Regulation of epithelial cell migration and macropinocytosis by septin GTPases

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Epithelial cancers, or carcinomas, account for approximately 90% of all cancers. Unregulated cellular proliferation drives tumor growth and expansion, while metastatic behavior, or the motility of tumor cells to distant sites, underlies the majority of cancer-related deaths. Thus, better understanding of epithelial cell biology will provide novel anti-cancer therapeutics.

Septin GTPases are a novel component of the mammalian cytoskeleton. Human septins are comprised of thirteen genes that are divided into four groups based on sequence similarity. Septins bind and hydrolyze GTP in order to assemble into heteropolymers and higher-order structures such as bundles, ring and patches. Septins bind directly to the actin and microtubule cytoskeleton and cell membranes. Through these interactions, septins are posited to regulate their organization and dynamics by acting as scaffolds and diffusion barriers. Importantly, septins are frequently overexpressed in carcinomas, but their role in tumorigenesis and metastasis is unknown.

In the first part of this thesis, I investigated how septins function in renal epithelial cell migration. Epithelial cell migration is driven by the actin cytoskeleton, which assembles into a network of stress fibers that exert contractile forces at focal adhesions with the extracellular matrix (ECM). Here, I discovered that septin 9 (SEPT9) crosslinks directly actin filaments and controls focal adhesions stability, which is essential for productive epithelial migration. Importantly, I found that SEPT9 expression is
upregulated during epithelial-to-mesenchymal transition (EMT), a critical step during tumor cell invasion, and SEPT9 overexpression promotes renal carcinoma and renal epithelial cell migration. These results are among the first evidence that septins are bona fide actin crosslinking and suggest that septin overexpression may promote tumor cell invasion.

In the second part of this thesis, I investigated the molecular interaction between SEPT9 and actin filaments. I identified that a basic region in the SEPT9 N-terminus is sufficient to crosslink actin filaments. In addition, SEPT9 binds to a common site on the actin filament and interferes with the binding of the myosin motor domain and the actin-severing protein cofilin. By inhibiting myosin binding and cofilin-mediated severing, SEPT9 may protect nascent actin filaments from depolymerization. These results provide the first evidence that septins regulate spatially the binding of actin-binding proteins along the actin filament.

In the last part of this thesis, I identified a novel role of septins in epithelial macropinocytosis, a form of clathrin-independent endocytosis that is frequently upregulated in cancer and provides essential molecular intermediates for carbon metabolism during tumor cell proliferation. Here, I found that septins localize to macropinosome contact sites with endosomes in a phosphoinositide-specific manner. Significantly, septins regulate the delivery of macropinocytic cargo to the lysosome by promoting membrane fusion. These results provide the first evidence of a role for septins in a clathrin-independent endocytic pathway and endocytic vesicle fusion.

Collectively, these findings advance our knowledge of septin cell biology and provide new evidence for how septin overexpression may promote tumor growth and metastasis through two different cellular pathways.
CHAPTER 1. Introduction

Similar to microtubules, actin microfilaments and intermediate filaments, septins constitute a network of filamentous polymers, which are essential for the development and physiological functions of eukaryotic organisms. Septin filaments associate with cell membranes and the actin and microtubule cytoskeleton, exhibiting a structural and functional interdependence that distinguishes them apart from the conventional cytoskeleton. Septin filaments function as diffusion barriers and scaffolds that affect the compartmentalization of cell membranes and the spatial organization and functions of the microtubules and actin cytoskeleton (Caudron and Barral, 2009; Spiliotis and Gladfelter, 2012).

Septins are guanosin-5'-triphosphate (GTP)-binding proteins that self assemble into non-polar oligomers and polymers (Weirich et al., 2008; Zhang et al., 2000). Similar to actin and microtubules, septin assembly is coupled to nucleotide-binding and hydrolysis (Gasper et al., 2009). Resembling the hetero-polymeric nature of intermediate filaments, septin filaments consist of various subunits, which in mammalian organisms are encoded by 13-14 different genes with alternative splicing and translation initiation sites that give rise to multiple septin isoforms (Caudron and Barral, 2009).

I. Septin structure, assembly and dynamics

Classified under the P-loop superfamly of nucleotide-binding and hydrolyzing proteins, which includes the Ras-like GTPases and the kinesin and myosin motors, septins possess a highly conserved GTP-binding domain (G domain), which is structurally characterized by α-helices and β-sheets that alternate between flexible loops (Leipe et al., 2002). Septin G domains contain the G1 Walker A (GxxxxGKS/T), G3 Walker B (DxxG) and G4 (NKxD) motifs, which interact directly with atoms of the
guanine nucleotide. In addition to these signature motifs, the N- and C-termini of the septin G domains are respectively characterized by a polybasic region, which binds phosphoinositides (Casamayor and Snyder, 2003; Zhang et al., 1999), and a sequence of variable length and unknown function termed the septin unique element (SUE) (Versele and Thorner, 2005). Beyond these homology domains, septins differ significantly in the length and sequence of their N- and C-terminal extensions, which flank their G domains and consist of proline-rich and α-helical domains, respectively (Luedek et al., 2005).

Based on amino acid similarity, mammalian septin paralogs are categorized into four groups: SEPT2, SEPT6, SEPT7 and SEPT9 (Dobbelare and Barral, 2004; Luedek et al., 2005). The majority of septins fall under the SEPT2 group, which includes SEPT1, SEPT2, SEPT4 and SEPT5, and the SEPT6 group, which consists of SEPT6, SEPT8, SEPT10, SEPT11 and SEPT14 (Figure 1.1). Lacking a critical threonine residue from their G domains, septins of the SEPT6 group are hydrolytically inactive and always bound to GTP (Gulesserian et al., 2007; Sirajuddin et al., 2009). The remaining septins are classified under the SEPT9 group, which comprises SEPT3, SEPT9 and SEPT12, and the SEPT7 group, which contains SEPT7 isoforms (Figure 1.1). SEPT2, SEPT7 and SEPT9 are nearly ubiquitously expressed, while SEPT1, SEPT3, SEPT12 and SEPT14 are tissue-specific. The remaining septins are expressed widely, but not present in every tissue (Dolat et al., 2014a).

Septin polymerization is driven by the propensity of their G domains to homo- and hetero-dimerize (Sirajuddin et al., 2007). In the presence of guanosine nucleotide, septin G domains dimerize by interacting directly through their GTP-binding pockets (G interface), which are oriented opposite to one another, or, alternatively, through their N- and C-terminal helices (NC interface). Using the G and NC dimerization interfaces in
tandem, septin monomers assemble linearly into non-polar oligomers (Sirajuddin et al., 2007) (Figure 1.1). In mammalian systems, the basic septin oligomer is a hetero-octamer composed of septins from each of the SEPT2, SEPT6, SEPT7 and SEPT9 groups at a 2:2:2:2 stoichiometry (SEPT9-SEPT7-SEPT6-SEPT2-SEPT2-SEPT6-SEPT7-SEPT9) (Kim et al., 2011; Sellin et al., 2011b). This mode of hetero-octameric assembly is conserved between mammals and yeast (Bertin et al., 2008; Farkasovsky et al., 2005), septin filaments could also consist of hetero-hexameric (SEPT7-SEPT6-SEPT2-SEPT2-SEPT6-SEPT7) and -tetrameric (SEPT6-SEPT7-SEPT7-SEPT6) units (Engidawork et al., 2003a; Kinoshita, 2003). In addition, “non-canonical” hetero-oligomers that consist of multiple subunits of the same septin group may also exist (Beites et al., 1999; Blaser et al., 2002; Hsu et al., 1998; Martinez et al., 2004; Shinoda et al., 2010). Overall, septin hetero-oligomers could vary in size and composition depending on cell and tissue type. Irrespective of subunit identity, presence of GDP and thus GTP hydrolysis favors oligomerization by stabilizing the G interface, while GTP-binding appears to destabilize the NC interface (Gulesserian et al., 2007). With the exception of the SEPT6 group septins, which cannot hydrolyze GTP, oligomeric septins are GDP-bound and septin polymers contain an excess of GDP relative to GTP (Farkasovsky et al., 2005; Sirajuddin et al., 2007). Therefore, in contrast to microtubules and actin microfilaments whose polymerization is favored by a non-hydrolyzed nucleotide, septin polymerization is favored by GTP hydrolysis.

Septin hetero-octamers polymerize into higher order structures such as linear and curved filaments, rings and gauze-like meshworks (Bertin et al., 2008; Garcia et al., 2011; Kinoshita, 2002) (Figure 1.1). End-to-end binding of septin hetero-octamers results in filaments, which are 5-10 nm wide and up to several microns long (Bertin et al., 2008; Bertin et al., 2010; Kinoshita, 2002). Septin filaments pair with one another by
interacting laterally through the C- (coiled coil domains) and possibly N-terminal extensions that stretch outwards from the plane of oligomerization (Bai et al., 2013; Bertin et al., 2008; de Almeida Marques et al., 2012; John et al., 2007). Thus, septin bundles form by lateral stacking of individual septin filaments and adaptor proteins further enhance septin bundling (Sadian et al., 2013). Owing to the flexibility of the SEPT2 NC interface, which behaves like a hinge, septin oligomers can bend within a 25-30° angle of one another generating a curvature, which allows for the formation ring-like structures with 0.5 – 0.7 µm diameter (Sirajuddin et al., 2007). Septin subunits that enhance the flexibility of NC interface favor the formation of septin rings and post-translational modifications can promote the assembly of a gauze-like meshwork of orthogonally arranged filaments (Garcia et al., 2011). The filamentous pattern of septins is further influenced by their association with membrane bilayers and the actin and microtubule cytoskeleton. Giant unilamellar vesicle membranes enriched with phosphatidylinositol-4,5-bisphosphate can promote the formation of tightly paired septin filaments (Tanaka-Takiguchi et al., 2009). Conversely, actin-templated assembly of septins favors the formation of linear filaments as opposed to septin rings, which become more prevalent upon actin depolymerization (Gulesserian et al., 2003; Kinoshita, 2002). Similarly, the disk-like organization of septins in non-adherent cells depends on microtubules (Engidawork et al., 2003b; Sellin et al., 2011a).

Septin filaments are less dynamic than microtubules and F-actin (Table 1.1). Half-times of subunit exchange and turnover could vary from 10s of seconds for cytoplasmic septin filaments in mammalian cells to 3 minutes for membrane-associated septin polymers in yeast organisms (Dobbelaeere et al., 2003; Engidawork and Lubec, 2003; Gulesserian et al., 2003; Hagiwara et al., 2011; Lubec and Engidawork, 2002). Recent studies show that membrane-bound septin filaments grow by end-to-end
annealing (Bridges et al., 2014). Rod-shaped septin oligomers diffuse, collide and anneal to the free ends of septin filaments, which do not exchange any subunits along their length (Bridges et al., 2014). In contrast to membrane-bound septin filaments, which incorporate new subunits after fragmentation, cytoplasmic septin filaments are more dynamic, exchanging subunits along their length (Bridges et al., 2014; Engidawork and Lubec, 2003; Gulesserian et al., 2003). Septin filament dynamics are affected by post-translational modifications such as phosphorylation and acetylation, and vary depending on the phase of the cell cycle (Hernandez-Rodriguez and Momany, 2012). For example, in the budding yeast *Saccharomyces cerevisiae*, membrane-bound septin filaments are dynamic in G1, but highly stable during the S, G2 and M phases of the cell cycle (Dobbelaere et al., 2003). These changes in septin dynamics are mediated by septin-dependent kinases and phosphatases that are recruited to septin filaments in the beginning and end of the cell cycle, respectively (Dobbelaere et al., 2003).

The molecular mechanisms that regulate septin assembly and dynamics are not fully understood. Recent advances indicate that regulation of GTP hydrolysis, septin synthesis and degradation as well as post-translational modifications and protein-protein interactions influence the filamentous organization and dynamics of septins. Septin folding is mediated by the cytosolic chaperonin containing TCP-1 (CCT), which also controls the folding of actin and tubulin, and septin degradation involves E3 ubiquitin ligases including parkin, the von Hippel-Lindau (VHL) tumor suppressor and the Ring Finger protein 8 (RNF8) (Chahwan et al., 2013; Craven et al., 2006a; Dekker et al., 2008; Zhang et al., 2000). While these factors could regulate the availability of septin subunits for polymerization, proteins that promote the GTPase activity of septins (e.g., Orc6) and cross-link septin filaments (e.g., Gic1) can directly enhance septin assembly (Huijbregts et al., 2009; Sadian et al., 2013). In contrast, Cdc42, a signaling molecule of
the Rho family of GTPases, induces the unbundling and dissociation of septin filaments (Sadian et al., 2013). Post-translational modifications such as phosphorylation, acetylation and sumoylation further modulate septin assembly and dynamics, but many of the details remain unknown (Hernandez-Rodriguez and Momany, 2012).

II. Septins and the membrane skeleton

Septin filaments assemble on the cytoplasmic leaflet of membrane bilayers forming a meshwork that resembles the spectrin-actin membrane skeleton. In yeast cells, septins form filamentous rings and gauze-like patches in actin-free areas of the cell cortex (Engidawork et al., 2001a; Engidawork and Lubec, 2001), while in mammalian cells septin filaments associate with the actin membrane skeleton (Hagiwara et al., 2011). These membrane-bound septin filaments restrict protein diffusion and affect the shape and rigidity of cell membranes (Caudron and Barral, 2009; Spiliotis and Gladfelter, 2012). Septins and are posited to interact with phosphoinositides, yet their specificity for different phosphoinositides, which regulate the intracellular localization of membrane-bound proteins, is not fully known.

In the budding yeast *Saccharomyces cerevisiae*, a membrane-bound septin ring forms at the base of the emerging bud early in the cell cycle and evolves to a collar that underlies the mother-bud neck (Engidawork et al., 2001a; Engidawork and Lubec, 2001). Septin filaments are positioned between the plasma membrane and the smooth endoplasmic reticulum (ER), impeding the lateral diffusion of cortical and ER membrane proteins during polarized bud growth (Luedeke et al., 2005) (Figure 1.2 A). In mitosis, splitting of the septin collar into two rings results in the formation of a membrane compartment, which enables the accumulation of membrane-associated factors (e.g., vesicle tethering complexes) without diffusing into the mother and bud cell membranes
(Barral, 2000). During mammalian mitosis, a septin diffusion barrier is posited to similarly limit the diffusion of inner leaflet membrane proteins across the equatorial plane of a dividing cell (Schmidt and Nichols, 2004a).

Septins form membrane-bound rings at the base of primary and motile cilia and the dendritic spines of neurons (Fliegauf et al., 2014; Gulesserian et al., 2001; Hu et al., 2010; Tada et al., 2007). At the interface of the plasma and ciliary membrane, septins control the localization of ciliary membrane proteins and thus, they are required for the formation and maintenance of the primary cilium (Engidawork et al., 2001e; Hu et al., 2010). Septins could similarly impede the lateral diffusion of post-synaptic proteins from the dendritic spines to the shaft, aiding to the compartmentalization of synaptic receptors and channels (Caudron and Barral, 2009). At the annulus of spermatozoa, septins form a barrier between the principal and mid pieces of the sperm, and are required for the proper localization of a protein that is essential for spermiogenesis (Engidawork et al., 2001c; Ihara et al., 2005; Kissel et al., 2005). The mechanism by which septins restrict lateral diffusion in cell membranes is unknown, but recent evidence indicates that septins influence the organization of lipid domains and interact with the cytoplasmic tails of membrane transporters (Hagiwara et al., 2011; Kinoshita et al., 2004; Sharma et al., 2013b). Protein-lipid compartmentalization and domain identity could be further reinforced by septin-mediated inhibition of vesicle delivery; septins interact with the exocyst, a vesicle tethering complex, and components of the SNARE machinery of vesicle fusion, and may introduce a physical barrier that interferes with vesicle delivery to the plasma membrane (Engidawork et al., 2001b; Engidawork et al., 2001d; Gulesserian et al., 2000; Yoo et al., 2001) (Figure 1.2 B). Hence, septins are an integral component of the structure of cell membranes, contributing to the organization of
membrane domains by inhibiting selectively the lateral diffusion and delivery of membrane proteins.

In addition to their diffusion barrier properties, membrane-bound septins affect the shape and physical properties of lipid bilayers. Similar to the membrane-remodeling functions of the Bin-Amphiphysin-Rvs (BAR)-domain proteins, septins have been shown to induce the tubulation of spherical liposomes made of phosphatidylcholine and phosphoinositides (Tanaka-Takiguchi et al., 2009) (Figure 1.2 C). These membrane tubules have the diameter of septins rings (~0.5 µm) and are circumferentially braced by septin filaments (Tanaka-Takiguchi et al., 2009). It is unknown, however, whether septins affect membrane curvature and induce membrane tubules in vivo (Figure 1.2 D).

Conversely, several studies have shown that septin depletion decreases the rigidity and stiffness of cell membranes (Mostowy et al., 2011; Tooley et al., 2009) (Figure 1.2 E). In T lymphocytes, septins suppress membrane blebbing and protrusive activity is spatially restricted to septin-free areas of the cell cortex (Gilden et al., 2012; Tooley et al., 2009). Measurements of membrane elasticity and viscosity show a marked decrease in the rigidity of cell membranes after septin depletion, which alters cell shape and morphology (Mostowy et al., 2011). Thus, like the spectrin-actin membrane skeleton, septin filaments contribute to the organization and mechanochemical properties of cell membranes.

III. Septins and the Actin Cytoskeleton

From yeast to mammals, septins are implicated in the organization and functions of the actomyosin cytoskeleton. Septins colocalize with actin filaments and interact with actin-binding proteins and signaling effectors that regulate the organization of the actin cytoskeleton (Kinoshita, 2002; Mavrakis et al., 2014; Nagata and Inagaki, 2005) (Figure
1.3 A). Notably, septins interact directly with myosin II, regulating its localization and activation in interphase and mitotic cells (Joo et al., 2007).

In the budding yeast *S. cerevisiae*, septins are essential for the recruitment of proteins involved in actin cable assembly during bud growth (Figure 1.3 D). At the site of bud formation, linear actin cables, which enable the polarized transport of vesicles, are polymerized by the formins Bni1 and Bnr1 (Bretscher, 2013). In the absence of the nonessential septin Shs1, Bnr1 is mislocalized and actin cables fail to assemble (Buttery et al., 2012; Gao et al., 2010). The actin assembly activity of Bnr1 appears to depend on Shs1 (Buttery et al., 2012) and the septin-interacting partner Bni5 is required for the recruitment of myosin II, which drives actin cable retrograde flow (Nam, 2007). Thus, septins scaffold and may activate the assembly of actin cables during bud growth. Interestingly, mammalian septins scaffold the recruitment of cortactin, an activator of Arp2/3, to actin patches in the axons of sensory neurons (Hu et al., 2012). Septin 6 (SEPT6) localizes to actin patches, which consist of branched actin filaments, and SEPT6 depletion reduces the recruitment of cortactin to actin patches and their transition to filopodia (Hu et al., 2012) (Figure 1.3 B and D). Reconstitution of branched actin assembly *in vitro* and in the presence of Arp2/3 shows that SEPT6 localizes preferentially to actin branch points (Hu et al., 2012). In agreement with this observation, overexpression of SEPT6 increases the recruitment of cortactin to the lamellipodium, a branched actin network at the leading edge of motile cells (Hu et al., 2012). Additional septins (e.g., SEPT1, SEPT4) are enriched in the lamellipodia of squamous carcinoma cells (Mizutani et al., 2013). Septins, therefore, have an evolutionarily conserved role in scaffolding proteins involved in the formation of linear and branched actin filaments.

More recently, septins were found to cross-link and bundle actin filaments directly, contributing to the assembly of contractile actin rings in *Drosophila* embryos
In developing *Drosophila* embryos, the blastoderm contains thousands of nuclei, which become individual cells. Cellularization requires the assembly of contractile rings of actomyosin filaments that furrow the plasma membrane resulting in membrane canals that envelope the nuclei of the syncytial embryo. Embryos expressing a septin mutant are characterized by a loss of circular actin filaments, which are less bundled and contract at slower rates (Mavrakis et al., 2014). *Drosophila* septins bundle actin filaments and copolymerization of actin with septins results in curved and circular actin bundles (Mavrakis et al., 2014) (Figure 1.3 C and F). Mammalian septins also bundle actin filaments *in vitro* and septin depletion results in loss of actin stress fibers (Kinoshita, 2002; Kremer et al., 2007). These findings suggest that septins are actin-binding and bundling proteins that regulate the organization of actin microfilaments. However, the function of septins during actomyosin assembly is not fully understood.

In addition to their direct roles in actin organization, septins influence actin organization and contractility by interacting with signaling factors and myosin II. Assembly of stress fibers is under the control of Rho GTPases, which activate actin nucleation factors and promote the contraction of myosin II (Chrzanowska-Wodnicka and Burridge, 1996). Several studies have indicated that septins are involved in the Rho signaling pathways that control stress fiber assembly. Septin 9 (SEPT9), which is positioned at the terminal ends of septin hetero-octamers, interacts directly with a Rho guanine exchange factor (GEF), termed the septin-associated Rho GEF (SA-Rho GEF), and Rhotekin, a secondary effector of Rho signaling (Ito et al., 2005; Nagata and Inagaki, 2005). Both SA-Rho GEF and Rhotekin regulate septin assembly and localization to stress fibers. Interestingly, SEPT9 appears to negatively regulate Rho activation by inhibiting GTP loading by the SA-Rho GEF (Nagata and Inagaki, 2005). Septins also interact with Binders of Rho GTPases (Borgs), which are effectors of Cdc42.
New studies show that Gic1, a yeast homologue of the human Borg, binds and cross-links septin filaments \textit{in vitro} in a Cdc42-dependent manner (Sadian et al., 2013). Binding of the active Cdc42-GTP to Gic1 results in the dissociation of Gic1 from septin filaments. The inactive Cdc42-GDP, however, also inhibits the assembly of septin filaments by competing with Gic1 for binding to septins (Sadian et al., 2013). In mammalian cells, Borgs have been shown to affect both septin and stress fiber assembly (Joberty et al., 1999; Joberty et al., 2001).

While more work is needed to elucidate how the interactions between septins and signaling molecules affect actin organization, structural and functional analysis of the septin-myosin II interaction has provided a novel mechanism for the activation of the non-muscle myosin II. The mammalian SEPT2 has been shown to interact directly with the heavy chain of the non-muscle myosin II and yeast two-hybrid data indicate that Myo1, the \textit{S. cerevisiae} type II myosin, binds the sporulation-specific septin Spr3 (Drees et al., 2001; Joo et al., 2007). Disruption of the SEPT2-myosin II interaction results in loss of stress fibers in interphase cells and incomplete ingression of the cleavage furrow during mitosis (Joo et al., 2007). These defects are accompanied by significant reduction in the phosphorylation of the myosin light chain, which stimulates myosin II contractility. Importantly, SEPT2 appears to scaffold the interaction of myosin II with the citron and Rho-activated myosin kinases, which in the absence of septins dissociate from the cleavage furrow (Joo et al., 2007). Thus, septins may control spatially and temporally the localization and activation of myosin II. Importantly, this function might be evolutionarily conserved as myosin activation and actin organization is also disrupted in \textit{Xenopus} embryos after Sept7 depletion, decreasing planar tension and protrusive activity during the collective cell migration that drives body axis elongation (convergent extension) (Shindo, 2014).
In summary, septins are integral components of the actomyosin cytoskeleton. Recent studies have revealed that septins interact directly with actin filaments and myosin II, but many of the molecular and functional details remain unknown. Future studies will address the mechanisms by which septins affect the formation of linear and branched actin filaments and myosin II-dependent contractility, and how septins interface with signaling pathways that affect actin organization and contractility.

IV. Septins and the Microtubule Cytoskeleton

Septin filaments colocalize with microtubules in a diversity of eukaryotic organisms and cells (Spiliotis, 2010). The extent of septin-microtubule colocalization varies between septin paralogs and isoforms, and depends on cell type, phase of the cell cycle and tubulin isoform expression and post-translational modifications. Recent studies have shown that septins interact directly with microtubules, microtubule-associated proteins (MAPs) and motors as well as enzymes that modify tubulin’s post-translational modification. Thus, septins have emerged as novel regulators of microtubule organization and dynamics, and may play a key role in the spatial regulation of microtubule-dependent transport.

