins in order to carry out specific functions. Consistent with this possibility, only SEPT2 was found to play a similar role to APC in chromosome congression and segregation (Figure 31), and only SEPT9 and APC could counteract each other’s siRNA-mediated mitotic defects (Figures 31, 36 and 37). Also, preliminary data from our lab suggests that SEPT11 may be involved in spindle positioning, one of the functions of APC during mitosis (Estey, personal communication; Draviam et al. 2006; Caldwell et al. 2007). Taken together, it will be necessary to further elucidate the SEPT-APC interaction by isolating each septin, starting by determining which one(s) can bind directly to APC.

4.2 Concluding remarks

In brief, we have determined that SEPT2, 9, 10 and 11 are novel interacting partners of APC. The interaction is confined to the N-terminal region of APC, the region maintained in cancers. The fact that this region is rarely deleted from cancers suggests that septins may be
involved in establishing and/or optimizing proliferation and genomic instability in tumour cells. Also, the interaction was observed on filamentous structures, possibly microtubules, at the cell membrane and at the midbody bridge during cytokinesis.

siRNA-mediated depletion of APC and SEPT2 increased the percentage of cells with lagging chromosomes, and more importantly, co-depleting these proteins did not enhance this phenotype, suggesting that they may function within the same pathway to regulate chromosome congression and segregation. SEPT9 co-depletion with APC rescued siAPC-mediated chromosome congression and segregation defects, probably through restoring microtubule stability. Furthermore, SEPT9 depletion significantly increased abscission times, which was rescued by co-depleting APC. This may be due to microtubule destabilization caused by APC loss, where the former has been shown to be crucial for abscission. Moreover, it has been abundantly demonstrated that SEPT9 and APC are deregulated in colon cancers, where the former is downregulated via promoter hypermethylation and the latter is truncated. Taken together, my rescue data may indicate that these two events are coupled in order to promote the “just right” signaling model previously mentioned in the introduction. Future studies will be aimed at elucidating the functional significance of the opposing roles of APC and SEPT9 in microtubule stability and determining if it is necessary for optimizing tumor progression.

Since there is emerging evidence that septins may not be functionally equivalent, it will be necessary to further investigate the significance of the SEPT-APC interaction by isolating each septin, including SEPT10. However, to proceed with these investigations, in vitro binding studies to narrow down the septin(s) which bind(s) directly to APC are indispensable. Lastly, it would be important to determine if septins play roles in other APC-related functions: kinetochore capture by microtubules and β-catenin degradation. These possibilities can be
explored by determining if septin depletion affects β-catenin degradation and if septins associate with kinetochore markers and checkpoint proteins during microtubule capture.
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