Mammalian SEPT2/6/7/9 complexes interact directly with microtubules through their SEPT9, SEPT7 and SEPT6 subunits (Figure 1.3 I); SEPT2 does not bind microtubules (Bai et al., 2013). It is unknown how SEPT6 and SEPT7 associate with microtubules, but SEPT9 interacts with the acidic tails of βII-tubulin via its N-terminal extension, which contains multiple repeats of the K/R-R/x-x-D/E motif (Bai et al., 2013). Similar to the microtubule-binding sequence repeats of filamentous MAPs (e.g., tau, MAP1), the SEPT9 repeat motifs consist of positively and negatively charged residues, which interact electrostatically with one another and the acidic C-terminal tails of α-
tubulin (Bai et al., 2013). Thus, the N-termini of SEPT9 mediate septin-septin and septin-microtubule interactions that cross-link microtubules into bundles. By forming filamentous cross-bridges that bind microtubules in a staggered arrangement, septins could increase the thickness and length of microtubule bundles as observed in vitro (Bai et al., 2013). Consistent with this function, septins colocalize preferentially with elongated microtubule bundles in cultured kidney epithelia (Bowen et al., 2011) (Figure 1.3 H). Moreover, expression of SEPT9 isoforms that lack N-terminal repeat motifs reduces both microtubule bundling and septin-microtubule colocalization (Bai et al., 2013). Hence, through the properties of SEPT9, mammalian septins function as bona fide MAPs that facilitate the bundling of microtubules.

Like most MAPs, septins affect not only microtubule bundling, but also microtubule dynamics. In kidney epithelia, SEPT2 knock-down increases microtubule catastrophe and reduces the rate of microtubule growth, indicating that septins are required for persistent microtubule growth (Bowen et al., 2011). Interestingly, loss of SEPT2 also affects microtubule-microtubule interactions and the overall directionality of microtubule growth (Bowen et al., 2011). In agreement with a septin requirement for microtubule growth and bundling, SEPT9 knock-down decreased the density of the microtubule network in mammary epithelia (Bai et al., 2013). Depletion of SEPT7, however, has the opposite effect in HeLa cells, increasing microtubule density and acetylation, a marker of microtubule stability (Kremer et al., 2005). A direct interaction between SEPT2 and MAP4 indicates that cytoplasmic SEPT2/6/7 complexes inhibit MAP4 from binding and bundling microtubules, but SEPT2/6/7 and MAP4 also compete for binding to microtubules and a new study shows that SEPT7 mediates the interaction of α-tubulin with the deacetylase HDAC6 (Ageta-Ishihara et al., 2013; Spiliotis et al., 2008). Surprisingly, SEPT7 is required for α-tubulin deacetylation and SEPT7-depleted
neurons have increased levels of acetylated tubulin and reduced rates of microtubule growth (Ageta-Ishihara et al., 2013). While the cause-and-effect relationship between microtubule “stability” and acetylation is unclear, septins appear to affect microtubule bundling, dynamics and post-translational modifications including acetylation and polyglutamylation. Given that septins interact with α-tubulin, MAP4 and HDAC6 in a paralog- and isoform-specific manner, it is possible that septins have distinctly different roles, targeting different aspects of microtubule organization and dynamics.

Microtubule post-translational modifications and MAPs affect the binding and processive movement of motors, generating a code for the spatial regulation of intracellular transport (Verhey and Gaertig, 2007). Presence of septins on select microtubule tracks (e.g., perinuclear microtubule bundles) raises the possibility that septins regulate microtubule-dependent transport (Spiliotis et al., 2008). In kidney epithelial cells, tubular vesicular carriers egress from the trans-Golgi network on septin-coated microtubule tracks (Spiliotis et al., 2008). Microinjection of septin function-blocking antibodies or SEPT2 depletion results in stalling of vesicle transport en route to the plasma membrane (Spiliotis et al., 2008). Competition between SEPT2 and MAP4 for binding to microtubules suggests that septins could block the microtubule binding of MAP4, which inhibits kinesin-dependent transport (Spiliotis et al., 2008). It is unknown whether septins affect directly the binding and/or processive movement of microtubule motors, but SEPT7 interacts directly with the mitotic motor CENP-E (kinesin 7) (Zhu et al., 2008). Interestingly, septins are required for the proper localization of CENP-E and consequently, for the stable capture and biorientation of the kinetochores of congressing chromosomes (Spiliotis et al., 2005). Because SEPT7 interacts with the C-terminal tail of CENP-E, which binds and inhibits the motor domain of CENP-E, SEPT7 could promote the activation and motility of CENP-E by relieving its autoinhibition. Alternatively, septins
may scaffold the interaction of CENP-E with the Aurora A and B kinases or the protein phosphatase 1 (PP1), which regulate CENP-E binding to microtubules; Aurora B has been reported to interact directly with SEPT1 (Qi et al., 2005).
**Figure 1.1. Septin filament assembly.** Mammalian septins are classified in four groups (SEPT2, SEPT6, SEPT7 and SEPT9) and characterized by highly conserved domains such as a phosphoinositide-binding polybasic domain (PB), a GTP-binding domain, a septin unique element (SUE) and C-terminal α-helical coiled-coil (CC) domains. Septins form non-polar hetero-oligomeric complexes by dimerizing in tandem using their GTP-binding domains, which interact with one another via their N- and C-terminal helices (NC interface) or their GTP-binding pockets (G interface). GTP hydrolysis favors oligomerization by stabilizing the G interface, but septins of the SEPT6 group do not hydrolyze GTP. End-to-end assembly of septin hetero-oligomers results in the formation of non-polar filaments, which interact laterally through the N- and C-terminal extensions of their septin subunits. Owing to the flexibility of the NC interface, paired septin polymers can bend into curved and ring-like filaments. In addition to septin bundles and rings, membrane-associated septins form gauze-like structures, which consist of orthogonally arranged filaments.
Table 1.1. Septin filaments in comparison to actin microfilaments, microtubules and intermediate filaments.

<table>
<thead>
<tr>
<th></th>
<th>Septins</th>
<th>F-actin</th>
<th>Microtubules</th>
<th>Intermediate Filaments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Subunit(s)</strong></td>
<td>SEPT1-14</td>
<td>α-β-γ-δ-ε-actin</td>
<td>α-β-tubulin</td>
<td>keratin, vimentin, lamin, desmin, peripherin, nestin, neurofilament proteins</td>
</tr>
<tr>
<td><strong>Nucleotide</strong></td>
<td>GTP</td>
<td>ATP</td>
<td>GTP</td>
<td>None</td>
</tr>
<tr>
<td><strong>Diameter</strong></td>
<td>~ 5 nm</td>
<td>~ 7 nm</td>
<td>~ 25 nm</td>
<td>~ 10 nm</td>
</tr>
<tr>
<td><strong>Length</strong></td>
<td>25-32 nm</td>
<td>several microns</td>
<td>several microns</td>
<td>several microns</td>
</tr>
<tr>
<td><strong>Structure</strong></td>
<td>Straight or curved paired filaments, rings and gauzes</td>
<td>right-handed helix</td>
<td>hollow tube</td>
<td>twisted filaments</td>
</tr>
<tr>
<td><strong>Polarity</strong></td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td><strong>Subunit turnover (t/2)</strong></td>
<td>10 – 200 seconds</td>
<td>10s of seconds</td>
<td>10s of seconds</td>
<td>100s of minutes</td>
</tr>
<tr>
<td><strong>Post-translational modifications</strong></td>
<td>phosphorylation, acetylation, sumoylation</td>
<td>phosphorylation, arginylation, ribosylation, methylation, ubiquitylation, glycosylation, carbonylation, sumoylation</td>
<td>phosphorylation, (de)acetylation, polyglutamylation, (de)tyrosination, palmitoylation, glycylation</td>
<td>farnesylation, phosphorylation, ubiquitylation, acetylation, sumoylation</td>
</tr>
</tbody>
</table>
Figure 1.2. Septins regulate membrane organization, shape and dynamics.

(A) Septins form barriers to lateral diffusion of membrane lipids (orange) and proteins (blue).

(B) Septins localize to membrane-membrane contact sites and regulate vesicle docking and fusion.

(C) Paired septin filaments induce the tubulation of giant unilamellar vesicles in vitro.

(D) Septins can sense membrane curvature.

(E) Septins rigidify cell membranes by localizing adjacent to membrane protrusions.
Figure 1.3. Septins interact with the actin and microtubule cytoskeleton. (A – E) Fluorescence images show purified recombinant septins tagged with the green fluorescent protein decorating linear (A), branched (B) and circular (C) actin filaments. Recent evidence indicates that septins crosslink actin filaments into curved and circular bundles (Mavrakis et al, 2014) and scaffold the recruitment of cortactin, an actin nucleation promoting factor, at Arp2/3-mediated branch points (Hu et al, 2012). In the budding yeast *Saccharomyces cerevisiae*, septins are required for the localization and activity of formins, which stimulate the formation of actin cables (D). In the axons of sensory neurons, septins accumulate to actin patches (E), which consist of Arp2/3-nucleated branched filaments, and in Drosophila embryos, septins are essential for the assembly of the contractile rings of actomyosin (F), which drives membrane invagination and furrow ingestion during cellularization. Scale bars, 1 µm. (G – L) Fluorescent images show colocalization of septin filaments with actin stress fibers (G) and microtubules (H) in Madin-Darby canine kidney cells, and *in vitro* decoration of pre-polymerized microtubules with purified recombinant SEPT7. Septins bundle microtubules and inhibit their interaction with other microtubule-associated proteins (J). Post-translational modifications (PTM) of tubulin such as acetylation and polyglutamylation are influenced by septins, which can scaffold the interaction of tubulin with the deacetylase HDAC6 (K). In addition, septins have been shown to guide spatially microtubule-microtubule interactions and may influence the localization of microtubule motors (L). Scale bars, 10 µm.
CHAPTER 2. Tissue-specific expression of septins in health and disease

Insights into the tissue expression and functions of mammalian septins have emerged from DNA microarray analyses and septin knock-out mice. Serial analysis of gene expression in a variety of human tissues shows unique expression of individual septins (Table 2.1). SEPT2, SEPT7 and SEPT9 are ubiquitously expressed (Cao et al., 2007; Connolly et al., 2011a; Hall et al., 2005a). While absent from some tissues, the septins SEPT4, SEPT8, SEPT10 and SEPT11 are expressed widely (Cao et al., 2007; Hall et al., 2005b). In contrast, expression of SEPT1, SEPT3, SEPT12 and SEPT14 is limited to specific tissues. SEPT1 and SEPT3 are expressed in lymphoid and nervous tissues, respectively (Hall et al., 2005a), and SEPT12 is a testis-specific septin (Steels et al., 2007; Xue et al., 2004). SEPT14 was originally identified as a novel testis-specific protein, but is also expressed in the nervous system (Peterson et al., 2007; Shinoda et al., 2010). Little is known about how septin expression varies throughout development and adult life, but a recent study showed that the levels of ten different septin proteins change dramatically during brain development (Tsang et al., 2011).

I. Septin expression and roles in embryogenesis

To date, knockout mice have been generated for seven of the thirteen septins. Genetic ablation of the ubiquitous Sept7, Sept9 and Sept11 resulted in embryonic lethality. Sept7−/− mice were never born, arresting early in development possibly due to mitotic failure (Kinoshita, 2008). Development of Sept11−/− mice ceased at day 11.5 in utero and the mice died by day 13.5 (Roseler et al., 2011). Despite the development of a healthy yolk sac, heartbeat and blood vessels, Sept9−/− embryos died by day 10 of gestation, exhibiting mesenchymal tissue degeneration and extensive cell death.
(Fuchtbauer et al., 2011). Surprisingly, loss of the neuron-specific Sept3 did not have any discernible effects on the physiology and function of Sept3\(^{-/-}\) mice (Tsang et al., 2008). Functional redundancy between Sept3 and Sept9 or compensation of Sept3 loss by Sept9 isoforms could account for the lack of phenotype. Similarly, Sept5\(^{-/-}\) and Sept6\(^{-/-}\) mice were reported to be normal, but newer behavioral assays indicate changes in the social interaction, rewarded goal approach and anxiety-related behavior of Sept5\(^{-/-}\) mice (Ono et al., 2005a; Suzuki et al., 2009). From all the septin knock-out mice, Sept4\(^{-/-}\) are the most well-studied and characterized. Male Sept4\(^{-/-}\) mice are sterile due to a morphologically altered and immotile sperm (Ihara et al., 2005; Kissel et al., 2005). Neurologically, the cerebellum of Sept4\(^{-/-}\) mice is mildly maldeveloped and hypodopaminergic phenotypes such as an increase in the inhibition of the startling response by weaker pre-stimuli have been reported (Kinoshita, 2008). Loss of Sept4 from mice that express the alpha synuclein mutant A53T, which is common among familial forms of Parkinson’s disease, increased amyloid deposition and neurodegenerative pathology, leading to shorter lifespans (Ihara et al., 2007). Moreover, Sept4\(^{-/-}\) mice are more susceptible to tumor growth and liver fibrosis, and contain high levels of hematopoietic and hair follicle stem cells (Fuchs et al., 2013; Garcia-Fernandez et al., 2010; Iwaisako et al., 2008).

The embryonic lethality phenotype of the Sept7\(^{-/-}\), Sept9\(^{-/-}\), Sept11\(^{-/-}\) mice suggests that septins play an essential role in embryogenesis. It is unknown how septins are involved in the embryonic development of mammals, but studies in model organisms such as the fruit fly Drosophila melanogaster, the roundworm Caenorhabditis elegans and the frog Xenopus laevis have shed some light on the developmental functions of septins. In early Drosophila development, multiple rounds of mitotic nuclear divisions lead to the formation of a multinucleated single cell embryo. The nuclei of this syncytial...
blastoderm are partitioned into individual cells by a process termed cellularization. In septin-deficient embryos, cellularization is incomplete resulting in multinucleated cells, less imaginal discs (precursors of epithelial tissues) and larval death (Adam et al., 2000; Neufeld and Rubin, 1994). In *C. elegans*, septins localize similarly to the cytokinetic furrows, but are not essential for embryonic development (Nguyen et al., 2000). Septin-depleted embryos rarely fail to complete cytokinesis, but the asymmetric geometry of furrow ingestion is disrupted and the contractile apparatus is less robust and more prone to stochastic errors (Maddox et al., 2007). Post-embryonic development of *C. elegans* tissues is more severely affected by septin mutations, which disrupt gonadogenesis and the formation of a functional sensory and motor nervous system (Finger et al., 2003; Nguyen et al., 2000). Recently, a study in *Xenopus* showed that septins are involved in the planar cell polarity (PCP) signaling pathway, which directs the collective cell movements of embryogenesis during gastrulation, axial elongation and organogenesis. The PCP signaling protein Fritz, which interacts directly with Sept2, was shown to control septin localization to the cortical membrane of gastrulating cells (Kim et al., 2010). Moreover, Fritz and septins synergized toward the formation of cilia, which are critical for the transduction of the Sonic Hedgehog signals that regulate organogenesis (Kim et al., 2010). Thus, septins are essential components of the molecular and cellular mechanisms that give rise to complex organ and tissue systems.

II. Cardiovascular System

The heart is the first organ to develop during embryogenesis. Terminal differentiation of cardiomyocytes occurs near birth and is characterized by the cessation of cell division and the development of contractile multinucleated cells. In mouse embryonic cardiac cells, the levels of septin 2, 6, 7 and 9 levels are the highest in early
development and decrease at birth and adulthood (Ahuja et al., 2006). In embryonic cardiomyocytes, septins localize to the cytokinetic ring and midbody of dividing cells and are absent from sarcomeric actomyosin (Ahuja et al., 2006). Thus, septins could function in early cardiac development by interacting with key components of the cytokinetic machinery (e.g., myosin, anillin). Moreover, septin downregulation could trigger mitotic arrest and the formation of terminally differentiated multinucleated cells. Interestingly, SEPT5 is located within the 22q11 locus that is commonly deleted in the DiGeorge/velocardio-facial syndrome (DGS), which is characterized by cardial malformations and lesions (Judith M. McKie, 1997). Studies in animal models suggest that 22q11 hemizygosity, which occurs in 90% of DGS cases, results in developmental impairment of the right ventricle and outflow tract (Scambler, 2000). It is unknown if partial loss of SEPT5 contributes to the pathology of DGS pathology, but decreased septin (SEPT8) expression is also implicated in the toxic effects that anti-inflammatory drugs have on the development of embryonic cardiomyocytes (Baek et al., 2010).

Septin expression and functions in the vascular network have been identified in platelets, which are specialized secretory cells that regulate haemostasis, thrombosis and injury repair. SEPT5 (CDCRel-1) localizes around platelet α-granules, storage vesicles that contain growth factors and effector molecules involved in adhesion and clot formation (Dent et al., 2002). Additionally, SEPT5 co-precipitates with syntaxin-4, a component of the SNARE-SNAP complex that mediates vesicle fusion with the plasma membrane (Dent et al., 2002). Platelets isolated from Sept5 null mice secrete serotonin more readily upon stimulation, suggesting that Sept5 negatively regulates α-granule fusion with the plasma membrane (Dent et al., 2002).

Abnormal SEPT5 expression has been linked to the Bernard-Soulier syndrome (BSS), a rare autosomal recessive blood disorder characterized by excessive bleeding
and abnormal platelet morphology, count and secretion (Lopez et al., 1998). BSS patients harbor mutations in the quaternary platelet glycoprotein (GP) Ibβ-IX receptor subunit, which regulates platelet adhesion, activation and aggregation. A mouse model lacking the IBβ gene recapitulates BSS pathology and isolated platelets contain abnormally large α-granules and elevated levels of SEPT5 (Kato et al., 2004). More recently, genotypic analysis of a juvenile patient with a rather severe case of BBS showed homozygous deletions of the Ibβ and SEPT5 genes, which are positioned directly next to one another (Bartsch et al., 2011; Zieger et al., 1997).

III. Immune System

The mammalian innate and adaptive immune systems encompass several tissues and specialized cell types that defend against infectious agents. Despite that the first mammalian septin (SEPT1) was cloned from lymphocytes twenty five years ago (Carol., 1990), it was not until recently that researchers began to investigate the immunological functions of septins.

Cells of the innate immunity (e.g. dendritic cells, macrophages) recognize and engulf foreign bodies, and elicit a larger response through antigen presentation. SEPT10 was initially identified in dendritic cells, where it is abundantly expressed (Sui et al., 2003). In contrast, SEPT10 is weakly expressed in the spleen, thymus and peripheral blood leukocytes. In dendritic cells SEPT10 localizes to the cytoplasm and the nucleus, and is transcriptionally upregulated upon stimulation with lipopolysaccharide (Sui et al., 2003). Septins 2, 6, 8, 10 and 11 are abundantly expressed in macrophages, and SEPT2 and SEPT11 localize to actin-based structures at the base and periphery of the phagocytic cup (Huang et al., 2008). Disrupting septin assembly by expressing the septin-binding domain of BORG3 or targeted knock down of SEPT2 or SEPT11 reduced
phagosome formation and uptake of IgG-coated beads (Huang et al., 2008). Further studies are necessary to understand the precise functions of septins in dendritic and macrophage cell biology.

T lymphocytes proliferate and mature in lymphoid tissues (e.g., thymus, spleen, lymph nodes) and migrate to peripheral tissues, searching for antigen presenting cells. Septins are essential for both T cell development and migration. During mouse T-cell development, the transition from a double negative (CD4−CD8−) to a double positive (CD4+CD8+) stage is accompanied by a 5- to 10-fold increase in Sept9 expression (Lassen et al., 2013). Sept9 deletion affects T-cell development in the thymus leading to an increase in stage 3 double negative cells (CD4−CD8−CD25−CD44+) at the expense of stage 4 double negative cells (CD4−CD8−CD25−CD44+). Peripheral CD8+ and CD4+ T-cells are reduced concomitantly and in vitro proliferation of Sept9-deleted CD8+ T-cells is impaired; the effects of Sept9 deletion in vivo are more severe on CD8+ than CD4+ T-cells (Lassen et al., 2013). These studies are the first to implicate septins in T-cell development, but more work is needed to determine the mechanism by which SEPT9 regulates T-cell maturation and selection.

Lymphocytes migrate by forming a leading pseudopod and a trailing uropod, both of which require polarized assembly of filamentous actin and contraction of the cortical membrane. In T-cells, septins localize to the middle of the cell cortex and are distinctly absent from the protruding pseudopod and uropod (Tooley et al., 2009). SEPT7-depleted lymphocytes have elongated uropods and show poor persistent migration despite maintaining normal levels of actin, microtubules and phosphorylated (active) myosin (Tooley et al., 2009). In addition, SEPT7 depletion results in excessive blebbing and reduces the rate of cortical retraction in a hydrostatic volume change assay, which measures the ability of cells to expand and retract their cell membrane (Gilden et al.,
In migrating lymphocytes, septins (SEPT6 and SEPT7) are absent from blebs and filopodia, but are recruited to plasma membrane sites adjacent to these protrusive structures (Gilden et al., 2012; Sellin et al., 2011a). Overall, the plasma membrane organization and dynamics of migrating T-cells appear to depend on septins, which affect persistent migration.

In non-immune cells, septins are essential for host defense against infectious bacteria. The invasive bacterium *Listeria monocytogenes* enters mammalian cells through the interaction of its surface proteins internalin A and B (InIA, InIB) with E-cadherin and the Met receptor, respectively (Cossart, 2011). Targeted depletion of SEPT2 inhibits bacterial invasion and impairs the InIB/Met signaling pathway (Mostowy et al., 2009). Septins also thwart the intracellular mobility of the *Shigella flexneri* bacterium, which propels itself through the cytoplasm by recruiting and activating the Arp2/3 complex, which induces the polymerization of an actin tail (Egile et al., 1999). Septin filaments are recruited to cytoplasmic *Shigella* and assemble cage-like structures that inhibit bacterial mobility and facilitate the formation of an autophagosome for bacterial degradation (Mostowy et al., 2010).

**IV. Nervous system**

Development of a functional nervous system involves the migration of embryonic stem cells and neural precursors, and their differentiation into neurons and glial cells. Neuronal morphogenesis requires the formation of axons, dendrites and synapses. Recent studies have shown that septins play essential roles in neuronal migration, axonal and dendritic arborization, and synaptic activity. In developing mouse embryos, SEPT4 and SEPT14 are highly expressed in the cortical plate of the developing cortex, which is formed by post-mitotic neurons that migrate tangentially from the subventricular
zone to the outer layers of the neocortex (Shinoda et al., 2010). In vivo knock down of SEPT14 or SEPT4 expression and inhibition of their interaction perturb neuronal migration to the cortical plate; neurons fail to extend leading processes and stall at the ventricular and intermediate zones without reaching the cortical plate (Shinoda et al., 2010). The sensory and motor neurons of the *C. elegans* septin mutants *unc-59* and *unc-61* exhibit similar migration defects during larval development (Finger et al., 2003).

In cultured hippocampal neurons, septins are required for the formation of dendritic spines (Cho et al., 2011; Tada et al., 2007; Xie et al., 2007). SEPT5/7/11 complexes localize to dendritic branch points and at the base of dendritic spines (Xie et al., 2007). Targeted depletion of SEPT7, SEPT11 or SEPT6 reduces dendritic arborization and alters the length, density and head morphology of dendritic spines (Cho et al., 2011; Tada et al., 2007; Xie et al., 2007). In contrast, SEPT7 overexpression increases dendritic protrusions and branchpoints (Tada et al., 2007; Xie et al., 2007). Because dendritic spines are enriched with membrane proteins (e.g., neurotransmitter receptors) that localize specifically at the post-synaptic cleft, septins are posited to form a diffusion barrier at the base of dendritic spines blocking free diffusion of membrane proteins in and out of the dendritic shaft (Caudron and Barral, 2009). In addition, septins may also synergize with the actin and microtubule cytoskeleton for the formation of dendritic spines.

Septin-mediated reorganization of the actin and microtubule cytoskeleton is required for the collateral branching of axons. In sensory neurons isolated from chicken dorsal root ganglia, SEPT6 and SEPT7 localize at the base of axonal filopodia and axon branch points (Hu et al., 2012). This localization is reminiscent of septin complexes at the base of dendritic spines, but in axons septins control the initiation and maturation of collateral branches (Hu et al., 2012). SEPT6 enhances the transition of axonal patches
of F-actin to filopodia by enhancing the recruitment of the actin-binding protein cortactin, an activator of Arp2/3-mediated actin polymerization (Hu et al., 2012). In contrast, SEPT7 is involved in the entry of axonal microtubules into nascent filopodia, enabling the formation of collateral branches (Hu et al., 2012). These regulatory roles of septins are likely to influence not only the outgrowth and differentiation of neurites into axons and dendrites, but also axon extension and growth cone motility. Indeed, septins have been reported to affect axon length (Vega and Hsu, 2003), but more studies are needed to understand their precise roles in axonal differentiation and growth.

Neurotransmission and organ innervation rely on the formation and function of chemical synapses, which release and receive neurotransmitter molecules through their pre- and post-synaptic clefts, respectively. Neurotransmitter secretion and uptake are coupled to ion channels that sense and generate membrane potential. Initial studies indicated that septins inhibit the release of synaptic vesicles (Beites et al., 2001), but new evidence suggests that septins could also regulate the localization of ion channels in membrane microdomains and their endocytosis from the cell membrane. The septins 3, 5, 6, 7 and 11 localize to the presynaptic cleft and their filamentous organization coincides with the filament-like strands, which have been observed by electron microscopy to connect synaptic vesicles with each other and the plasma membrane (Hirokawa et al., 1989; Kinoshita et al., 2000; Tsang et al., 2011). SEPT5 and SEPT8 have been reported to interact with components of the molecular machinery of synaptic vesicle fusion (Beites, 2005; Beites, 1999; Ito et al., 2009). SEPT5 and SEPT7 are posited to inhibit vesicle fusion by inhibiting the formation of productive SNARE complexes (Beites, 2005; Wasik et al., 2012). Interestingly, however, synaptic transmission and morphology were not altered in Sept5−/− and Sept3−/− mice (Tsang et al., 2008). Further analysis of the developing calyx of Held, which is a large glutamatergic
synapse of the auditory nervous system, showed that Sept5 filaments play a key role in the positioning of pre-docked synaptic vesicles relative to the voltage gated calcium channels (VGCCs) of the active zone (Yang et al., 2010). In Sept5-/− synapses, synaptic vesicles are more tightly docked to the VGCCs. In addition, synaptic vesicles are more readily and cooperatively released requiring a lower number of calcium channels to trigger single fusion events (Yang et al., 2010). Thus, SEPT5 introduces a physical barrier between synaptic vesicles and VGCCs regulating the efficiency of synaptic transmission. A new study, however, suggests that SEPT5 and SEPT4 may modulate synaptic activity by controlling the membrane organization and clustering of calcium and possibly other ion channels (Sharma et al., 2013b). Although this hypothesis has yet to be tested in neurons, SEPT4 is required for the organization of phosphatidyl-inositol-(4,5)-bisphosphate-rich domains in the plasma membrane of HeLa cells and the clustering of the calcium channel ORAI1, which triggers calcium entry and signaling when calcium concentration is low in the endoplasmic reticulum (Sharma et al., 2013b).

Interestingly, the astrocyte glutamate transporter GLAST has been shown to interact directly with SEPT2 (Kinoshita et al., 2004). SEPT2 mutations increase GLAST internalization and decrease glutamate uptake (Kinoshita et al., 2004). Interaction of septins (SEPT5, SEPT11) with dynamin, which mediates scission of vesicles from the plasma membrane, raises the possibility that septins regulate endocytosis at synaptic terminals, affecting the recycling of synaptic vesicles and ion transporters (Maimaitiyiming et al., 2013; McNiven, 1998).

In summary, septins have fundamental roles in the development, activity and connectivity of the nervous system. Thus, abnormalities in septin expression are likely to compromise the function of the nervous system, contributing the pathology of neurodevelopmental and neurodegenerative disorders. Hereditary neuralgic
amyotrophy, a disorder characterized by shoulder and arm pain and muscle atrophy, is genetically linked to missense mutations and duplications in the N-terminal sequence of SEPT9 (Chance and Windebank, 1996; Kuhlenbaumer et al., 2005). These abnormalities may cause the axonal degeneration and focal demyelination observed in the brachial plexus nerves of HNA patients (Ueda et al., 2010). In the maldeveloped brain of fetuses with Down Syndrome, expression of SEPT6 and SEPT7 is markedly reduced and SEPT4 might be hyper-phosphorylated due to over-expression of the DYRK1A kinase (Sitz et al., 2008). Decreases in septin 7 and 11 expression were also observed in Schizophrenia and bipolar disorders, and SEPT9 was abnormally overexpressed in a mouse model of demyelinating neuropathy (Patzig et al., 2011; Pennington et al., 2008). Histological analysis of brains from Alzheimer’s patients identified SEPT1/2/4 in neurofibrillary tangles (Kinoshita et al., 1998), which are intracellular aggregates of the hyper-phosphorylated tau protein. In addition, allelic variations in SEPT3 isoforms have been observed in Alzheimer’s tissue samples indicating a potential role or risk factor in AD pathogenesis (Takehashi et al., 2004). In the brains of patients with Parkinson’s disease, SEPT4 is found in the Lewy body aggregates of α-synuclein (Ihara et al., 2003). Interestingly, SEPT4 interacts with α-synuclein directly, and loss of SEPT4 expression in the A53T familial Parkinson’s mouse model increases amyloid-like aggregation and neurodegeneration (Ihara et al., 2007).

V. Reproductive system

Septins have been genetically linked to male infertility and ovarian cancer. The importance of septins for sperm development was discovered by two independent studies of SEPT4 knock-out mice. Male Sept4−/− mice are sterile and their sperm is immotile and morphologically bent with an abnormal L-shape (Ihara et al., 2005; Kissel
et al., 2005). This phenotype correlates with Sept4 localization to the annulus, a cortical ring separating the middle and principle pieces of spermatozoa. In Sept4−/− sperm, the annular structure is abolished (Ihara et al., 2005; Kissel et al., 2005). Moreover, Sept4−/− sperm mitochondria have abnormal size, cristiae and membrane morphology, and an excess of cytoplasmic droplets is observed in the head and neck regions of the sperm (Kissel et al., 2005). While these data suggest that Sept4 functions in mitochondrial division and caspase-mediated removal of the cytoplasm during spermiogenesis, follow-up studies explored the hypothesis that Sept4 maintains a cortical diffusion barrier at the annulus. Hunnicutt and colleagues tracked the localization of the diffusing membrane protein basigin, which localizes to the principle piece during spermatogenesis and shifts to the middle piece during epididymal maturation (Kwitny et al., 2010). In the sperm of Sept4−/− mice, basigin fails to localize to either the principle or middle pieces, indicating that Sept4 is critical for maintaining the proper localization of sperm proteins through the establishment of an annular diffusion barrier (Kwitny et al., 2010). Because a defective annulus is common among patients with asthenospermia, screening for Sept4 localization is now used as a diagnostic tool for sperm malformation (Sugino et al., 2008).

In addition to Sept4, septins 1, 6, 7 and 12 also localize to the annulus of the sperm (Toure et al., 2011). Notably, Sept12 is expressed only in the testis (Steels et al., 2007) and similar to the Sept4−/− mice, Sept12+/− chimeric mice are sterile and their sperm is immotile with nuclear defects and distorted shape (Lin et al., 2009). In vitro fertilization of mouse oocytes with Sept12+/− sperm has low success rates and fertilized oocytes do not progress past the morula stage (Lin et al., 2011). Interestingly, genetic analysis of infertile men revealed two missense SEPT12 mutations, T89M and D197N, which map to the switch I and GTP recognition motifs of Sept12, respectively (Kuo et al., 2012).
Biochemical analysis showed that the SEPT12-T89M mutant had reduced rates of GTP hydrolysis and SEPT12-D197N did not bind GTP (Kuo et al., 2012). Both mutants failed to form filaments in tissue culture cells and SEPT12 was absent from the sperm annulus of SEPT2-D197N patients (Kuo et al., 2012). Thus, mutations in the testis-specific SEPT12 are genetically linked to male infertility.

Septin expression in ovarian tissue was first identified in *Drosophila* where *sep1* localizes to follicle cells during embryonic development (Fares et al., 1995). The precise function of septins in ovarian physiology is unknown. Misregulation of septin expression has been identified in ovarian tumors and is discussed in the next chapter.

**VI. Urinary and digestive systems**

The tissue and organ surfaces of the urinary and digestive systems are composed of epithelial cells. The epithelial plasma membrane is biochemically differentiated into the apical and basolateral membrane domains. The directional transport of water, ions and solutes depends on the proper localization of specific channels (e.g., aquaporins, Na/K pump) and transporters (e.g., p-glycoprotein) to the epithelial membrane domains. Establishment and maintenance of the polarized distribution of these proteins is key for the development and function of organs such as the kidney, intestine and lung.

In Madin-Darby canine kidney cells (MDCKs), a well-established model for studying renal epithelial morphogenesis in 2D and 3D cultures, SEPT2 is essential for the organization of the microtubule cytoskeleton and the transport of membrane vesicles from the Golgi to the plasma membrane (Bowen et al., 2011). During the establishment of columnar epithelial morphology, SEPT2 guides microtubule growth and microtubule-microtubule interactions for the establishment of the subapical microtubule network.
Moreover, SEPT2 localization to a subset of perinuclear, microtubule bundles enables the egress of Golgi-derived vesicles by hindering the binding of the microtubule-associated protein 4 (MAP4), which impedes microtubule motor-driven transport. Without proper microtubule architecture and post-Golgi membrane traffic, SEPT2-depleted epithelia fail to assume a columnar shape and most apical and basolateral membrane proteins accumulate in the cytoplasm. New studies suggest that septins are also important for the two-dimensional expansion of the renal epithelium, which occurs by planar division and asymmetric ingression of the cleavage furrow from the basal to the apical membrane. In Drosophila epithelia, septins enable the closure of the cytokinetic furrow by strengthening the cytokinetic actomyosin ring, which must overcome the tensile forces exerted by the apical adherens junctions (Founounou et al., 2013; Guillot and Lecuit, 2013).

Renal epithelia not only maintain water and ion homeostasis, but also monitor the flow of fluid in the lumen of the nephron, which in turn can affect cell morphology and proliferation. Fluid flow is sensed by the primary cilium, a microtubule-based antenna-like organelle that projects from the epithelial apical membrane into the lumen of the nephron (Pazour and Witman, 2003). Septins are integral and essential components of the primary cilium (Chih et al., 2012; Ghossoub et al., 2013; Hu et al., 2010). In the inner medullary collecting duct cells IMCD3, SEPT2 localizes to the base of the primary cilium and is required for the formation of functional cilia (Hu et al., 2010). SEPT2 depletion results in complete loss of primary cilia and the formation of shorter cilia, in which ciliary membrane proteins diffuse freely into the surrounding plasma membrane (Hu et al., 2010). Thus, SEPT2 is thought to be part of a membrane diffusion barrier, which maintains the localization of ciliary membrane proteins. Recent studies, however,
indicate that SEPT2/7/9 complexes may also interact with axonemal microtubules controlling the length of cilia (Ghossoub et al., 2013).

Podocytes are specialized epithelial cells that wrap around the capillaries of the kidney’s glomeruli and respond to insulin. Podocytes prevent serum albumin and other macromolecules from leaving the blood, while allowing water, salts and glucose to enter the kidney lumen. Loss of podocyte function is prevalent in diabetic nephropathy, the most common cause of renal failure (Pagtalunan et al., 1997). A new study shows that SEPT7 colocalizes with the glucose transporter isoform 4 (GLUT4) storage vesicles and interacts with nephrin, the CD2-associated protein CD2AP and the vesicle SNARE protein VAMP2 (Wasik et al., 2012). SEPT7 knock-down increased the interaction of VAMP2 with nephrin and syntaxin, and increased glucose uptake (Wasik et al., 2012). Thus, SEPT7 could be involved in the regulation of glucose transport in the insulin-sensitive podocytes.

VII. Respiratory System

Respiratory epithelia perform a number of key functions including gas exchange and protection from particulate matter. In the lower respiratory tract, the multiciliated bronchial epithelia protect the underlying tissue from noxious agents and push the mucus toward the pharynx (Fahy and Dickey, 2010). Maintenance of the epithelial cell-cell junctions is critical for limiting paracellular permeability, subepithelial inflammation and injury (Frank, 2012). Physiological stresses in bronchial epithelia (e.g. particulate matter) stimulate dynamic reorganization of the actin cytoskeleton at the cell cortex, strengthening cell-cell junctions and restricting paracellular permeability (Youakim and Ahdieh, 1999). In shear-stressed bronchial epithelia, SEPT2 is recruited to the apical plasma membrane and interacts with the F-actin network more strongly (Sidhaye et al.,
In SEPT2-depleted MDCK and bronchial cells challenged with shear stress or particulate matter, cortical F-actin is reduced and low-molecular-weight dextran enters into the basolateral space more readily (Sidhaye et al., 2011). Moreover, apical application of EGF in SEPT2-depleted cells results in elevated activation of the basolaterally-confined EGF receptor (Sidhaye et al., 2011). Taken together, these data indicate that SEPT2 is critical for the maintenance of epithelial integrity during respiratory stress.

### VIII. Endocrine system

Tissues and cells of the endocrine system secrete hormones that regulate the physiology and function of peripheral tissues and organs. In the pancreas, SEPT5 is highly expressed in the islet of Langerhans, while its expression is markedly reduced and absent from the acinar and ductal epithelia, respectively (Capurso et al., 2005). The islet of Langerhans consists of α- and β-cells that secrete glucagon and insulin, respectively, stimulating glucose release and uptake from the blood. The role of SEPT5 in exocytosis was tested in the HIT-T15 insulinoma cell line by assaying for the release of exogenous human growth hormone (hGH) in the presence of wild-type or dominant-negative SEPT5. Secretion of hGH was significantly reduced in cells expressing wild-type SEPT5 indicating that SEPT5 inhibits exocytosis (Beites, 1999). In pancreatic β-cells, SEPT2 interacts with EXO70, a component of the octameric exocyst complex that mediates vesicle tethering to the plasma membrane (Rittmeyer et al., 2008). The SEPT2-EXO70 complex localizes to the β-cell membrane and interacts directly with the Rho GTPase effector, IQGAP1 (Rittmeyer et al., 2008). Interestingly, overexpression of the SEPT2-EXO70 binding domain of IQGAP1 displaces SEPT2 from the plasma membrane, and the constitutively-active form of CDC42 inhibits the interaction of
IQGAP1 with the SEPT2-EXO70 complex and blocks insulin secretion (Rittmeyer et al., 2008). Although the precise role of septins in vesicle docking and fusion is unknown, regulation of septin localization and function by IQGAP and Rho signaling might be important for the secretion of insulin from pancreatic β-cells.

IX. Integumentary system

The integumentary system consists of the skin, hair follicles and sebaceous glands. Collectively, these structures make up the largest organ system of the body protecting all other organs from physical damage, infectious agents and water loss. Regeneration of the epidermis after injury relies on skin and hair follicle stem cells, whose growth and differentiation are tightly regulated so that tissue structure is preserved without overgrowth. A recent study showed that the SEPT4 splice variant ARTS (SEPT4_v2), is specifically expressed in hair follicle stem cells (HFSCs) (Fuchs et al., 2013). Hair follicle bulges in mice lacking Sept4/ARTS are morphologically normal, but contain twice as many CD34+ progenitor cells (Fuchs et al., 2013). Sept4/ARTS-deficient CD34+ cells grow faster in culture without feeder cells and are highly-resistant to pro-apoptotic stimuli, suggesting that SEPT4/ARTS restricts HFSC proliferation (Fuchs et al., 2013). Remarkably, wound recovery in Sept4/ARTS-deficient mice is extraordinarily fast. Sept4/ARTS has previously been shown to promote apoptosis by targeting XIAP, an inhibitor of apoptosis, for degradation (Larisch et al., 2000). HFSCs from mice deficient in both SEPT4/ARTS and XIAP showed reduced wound repair (Fuchs et al., 2013) supporting the hypothesis that SEPT4/ARTS-mediated apoptosis can limit stem cell growth.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus</th>
<th>Tissue Expression</th>
<th>Mouse model</th>
<th>Binding Partner</th>
<th>Disease Association</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEPT1</td>
<td>16p11.1</td>
<td>lymphoid - skin</td>
<td>not done</td>
<td>aurora B kinase</td>
<td>Alzheimer’s; oral, skin and colorectal cancers, leukemia</td>
<td>Qi et al., 2005</td>
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<td>SEPT2</td>
<td>2q.37.3</td>
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<td>GLAST, MAP4, snailin, myosin II, exo70, IQGAP, p85</td>
<td>Alzheimer’s, renal cell carcinoma, brain cancer, leukemia, systemic lupus erythematosus</td>
<td>[Bettges et al., 1999; Garcia et al., 2008; Joo et al., 2007; Kinoshita et al., 2002; Kinoshita et al., 2004; Kremer et al., 2005; Voga and Hsu, 2003]</td>
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<tr>
<td>SEPT3</td>
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<td>neuronal</td>
<td>Viable</td>
<td>cGMP-dependent protein kinase-1 (PKG-I)</td>
<td>Alzheimer’s, Down syndrome, brain cancer</td>
<td>Xue et al., 2004</td>
</tr>
<tr>
<td>SEPT4</td>
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<td>Viable; sterile</td>
<td>X-linked inhibitor of apoptosis (XIAP), α-synuclein, DYRK1A kinase, Kaposin A</td>
<td>Alzheimer’s, Parkinson’s, melanoma, leukemia, urothelial cancers, schizophrenia, male infertility</td>
<td>[Gottfried et al., 2004; Ihera et al., 2003; Lin et al., 2007; Sitz et al., 2008]</td>
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<td>22q13.2</td>
<td>widely expressed</td>
<td>Viable; behavioral defects</td>
<td>syntaxin-1A, syntaxin-4, parkin, dynamin-1</td>
<td>Pancreatic cancer, leukemia, Bernard-Soulier syndrome, Parkinson’s, schizophrenia, bipolar disorder</td>
<td>[Bettges et al., 1999; Choi et al., 2003; Dent et al., 2002; Malmqvist et al., 2013]</td>
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<tr>
<td>SEPT6</td>
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<td>Viable</td>
<td>SOCS7, binder of Rho GTPases (BORGs), hnrNPA1, NSSb, myelin and lymphocyte protein (MAL)</td>
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<td>Embryonic lethal</td>
<td>CENP-E, nephrin, CD2AP, ERK3</td>
<td>Alzheimer’s, Down Syndrome</td>
<td>Brind et al., 2012; Wasik et al., 2012; Zhu et al., 2008</td>
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<tr>
<td>SEPT8</td>
<td>5q31</td>
<td>widely expressed</td>
<td>not done</td>
<td>VAMP2, mitogen-activated protein kinase activated protein kinase 5 (MK5), CDX14 (PFTARE1)</td>
<td>Retinal degeneration</td>
<td>[Ito et al., 2009; Shinaev et al., 2012; Yong et al., 2001]</td>
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<td>Embryonic lethal</td>
<td>microtubules, rhoetkin, septin-associated (SA)-RhoGEF, HIF1-α, internalin B</td>
<td>Down syndrome, hereditary neurologic amyotrophy, colorectal, breast, ovarian, liver and head and neck cancers, leukemia</td>
<td>[Amir et al., 2006; Ito et al., 2005; Nagata and Napaki, 2015; Nagata et al., 2003; Picarra-Clenta et al., 2020]</td>
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<td>not done</td>
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<td>widely expressed</td>
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<td>dynamin-1</td>
<td>Schizophrenia, bipolar disorder, renal cell carcinoma, leukemia</td>
<td>(Maimaitiying et al., 2013)</td>
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<td>not done</td>
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<td>7p11.2</td>
<td>testicular - neuronal</td>
<td>not done</td>
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Figure 2.1 Septin functions in organ systems and their connection to human disease
Schematic diagram depicts main organ systems of the human body and outlines organ- and cell type-specific structures and processes that require septins. Color codes indicate the intracellular involvement of septins, which includes septin functions in the organization of actin (red), microtubules (green) and cell membranes (grey), vesicle fusion (blue) and apoptosis (yellow). Human diseases are listed based on genetic, histological, genomic and proteomic evidence that implicates septins in the pathology of various organ disorders.
CHAPTER 3. Septins in cancer

Septin expression is frequently altered in cancer, including tumors of epithelial origin and hematological malignancies. On the other hand, increased assembly of septins with actin stress fibers in cancer-associated fibroblasts, resident cells of the tumor microenvironment, was shown recently to promote ECM remodeling, angiogenesis and tumor cell invasion in a mouse model of breast cancer (Calvo et al, 2015). Septins are also emerging biomarkers for urological and colon cancers, but their role in tumorigenesis and metastasis is poorly understood.

Septins in hematological malignancies

Aberrant septin expression has been implicated in blood disorders including acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) (Cerveira et al., 2011). MLL is a histone methyltransferase that regulates homeobox (HOX) gene expression during hematopoiesis (Muntean and Hess, 2012). Chromosomal translocations of the mixed lineage leukemia (MLL) gene account for ~10% of the de novo and therapy-induced leukemias (Muntean and Hess, 2012). Septins 2, 5, 6, 9 and 11 have all been identified as MLL fusion partners, accounting for the largest known protein family associated with MLL rearrangements (Cerveira et al., 2011). The MLL-septin chimera results from double-stranded intronic breaks in the MLL and septin genes, producing in-frame fusions of the MLL N-terminus and almost the entire open-reading frame of the septin (Cerveira et al., 2011). Ectopic expression of the MLL-SEPT6 chimera has been shown to immortalize hematopoietic progenitor cells, upregulating the expression of the Hox-7a and Hox-9a genes (Ono et al., 2005b). Although MLL-SEPT6 expression in mice was not sufficient to induce leukemogenesis (Ono et al., 2005a), mice transduced with an active FMS-like receptor tyrosine kinase 3,
which is frequently overexpressed in AML, required MLL-SEPT6 to stimulate leukemogenesis (Ono et al., 2005b). MLL-SEPT6 expression also led to a lethal myeloproliferative disease with long latency (Ono et al., 2005b).

Recent studies have shown that hematopoietic stem cell maturation and malignancy are affected by the SEPT4 splice variant ARTS (apoptosis-related protein in the TGF-beta signaling; SEPT4_v2) (Garcia-Fernandez et al., 2010). SEPT4/ARTS binds to inhibitors of apoptosis proteins, initiating caspase activation and cell death, 2004(Gottfried et al., 2004; Larisch et al., 2000). Sept4-deficient mice contain elevated levels of hematopoietic stem cells, including progenitor and immature B-cells that are resistant to pro-apoptotic stimuli (Garcia-Fernandez et al., 2010). Genetic crosses between Sept4^-/- and the leukemogenic Eµ-Myc mice showed that SEPT4 ablation enhances lymphomagenesis and reduces lifetime by 50% (Garcia-Fernandez et al., 2010). Significantly, ARTS expression is frequently altered in bone marrow lymphocytes derived from patients with childhood ALL, the most common malignancy found in children (Elhasid et al., 2004). Lymphocytes from ALL patients exhibit a significant reduction in SEPT4/ARTS mRNA levels, and SEPT4/ARTS-deficient lymphocytes are highly-resistant to apoptosis when treated with the chemotherapeutic agent, ara-C (Elhasid et al., 2004). DNA methylation inhibitors can rescue SEPT4/ARTS expression suggesting that aberrant methylation downregulates its expression during tumorigenesis (Elhasid et al., 2004). Clinically, SEPT4/ARTS expression was rescued in patient lymphocytes following therapy, indicating that SEPT4/ARTS expression suppresses growth and possibly malignant transformation of hematopoietic cells.

**Altered septin expression in solid tumors**

Septins are overexpressed in renal cell carcinoma (RCC), which is an aggressive and highly metastatic cancer of the kidney with poor response rates to therapeutics
SEPT2 and SEPT11 are upregulated in RCCs defective in the von-Hippel Lindau (VHL) tumor suppressor, a ubiquitin E3 ligase that targets the hypoxia-inducible factor 1 (HIF-1) transcription factor for degradation (Craven et al., 2006a). It is unknown if SEPT2 and SEPT11 are transcriptionally regulated by HIF1 or directly degraded by VHL. Moreover, it is unclear if and how septin upregulation contributes to RCC growth and metastasis.

SEPT9 is over-expressed in hormonally regulated breast, prostate and ovarian cancers and endometrial cancer. SEPT9 binds directly to HIF-1 and increases its transcriptional activity in prostate cancer cells by inhibiting HIF-1 degradation. Septin 9 expression, however, is significantly altered in ovarian cancers. Initial studies in sporadic ovarian tumor samples showed that the chromosomal locus 17q25.3, which includes the SEPT9 gene, was partially deleted leading to allelic imbalances and loss of heterozygosity (Kalikin et al., 2000; Russell et al., 2000). Subsequent studies revealed that SEPT9 has a complex genomic organization encoding for up to 18 different mRNA transcripts and 15 protein isoforms (McIlhatton et al., 2001). Analysis of SEPT9 mRNA transcripts in benign, malignant and borderline, low-grade ovarian carcinomas showed that the SEPT9_v1 and SEPT9_v4* transcripts were overexpressed in serous and mucinous borderline tumors (Scott et al., 2006). The enhanced translational efficiency of SEPT9_v4* relative to SEPT9_v4 suggested that increased levels of SEPT9_i4 could promote the malignant and metastatic potential of ovarian carcinomas (McDade et al., 2007). Consistent with this possibility, over-expression of SEPT_i4 enhances cell motility and resistance to anti-cancer drugs that target microtubules (Chacko et al., 2012). Upregulation of SEPT9_i1 could also enhance tumor growth and angiogenesis by slowing down the degradation of the c-Jun-N-terminal kinase (JNK) and the hypoxia-inducible factor HIF1a (Amir et al., 2009; Gonzalez et al., 2009). While the cause of the
amplification of certain SEPT9 mRNA transcripts is not understood, epigenetic changes such as the hypermethylation of alternative intragenic promoters could alter the relative expression of mRNA transcripts (Connolly et al., 2011b).

An increase in the mRNA and protein levels of the Bradeion isoform of SEPT4 has also been detected in urine samples from patients with RCCs and transitional bladder cancers (Bongiovanni et al., 2012; Tanaka et al., 2003). Quantification of SEPT4 Bradeion expression by immunochromatography and RT-PCR of urine samples has been introduced for the diagnosis of urothelial cancers in Japan and Europe (Bongiovanni et al., 2012; Tanaka et al., 2003). Abnormal expression of SEPT4 has also been observed in colorectal cancer (CRC). While absent from patient-matched healthy tissues, two SEPT4 isoforms (Bradeion α and β) were identified in colorectal tissue isolated from human patients with mucinous carcinoma and rectal malignant melanoma (Tanaka et al., 2001). Ribozyme-mediated cleavage of either or both isoforms arrests CRC growth in the G2 phase of the cell cycle in vitro and prevents tumor growth in mice injected with CRC cells (Tanaka et al., 2002). The SEPT4 isoforms exhibit cytoplasmic and nuclear localization (Tanaka et al., 2001), but further studies are necessary to elucidate their cellular functions and the molecular mechanisms that contribute to their abnormal expression in CRC.

In squamous cell carcinoma (SCC) and malignant melanoma tissue sections, SEPT1 is significantly upregulated (Mizutani et al., 2013). In SCC cells, SEPT1 and SEPT5 coimmunoprecipitate and colocalize with SEPT4 in the lamellipodia, a dendritic actin network at the leading edge of migrating cells (Mizutani et al., 2013). SEPT1-depleted DJM-1 cells exhibit defective cell spreading, a process that depends on actin polymerization and cell adhesion with the ECM. While absent from healthy epidermis,
SEPT9 is highly expressed in cultured SCC cells and coprecipitates with SEPT7/10/11/14 (Mizutani et al., 2013).

Conversely, SEPT9 is highly expressed in normal colonic glandular and surface epithelia, but is markedly reduced in adenomas and almost completely absent from tumor tissues, indicating that SEPT9 levels decrease progressively during tumorigenesis (Toth et al., 2011). Treatment of HT29 CRC cells with a demethylating agent results in increased levels of SEPT9, suggesting that SEPT9 expression is downregulated by gene methylation during CRC progression (Toth et al., 2011). Epigenetic modifications in stem cell DNA within the colonic crypt are known to change with age and have been identified in a subset of CRCs (Humphries and Wright, 2008; Taylor et al., 2003). More specifically, hypermethylation of CpG islands (GC-rich regulatory sequences found in the promoters of almost half of all human genes) are common in many cancers (Herman and Baylin, 2003). Plasma DNA from CRC patients contains abnormally high levels of methylated SEPT9 (Grutzmann et al., 2008). Significantly, SEPT9 is hypermethylated in 87% of early stage I and II colorectal tumors (Warren et al., 2011). These findings have led to the development of the septin 9 test, which is a commercial non-invasive, blood-based test that screens for the methylation of the SEPT9 gene as a diagnostic of early-stage colon cancer.

**Septins in the tumor microenvironment**

Surrounding the tumor mass lies a complex environment referred to as the tumor microenvironment (TME), whose organization and constituent cells become altered during tumor growth and metastasis (Balkwill et al., 2012). Cells of the immune, vasculature and lymphatic systems, along with resident cells of the ECM (e.g.
fibroblasts) can function in part to support tumor growth by promoting angiogenesis, growth and motility.

In particular, cancer-associated fibroblasts (CAFs) are a subpopulation of “activated” cells that reside within the tumor microenvironment that promote tumor cell transformation, angiogenesis and invasion (Kalluri and Zeisberg, 2006). During tumor progression, CAFs become highly contractile through the increased assembly of actomyosin stress fibers, which promotes ECM remodeling by protein deposition and upregulating cell-ECM adhesions (Butcher et al., 2009). Furthermore, tumor-associated macrophages (TAMs) also promote cell invasion by localizing to the invasive edge of tumors where they sustain a paracrine signaling loop that stimulates cell motility (Patsialou et al., 2009).

In normal mouse NIH3T3 fibroblasts, septins colocalize with subnuclear actin stress fibers (Kinoshita et al., 2002). Septin knockdown results in stress fiber disassembly, while pharmacological targeting of actin stress fibers induces septin filament assembly (Kinoshita et al., 2002). Conversely, septins and actin are largely cytoplasmic in fibroblasts derived from mouse mammary glands, but co-assemble into actomyosin stress fibers in CAFs (Calvo et al., 2015). Septin depletion inhibits actomyosin stress fiber assembly in CAFs and reduces their ability to promote tumor growth in vivo (Calvo et al., 2015). Thus, septins and actin stress fibers show an interdependent relationship, but whether septins bind directly to actin filaments and how septins regulates the organization of the actin stress fiber network during cell migration has remained unknown.
CHAPTER 4. Septin overexpression in tumor cell migration, trafficking and metabolism

As discussed in the previous chapter, septin overexpression is frequently reported in solid tumors of epithelial origin including renal, breast, ovarian and skin; yet, how septins contribute to tumorigenesis and tumor metastasis at the molecular level is unknown. I hypothesize that septin overexpression promotes tumor growth and invasion through at least two distinct mechanisms: i) promoting the mesenchymal-like motility of renal epithelia, and ii) regulating the intracellular trafficking of macropinocytic cargo.

Because septins are multi-functional proteins that interact directly with the cytoskeleton and cell membranes, it is not surprising that these findings implicate septins in different cellular processes. Furthermore, tumorigenesis is a complex process that stems largely from changes in the genome, which can alter many different cellular pathways (Hanahan and Weinberg, 2000). Tumor cells proliferate unchecked by evading cell death signals and adapt to growing metabolic needs by changing the mechanisms by which energy is produced in order to sustain proliferation (Hanahan and Weinberg, 2011). Through changes in cell polarity, cytoskeletal organization and the trafficking of intracellular cargo, tumor cells can invade the surrounding ECM and migrate towards tissues of the blood and lymph to initiate metastasis (Bravo-Cordero et al., 2012).

Septins functions in tumor cell migration

Epithelial tumor cell invasion is activated by the aberrant induction of epithelial-to-mesenchymal transition (EMT), a developmental transcriptional program that regulates the patterning of epithelial tissues (Thiery et al., 2009). During this process, epithelia breakdown cell-cell contacts, upregulate the attachment and turnover of adhesions with the ECM, and adopt a front-rear, mesenchymal-like polarity (Thiery et al.,
Tumor cell invasion into the ECM is initiated by membrane protrusions, such as invadopodia and pseudopodia, which are driven by the assembly of actin filaments against the ventral cell membrane (Murphy and Courtneidge, 2011). Within these protrusions, actin-binding proteins, cell-ECM receptors and ECM-degrading enzymes are enriched (Linder, 2007).

Tumor cell invasion into the ECM is a dynamic process. Tumor cells adapt to ECM organization and topography, which becomes stiffer and more crosslinked during tumorigenesis, and can interconvert between different modes of motility (Figure 4.1): a) mesenchymal-like; b) amoeboid; c) lobopodial (Friedl and Wolf, 2010; Petrie et al., 2012). The mesenchymal-like motility of epithelia has been characterized extensively (Figure 4.1). At the leading cell edge, actin polymerization by the Arp2/3 complex drives the formation of protrusions termed lamellipodia, which make new contacts with the ECM through integrin receptors (Parsons et al., 2010; Svitkina and Borisy, 1999). These focal complexes mature into focal adhesions, which is induced under the tension and contractile forces of a network of actin stress fibers. Radial actin stress fibers emanate from growing focal adhesions and link up with the contractile transverse arcs in the leading lamella, which transmits forces to focal adhesions to promote forward translocation (Gardel et al., 2010). Assembly of this dynamic network involves proteins that nucleate, bundle and cross-link actin filaments (Choi et al., 2008). Force transduction is regulated by the myosin II regulatory light chain, which modulates the ATPase activity and step rate of myosin II motor in response to ECM stiffness (Davis et al., 2002; Schwartz, 2010). Thus, the force-generating mechanisms that drive cell motility are in a direct crosstalk with the extracellular microenvironment. In contrast, bleb and lobopodial-based cell migration rely upon the assembly of contractile actomyosin fibers at the cell cortex, which induces the formation of membrane protrusions that help
tumor cells navigate through gaps in the ECM (Lammermann and Sixt, 2009; Petrie and Yamada, 2012) (Figure 4.1). The conversion to amoeboid and lobopodial-based cell motility correlates with the expression of actin-binding proteins and ECM-degrading enzymes and ECM topography (Petrie and Yamada, 2012), but the mechanism by which tumor cells interconvert between these motility modes is not fully known.

Importantly, invasive tumor cells frequently show elevated expression of actin-binding proteins (Wang et al., 2004), which control the assembly and organization of the actin cytoskeleton during cell migration (Parsons et al., 2010). Septins have been reported previously to promote cell migration, but the mechanism has remained elusive. In metastatic cancer cell lines, SEPT9 expression is elevated and essential for the formation of actin-rich pseudopods and cell migration (Shankar et al., 2010). In agreement with this study, overexpression of five different SEPT9 isoforms in the non-invasive MCF-7 breast cancer cells promotes cell migration (Connolly et al., 2011b). Thus, septins promote tumor cell migration, but how septins influence the organization of the actin cytoskeleton at the molecular level during cell motility has been unclear.

Septins have been linked to the assembly of branched actin networks in epithelia and branched actin patches and neurons. SEPT1 knockdown in squamous cell carcinoma cells inhibits cell spreading, which is dependent upon the formation of the lamellipodia by the Arp2/3 complex (Mizutani et al., 2013). Interestingly, SEPT6 overexpression promotes the recruitment of cortactin, an activator of the Arp2/3 complex, to actin patches in neurons and renal epithelial lamellipodia (Hu et al., 2012). Experiments in vitro with purified actin filaments and the Arp2/3 complex show that recombinant SEPT6 localizes to actin branch points (Hu et al., 2012), which correlates with Arp2/3 localization. Despite these findings, how septins interact with and regulate actin filament assembly remained unknown.
In the first part of this thesis, I present a novel role for septins in promoting the mesenchymal-like motility of renal epithelia undergoing EMT by directly crosslinking actin filaments and regulating focal adhesion stability. Septins localizes to transverse actin arcs and their orthogonal junctions with radial stress fibers, which are anchored to focal adhesions. By disrupting the actin stress fiber network, septin depletion reduces focal adhesion stability and cell migration. I identified further that SEPT9 expression specifically increases during EMT, suggesting that SEPT9 may be part of the EMT program that promotes the mesenchymal-like motility of epithelia. Indeed, SEPT9 overexpression promotes the migration of renal carcinoma cells and the collective migration of renal epithelia undergoing EMT in 3D collagen matrix. Conversely, SEPT9 depletion in single cells migrating in a 3D matrix inhibits motility and induces a transition to a more rounded and protrusive shape, indicating that SEPT9 expression promotes specifically the mesenchymal-like motility mode.

Because stress fiber assembly during mesenchymal motility is regulated by plethora of actin-binding proteins, whose spatial and temporal recruitment is tightly regulated, I investigated further the interaction between SEPT9 and actin filaments at the molecular level. SEPT9 binds to three specific sites on the actin filament, two of which are shared by other actin-binding proteins. Strikingly, SEPT9 interferes with the binding of the myosin motor domain in its low-affinity state and the actin-severing protein cofilin, which could stabilize and protect filaments from depolymerizing forces. Thus, I hypothesize that SEPT9 protects actin filaments by regulating spatially the recruitment of actin-binding proteins during filament assembly. In agreement with this hypothesis, I investigated the assembly and stability of nascent actin stress fibers in live renal epithelia. Indeed, septin depletion results in unstable radial actin stress fibers that disassemble at a faster rate.
At the molecular level, septins operate collectively as hetero-polymers and filaments. In renal epithelia, SEPT9 colocalizes and immunoprecipitates with SEPT2, SEPT6 and SEPT7, indicating that they co-assemble into complexes and filaments. While SEPT6 and SEPT9 bind directly to actin, SEPT2 does not interact with actin filaments, but it binds directly to myosin II and is posited to control the phosphorylation of its regulatory light chain and myosin contraction (Joo et al., 2007). These differential interactions may regulate spatially the binding and activation of myosin along the actin filament and contraction of actomyosin stress fibers during cell motility. In the absence of septins, myosin motor proteins may bind and contract uncrosslinked actin filaments, which can induce filament buckling and disassembly. Conversely, actin filaments lacking septins may contain more available binding sites for the actin-severing protein coflin, which is enriched in the leading cell edge. Thus, overexpression of septins in cancer may reinforce the mesenchymal-like motility mode by controlling the interaction of actin-binding proteins with actin filaments during stress fiber assembly and contraction.

**Septins in membrane traffic and metabolism**

Septins also associate with the microtubule cytoskeleton and cell membranes, regulating the intracellular trafficking of membrane cargo (Spiliotis and Gladfelter, 2012). Importantly, membrane traffic is an emerging regulator of tumor cell migration, growth and metabolism (Jones et al., 2006). Intracellular vesicles are derived from organelles and the plasma membrane via endocytosis. Through the recruitment of adaptor and motor proteins, vesicles move along actin filaments and microtubules to distinct subcellular compartments (Fu and Holzbaur, 2014). Intracellular vesicle dynamics are controlled by the large family of Rab GTPases, which can recruit motor proteins (e.g. dynein) and promote vesicle docking, tethering and fusion (Stenmark, 2009). Moreover,
intracellular vesicles can serve as signaling hubs that activate pathways to promote cell motility and growth. Rab-dependent recycling of integrins to focal adhesions (Caswell et al., 2008) and active members of the Rho family of GTPases (Palamidessi et al., 2008), which regulate actin polymerization at the leading cell edge, promotes tumor cell motility and endocytosis. Mutations present in human cancers that render Rab GTPases (e.g. Rab35) constitutively active were identified recently to stimulate tumor cell proliferation via aberrant trafficking of growth factor receptors to lysosomes and activation of the PI3K/AKT/mTOR signaling pathway (Wheeler et al., 2015).

Septins regulate the intracellular delivery of golgi-derived to the plasma membrane domains in renal epithelia, which is important for epithelial polarity and morphogenesis (Spiliotis, 2008). Septin knockdown or inhibition with a function-blocking antibody impedes the transport of plasma membrane proteins through the exocytic pathways (Spiliotis, 2008). Septins colocalize with polyglutamylated microtubules and regulate the organization and dynamics of the microtubule network (Bowen et al., 2011; Spiliotis, 2008).

In contrast, little is known about the function of septins in endocytic pathways. Recent evidence shows septins regulate epidermal growth factor receptor (EGFR) surface levels and degradation (Diesenberg et al., 2015), which occurs through endocytic recycling, and promote the formation of multivesciular bodies (Traikov et al., 2014). Importantly, altered endocytosis is commonly observed in tumor cells. Upregulation of the endocytic mechanisms that internalize adhesion proteins promote the breakdown of cell-cell junctions (Yang et al., 2006), and increased macropinocytosis, a form of clathrin-independent endocytosis, promotes tumor cell growth and possibly migration (Commissso et al., 2013).
Macropinosomes are derived from plasma membrane ruffles, which fold back on themselves forming large vesicles that can capture membrane proteins, extracellular fluids and solutes (Lim and Gleeson, 2011). Following ruffle closure, macropinosomes undergo a maturation process that involves changes in lipid composition, the recruitment of Rab GTPases and fusion with late endosomes, which can recycle proteins back to the plasma membrane, and lysosomes, which degrade proteins for catabolic pathways (Bright et al., 2005; Swanson, 2008) (Figure 4.2). In motile fibroblasts, growth factor stimulation induces the formation of macropinosomes that recycle integrins back to nascent focal adhesions in the leading cell edge; and inhibition of macropinocytosis or Rab-dependent integrin trafficking attenuates fibroblast migration (Gu et al., 2011). Thus, macropinocytic trafficking of cell adhesion molecules may be necessary for tumor cell invasion.

In addition, recent evidence also supports a role for macropinocytosis in tumor cell growth via carbon metabolism (Commisso et al., 2013). Uncontrolled tumor cell proliferation requires increased energy metabolism in order to sustain growth. Tumor cells frequently bias energy production towards glycolysis and glutamine metabolism, or glutaminolysis, which have emerged as hallmarks of tumor cell metabolism (Deberardinis et al., 2008). Notably, the glycolytic and glutamine metabolic pathways are frequently linked to cellular transformation by oncogenes, such as Ras GTPases (White, 2013). In addition, pancreatic tumor cells expressing a constitutively-active mutation in the KRas oncogene show increased uptake of amino acids through macropinocytosis and the delivery of glutamine to the central metabolic pathway (Commisso et al., 2013). Strikingly, inhibition of macropinocytosis and subsequent glutamine metabolism reduces tumor cell proliferation in vitro and tumor growth in vivo (Commisso et al., 2013).
In the last part of this thesis, I discovered a population of membrane-associated septins that localize to macropinosomes and their contact sites with endosomes by interacting directly with Pi(3,5)P2, a low-abundant lipid that concentrates on late endosome and lysosomes. Septin knockdown results in clusters of macropinosomes and abrogates macropinosome fusion with lysosomes. Conversely, septin overexpression enhances the trafficking of macropinocytic cargo to the lysosomes. Using an in vitro vesicle fusion assay, I found that septins promote membrane fusion independently of the actin and microtubule cytoskeleton.

Membrane-associated septins control the organization of plasma membrane lipids and proteins (Spiliotis and Gladfelter, 2012). In addition, septins have been reported to interact directly with exocytic SNAREs, whose clustering into membrane domains is required for fusion. (Aoyagi et al., 2005). Based on this knowledge, septins may promote vesicle fusion by controlling SNARE organization in membrane domains or the dynamic assembly and disassembly of SNARE complexes during macropinosome fusion with lysosomes. Through these potential mechanisms, septin overexpression may promote tumor cell proliferation by promoting the trafficking of macropinocytic cargo to the lysosome, which can supply molecular intermediates for the central metabolic pathway.

In a similar manner, autophagy is a process by which stressed or starved cells capture cellular material (e.g. organelles, proteins) into autophagosomes, which are targeted to the lysosome for degradation (Kaur and Debnath, 2015; Levine and Klionsky, 2004). Autophagy has been linked to prolonged tumor cell survival, but also defects in the autophagic machinery are associated with tumorigenesis (Mathew et al., 2007). During microbial infection, septins target intracellular bacteria (e.g. Shigella) to autophagosomes, which restricts their replication and reduces infection in vitro and in
vivo (Mostowy et al., 2010; Mostowy et al., 2013). Septins are posited to interact directly with the autophagy machinery, which also targets autophagosomes to lysosomes by interacting with lysosomal SNAREs (Liu et al., 2015; Mostowy et al., 2010). How septins function in autophagy is not fully understood, and whether septin overexpression may support autophagy-mediated cell survival in tumor cells is unknown. However, I speculate that septins may have a wider role in targeting intracellular vesicles to lysosomes.
Fig 4.1 Modes of single tumor cell migration.

(A) Mesenchymal motility is characterized by the formation of focal adhesions and an actin stress-fiber network containing radial stress fibers (SFs) and transverse arc SFs that link up to promote focal adhesion growth.

(B) Amoeboid motility is characterized by the assembly of actomyosin fibers at the cell cortex and the formation of protrusive blebs.

(C) Lobopodial motility is characterized by the assembly of an actomyosin network at the cell cortex that connects with a network of intermediate filaments near the nucleus. Through this cytoskeletal connectivity, the nucleus pressurizes the cell front in a piston-like mechanism that promotes the extension of blunt membrane protrusions termed lobopodia.
Figure 4.2 Macropinosome formation, maturation and traffic to endolysosomes.

Plasma membrane ruffles fold back upon themselves to engulf soluble cargo (red) into macropinosomes. Macropinosomes undergo a maturation process that is controlled by changes in phosphoinositide lipid composition and the recruitment of Rab GTPases, which collectively regulate macropinosome retrograde movement and fusion with late endosomes and lysosomes.
CHAPTER 5. Septins promote stress fiber-mediated maturation of focal adhesions and renal epithelial motility.

Abstract

Organogenesis and tumor metastasis involve the transformation of epithelia to highly motile mesenchymal-like cells. Septins are filamentous G proteins, which are over-expressed in metastatic carcinomas, but their functions in epithelial motility are unknown. Here, we show that a novel network of septin filaments underlies the organization of the transverse arc and radial (dorsal) stress fibers at the leading lamella of migrating renal epithelia. Surprisingly, septin depletion resulted in smaller and more transient and peripheral focal adhesions. This phenotype was accompanied by a highly disorganized lamellar actin network and rescued by the actin bundling protein α-actinin-1. We show that pre-assembled actin filaments are crosslinked directly by SEPT9, whose expression is increased after induction of renal epithelial motility with the hepatocyte growth factor. Significantly, SEPT9 over-expression enhanced renal cell migration in 2D and 3D matrices, while SEPT9 knock-down decreased migration. These results suggest that septins promote epithelial motility by reinforcing the cross-linking of lamellar stress fibers and the stability of nascent focal adhesions.

Introduction

Embryonic patterning and organogenesis depend on the morphogenetic movements of epithelial cells, which transition to migratory mesenchymal-like cells (Thiery et al., 2009). In the developing kidney, extensive epithelial movements accompany the formation, branching and tubulogenesis of the ureteric bud (Dressler, 2002; Zegers et al., 2003), and epithelial-to-mesenchymal transition (EMT) underlies
renal fibrosis and the metastasis of renal cell carcinomas (RCCs) (De Craene and Berx, 2013; Liu, 2010). EMT is characterized by disruption of cell-cell adhesions, cell scattering and the enhancement of invasive cell motility with a front-rear polarity (Guarino et al., 2007; Lim and Thiery, 2012).

Mesenchymal motility is driven by the actomyosin cytoskeleton and involves adhesion to the ECM and its degradation (Bravo-Cordero et al., 2012; Friedl and Wolf, 2010). Nascent adhesions are stabilized and grow to focal adhesions (FAs) under the tension exerted by a network of actin stress fibers (Geiger and Yamada, 2011; Schwartz, 2010). At the leading lamellae of migrating cells, forces are transduced to adhesions by the radial (dorsal) stress fibers (RSFs), which extend dorsally from FAs and connect with the transverse arc (TA), a contractile network of curved actin filaments (Burridge and Wittchen, 2013; Gardel et al., 2010; Parsons et al., 2010). Actin nucleating and cross-linking proteins and myosin II, whose activity is regulated by the phosphorylation of its regulatory light chain (RLC), mediate the assembly, organization and contraction of actin stress fibers (Burridge and Wittchen, 2013; Parsons et al., 2010). Actomyosin organization and contractility are regulated by the small GTPases of the Ras superfamily, which affect cell motility (Parri and Chiarugi, 2010; Raftopoulou and Hall, 2004).

Septins are evolutionarily and structurally related to the Ras GTPases (Leipe et al., 2002), but unlike the monomeric small GTPases, septins form filamentous polymers that consist of non-polar hetero-octameric complexes (Mostowy and Cossart, 2012; Weirich et al., 2008). Septins interact with the actomyosin cytoskeleton (Joo et al., 2007; Kinoshita, 2002; Mavrakis et al., 2014) and are abnormally over-expressed in renal cell carcinomas, but their precise function in stress fiber organization and mesenchymal-like
motility is unknown. Here, we show that in the leading edge of motile renal epithelia, a novel network of septin filaments promotes cell motility by reinforcing the organization of the lamellar stress fiber network and the stability of nascent FAs.

**Materials and methods**

**Tissue culture and transfections.**

MDCK II/G cells and the stable MDCK-SEPT2-YFP (Spiliotis et al., 2005), MDCK-paxillin-GFP (Yamada and Nelson, 2007), MDCK-SEPT9-mCherry, MDCK-ABD-ABP140-mCherry cell lines were cultured in low glucose DME with 1g/liter NaHCO₃, 10% FBS (Cell Generation), and 1% PSK (penicillin, streptomycin, kanamycin) at 5% CO₂. The stable MDCK-SEPT9-mCherry and MDCK-ABP140-mCherry cells were generated by standard selection with G418 sulfate (0.8 mg/mL; Corning) and stable transfectants were cloned in 96-well plates by serial dilutions and levels of expression were analyzed by western blotting. The renal cell carcinoma 786-O cell line was purchased from ATCC and maintained in RPMI with 1 g/liter NaHCO₃, 10% FBS, and 1% PSK. Coverslips were acid-washed with 1 M HCl for 4h at 50°C and coated with 10-20 µg/mL fibronectin (Invitrogen) for 2h at 37°C or rat tail collagen. Cells were transfected with 1.5 µg of plasmid or co-transfected with 1.5 µg plasmid and 1µg shRNAs with Lipofectamine 2000 (Invitrogen). Prior to fixation or live-imaging, MDCK cells were stimulated with 20 ng/mL recombinant HGF (Sigma) in DME supplemented with 0.5% FBS for at least six hours. Live imaging studies were performed in phenol red-free media containing 20 mM HEPES.

To generate polarized cysts, MDCK cells and cells that express stably SEPT9-mCherry at low and high levels were cultured in collagen gels composed of 2mg/mL type I collagen (Advanced Biomatrix) on transwell filters (0.4 µm pore; BD Biosciences) for ten days. Polarized cysts were stimulated with 30 ng/mL HGF for three days (Fig. 4 C-E)
and with or without 50 µm FCF (Fig. 4E). To image and quantify single cell migration in 3D (Fig. 4 H and I), MDCK cells were transfected with control or SEPT9-targeting shRNAs for 24h on tissue culture plates. Cells were dissociated by trypsinization and cultured in 2 mg/mL type I collagen gels on 35 mm glass-bottom dishes (live imaging of MDCK-ABP140-ABD-mCherry; Fig. 4 H) or transwell filters (morphometric analysis of fixed MDCKs; Fig. 4I) and treated with 40 ng/mL HGF, 16 h prior to imaging.

**Plasmids and shRNAs.**

Canine SEPT2 was targeted with control (5'-TGA GTTCACA CTAATGGTGTCG-3'), SEPT2 shRNA #1 (5'-CCTTAGACGTCGCA TCATGAA-3') and SEPT2 shRNA #2 (5'-GCAACTACAAGCCCCGAAGAATAA-3') (Bowen et al., 2011). To target human SEPT2, the SEPT2 shRNA 5'-AAGGTTGAATATTGTGCCTGTC-3' was subcloned into the GFP-expressing pG-SUPER vector. The SEPT9 shRNA construct was made by inserting the SEPT9 shRNA 5'- GACCGGCTGGTGAACGAGAAGTT-3' into mCherry-expressing pSuper vector. The shRNA constructs against canine and human SEPT9 were made by subcloning the shRNAs 5'-GACCGGCTGGTGAACGAGAAGTT-3' and 5'-GACCATCGAGATCAAGTCC-3' into the mCherry-expressing pSuper and GFP-expressing pGFP-V-RS vectors, respectively. ABP140-ABD-mCherry was generated by annealing the primers 5'-ATCCTCCTCCTTGCCTGATGCTTCGAACTTCTTGATCAGGGCCACGC CGCATGGTGGCG-3' (forward) and 5'-AATTCGCCACCATGGGCGTGGCCGACCTGATCAAGAAGTGAGAG CATCAGCAAAGGAGG-3' (reverse), which correspond to the first 17 amino acids (Lifeact) of ABP140, and inserting the sequence into the N1-mCherry vector (Invitrogen) using the Ncol restriction site. SEPT9_mCherry was constructed by PCR amplification of SEPT9_i1 from pET28a-SEPT9_i1 (Bai et al, 2013) using the primers 5'-
CGTAAGCTTGCATGAAGAAGT-3’ and 5’-GTAGTCGACCTACATCTCTGGGGC-3’ and the product was ligated into the HindIII and SalI sites of the pmCherry-C1 vector. Mouse SEPT2 was inserted into N1-YFP (Spiliotis et al, 2005). Avian paxillin was inserted into C1-EGFP (West et al, 2001). Mouse myosin IIA-GFP, human α-actinin-1-GFP and human myosin II RLC-DD-GFP (pEGFP-MRLC1 T18D, S19D) were purchased from Addgene. The actin-binding domain of α-actinin-1 (amino acids 30-253) was deleted from α-actinin-1-GFP (Roca-Cusachs et al., 2012). The myosin IIA-N93K-GFP mutant was generated by site-directed mutagenesis using the primers 5’-CTCACGTGCCTCAAGGAAGCTTCGGTG-3’ (forward) and 5’-CACCAGCGCTTCTCTTGAGGCGACGTCGAG-3’ (reverse).

**Protein expression and purification.**

SEPT2/6/7 was expressed by co-transforming E. coli BL21(DE3) cells with a bicistronic, kanamycin-resistant pET-28a(+) plasmid that expresses SEPT6 and His-SEPT7 in tandem, and an ampicillin resistant pET-15b(+) plasmid that expresses untagged SEPT2 (Bai et al., 2013). His-SEPT9_1 was expressed by transforming E. coli BL21(DE3) cells with pET28a-SEPT9_i1 (Bai et al, 2013). Bacterial cultures of at least OD600 of 0.6 were induced with 0.5 mM IPTG for 16h at 18C, centrifuged at 5,000 rpm for 5 m at 4ºC, and resuspended in buffer containing 1% Triton X-100, 50 mM Tris, pH 8.0, 150 mM NaCl, 10% glycerol and 10 mM imidazole. Bacteria were lysed with a French pressure cell at 1,280 psi. Cell lysates were cleared using centrifugation (14,000 x g) for 30min at 4ºC and the supernatants were passed through Ni-NTA beads by gravity flow. The columns were washed with 10mL washing buffer (50mM Tris, pH 8.0, 300mM NaCl, 10% glycerol, 25 mM imidazol) and proteins were eluted with elution buffer (50 mM Tris, pH8.0, 300 mM NaCl, 10% glycerol, and 250 mM imidazole). Purification of SEP2/6/7
was verified by size exclusion chromatography using an AKTA FPLC system (GE Healthcare). Purified proteins were dialyzed overnight in buffer containing 50mM Tris, pH 8.0, 150 mM NaCl, and 10 mM imidazole.

**Immunoprecipitations and immunoblots.**

For immunoprecipitations, equal number of MDCK and SEPT2-YFP MDCK cells were lysed with buffer containing 50 mM HEPES pH 7.4, 100 mM NaCl, 0.5% Triton X-100, 1 mM PMSF, and protease inhibitors (Millipore) and incubated with anti-GFP antibody (3E6; Invitrogen) overnight at 4°C. Protein A beads (Thermo Scientific) were incubated for 4 h at 4°C and were stripped with SDS buffer at 95°C for 10min. Equal volumes were resolved on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane using 100 V for 1 h at 4°C. Membranes were blocked with buffer containing 5% milk and 1% BSA for 1h at 25°C. Primary antibodies targeting rabbit anti-SEPT2 (1:5000, Sigma), rabbit anti-SEPT6 (1:5000, Santa Cruz Biotechnology, Inc.), rabbit anti-SEPT7 (1:8000, ImmunoBiological Laboratories, Inc.) and rabbit anti-SEPT9 (1:2500, ITG Labs), and anti- rabbit or mouse secondary antibodies (LiCor) conjugated to infrared dyes were diluted in buffer containing 2% BSA, 0.1% Tween-20, and 0.025% sodium azide and sequentially incubated on each membrane prior to scanning with an Odyssey Li-Cor imaging system.

To test for changes in SEPT9 expression following HGF treatment, MDCK cells were cultured on collagen-coated plates and stimulated with HGF for the indicated times. Cell lysates of equal protein concentration were run on a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane and probed using primary antibodies targeting rabbit anti-SEPT9 (PTG Labs), mouse anti-actin (1:8000, Sigma) and mouse anti-GAPDH (1:8000, Abcam). The western blot shown in Fig. 4 A is representative of three
different experiments, which were reproduced with no limitations. To quantify SEPT9 overexpression in MDCK cells, untransfected cells and cells that express stably SEPT9-mCherry at high levels were lysed, processed and blotted as described above (Fig. S2 D).

Immunofluorescence microscopy and quantifications.

Cells were simultaneously fixed and extracted with 4% PFA in warm PBS buffer containing 0.1% Triton X-100 for 20 min, or fixed with 4% PFA in cytoskeleton buffer (10 mM MES pH 6.1, 0.32 M sucrose, 138 mM KCl, 3 mM MgCl2, 2 mM EGTA) for 20 min and extracted with 0.5% Triton X-100 for 10 min (Fig. 3 D). Excess PFA was quenched with 0.25% ammonium chloride. Prior to antibody incubation, cells were blocked with PBS containing 2% BSA for 20 min. Primary antibodies to rabbit anti-SEPT2 (N5N; gift from M. Kinoshita, Nagoya University, Nagoya, Japan), rabbit anti-SEPT6 (Tada et al., 2007), rabbit anti-SEPT7 (IBL, Inc.), SEPT9 (ITG Labs), mouse anti-paxillin (BD, 349), rabbit anti-pY118-paxillin (Millipore), rabbit anti-myosin-IIA (Sigma), mouse anti-pSer19-MLC (Cell Signaling Technologies), and secondary donkey DyLight 488-, 594-, 647-conjugated F(ab’)2 to mouse or rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) were diluted in PBS with 2% BSA and 0.1% Triton X-100. F-actin was visualized using phalloidin conjugated to Alexa-488 or -647 (Life Technologies). Coverslips were mounted on slides using Vectashield (Vector Laboratories) or FluorSave (EMD) and imaged using a confocal laser-scanning microscope (FluoView 1000; Olympus) with a Plan Apochromat 60x/1.42NA objective. Optical sections (0.2 µm thick) were exported to the Slidebook 5.0 (Intelligent Imaging Innovations), ImageJ or Volocity (Perkin Elmer) softwares for processing and quantification. Kymographs were generated in Slidebook by drawing a six pixel wide line down the center of a growing FA marked by paxillin or
ABP140-ABD. Super-resolution structured illumination microscopy was performed using the DeltaVision OMX V4 (GE Healthcare) microscope with a 60x/1.42NA objective and immersion oil with a refractive index of 1.514. A z-step size of 0.125 µm was used and images were acquired with sCMOS pco.edge cameras (PCO) and reconstructed in the softWoRx software.

FA sizes were quantified using masks of paxillin fluorescence intensity segmentation; the area of individual masks were exported to excel and quantified. FA distance from the cell edge was measured by converting the phalloidin-stained cell into a binary image, which was subsequently inverted and transformed into a Euclidean distance map (Schober et al., 2007). FAs were overlaid on the map and the distance from the FA center to the cell edge was measured. To assess relative levels of pTyr118 paxillin to total paxillin, confocal images were obtained using identical laser settings between control and septin-depleted samples. Individual FAs were masked using intensity segmentation and the ratios were derived from the sum intensities.

Colocalization of septins with actin in the leading cell edge was quantified in background subtracted images using the Mander’s Coefficient analysis in Slidebook. Fluorescence intensity of actin as a function of the distance from the cell edge was quantified from confocal images, which were acquired in during one sitting at identical laser intensities. Protrusions at the leading cell edge were sampled 2-3 times using a 10 µm x 1 µm line scan drawn perpendicular from the cortex and were averaged and plotted using custom software written in MATLAB (MathWorks). The number of RSFs per micron was calculated by drawing a line parallel to the cell edge and counting the number of radial fibers that intersected with the line. Fluorescence intensity ratios of pSer19-myosin II light chain to myosin IIA heavy chain (Fig. S3) was quantified by
masking 5 µm segments of the TAs parallel to the leading cell edge. In each cell, the sum intensities of at least six different segments were analyzed.

To visualize 3D cyst structure (Fig. 4 C), gels were briefly treated with collagenase (Sigma) in PBS at 37°C and fixed with 4% PFA in PBS with 0.5% Triton X-100. Gels were incubated with phalloidin for 1 h under gentle agitation and mounted in Vectashield. Optical sections (1.25 µm thick) were stacked as mass projections. The number of extensions per cyst and the number of cells per extension were quantified and plotted. Extensions were defined as protrusions from the main cyst body consisting of three cells or more. To quantify single cell morphology in 3D (Fig. 4 I), gels were fixed, stained and mounted using the same method but without the initial collagenase treatment. The long and short axes were measured in Slidebook to derive the axial ratio of each cell. To measure the velocity of cell movement in a 3D matrix (Fig. 4 H), time-lapse optical sections were imported into the Volocity software, which identified, tracked and quantified the movement of the cell centroid.

**Time-lapse imaging.**

All live imaging experiments were performed at 37°C in DME lacking Phenol red supplemented with 0.5% FBS, 1% PSK, and 20mM HEPES. Widefield microscopy was performed with an inverted microscope (IX-81; Olympus) equipped with a motorized stage (ProScanII; Prior), a camera (OrcaR2; Hamamatsu Photonics), a custom built stage-top chamber and temperature controller (Air-Therm ATX; World Precision Instruments) and the Slidebook 5.0 software. Fluorescent images were captured every 20s with a Plan Apochromat 60x/1.40 NA objective (Olympus). Spinning disk confocal microscopy was performed at 37°C using an inverted microscope (IX-71; Olympus) equipped with a camera (ImagEM; Hamamatsu Photonics), an airstream incubator
(LiveCell; Pathology Devices), scan head (CSU10; Yokogawa), and the MetaMorph software (Molecular Devices). Fluorescent images were captured every 20s with a 60x 1.49 NA objective. TIRF microscopy was performed at 37ºC with the DeltaVision OMX V4 inverted microscope (GE Healthcare) equipped with 60x/1.49NA TIRF objective (Olympus), motorized stage, sCMOS pco.edge cameras (PCO), stage-top incubator with temperature controller and the softWoRx software. 3D cell migration experiments were performed using a laser scanning confocal microscope (LSM700; Carl Zeiss) equipped with a stage-top incubator and temperature controller (XL modules; Carl Zeiss), and the Zen software (Carl Zeiss). One micron optical sections were acquired every two minutes with a 40x C-Apochromat/1.2 NA water-objective (Carl Zeiss). Supplemental videos were rendered using the Photoshop software (Adobe).

Quantification of FA dynamics.

FA dynamics were obtained using the stable MDCK-paxillin-GFP cell line and TIRF microscopy; background subtraction and photobleach correction algorithms in the Slidebook software were applied to normalize fluorescence intensities. Individual FA lifetimes were quantified from the length of each adhesion signal in three cells using the open-source Focal Adhesion Analysis Server (Berginski and Gomez, 2013). FA assembly and disassembly rates were quantified as previously described (Webb et al., 2004). In brief, individual FAs were masked in Slidebook and a semilogrithmic plot of paxillin intensity as a function of time was generated. The rate constants were derived from the slopes of 45 adhesions from the leading edge (three cells per condition).

Low speed co-sedimentation assay.

Human platelet actin (Cytoskeleton, Inc.) in G-buffer (5mM Tris-HCl, 0.2mM CaCl2) was polymerized using F-buffer (20 mM HEPES, 100 mM KCl, 1 mM MgCl2) supplemented
with 0.5 mM ATP and 4 mM DTT for 1h at 25°C to generate F-actin. 2 µM recombinant SEPT2/6/7 and 4 µM recombinant his-tagged SEPT9 were incubated with 2 µM F-actin for 1h at 25°C and centrifuged atop a glycerol cushion (20 mM HEPES, 100 mM NaCl, 1 mM MgCl₂, 10% glycerol) at 8,000 x g for 20min at 25°C. Equal volumes of the supernatant and pellet fractions were resolved using a 7.5% SDS-PAGE gel and stained with Coomassie brilliant blue.

**Electron Microscopy.**

Skeletal striated muscle G-actin (2.5 μM) was polymerized in 10 mM HEPES buffer (pH 7.4), 100 mM KCl, 1 mM MgCl₂, 0.5 mM DTT, and 0.5 mM ATP for 2 h. Actin filaments were incubated with 14 μM His-SEPT6 or His-SEPT9 for 15-30 min at 4°C. To visualize complexes of F-actin with septins, aliquots (10 µl) were applied to glow-discharged carbon-coated grids (Electron Microscopy Sciences) and stained with 2% uranyl acetate. The grids were examined in a JEOL 1200EXII transmission electron microscope (TEM) (JEOL Inc, Peabody, MA) under regular-dose conditions at an accelerating voltage of 70 keV. The images shown in Fig. 3 F and G are representative of similar results obtained from three independent experiments.

**Transmigration Assays.**

786-O cells were transfected with plasmids encoding for the indicated constructs for 48h, trypsinized and plated on collagen-coated transwell filters (8 µm pore) for 12h. Filters were fixed with 4% PFA in PBS and stained for nuclei with DAPI. Apical and basolateral sections were imaged at 20x magnification using confocal microscopy. The number of transfected cells in each section were quantified and plotted in excel.

**Statistical Analyses**

Data sets were analyzed using R and the Excel software. Normal distribution and equal variance were assessed using the Shapiro and Levene tests, respectively. Data sets
with normal distribution and variance were analyzed using an unpaired student’s t-test. Data sets that did not exhibit normal distributions or variance were further analyzed using the Kruskal-Wallis non-parametric ANOVA. All values represent the mean +/- SEM.

Results and Discussion

A network of septin filaments interfaces with RSF and TA stress fibers in the leading lamella of renal epithelia.

To examine septin functions in the motility of renal epithelia, we used the MDCK model of partial EMT, which allows the study of cell migration in 2D and 3D matrices after stimulation with the hepatocyte growth factor (HGF) (Zegers et al., 2003). Treatment of MDCKs with HGF disrupted cell-cell adhesions and induced scattering and development of front-rear cell polarity as previously shown (de Rooij et al., 2005). Resembling the stress fiber organization of migratory cells, the lamellae of fan-shaped MDCK cells contained radial stress fibers that extended dorsally from FAs and connected with transverse arc (TA) filaments (Fig. 1 A; arrows). Staining for endogenous SEPT2, SEPT6, SEPT7 and SEPT9 revealed an extensive network of septin filaments that localized at the interface of TA filaments and RSFs (Fig. 1 A, Fig. S1). Colocalization and co-immunoprecipitation of SEPT2, SEPT6, SEPT7 and SEPT9 (Fig. S2, A and B) indicated that these septin filaments consist of SEPT2/6/7/9 complexes, which are the basic structural units of mammalian septin filaments (Kim et al., 2011; Sellin et al., 2011b). Thus, we used SEPT2 as a marker for the lamellar network of septin fibers, and SEPT2 and SEPT9 shRNAs were interchangeably applied to perturb the localization and function of septins; knock-down of SEPT2 or SEPT9 affected the
expression and localization of both of these septins as well as SEPT6 and SEPT7 (Fig. S2, C-D) as reported previously (Kim et al., 2011).

To determine the precise localization of septins with respect to the lamellar network of stress fibers and FAs, we used confocal microscopy in combination with 3D image reconstructions and super-resolution structured illumination microscopy (SIM). Septins localized mainly on and between the curved actin bundles of the TA network and on a subset of RSFs (Fig. 1 B-E; Video 1). Septins were present on the dorsal ends of 80% ± 2% RSFs \( (n = 72; 12 \text{ cells}) \) that connected with the TA (Fig. 1, B and D). On the quartile end of these RSFs, 1.9 ± 0.16 µm away from FAs \( (n = 58; 12 \text{ cells}) \), septins interdigitated with actin filaments as they flared from their FA-anchored bundle toward the TA (Fig. 1, E; panel 1). Time-lapse microscopy showed that septins are recruited to the distal ends of RSFs after the assembly of nascent FAs (Fig. 1 F and G). In live cells, septins persisted on RSFs during their interactions with the TA, spanning segments of both RSF and TA stress fibers (Fig. 1 H). Taken together, these data indicate that septins localize at the interface of the contractile TA and RSFs, and thus, may be part of the actomyosin machinery that drives the growth and turnover of nascent FAs in motile renal epithelia.

**Septins are required for the stabilization of nascent FAs and the organization of the lamellar stress fiber network.**

To test whether septins affect the formation and dynamics of FAs, HGF-treated MDCK epithelia were depleted of SEPT2 or SEPT9. Septin knock-down with two different SEPT2 shRNAs and a SEPT9 shRNA increased the number of FAs, which were smaller and localized closer to the cell edge compared to control cells (Fig. 2 A-D). Time-lapse imaging of paxillin-GFP showed that septin knock-down reduces the life time
of nascent FAs (Fig. 2E), which fail to grow significantly in size and do not move closer to the cell body (Video 2). Analysis of the kinetics of paxillin-GFP fluorescence revealed no differences in the rates of FA assembly and disassembly (Fig. 2 F), but septin depletion abolished the stabilization phase of the FA life cycle (Fig. 2 G). Consistent with lack of FA stabilization, which is characterized by dephosphorylation of paxillin’s Tyr118 (Zaidel-Bar et al., 2007), septin depletion increased the levels of pTyr118-paxillin relative to total paxillin in MDCK and 786-0 RCC cells (Fig. 2 H-I).

Maturation of nascent FAs is mediated by stress fibers, which exert mechanical forces and provide a template for the growth of cell-ECM adhesions (Burridge and Wittchen, 2013; Gardel et al., 2010; Vicente-Manzanares et al., 2009). While myosin-mediated tension is thought as the main trigger of FA maturation, recent work shows that myosin II-dependent tension is necessary but not sufficient for FA maturation, which requires actin-binding proteins that mediate the formation and maintenance of the lamellar actin network (Choi et al., 2008; Oakes et al., 2012; Roca-Cusachs et al., 2013; Stricker et al., 2013).

Next, we examined the organization and dynamics of the lamellar actin network in septin-depleted cells. Strikingly, the lamellar actin meshwork was thin and devoid of an organized TA (Fig. 2 J). F-actin was significantly reduced in the lamellar regions of the cell (5-10 µm from the cell edge; Fig. 2 K), and the length and density of RSFs decreased by ~50% (Fig. 2 L and M). In live SEPT2-depleted cells, RSFs were short-lived, dissipating quickly after their formation, while in control cells the actin fluorescence of RSFs persisted and increased over time (Fig. 2 N; Video 3). Taken together with the localization of septins to the TA and distal ends of RSFs, these data suggest that septins
are required for the stabilization and maturation of nascent FAs by maintaining the organization and possibly contractility of the lamellar stress fiber network.

**Septins function as actin cross-linking proteins in FA maturation.**

Because SEPT2 binds directly myosin II and this interaction is posited to facilitate the activation of the myosin RLC (Joo et al., 2007), we sought to determine whether septins affect FA maturation through their role in the organization of the lamellar actin network or by a possible involvement in myosin-mediated tension. To distinguish between these possibilities, we tested first whether septins affect the levels of activated myosin RLC on the contractile TA, and second whether the actin bundling protein α-actinin-1 or the constitutively active di-phosphomimetic myosin RLC-DD can rescue septin loss of function. Analysis of the fluorescence intensity ratio of the phosphorylated pSer19 myosin RLC to total myosin IIA showed no difference between control and SEPT2-depleted cells (Fig. S3 A and B). In agreement, RLC-DD did not rescue FA size and number in SEPT2-depleted cells (Fig. 3 A-C). In contrast, α-actinin-1 restored the number and size of FAs to near control levels (Fig. 3 A-C). This effect of α-actinin-1 was also accompanied by a rescue of the lamellar actin network in septin-depleted cells, which now contained a network of TA filaments and RSFs (Fig. 3 D). Notably, α-actinin-1-∆ABD, which lacks its actin-binding domain, did not rescue FAs (Fig. 3 B and C), while the actin-binding motor-dead myosin II-N93K did (Fig. S3 C-E). Thus, loss of septin function in FA maturation is specifically rescued by the actin-binding and cross-linking properties of α-actinin-1 and myosin IIA heavy chain.
Previous studies have reported that septin complexes comprising of SEPT2, SEPT6 and SEPT7 do not bind actin filaments directly (Kinoshita et al., 2002), but new work shows that copolymerization of actin with SEPT2/6/7 results in circular actin filaments (Mavrakis et al., 2014). Because in the lamellar regions of motile MDCK epithelia, septins interact with pre-assembled RSFs and TA filaments, we examined whether septins have the ability to cross-link pre-polymerized actin filaments using low speed actin sedimentation assays and negative stain EM. We found that SEPT9 directly cross-links pre-polymerized actin filaments into bundles, while SEPT2/6/7 does not (Fig. 3 E-G). These results suggest that septins promote the maturation of nascent FAs by cross-linking the lamellar stress fibers of motile epithelia.

**SEPT9 is up-regulated in renal epithelia undergoing EMT and enhances renal cell migration.**

Given that septins are over-expressed in renal cell carcinomas and other cancers (Connolly et al., 2011a; Craven et al., 2006a; Dolat et al., 2014a), we asked whether SEPT9 is upregulated during renal EMT. Treatment of MDCK cells with HGF increased the levels of SEPT9 relative to actin, while actin levels did not change (Fig. 4 A and B). To test if increased SEPT9 expression promotes cell motility, we established stable MDCK cell lines expressing SEPT9-mCherry at low endogenous-like and high levels of expression, and cultured these cells in a 3D ECM, which enables the morphogenesis of renal cysts. Treatment of the 3D MDCK cysts with HGF causes tubulogenesis, a process that mimics the collective and invasive migration of metastatic tumors (Debnath and Brugge, 2005). Over-expression of SEPT9 doubled the number of multicellular extensions that protruded from the main cyst and the extensions were longer and contained more cells (Fig. 4 C and D). In contrast, treatment of cysts with
forchlorfenuron (FCF), a septin-targeting compound (Hu et al., 2008), reduced significantly the number of HGF-induced extensions (Fig. 4 E). Moreover, dissociated single MDCK cells migrated slower in 3D and were less elongated and fibroblast-like after SEPT9 depletion (Fig. 4 H and I; Video 4). To corroborate the effects of SEPT9 over-expression on renal cell motility, we performed a transmigration assay using the 786-0 renal carcinoma cells. SEPT9 over-expression increased the migration of 786-0 cells along a serum gradient, while SEPT9 knock-down decreased migration (Fig. 4 F and G). These results, therefore, indicate that SEPT9 promotes the mesenchymal-like migration of renal epithelia.

Previous studies have implicated septins in the amoeboid motility of T-cells and neuronal migration (Shinoda et al., 2010; Tooley et al., 2009), which are mechanistically different from the stress fiber-driven mode of mesenchymal migration. Septins have been reported to colocalize with stress fibers in fibroblasts (Kinoshita et al., 1997; Kremer et al., 2007; Schmidt and Nichols, 2004b), but this interaction has been considered to be indirect (Joo et al., 2007; Kinoshita et al., 2002), and the precise function(s) of septins in epithelial and mesenchymal motility are unknown (Chacko et al., 2005; Connolly et al., 2011b). Here, we have found that septins are required for the stabilization of nascent FAs. Our results indicate that septins cross-link stress fibers, maintaining the organization of the lamellar actin network, and thereby, promoting the transmission of forces from the contractile TA to FAs. This septin function relies on the ability of SEPT9 to cross-link preassembled actin filaments. Thus, septin upregulation might be part of the EMT program that retools the actin cytoskeleton for a migratory mesenchymal-like phenotype.
Figure 5.1. Septin filaments interface with RSF and TA stress fibers in the leading lamella of renal epithelia. (A) Confocal images of SEPT2 (green), actin stress fibers (phalloidin; red) and FAs (paxillin; white) in HGF-treated (16 h) MDCK epithelia. White arrows point to RSFs that connect with the TA. (B) 3D rendering of the lamellar region outlined in panel A. Arrows point to SEPT2 fibers that localize on FA-anchored RSFs. Scale bars, 1 µm. (C) Quantification of SEPT2 colocalization with F-actin (green) and vice versa (red). (D) SIM images show ventral and dorsal optical sections from the lamellar region of an HGF-treated MDCK cell. Arrowhead points to SEPT9 localization on the dorsal segment of an RSF that connects with the TA. (E) SIM images show an HGF-treated MDCK cell stained for F-actin (phalloidin; red), SEPT2 (green) and paxillin (white). An FA-anchored RSF (1) and TA filaments (2) are shown in higher magnification. Arrowheads point to SEPT2 localization on and between actin filaments. (F) Still frames show FA formation (paxillin-mCherry; red) and subsequent recruitment of SEPT2-YFP (green). (G) Total internal reflection fluorescence microscopy kymograph shows recruitment of SEPT2-YFP (green) to a nascent RSF (ABP140-ABD-mCherry, red). (H) Inverted monochrome images show frames from spinning disk confocal time-lapse microscopy of HGF-treated MDCK cells expressing ABP140-ABD-mCherry and SEPT2-YFP. A subset of TA filaments and an RSF were pseudo-colored red and superimposed onto the inverted monochrome image of the SEPT2-YFP channel. SEPT2-YFP elements recruited to the junction of the RSF with the TA were pseudo-colored green. Overlay image shows the pseudocolored stress fibers and SEPT2 outlined in the inverted monochrome frames. Arrow points to the RSF end and its junction point with the TA.
Figure 5.2. Septins regulate the organization of the lamellar actin network and are required for the stabilization of nascent FAs. (A) Confocal images of MDCK cells, which were transfected with mCherry-expressing plasmids that encode for control or SEPT2 shRNAs, and stained for paxillin after treatment with HGF. Red dashed line highlights the cell edge. (B and C) Graphs show FA size and number from MDCK cells ($n = 20$) treated with control, SEPT2 and SEPT9 shRNAs. (D) Graph shows FA distance from the cell edge of MDCK cells ($n = 10$) treated with control ($n = 903$ FAs), SEPT2 # 1 ($n = 749$ FAs), SEPT2 # 2 ($n = 512$ FAs) and SEPT9 shRNAs ($n = 840$ FAs). (E) Graph shows FA lifetime in three different MDCK cells treated with control ($n = 6,066$ FAs) and SEPT2 shRNAs ($n = 10,528$ FAs). (F) Graph shows the rates of FA ($n = 45$) assembly and disassembly in cells treated with control and SEPT2 shRNAs. (G) Representative profiles of the kinetics of paxillin-GFP fluorescence in MDCK cells treated with control and SEPT2 shRNAs. (H) Confocal images show 786-O cells stained for total paxillin (red) and phosphorylated pY118-paxillin (green). Insets show GFP expression from plasmids encoding for control and SEPT2 shRNAs. (I) Bar graphs show the ratio of the fluorescence intensity of pY118-paxillin to total paxillin in MDCK cells ($n = 10$) treated with control ($n = 740$ FAs) and SEPT2 shRNAs ($n = 962$ FAs), and 786-O cells ($n = 10$) treated with control ($n = 757$ FAs) and SEPT2 shRNAs ($n = 602$ FAs). (J) Confocal images of control and SEPT2-depleted MDCK cells stained for F-actin (phalloidin; red) and paxillin (white). Insets show GFP expression from plasmids encoding for control and SEPT2 shRNAs. (K) Plot shows line scans of phalloidin fluorescence across the leading edges of MDCK cells ($n = 13$) treated with control ($n = 54$ line scans) and SEPT2 shRNAs ($n = 56$ line scans). (L and M) Graphs show the mean number of RSFs per lamellar length (L) and mean length of RSFs (M) in MDCK cells treated with control ($n = 43$) and SEPT2 shRNAs ($n = 72$). (N) Inverted monochrome frames show ABP140-ABD-
mCherry from total internal reflection fluorescence (TIRF) microscopy of MDCK cells treated with control or SEPT2 shRNAs and HGF. Arrows point to RSFs that persist and grow overtime in control cells, and stress fibers that dissipate in SEPT2-depleted cells.
Figure 5.3. SEPT9 functions as an actin cross-linking protein in FA maturation. (A) Confocal images of paxillin-stained MDCK cells transfected with mCherry-expressing plasmids that encode for control or SEPT2 shRNAs, and RLC-DD-GFP, α-actinin-GFP or GFP vectors. Scale bars, 10 μm. (B and C) Graphs show FA size (B) and number per μm² (C) in control cells expressing GFP (n = 1,650 FAs; 25 cells), and SEPT2 knock-down cells expressing GFP (n = 1,567 FAs; 24 cells), α-actinin-1-GFP (n = 1,556 FAs; 20 cells), RLC-DD-GFP (n = 1,962 FAs; 18 cells) and α-actinin-1-ΔABD-GFP (n = 1,962 FAs; 20 cells). (D) Confocal images of MDCK cells transfected with mCherry-expressing plasmids encoding for control or SEPT2 shRNAs, and α-actinin-1-GFP or GFP vectors. Cells were stained for F-actin (phalloidin). Scale bars, 10 μm. (E) Coomassie-stained gel shows equal volumes of supernatant (S) and pellet (P) fractions from low speed sedimentation of pre-polymerized actin filaments in the presence of recombinant SEPT2/6/7 and SEPT9. (F-G) Negative stain EM images of actin filaments in the absence and presence of recombinant SEPT6 or SEPT9. Outlined regions (F) are shown in higher magnification in G. Scale bars, 0.5 μm.
Figure 5.4. **SEPT9 promotes the motility of renal epithelia during EMT.** (A) Western blot of HGF-treated MDCK cell lysates probed for SEPT9, actin and GAPDH. (B) Graphs show the median, lowest and highest (error bars) ratio values of the SEPT9, actin and GAPDH band intensities from three independent experiments. Values were normalized to the protein band intensity ratios at 0 h of HGF treatment. (C) Maximum intensity projections from 3D confocal microscopy images of HGF-treated renal cysts of stable MDCK cells expressing SEPT9-mCherry (green) at low and high levels of expression. 3D renal cysts were grown in collagen and stained for nuclei (DAPI; blue) and F-actin (phalloidin; red). (D) Graphs show number of extensions per MDCK cyst ($n = 23$) and number of cells per extension from untransfected ($n = 18$), low- ($n = 35$) and high-expressing ($n = 51$) MDCK cysts ($n = 15$). (E) Mean number of extensions per cyst after treatment with DMSO ($n = 23$) and FCF ($n = 19$). (F and G) Graphs show the mean percentage of transmigrated 786-0 cells expressing GFP and SEPT9-GFP (F), and control and SEPT9 shRNAs from 20 different areas of a transwell filter. (H and I) Graphs show the mean velocity (H; $n = 16$) and axial ratio of the long to short axes (I; $n = 31$) of MDCK cells migrating in a 3D matrix in the presence of HGF after transfection with control or SEPT9 shRNAs.
Figure 5.5. Lamellar septin fibers are composed of SEPT2/6/7/9. (A-C) Confocal images of MDCK cells stained for paxillin (white), F-actin (phalloidin; red) and endogenous SEPT7, SEPT9 and SEPT6 (green).Outlined lamellar regions (dashed rectangles) are shown in higher magnification. (D-F) Fluorescence images show MDCK cells that expresses SEPT9-mCherry at endogenous-like levels after staining with antibodies against SEPT2, SEPT6 and SEPT7. Scale bars, 10 µm. (G) Cell lysates from a stable cell line that expresses SEPT2-YFP at subendogenous levels (see relative intensity of SEPT2-YFP and SEPT2 bands in the “input” lanes) and untransfected MDCKs were incubated with mouse anti-GFP antibody and immunoprecipitates were blotted for rabbit antibodies against SEPT2, SEPT6, SEPT7 and SEPT9.
Figure 5.6. Quantification of septin knockdown and overexpression in MDCK cells

(A) Confocal microscopy images show MDCK cells transfected with mCherry-expressing plasmids encoding for scrambled control, SEPT2- and SEPT9-targetting shRNAs. Cells were stained with antibodies against endogenous SEPT2, SEPT9, SEPT7 and SEPT6. Scale bars, 10 µm. (B) Bar graphs show quantification of SEPT2 and SEPT9 fluorescence intensity (arbitrary units; AU) per µm² in cells transfected for 48 h with control or SEPT2 shRNA # 1 (n = 20), SEPT2 shRNA #2 (n = 15) and SEPT9 shRNA (n = 15). (C) Cell lysates from untransfected MDCKs and MDCKs that overexpress SEPT9-mCherry stably were blotted for rabbit antibodies against SEPT2, SEPT6, SEPT7 and SEPT9, and a mouse antibody against GAPDH as a loading control. (D) Bar graph shows the ratio of fluorescence intensity (arbitrary units; AU) for each septin relative to GAPDH.
Figure 5.7. b. (A) Confocal microscopy images show MDCK cells treated SEPT2 and control shRNAs and stained for myosin-IIA heavy chain (red) and phosphorylated Ser19-myosin II regulatory light chain (green). Scale bars, 10 µm. (B) Bar graph shows quantification of the ratio of pSer-myosin II light chain to myosin IIA heavy chain fluorescence from 5 µm-long myosin filament segments, which were blindly selected from the lamellar regions of cells treated with control ($n = 93; 12$ cells) and SEPT2 ($n = 74; 11$ cells) shRNAs. (C) Confocal microscopy images of paxillin-stained MDCK cells transfected with mCherry-expressing plasmids that encode for control or SEPT2 shRNAs, and GFP or the GFP-tagged non-muscle myosin IIA heavy chain mutant N93K. Scale bars, 10 µm. (D and E) Bar graph shows FA size (D) and number per µm$^2$ (E) in control cells ($n = 16$) expressing GFP ($n = 1,722$ FAs), and SEPT2 knock-down cells ($n = 16$) expressing GFP ($n = 1,515$ FAs) and myosin IIA N93K-GFP ($n = 1,139$ FAs).
CHAPTER 6. Septin 9 exhibits polymorphic binding to F-actin and inhibits myosin and cofilin activity

Abstract

Septins are a highly conserved family of proteins in eukaryotes that is recognized as a novel component of the cytoskeleton. Septin 9 (SEPT9) interacts directly with actin filaments and functions as an actin stress fiber cross-linking protein that promotes the maturation of nascent focal adhesions and cell migration. However, the molecular details of how SEPT9 interacts with F-actin remain unknown. Here, we use electron microscopy and image analysis to show that SEPT9 binds to F-actin in a highly polymorphic fashion. We demonstrate that the basic domain (B-domain) of the N-terminal tail of SEPT9 is responsible for actin cross-linking, while the GTP-binding domain (G-domain) does not bundle F-actin. We show that the B-domain of SEPT9 binds to three sites on F-actin, and the two of these sites overlap with the binding regions of myosin and cofilin. SEPT9 inhibits actin-dependent ATPase activity of myosin and competes with the weakly-bound state of myosin for binding to F-actin. At the same time, SEPT9 significantly reduces the extent of F-actin depolymerization by cofilin. Taken together, these data indicate that SEPT9 protects actin filaments from depolymerization by cofilin and myosin, and suggest a mechanism by which SEPT9 could maintain the integrity of growing and contracting actin filaments.

Introduction

Septins were first discovered in the budding yeast as genes that are crucial for cell division (Hartwell, 1971). Since the discovery of septins in yeast, proteins with homologous sequences have been found in almost all eukaryotic cells. The number of septin genes per organism is variable and ranges from two isoforms in Caenorhabditis
*elegans* to 13 isoforms in humans (Russell and Hall, 2011). Based on sequence similarity, human septins are classified into four homology groups named after their founding members: SEPT2, SEPT3, SEPT6 and SEPT7. In this manuscript we focus on SEPT9 which belongs to the SEPT3 subgroup and has a unique N-terminal domain (NTD) consisting of a basic domain (B-domain) and an acidic domain (A-domain) (Fig. 1a) (Bai et al., 2013). All septins have a GTP-binding domain (G-domain), which is evolutionarily and structurally related to the Ras GTPases (Leipe et al., 2002). Unlike the monomeric small GTPases, septins form polymers that consist of non-polar oligomeric complexes(Bertin et al., 2008; Sirajuddin et al., 2007)

Septins have been recognized as the fourth component of the cytoskeleton because of their filamentous appearance and their interdependence with actin filaments and microtubules. The N-terminal domain of SEPT9 has been shown to bind and bundle microtubules by interacting with the acidic C-terminal tails of β-tubulin (Bai et al., 2013). Septins have been shown to bind actin filaments indirectly via anillin or the motor protein myosin II(Joo et al., 2007; Kinoshita, 2002). Recent data, however, demonstrated that *Drosophila* septin complexes (Sep1-Sep2-pnut) can directly interact with actin and cross-link actin filaments into curved bundles, which are critical for the organization and function of contractile actin rings during *Drosophila* embryonic cleavage (Mavrakis et al., 2014). Moreover, we recently found that human SEPT9 directly binds and bundles F-actin, and that cross-linking of actin filaments by SEPT9 promotes focal adhesion maturation and epithelial cell motility (Dolat et al., 2014b).

Despite the new evidence that septins can directly interact with the actin cytoskeleton, the molecular details of these interactions and how septins interact with the surface of the actin filament remain unknown. Here we use electron microscopy,
image analysis and co-sedimentation assays to identify how SEPT9 interacts with F-actin. Our data indicate that the B-domain of SEPT9 is responsible for its F-actin bundling activity, while the G-domain of SEPT9 does not bundle actin filaments. We demonstrate that SEPT9 binds to three sites on the surface of F-actin that are commonly bound by other actin binding proteins. Importantly, two sites overlap with the regions of the actin molecule involved in the binding of myosin and coflin. As predicted by the structural data, SEPT9 inhibits F-actin depolymerization by coflin, which severs actin filaments, and reduces the actin-dependent ATPase activity of myosin. Our results suggest that SEPT9 could protect growing actin filaments from the combined depolymerizing activity of coflin and myosin during actin filament assembly and contraction.

**Materials and Methods**

**Protein expression and purification.** G-Mg\(^{2+}\)-actin was prepared as described (Orlova and Egelman, 1995). SEPT9 constructs were prepared as follows. E. Coli BL21 (DE3) cells were transformed with SEPT9_i1, SEPT9-NTD, SEPT9-G, SEPT9-A, or SEPT9-B in kanamycin-resistant pET-28a (Bai et al., 2013). Plasmids expressing His-tagged SEPT9-N (aa 1–283 of SEPT9_i1), SEPT9-B (aa 1–142 of SEPT9_i1), SEPT9-A (aa 143–283 of SEPT9_i1), SEPT9-G (aa 284–586 of SEPT9_i1) were made with the QuikChange II Site-Directed Mutagenesis kit (Agilent Technologies) using the pET-SEPT9_v1 and pET-SEPT9_v3 plasmids. Bacterial cultures of OD600 were induced with 0.5 mM IPTG for 16h at 18°C, collected by centrifugation and resuspended in lysis buffer containing 50 mM Tris, pH 8.0, 1% Triton X-100, 150 mM NaCl, 10% glycerol, and 10 mM imidazole. Bacteria were lysed using a French pressure cell at 1,280 psi, and lysates were cleared using centrifugation (14,000 g) for 30 min at 4°C. Columns
containing Ni-NTA beads were equilibrated with lysis buffer prior to passing supernatants through by gravity flow. The beads were washed with buffer containing 50mM Tris, pH 8.0, 300 mM NaCl, 10% glycerol, and 10 mM imidazole, and proteins were eluted with buffer containing 50mM Tris, pH 8.0, 300 mM NaCl, 10% glycerol, and 250 mM imidazole.

MyosinV-S1 heavy chain, 907 amino acid residues including 6IQ domains was expressed in a baculovirus/Sf9 cell system. The proteins were purified using FLAG-affinity chromatography and then concentrated and fractionated on a MonoQ ion-exchange column with a linear gradient of 0.1−0.5 M NaCl in 10 mM MOPS, 0.1 mM EGTA, 3 mM NaN₃, and 1 mM DTT, pH 7.2, using HPLC. The concentration of myosinV-S1 was determined from the A280 after correction for light scattering (A280~1.5A320). The extinction coefficient was calculated to be 1.1 × 10⁵ M⁻¹ cm⁻¹ from the number of tyrosine and tryptophan residues and their molar extinction coefficients: ε = (n = 10)trp × 5690 + (n = 42)tyr × 1280. All preparations were analyzed by SDS protein gel electrophoresis and by active site titration with deac-aminoATP. Cofilin-1 was a generous gift from Dr. Emil Reisler (UCLA).

Electron microscopy of negatively stained SEPT9 domains. G-actin was polymerized for 1–2 hours in F-buffer (10mM HEPES, pH 7.4, 100mM KCl, 1mM MgCl₂, 0.5mM ATP). For EM samples shown in Fig. 1c-g, 2 µM F-actin was incubated for 2 min in tube with 34 µM NTD, B-domain, A-domain, or G-domain. Samples were blotted and negatively stained with 2% (wt/vol) uranyl acetate. To obtain single actin filaments decorated with SEPT9 domains was 2 µM F-actin was incubated for 1 min on carbon coated glow discharged grids in a humidified chamber, gently blotted and incubated again with 34 µM NTD, B-domain, A-domain, or G-domain. Samples were negatively
stained with 2% (wt/vol) uranyl acetate. A JEM-2100F (JEOL) Field Emission Transmission Electron Microscope, equipped with 11-megapixel Gatan SC1000 ORIUS CCD camera, was used at an accelerating voltage of 200 kV, and a nominal magnification of 60,000x to record micrographs at a raster of 1.58 Å/pixel.

**Electron microscopy of SEPT9 in presence of cofilin-1 and SEPT9.** F-actin (2 µM) was mixed with 10 µM of cofilin-1, or preincubated with 10 µM of SEPT9 before addition of 10 µM of cofilin-1. Samples were left at room temperature and analyzed by TEM at 2 and 30 min time points.

**Image analysis.** The SPIDER software package (Galkin et al., 2001) was used for most image processing, while the EMAN package (Galkin et al., 2003) was used to extract filament images from micrographs. Images were corrected for the contrast transfer function (CTF) using theoretical CTF obtained with the CTFIND software (Frank, 1984) and decimated to 3.16 Å/pixel scale. From these CTF-corrected images segments (each 120 pixels long) of F-actin decorated with the NTD (n=8,193), B-domain (n=2,708), A-domain (n=5,435) constructs were extracted. Segments of filament decorated with the NTD were reconstructed using IHRSR approach (Ludtke et al., 1999). The resultant reconstruction showed three additional densities bound to F-actin - one at the front interface between SD1 and SD2 of two adjacent actin protomers, the other one at the side of SD1, and one on the top of SD4 (Fig. S1). Three model volumes were created by using pseudo-atomic model of F-actin (Mindell and Grigorieff, 2003) having model density attached to the first site, second site, or third site. These volumes, along with a naked F-actin volume, were projected onto 120 × 120-pixel images with an azimuthal rotational increment of 4°, and the resultant 360 reference projections (4 × 90) were cross-correlated with the 8,193 NTD-decorated actin filament segments. Each of the four
classes was reconstructed starting from the two independent starting models (Fig. S2). The naked class (n=4,222) yielded a symmetry of 166.6°/27.3 Å, the mode1 class (n=1,590) yielded a symmetry of 166.7°/27.2 Å, the mode 2 class (n=1,305) converged to 166.1°/27.2 Å, while a set of images classified as mode 3 (n=1,079) reached a stable solution having 167.0°/27.2 Å symmetry values. The same sorting procedure was applied to actin filaments decorated with the B-domain. The naked segments extracted from the filaments decorated with the B-domain (n=1,571) converged to a 166.6°/27.2 Å solution, the mode1 class from that set (n=473) yielded 166.3°/27.1 Å, the mode 2 class (n=332) converged to a symmetry of 166.1°/27.3 Å, while the mode 3 class (n=332) yielded a symmetry of 166.5°/27.2 Å. Segments of filaments incubated with the A-domain were mostly naked actin (n=4,935) with only 218 segments assigned to mode 1 class, 135 segments assigned to the mode 2 class, and 147 segments assigned to the mode 3 class. Neither of these classes yielded reasonable 3D reconstruction (data not shown). The naked class converged to the stable solution of 166.5°/27.1 Å. Fourier Shell Correlation approach was used to determine the resolution of the 3D-reconstructions. Each image set was split into two non-overlapping subsets, and these subsets were reconstructed independently. Resultant volumes were used for resolution determination. Using 0.5 criterion all maps yielded 27-28 Å resolution (Fig. S7). All the 3D-reconstructions shown in the paper were filtered to the measured resolution. The Chimera software (Egelman, 2000) was used to dock a protomer from the atomic model of F-actin into the reconstruction manually. Figures 2, 3 and 4 were produced using the Chimera software.

**Actin activated steady-state ATPase experiments.** Actin activated steady-state experiments were done by the NADH coupled assay at 25 °C. 20 nM Myosin V-S16IQ was mixed with 10 µM actin and increasing concentrations of SEPT9 A- domain , B-
domain or full length SEPT9 in a buffer containing 10 mM MOPS (pH 7.0), 2 mM MgCl₂, 25 mM KCl, 0.15 mM EGTA, 2 mM ATP, 40 units/ml lactate dehydrogenase, 200 units/ml pyruvate kinase, 1 mM phosphoenolpyruvate, and 200 μM NADH (Fujii et al., 2010). Changes in NADH absorption at 340 nm (ε340 = 6220 M⁻¹cm⁻¹) were followed in a Beckman DU640 spectrophotometer. The ATPase activity of blanks containing actin but no myosin was subtracted from the actomyosin data. The data was fit to the equation $F(x) = \frac{V_{\text{max}}}{1+x/K_i}$.

**Low-speed actin pelleting assays.** G-actin in G buffer (5 mM Tris-HCl and 0.2 mM CaCl₂) was polymerized in buffer containing 20 mM Hepes, pH 7.4, 100 mM KCl, and 1 mM MgCl₂ supplemented with 0.2 mM ATP and 4 mM DTT for 1 h at 25°C. In Figure 1, 8 μM of recombinant SEPT9-N, SEPT9-G, SEPT9-A, or SEPT9-B were incubated with 2 μM F-actin for 1 h at 25°C. In Supplemental Figure 6, increasing concentrations of full-length SEPT9 or SEPT9-B were incubated with 2 μM F-actin for 1 h at 25°C. Reactions were subsequently centrifuged on top of a glycerol cushion (20 mM Hepes, 100 mM NaCl, 1 mM MgCl₂, and 10% glycerol) at 8,000 g for 20 min at 25°C. Supernatant and pellet fractions were collected in SDS sample buffer, and equal volumes of each fraction were resolved with a 10% or 6-12% gradient SDS-PAGE gel and stained with Coomassie brilliant blue. Protein band densities from scanned gels were quantified using the Odyssey infrared scanning system (LI-COR Biosciences) and analyzed in excel. All experiments were repeated 3 times.

**High-speed actin pelleting assays.** Competition between SEPT9 and the myosin V-S1 heavy chain for F-actin binding were performed in buffer containing 10mM MOPS, pH 7.0, 100 mM KCl, 2 mM MgCl₂, 0.5 mM EGTA, and 2 mM DTT with 1.25 mM ATP or without ATP. For experiments without ATP, the F-actin stock was dialyzed in F-buffer
without ATP. F-actin (2 μM) was pre-incubated with increasing concentrations of SEPT9 for 30 minutes at 25°C. Reactions were incubated with myosin V-S1 heavy chain (0.75 – 1 μM) for an additional 30 minutes at 25°C, placed on a glycerol cushion (10mM MOPS, pH 7.0, 100 mM KCl, 2 mM MgCl₂, 0.5 mM EGTA, 20% glycerol) and centrifuged at 200,000 g for 30 minutes at 21°C. Equal volumes of supernatant and pellet fractions were collected in SDS sample buffer, and equal volumes of each fraction were resolved using a 10% SDS-PAGE gel and stained with Coomassie brilliant blue. Protein band densities were quantified using the Odyssey infrared scanning system and analyzed in excel. Each experiment was repeated 3 times.

Co-sedimentation assays (Fig. 4) that tested for cofilin-mediated depolymerization were performed by pre-incubation of aged F-actin with recombinant SEPT9_i1 (5 μM) or 5 μM SEPT9 fragments (NTD, B-domain or A-domain) in buffer containing 10 mM Hepes, pH 7.6, 100 mM KCl, 1 mM MgCl₂, and 2 mM DTT for 30 min at 25°C. Reactions were incubated with 5 μM recombinant human cofilin-1 for an additional 30 min at 25°C, placed on a glycerol cushion (20 mM HEPES, pH 7.6, 100 mM NaCl, 1 mM MgCl₂, and 10% glycerol) and centrifuged at 200,000 g for 30 min at 21°C in an ultracentrifuge (Optima TL100, Beckman Coulter). Supernatant and pellet fractions were collected in SDS sample buffer, and equal volumes of each fraction were resolved using a 8-15% gradient SDS-PAGE gel and stained with Coomassie brilliant blue. Protein band densities from scanned gels were quantified using the Odyssey infrared scanning system (LI-COR Biosciences) and analyzed in excel. Each experiment was repeated 3 times.

High-speed pelleting assays between SEPT9 and F-actin (Fig. S5) were performed by incubating full-length SEPT9 (1 μM) with increasing concentrations of F-
actin for 30 minutes at 25°C in buffer containing 20 mM Hepes, pH 7.6, 100 mM KCl, and 1 mM MgCl$_2$ supplemented with 0.2 mM ATP and 4 mM DTT. Reactions were placed on a glycerol cushion (20 mM HEPES, pH 7.6, 100 mM NaCl, 1 mM MgCl$_2$, and 20% glycerol) and centrifuged at 200,000 g for 30 min at 21°C. Supernatant and pellet fractions were collected in SDS sample buffer, and equal volumes were resolved using a 10% SDS-PAGE gel. Gels were stained with Coomassie brilliant blue, and protein band densities from scanned gels were quantified using the Odyssey infrared scanning system. The fraction of SEPT9 protein in each pellet fraction was plotted against the concentration of actin in GraphPad and fitted with a one-site specific binding curve to determine the dissociation constant.

RESULTS

The B-domain of SEPT9 promotes F-actin bundling.

Using negative stain EM and low-speed pelleting assays, we sought to understand how SEPT9 interacts with F-actin by expressing and purifying each of the three SEPT9 domains; the domain composition of SEPT9 is shown in Fig. 1a. Full-length SEPT9 (isoform 1) as well as the N-terminal domain (NTD) of SEPT9 effectively assemble F-actin into bundles (Fig. 1c and d, respectively). Strikingly, the B-domain alone promotes formation of ordered thick bundles (Fig. 1e), while the A-domain does not possess any bundling activity (Fig. 1f). Similarly to the A-domain, the G-domain of SEPT9 does not possess any bundling activity (Fig. 1g). Our results indicate that the B-domain of SEPT9 is responsible for the SEPT9 F-actin bundling activity. To independently corroborate these EM observations, we examined the ability of individual SEPT9 domains to cross-link actin filaments using a low speed actin sedimentation
assay (Fig. 1h and i). In agreement with the EM data, the NTD (Fig. 1h) and B-domain (Fig. 1i) of SEPT9 pelleted F-actin at low speed, while the G-domain (Fig. 1h) and the A-domain (Fig. 1h) did not. Taken together, these data indicate that the B-domain of the NTD of SEPT9 is responsible for the actin-bundling activity of SEPT9.

**SEPT9 binds to three sites on the surface of F-actin.**

To identify the binding site of SEPT9 on the surface of F-actin we performed a 3D-reconstruction of actin filaments decorated with the two SEPT9 actin bundling fragments – the NTD and the B-domain (Fig. 2). In order to obtain single actin filaments suitable for image analysis, the NTD or B-domain were incubated with actin filaments that were pre-bound to a carbon film to reduce bundle formation. The overall 3D-reconstruction of F-actin segments (each segment contained ~14 actin subunits) decorated with the NTD of SEPT9 revealed three sites of interaction of the NTD with the actin filament (Fig. S1). We used a modeling approach and cross-correlation sorting (see Methods) to compute individual 3D-reconstructions for each of the three modes of binding of the NTD to F-actin revealed in the overall reconstruction (Fig. 2a). Since model-based sorting may be biased, we used two independent approaches to confirm that our 3D-reconstructions reflected the intrinsic properties of the images. First, we computed two independent 3D-reconstructions for each of the three structural modes starting the IHRSR algorithm from a featureless solid cylinder, or from a 3D-reconstruction of a different structural mode (Fig. S2). This approach proved that the resultant EM map was independent from the starting model and hence all its features reflected the intrinsic properties of the raw images (Fig. S2). Second, we calculated reference free 2D-averages for each class of NTD-decorated F-actin segments to confirm that all three 2D averages were different from each other and hence, the
differences in the 3D reconstructions reflected the differences in the raw images (Fig. S3).

The NTD of SEPT9 binds to three distinct sites on the surface of F-actin. In mode 1 (Fig. 2a, magenta arrows), the NTD binds to the side of the SD1/SD2 interface of the actin molecule which includes actin residues 80-101, 125-130, and 357-360 (Fig. 2d, shown in magenta). In mode 2 (Fig. 2a, red arrows), the NTD is bound to the front portion of the SD1/2 interface, which involves residues 1-7, 21-30, 56-60, and 92-103 (Fig. 2d, shown in red). In mode 3 (Fig. 2a, cyan arrows), actin residues 223-238 and 248-251, which belong to the SD4 of F-actin (Fig. 2d, shown in cyan), are located at the interface with the NTD.

We used the same set of references to determine how the B-domain of SEPT9 binds to F-actin (Fig. 2b). We found the B-domain is bound to the same three sites on the surface of the actin molecule. We applied the same sorting procedure to F-actin incubated with the A-domain of SEPT9 and compared the frequency of decorated and naked actin segments between the three SEPT9 domains (Fig. 2c). In contrast to the NTD and the B-domain, more than 90% of actin segments incubated with the A-domain yielded a best match with naked F-actin. The remaining 10% generated an uninterpretable 3D-reconstruction (data not shown). To exclude any possibility that the A-domain binds to F-actin, we used the same set of references to run cross-correlation sorting of pure F-actin segments (Fig. S4). Not surprisingly, the sorting showed a very similar distribution of the pure F-actin segments between the four classes which reflected the margin of error in the sorting procedure.

We did not find a predominant mode of NTD or B-domain binding to F-actin as the frequencies of the three structural modes were quite similar (Fig. 2c). Because we
did not find any segments with all three sites occupied simultaneously, individual binding modes of SEPT9 appear to be highly cooperative and mutually exclusive; note that full length SEPT9 exhibited the same three modes of actin binding as the NTD and B-domains (data not shown). It is therefore likely that if a SEPT9 molecule is bound to an actin protomer in either mode 1, 2 or 3, adjacent SEPT9 molecules would also bind actin protomers in the same mode. Such a cooperativity has been reported for many actin binding protein and relies on cooperative structural transitions that can propagate along the actin filament (Galkin et al., 2005; Galkin et al., 2010; Orlova and Egelman, 1997). In addition to the structural cooperative transitions within the actin filament, the spatial arrangement of the SEPT9 domains on the surface of F-actin could result in mutually exclusive modes of SEPT9 binding to F-actin. Binding modes 1 and 2 have an overlapping interface on the surface of F-actin (Fig. 2d, 80-101 helix in mode 1 and 92-103 in mode 2), and modes 2 and 3 would clash spatially if present simultaneously within the same actin filament. Taken together, our results indicate that SEPT9 interacts with F-actin via the B-domain of its NTD in a polymorphic fashion. Importantly, this interaction involves actin-binding sites, which are commonly bound by actin-binding proteins including myosin and cofilin (Behrmann et al., 2012; Galkin et al., 2011).

**SEPT9 inhibits actomyosin activity**

The localization of myosin S1-fragment on the surface of F-actin in the absence of ATP (rigor state) has been previously resolved (Behrmann et al., 2012). While the position of myosin on the surface of the actin filament in the presence of ATP (weak binding mode) has not been visualized, modeling studies based on the X-ray diffraction from permeabilized muscles suggest that the N-terminus of F-actin is at the interface with the weakly bound myosin head (Gu et al., 2002). Our data suggest that the position
of myosin in a rigor state would clash with the position of the NTD of SEPT9 in two of its three binding modes (Fig. 3a-b, red arrows); the N-terminus of F-actin (denoted with red asterisk in Fig. 3a-c) is also at the interface of NTD with F-actin. Thus, binding of SEPT9 to F-actin in modes 1 or 2 could interfere with the actin binding of myosin in both rigor and weak states (Fig. 3a-b), while binding of SEPT9 to F-actin in mode 3 may not interfere with the acto-myosin interaction (Fig. 3c).

To test these structural predictions, we first used an actin-activated steady-state ATPase assay. In this assay, myosin ATP hydrolysis is coupled with its interaction with F-actin. Thus, any protein that interferes with the actomyosin interaction will reduce the ATPase activity of the myosin head. To evaluate the effect of SEPT9 on the actomyosin cross-bridge cycle, we carried out the actin activated steady-state ATPase assay using the NADH coupled method (Fig 3d). The S1 fragment of myosin V and F-actin were mixed with increasing concentrations of full length SEPT9, or its individual B- and A-domains domains (see Methods for details). The full length SEPT9 exhibited the largest inhibition with an apparent Ki of 0.46 µM followed by the B-domain with a Ki of 2.44 µM. The A-domain had almost no effect on myosin ATPase activity with an apparent Ki of 185 µM.

Since bundling would reduce the number of F-actin sites that are available for myosin binding, we tested whether the reduction in myosin ATPase activity correlated with an increase in F-actin bundling. Low-speed actin sedimentation assays showed that both the full length SEPT9 and SEPT9 B-domain did not significantly affect F-actin bundling over concentration ranges that caused a drastic reduction in myosin activity (Fig. S5). Hence, the bundling activity of SEPT9 by itself could not account for full inhibition of the acto-myosin ATPase activity. Taken together with our structural data that
predict a mutually exclusive binding of myosin and SEPT9 in modes 1 and 2 (Fig. 3a,b), our results indicate that SEPT9 inhibits actin-dependent myosin ATPase activity by competing with myosin for binding to the same sites of F-actin.

ATP hydrolysis and subsequent dissociation of the nucleotide from the myosin head changes acto-myosin interaction from the initial weakly-bound state to a rigor-bound state. This transition in the acto-myosin interaction is an essential part of myosin’s ATPase cross-bridge cycle. To test how SEPT9 inhibits acto-myosin activity, we performed competitive high-speed actin pelleting assays of myosin V S1-fragment with the full length SEPT9 in the presence or absence of ATP, so we can distinguish between the weakly-bound (ATP) and rigor-bound (no ATP) states of myosin. In the presence of ATP, full length SEPT9 efficiently competed with the myosin S1-fragment for binding to F-actin (Fig. 3e). Interestingly, in the absence of ATP (only ADP was present in solution) SEPT9 co-sedimented with myosin S1-fragment (Fig. 3f). The affinity of the ADP-bound myosin V to F-actin (~7.6 nM) is ~1000 fold higher than its affinity to F-actin in the presence of ATP (~13 µM) (De La Cruz et al., 1999; Yengo et al., 2002). Taking into account that the affinity of septins to F-actin is in the micromolar range (Mavrakis et al., 2014) (Fig. S6), SEPT9 molecules that are bound to F-actin in modes 1 and 2 are likely to be displaced by ADP-bound myosin, which has a high affinity for F-actin. Cosedimentation of SEPT9 with rigor-bound myosin V suggests that SEPT9 is bound to F-actin in mode 3 (Fig. 3c), which does not clash with the position of rigor-bound myosin. In the presence of ATP, however, SEPT9 is likely to compete with myosin for the binding modes 1 and 2, which account for ~70% of the binding of SEPT9 to F-actin (Fig. 2c), and thereby, SEPT9 can inhibit the interaction of myosin with F-actin. To summarize, our data indicate that SEPT9 inhibits the weak acto-myosin interaction through its B-domain, while it can bind to F-actin in the presence of rigor-bound myosin.
SEPT9 reduces cofilin-driven F-actin depolymerization.

Superimposition of the 3D reconstructions of SEPT9-decorated F-actin in mode 1 (Fig. 4a, top) and mode 2 (Fig. 4a, bottom) revealed significant clashes between the SEPT9 density (Fig. 4a, black arrows) and cofilin (Fig. 4a, shown as solid red surface). Similar to our results with myosin, we hypothesized that SEPT9 may inhibit cofilin-actin binding. Using electron microscopy and high-speed pelleting assays, we sought to test whether SEPT9 affects cofilin-driven F-actin depolymerization.

EM imaging of actin filaments showed that within 2 min of incubation, cofilin disrupts the meshwork of naked actin filaments, which are disintegrated into short twisted filaments (Fig. 4b, 2 min). After 30 min, only a few cofilin-decorated actin filaments remain (Fig. 4b, 30 min). In the presence of full length SEPT9, F-actin bundles (Fig. 4c, 0 min) remain intact after 2 min of incubation with equimolar concentration of cofilin-1 (Fig. 4c, 2 min). Even after 30 min of incubation with cofilin-1, F-actin bundles are still present (Fig. 4c, 30 min). To further test whether SEPT9 protects against actin depolymerization by cofilin, we performed high-speed sedimentation assays in the presence of full length SEPT9 or its domains and cofilin-1 (Fig. 4,d-f). In our experiments we used equimolar concentrations of SEPT9 and cofilin, because the affinity of human ADF/cofilin to ADP-F-actin is similar to septins (Mavrakis et al., 2014) (Fig. S6). After 30 min incubation of F-actin with cofilin-1, ~90% of actin is recovered in the supernatant (Fig. 4d). Pre-incubation of F-actin with SEPT9 reduced the amount of actin in the supernatant to ~30% (Fig. 4d,). The NTD (Fig. 4e) and the B-domain (Fig. 4f) of SEPT9 also reduced the amount of depolymerized actin in the supernatant to ~50-60% of total actin, while the A-domain had no effect (Fig. 4f). Thus, similar to the inhibition of
myosin binding to F-actin, SEPT9 appears to inhibit cofilin binding, and thereby, protect actin filaments from cofilin-mediated depolymerization.

Discussion

*Drosophila* septins have been recently reported to directly bind and bundle F-actin (Mavrakis et al., 2014). Mammalian septins (SEPT2/6/7 oligomers) have been proposed to interact with F-actin indirectly via anillin and myosin II, but it was recently shown that the mammalian SEPT9 can bind and bundle actin filaments (Dolat et al., 2014b; Joo et al., 2007; Kinoshita et al., 2002). Our data show that the actin bundling activity of SEPT9 resides in the NTD – a unique domain that is not present in other septin paralogs. SEPT9 interacts with F-actin in a highly polymorphic fashion (Fig. 2a-b). Such multiplicity in binding modes is not unique to SEPT9 and has been reported for other actin binding proteins (ABPs) such as Arg/Abl kinase, drebrin, myosin binding protein C, utrophin, and a giant muscle protein nebulin (Galkin et al., 2005; Galkin et al., 2002; Grintsevich et al., 2010; Lukoyanova et al., 2002; Orlova et al., 2011) Since, the N-terminal domain of SEPT9 does not possess any detectable amino acid identity with these ABPs, it suggests that multiplicity of actin-binding modes is a common feature of ABPs independently of their actin-binding sequences and domains.

Recently we showed that F-actin can exist in multiple structural states and that the structural transitions in the mobile regions of the actin protomers are coupled (Galkin et al., 2010). SEPT9 binds to the two mobile regions of actin molecule including residues 1-7 of the N-terminus and residues 56-60 of the SD2 (Fig. 2d). It is plausible that in the cell the structural mode of SEPT9 binding to F-actin is determined by the structural state of the actin filament and the presence of other actin-bound ABPs. Our results suggest that SEPT9 may switch from mode 1 and 2, where it interferes with the
actin-myosin interaction (Fig. 3a-b), to mode 3 where both rigor-bound myosin and SEPT9 can simultaneously interact with F-actin. By switching between these structural modes of binding, SEPT9 may alter acto-myosin interactions in migrating cells, where tensile forces are tightly controlled and are linked to focal adhesion turnover (Parsons et al., 2010; Peacock et al., 2007) and cell-ECM adhesion (Vicente-Manzanares et al., 2007). Thus, SEPT9 may influence actomyosin activity at stress fibers and focal adhesions.

SEPT9-dependent actin bundling activity is required for the maintenance of a functional stress fiber network in the leading edge of migratory epithelial cells (Dolat et al., 2014b). Interestingly, our results indicate that SEPT9 protects actin filaments from depolymerization by coflin. Actin cross-linking proteins have been shown to protect actin filaments from depolymerization in vitro (Cano et al., 1992; Lebart et al., 2004; Zigmond et al., 1992). In living cells, varying levels of expression of actin bundling proteins have been shown to alter the rates of actin filaments turnover (Mukhina et al., 2007; Tilney et al., 2003) and myosin has been shown to regulate the turnover of actin bundles in neuronal growth cones (Ishikawa et al., 2003; Medeiros et al., 2006).

In addition, recent studies show that actin depolymerization by ADF/cofilin is differentially regulated by actin motors and actin-cross-linking proteins (Schmoller et al., 2011). While myosin II enhances the disintegration of F-actin bundles, cross-linking proteins inhibit the disassembly of actin bundles by coflin (Schmoller et al., 2011). Notably, cross-linking proteins such as fascin may affect the severing activity of coflin by reducing the flexibility of bundled F-actin, and thereby, limit the torsional twist that coflin triggers on F-actin (Breitsprecher et al., 2011). Because the efficiency of coflin severing is the highest between decorated and non-decorated segments of F-actin, the severing
activity of cofilin will be the highest on more rigid bundled actin filaments, which do not allow for a uniform, cooperative binding that is enabled by a torsional twist (Breitsprecher et al., 2011). Thus, depending on the ABP composition of F-actin bundles and their flexibility, cross-linking proteins such as fascin may either increase or decrease the activity of cofilin (Breitsprecher et al., 2011; Elkhatib et al., 2014).

Our results indicate that SEPT9 inhibits both myosin and cofilin interaction with F-actin, and hence may work as a protective factor for growing actin bundles. Given that both myosin (Juanes-Garcia et al., 2016) and cofilin (Bernstein and Bamburg, 2010) are involved in the regulation of actin organization and contractility in a diversity of cellular processes and structures, our results suggest that the ubiquitously expressed SEPT9 (Dolat et al., 2014a) may be a key player in the regulation of F-actin organization and function.
### (a) NTD

| Domain | 1 | 143 | 283 | 546 |

### (b) - (d)

- (b) B-domain
- (c) A-domain
- (d) G-domain

### (e) - (g)

- (e) B-domain
- (f) A-domain
- (g) G-domain

### (h)

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### (i)

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Figure 6.1. **SEPT9 bundles F-actin through the B-domain of its NTD.** (a) Schematic representation of SEPT9 domains. In addition to the GTP-binding domain (G-domain) found in all septin paralogs, SEPT9 has a unique N-terminal domain (NTD) consisting of a basic domain (B-domain) and an acidic domain (A-domain). (b) Pure F-actin, scale bar: 1 nm. (c) Full-length SEPT9 efficiently bundles actin filaments within 2 min of incubation. (d) NTD consisting of the B- and A-domains packs F-actin into tight bundles. (e) The B-domain bundles F-actin into tight bundles morphologically similar to those shown in (d). (f) The A-domain does not bind or bundle F-actin. (g) The G-domain does not bundle F-actin. (h and i) Low-speed sedimentation of F-actin in the presence of SEPT9 domains. Coomassie-stained gels with the corresponding bar graphs show that F-actin is recovered in the pellet in the presence of the NTD [(h), SEPT9-NTD] and the B-domain [(i), SEPT9-B]. Error bars correspond to the maximum and minimum values from three independent experiments. In the presence of the G-domain [(h), SEPT9-G] and the A-domain [(i), SEPT9-A], most of the actin is recovered in the supernatant.
Figure 6.2. The NTD and B-domain of SEPT9 bind to three sites on the surface of F-actin. (a and b) 3D reconstructions show the three modes of binding of the NTD (a) and B-domain (b) to F-actin. Three sites on the surface of the actin filament are shown: side of actins SD1/SD2 (magenta arrows), front portion of the same subdomains (red arrows) and the top of SD4 (cyan arrows). Actin molecules are shown as blue ribbons while non-filled empty envelopes represent additional mass on the surface of the actin filament that corresponds to the sites of binding of SEPT9 constructs to F-actin. (c) Cross-correlation sorting of segments of actin filaments decorated with SEPT9 domains shows that the NTD and B-domain bind to the three sites on F-actin with similar frequency, while the A-domain does not bind to F-actin. (d) Actin residues that are likely to be involved in the interaction with SEPT9 are marked on the actin monomer (yellow ribbons) for mode 1 in magenta, mode 2 in red and mode 3 in cyan.
Figure 6.3. **SEPT9 inhibits the activity and interaction of myosin with actin.** (a–c) An atomic model of the actomyosin complex in rigor state [14] was docked onto the 3D reconstructions of the three modes of binding of the SEPT9 NTD to F-actin (Fig. 2a) using the best fit of the actin molecules. Actin molecules are shown as blue ribbons and myosin head is depicted as solid surface. The clashes between the myosin head and the NTD density (red arrows) in modes 1 and 2 are marked with red arrows (a and b). There are no such clashes in mode 3 (c). The position of the N-terminus of F-actin that resides at the interface between the NTD and F-actin is denoted with red asterisks. (d) Plot shows the ATPase activity of the S1 fragment of myosin V in the absence and presence of full-length SEPT9 and the A- and B-domains of SEPT9. As predicted by the structural data, full-length SEPT9 and the B-domain inhibit the ATPase activity of myosin, while the A-domain does not have a significant effect. (e) Coomassie-stained gels show the pellet (P; top) and supernatant (S; bottom) fractions from a competitive high-speed actin pelleting assay in the presence of ATP (1.25 μM). F-Actin (2 μM) was incubated with increasing concentrations of recombinant full-length SEPT9 and the myosin S1 fragment was added at a constant concentration (0.75 μM). (f) Bar graphs show the fraction of total S1 fragment in the pellet and supernatant. Error bars correspond to the minimum and maximum values obtained from three independent experiments. (g) Coomassie-stained gels show the pellet (P; top) and supernatant (S; bottom) fractions from a competitive high-speed actin pelleting assay in the absence of ATP. F-Actin (2 μM) was incubated with increasing concentrations of recombinant full-length SEPT9 and the myosin S1 fragment was added at a constant concentration (1 μM). (f) Bar graphs show the fraction of total S1 fragment in the pellet and supernatant. Error bars correspond to the minimum and maximum values obtained from three independent experiments.
Figure 6.4. **SEPT9 reduces the extent of cofilin-driven F-actin depolymerization.** (a) Atomic model of actin–cofilin complex [15] docked into the 3D reconstructions of modes 1 and 2 (transparent gray surfaces) shows a clash between the cofilin molecule (red surface) and SEPT9 density (marked with black arrows). (b) Long F-actin filaments formed after 2 h of polymerization (b; 0 min) are disrupted into short segments within 2 min of incubation with cofilin-1 (b; 2 min); after a 30-min incubation with cofilin (b; 30 min), only few filaments that are heavily decorated with cofilin remain. (c) F-actin forms tight bundles in the presence of SEPT9 (c; 0 min). After 2 min of incubation with cofilin-1, SEPT9-induced F-actin bundles still remain intact (c; 2 min), and even after 30 min of incubation, F-actin bundles still persist (c; 30 min). (d–f) High-speed sedimentation assay of F-actin in the presence of SEPT9 domains and cofilin-1. Actual gels with the corresponding bar graphs (d–f) show that, in the presence of cofilin-1, most of the actin is found in the supernatant (d), while in the presence of full-size SEPT9 (d), NTD (e) or the B-domain (f), more than half of the actin is recovered in the pellet. In the presence of the A-domain, most of the actin remains in the supernatant (f).
Figure 6.5. Overall 3D reconstruction of F-actin decorated with the NTD suggest the sites of SEPT9 binding to the actin filament. Segments of actin filaments decorated with the NTD of SEPT9 were reconstructed using IHRSR approach starting from a solid cylinder. The resultant reconstruction possessed three additional densities on the surface of F-actin. The first one is at the front interface between SD1 and SD2 of the two adjacent actin protomers (red arrow). The second density is at the side of SD1 (magenta arrow), while the third one is on the top of SD4 (cyan arrow)
Figure 6.6. Convergence of structural classes validates model-based crosscorrelation sorting. Every structural class was independently reconstructed using IHRSR algorithm starting from either a solid featureless cylinder (purple) or 3D reconstruction obtained from another structural class. 3D reconstructions of mode 1, mode 2 and mode 3 are shown in yellow, cyan and green, respectively. Each class converged to the same structural solution regardless of the starting model.
Figure 6.7. Reference free 2D averages of occupied classes. SPIDER1 AP SR routine was used to generate reference free 2D averages of the three structural modes recovered in the NTDdecorated actin filaments. Red arrows indicate visible differences in the SEPT9-NTD positions.
Figure 6.8. **Comparison of the frequencies of structural classes upon cross-correlation sorting of F-actin segments in the presence or absence of the A-domain of SEPT9.** We performed cross-correlation sorting of pure F-actin segments and F-actin segments in the presence of the A-domain using the same set of references that was employed in the sorting of the NTD-decorated F-actin segments. Comparison of the classes’ frequencies does not reveal any difference between the two data sets and suggests that the small number of F-actin segments assigned to the occupied classes in the presence of A-domain is not due to the decoration by the A-domain, but rather reflects the margin of error in the sorting procedure.
Figure 6.9. Full length SEPT9 and SEPT9 B-domain do not significantly affect F-actin bundling over concentration ranges that inhibit myosin activity. (a - b) Coomassie-stained SDS-PAGE gel shows the supernatant (S) and pellet (P) fractions from a low-speed actin sedimentation assays in the absence and presence of recombinant full length SEPT9 (a) or B-domain (b). (c - d) Bar graph shows the percentage of F-actin in the supernatant and pellet fractions in the absence and presence of SEPT9 (c) or B-domain (d). Error bars indicate the lowest and highest values from three independent experiments. Note that the percentage of pelleted actin is similar between 1 and 3 µM of full length SEPT9, while this concentration increase results in a 2-3-fold decrease in myosin ATPase activity (Fig. 3d). The percentage of pelleted actin is also similar in the range of 3 – 12 µM of B-domain, which causes >5-fold decrease in myosin ATPase activity (Fig. 3d).
Figure 6.10. **Cosedimentation assay of full length SEPT9 with F-actin.** (a) Coomassie-stained gels show the pellet and supernatant fractions (equal volumes) from a representative high-speed sedimentation assay. SEPT9 (1 µM) was incubated with increasing concentrations of F-actin for 30 min at room temperature and was centrifuged at 200,000 g. (b) Binding curve shows the fraction of F-actin-bound SEPT9 as a function of increasing concentrations of F-actin. Data were fitted in GraphPad with one site specific binding curve and an estimated K_d of 2.23 ± 0.60 µM was derived based on three independent experiments.
Figure 6.11. Resolution determination. Each structural class was split in two non-overlapping sets and the IHRSR procedure was used on each set. The correlation between the corresponding Fourier shells of the two resultant volumes was calculated for each data set.
CHAPTER 7. Septins promote macropinosome maturation and traffic to the lysosome by facilitating membrane fusion

Abstract
Macropinocytosis, the internalization of extracellular fluid and material by plasma membrane ruffles, is critical for antigen presentation, cell metabolism and signaling. Macropinosomes mature through homotypic and heterotypic fusion with endosomes, and ultimately merge with lysosomes. The molecular underpinnings of this clathrin-independent endocytic pathway are largely unknown. Here, we show that the filamentous septin GTPases associate preferentially with maturing macropinosomes in a phosphatidylinositol 3,5-bisphosphate-dependent manner, and localize to their contact/fusion sites with macropinosomes/endosomes. Septin knock-down results in large clusters of hemifused macropinosomes, which persist longer and exhibit less fusion events. Septin depletion and over-expression down-regulate and enhance, respectively, the delivery of fluid-phase cargo to lysosomes, without affecting Rab5 and Rab7 recruitment to macropinosomes/endosomes. In vitro reconstitution assays show that fusion of macropinosomes/endosomes is abrogated by septin immunodepletion and function-blocking antibodies, and is induced by recombinant septins in the absence of cytosol and polymerized actin. Thus, septins regulate fluid phase cargo traffic to lysosomes by promoting macropinosome maturation and fusion with endosomes/lysosomes.

Introduction
Macropinocytosis is a clathrin-independent pathway of endocytosis that originates from the closure of plasma membrane ruffles into large vesicles, which
internalize extracellular fluid and particles (Kerr and Teasdale, 2009; Swanson, 2008). Macropinocytosis is upregulated by growth factors and oncogenes, which stimulate the uptake of nutrients for cell proliferation and growth (White, 2013). Many bacterial and viral pathogens invade cells by hijacking macropinocytosis, which is critical for the antigen presentation (de Carvalho et al., 2015; Lim and Gleeson, 2011; Mercer and Helenius, 2012). By modulating the internalization of plasma membrane receptors and adhesion molecules, macropinocytosis is also implicated in cell signaling, adhesion and motility (Bryant et al., 2007; Gu et al., 2011; Schmees et al., 2012).

Macropinosomes traffic to lysosomes while undergoing a process of maturation, which is characterized by size reduction concomitant with membrane tubulation and fusion with other macropinosomes and early/late endosomes (Bright et al., 2005; Racoosin and Swanson, 1993). Macropinosome maturation is accompanied by changes in phosphoinositide and Rab GTPase content (Levin et al., 2015). Nascent macropinosomes become enriched with phosphatidylinositol 3-phosphate (PI3P) and Rab5 (Feliciano et al., 2011; Yoshida et al., 2009), while maturing macropinosomes contain phosphatidylinositol 3,5-biphosphate (PI(3,5)P2) and Rab7 (Kerr et al., 2006; Kerr et al., 2010). Despite our knowledge of these transitions, the molecular basis and regulation of the membrane fusion events that underlie macropinosome maturation and merging with the lysosome remain unknown.

Septins are filamentous heteromeric GTPases that associate with cell membranes and the cytoskeleton (Fung et al., 2014; Mostowy and Cossart, 2012). Plasma membrane septins are essential for the maintenance of diffusion barriers and modulate exocytosis (Bridges and Gladfelter, 2015; Caudron and Barral, 2009). Much less is known about how septins function in the endomembrane system of organelles and their communication. Septin mRNAs and proteins associate with endosomes
Baumann et al., 2014; Baust et al., 2008) and are implicated in multivesicular body formation (Traikov et al., 2014). However, how septins function in endocytic membrane traffic is not known.

Materials and methods

Plasmids

Mouse SEPT2 was inserted into N1-YFP (Spiliotis et al., 2005). Mouse SEPT2 was inserted into N1-GFP (Bowen et al., 2011). PM-mCherry was constructed by inserting the membrane-binding domain of lyn kinase in pcDNA 3.1-mCherry (Benjamin et al., 2010). Rab5A was inserted into C1-GFP using the HindIII and Sall restriction sites, and Rab7A was inserted into C1-GFP using the Xho1 and Kpn1 restriction sites. Rab5B-mCherry and Rab7A-mCherry were purchased from addgene. Rat SEPT7v2 and human SEPT6v3 were inserted into the pET-28a(+) vector, which expresses in tandem His-SEPT7 and an untagged SEPT6 (Bai et al., 2013). Mouse SEPT2 was inserted into pET-15b and expresses an untagged SEPT2 (Bai et al., 2013).

For experiments using shRNAs, the non-targeting control (5′-TGAGTTTCACTAITGTTGTCG-3′) was inserted in pG-SUPER-GFP (Bowen et al., 2011) and pSUPER-mCherry (Dolat et al., 2014b). To target canine SEPT2, the shRNA #1 (5′-CCTTAGACGCATTGTCGATTGTC-3′) was inserted in pSUPER-mCherry (Dolat et al., 2014b) and SEPT2 shRNA #2 (5′-GCAACTACAAGGCTCCGGAAGAATAA-3′) was inserted in pG-SUPER-GFP. Human SEPT2 was targeted using the SEPT2 shRNA 5′-AAGGGAATATTTGTGCGCTGTC-3′, which was inserted in pG-SUPER-GFP (Bowen et al., 2011). For siRNA experiments (Fig. 5), the SEPT2 siRNA oligonucleotides (5′AAGGUGAAUAUUGUGCCUGUC-3′) and mock GFP siRNA oligonucleotides (5′-
GGCUACGUCCAGGAGCGCACC-3’) were purchased from Dharmacon Research (LaFayette, CO).

Tissue culture and transfections

MDCK II/G cells and the stable cell lines MDCK-SEPT2-YFP (CMV promoter; N1-YFP) (Spiliotis et al., 2005) and MDCK-PM-mCherry (CMV promoter; pcDNA3.1) were cultured in low glucose DME with 1 g/L NaHCO₃, 10% FBS (Cell Generation), and 1% PSK (penicillin, streptomycin and kanamycin) at 37 ºC with 5% CO₂. HT1080 cells (CCL-121) were purchased from ATCC and cultured in high glucose DME with 1 g/L NaHCO₃, 10% FBS (Cell Generation), and 1% PSK (penicillin, streptomycin and kanamycin) at 37 ºC with 5% CO₂. Cells were plated on No. 1.5 glass coverslips (VWR), which were pre-coated with 10 µg/mL type I collagen (PurCol; BioMatrix) in 0.1% acetic acid for five minutes, and subsequently were dried and rinsed twice with media before cell plating. Cells were transfected with 1.5 µg plasmid or co-transfected with 1.0 µg of one plasmid and 1.5 µg of the second plasmid using lipofectamine 2000 (Invitrogen).

Dextran uptake and pulse-chase assays

Cells were cultured in low-serum media (0.5% FBS) overnight and switched to media with 10% FBS to stimulate macropinocytosis for the duration of incubations and pulse-chase treatments with fluorescent dextrans. Pulse-chase experiments were performed by incubating cells with media containing 10% FBS and 0.2 mg/mL FITC- or TR-dextran (10,000 MW, ThermoFisher Scientific) for 5 min and then washing three times and incubating cells in dextran-free media for the indicated times. In Fig. 2C, cells were incubated with 800 nM YM201636 (SelleckChem, Inc.) or vehicle control (DMSO)
for 2 h in low-serum media and then stimulated with media containing 10% FBS and 0.2 mg/mL TR-dextran with YM201636 or DMSO for 10 min.

**Immunofluorescence microscopy**

Cells were fixed with warm (37 °C) 2% paraformaldehyde (PFA) in PBS (phosphate-buffered saline) and simultaneously permeabilized and blocked with 2% BSA (bovine serum albumin; Sigma) and 0.1% saponin (Calbiochem) for 30 min. In Fig. 1A, cells were extracted prior to fixation by incubating for 30 s with warm (37 °C) cytoskeleton buffer (CB) containing 10 mM MES (pH 6.1), 138 mM KCl, 3 mM MgCl2, 2 mM EGTA, 320 mM sucrose and 0.1% Triton X-100. Cells were subsequently rinsed twice with CB and fixed with 2% PFA in CB.

Rabbit antibodies against SEPT2 (N5N; a gift from M. Kinoshita, Nagoya University, Nagoya, Japan) and Rab5 (Stressgen), and mouse antibodies against Rab7A (Abcam), LAMP1 (H4A3, Developmental Studies Hybridoma Bank; University of Iowa), Sec6 (Exoc3; 10C2), Sec8 (Exoc4; 9H5), Exo70 (Exoc7; 13F3), gifts from C. Yeaman (University of Iowa), were diluted in PBS containing 2% BSA and 0.1% saponin. Secondary donkey Alexa Fluor 488-, AlexaFluor 594- or Alexa Fluor 647-conjugated F(ab’) to mouse or rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) were diluted in PBS containing 2% BSA and 0.1% saponin. Cells were stained for F-actin using phalloidin conjugated to Alex Fluor 555 (ActiStain; Cytoskeleton, Inc.). Coverslips were mounted on coverslides using Vectashield (Vector Laboratories).

Slides were imaged using a confocal laser-scanning microscope (FluoView 1000; Olympus) equipped with three Hamamatsu R7862 photomultiplier tubes (PTMs) and multi-line Argon ion (458 nm, 488 nm, 515 nm), Helium-Neon (543 nm) and diode 401 nm and 635 nm lasers. Images were acquired using 60x/1.42 NA oil objective and the
FV10-ASW 02.01 software. 3D rendering of confocal stacks was performed in the Volocity software (Perkin Elmer).

**Time-lapse imaging**

All time-lapse microscopy experiments were performed at 37°C using DME without Phenol red supplemented with 10% FBS, 1% PSK and 20 mM Hepes. In Fig. 1 and 3A, PM-mCherry dynamics were imaged with 3D deconvolution wide-field microscopy using an inverted microscope (DeltaVision OMX v4; GE Healthcare) equipped with a motorized stage, stage-top incubator with temperature controller, and three pco.edge sCMOS cameras (custom version). 3D stacks (1 – 2 µm; 0.125 nm slices) were acquired every 10-20s using a 60x/1.49 NA objective lens (Olympus) and were deconvolved in the softWoRx 6.1.1 software. In Fig. 3B-D and 3H, macropinosome dynamics were imaged using an inverted microscope (IX81; Olympus) equipped with a motorized stage (ProScanII; Prior), an OrcaR2 CCD camera (Hamamatsu Photonics), a custom-built stage-top chamber and temperature controller (Air-Therm ATX; World Precision Instruments) and the Slidebook 6.0 software. Fluorescent images were acquired every 5 s for 8 min with a Plan-Apochromat 60x/1.40 NA objective. Time-lapse videos were rendered in Photoshop (Adobe).

**Protein expression and purification**

Recombinant SEPT2/6/7 was expressed by co-transforming *Escherichia coli* BL21(DE3) cells with a kanamycin-resistant, bicistronic pET-28a(+) that expresses His-SEPT7 and SEPT6, and an ampicillin-resistant pET-15b(+) plasmid that expresses an untagged SEPT2. Bacterial cultures were grown to 0.6 OD and induced with 0.5 mM IPTG for 16 hours at 18 °C. Cultures were centrifuged (4,000 g) for 10 min at 4 °C, lysed
in buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM PMSF, 1 mM DTT, 10 mM imidazole, and 10% glycerol using a French pressure cell at 1,280 psi. Cell lysates were centrifuged (14,000 g) for 30 min at 4 °C and passed through a 0.45 µm filter before applying to a gravity flow column with Ni-NTA beads. The columns were washed with buffer containing 50 mM Tris pH 8.0, 300 mM NaCl, 1 mM DTT, 20 mM imidazole, and 10% glycerol, and SEPT2/6/7 complexes were eluted using buffer containing 50 mM Tris pH 8.0, 150 mM NaCl, 10% glycerol, and 250 mM imidazole. Protein preps were dialyzed overnight in dialysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM DTT, and 10% glycerol).

**Liposome flotation assays**

All lipids were purchased from Avanti Polar Lipids, except PI(4,5)P2 diC8 (Echelon Biosciences; p-4508). L-α-phosphatidylcholine (840051) and L-α-phosphatidylinositol (840042) were diluted in chloroform. PI(3,4,5)P3 (850156), PI(3,5)P2 (850154), PI(3)P (850150), PI(4)P (850151), and PI(5)P (850152) were diluted in a solution made of chloroform, methanol and water (20:9:1). PI(4,5)P2 was diluted in water. To generate small unilamellar vesicles, lipids were mixed to a final concentration of 75 %mol L-α-phosphatidylcholine, 20 %mol L-α-phosphatidylinositol, and 5 %mol of each phosphoinositide. Lipid mixtures were dried completely under a nitrogen stream, rehydrated in liposome buffer (50 mM Hepes pH 7.2, 50 mM KCl) for 30 min at 25 °C, freeze/thawed five times, and passed through a mini-extruder using 0.1 µm pore filter at least 12 times to isolate small unilamellar vesicles (SUVs). SUVs were stored at 4°C and used within 1-2 days.

To test for septin binding, 0.5 mM SUVs were mixed with 0.5 µM recombinant SEPT2/6/7 in 50 µl liposome buffer containing 1 mM MgCl₂ and incubated with rotation
at 25 °C for 1 h. Reactions were mixed with 200 µl liposome buffer with sucrose (75%) and placed at the bottom of a polycarbonate tube (Beckman Coulter). Liposome buffer (150 µl) with 25% sucrose was gently overlaid and on top of this layer, 50 µL of liposome buffer was gently placed. Samples were centrifuged (220,000 g) in a SW60 Ti rotor (Beckman Coulter) for 1 h at 20 °C. Equal volumes of the top and bottom fractions were collected with a thin pipette tip, run on a 10% SDS-PAGE gel and stained with Coomassie Brilliant Blue. Gels were scanned using the Odyssey imaging system (LI-COR Biosciences).

**In vitro fusion assay**

Reconstitution of endosome fusion was performed in a cell-free fluorescence-based assay as previously described (Barysch et al., 2010). MDCK cells were cultured on 15 cm plates to 80% confluence, trypsinized and centrifuged (300 g) for 5 min. Cells were resuspended in cold PBS containing 5 mg/mL BSA (PBS-BSA) and centrifuged (250 g) for 5 min at 4°C. The cell pellet was resuspended in cold internalization buffer (0.2 mg/mL glucose in OPTI-MEM) and centrifuged (250 g) for 5 min at 4°C. The supernatant was discarded and the pellets were incubated at 37°C for 5 min before resuspending in warm internalization buffer with either FITC- or TR-dextran (0.5 mg/ml) and incubating for an additional 5 min at 37 °C. Cells were transferred to ice and washed three times with cold PBS-BSA and once with homogenization buffer (250 mM sucrose, 3 mM imidazole pH 7.4) while centrifuging (250 g) for 5 min at 4 °C in between washes. The cell pellet was resuspended in 1 ml homogenization buffer containing protease inhibitors (Calbiochem), passed through a ball-bearing homogenizer 20 times, and centrifuged (1,200 g) for 15 min at 4 °C. The post-nuclear supernatant (PNS) was recovered and protein concentration was determined using a Bradford assay (Thermo
Small aliquots of the post-nuclear supernatants were flash frozen in liquid nitrogen and stored in -80 °C. Canine kidney cytosol (gift from Dr. James Nelson) was isolated as previously described (Davis et al., 1974). Canine kidney cortex was washed in cold PBS, cut into 1 cm cubes and homogenized using a Waring blender in a PBS containing 250 mM sucrose. The homogenate was filtered, centrifuged (100,000 g) to remove cell membranes, and aliquots of the supernatant were drop flash frozen in liquid nitrogen and stored at -80°C.

Fusion reactions were assembled by mixing 5 µg canine kidney cytosol with 0.1 µg of post-nuclear extracts with FITC- and TR-dextran-labeled macropinosomes/endosomes in homogenization buffer (50 µl) with protease inhibitors and with or without 3.3 mM ATP. All reactions were incubated at 37 °C for 45 min. To inhibit SEPT2 function, the cytosol was pre-incubated in homogenization buffer containing 0.5 mg/ml rabbit anti-SEPT2 (N5N) or rabbit IgG (Sigma) for 1 h at 4 °C. SEPT2 was immunodepleted from the cytosol as previously described (Pagano et al., 2004). Cytosol was diluted to 1 mg/ml in buffer containing 20 mM Hepes pH 7.2, 90 mM potassium acetate and 2 mM magnesium acetate with protease inhibitors and subsequently incubated with 5 µg rabbit anti-SEPT2 or rabbit IgG overnight with rotation at 4 °C. Protein A beads (Thermo Scientific) were blocked with 2% BSA overnight, washed five times with homogenization buffer, and added to the cytosol for 3 hours at 4°C. Samples were centrifuged (1,000 rpm) for 5 minutes at 4°C and the supernatant was collected. SEPT2 protein levels in the cytosol samples were assessed by western blots.

To assess fusion microscopically, reactions (30 µl) were floated into chambers created by immobilizing a glass coverslip on a slide with two strips of a double-side tape; prior to mounting, coverslips were cleaned with 70% ethanol and flame-dried. Chambers
were washed with homogenization buffer prior to adding each reaction mix and sealing with vacuum grease. Slides were centrifuged (300 g) upside-down for 10 min at 25 °C using a swing-bucket rotor with microtiter adapters. Samples were imaged using an inverted Zeiss AxioObserver Z1 wide-field microscope equipped with a Hamamatsu Orca-R2 digital CCD camera, X-cite 120 metal halide lamp and a motorized z-drive. Images were acquired using a 63X/1.4 NA objective and the Slidebook 6.0 software (Intelligent Imaging Innovations).

**Western blots**

Cells were lysed in buffer containing 50 mM Hepes, pH 7.4, 100 mM NaCl, 0.5% Triton X-100, 1 mM MgCl2, 1 mM PMSF and protease inhibitors. Samples were centrifuged at 8,000 rpm for 10 min at 4°C and the supernatants were collected. Equal volumes were resolved with a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane at 100V for 1 h at 4°C. Membranes were blocked with 5% dried milk (w/v) in PBS containing 0.1% Tween-20. A rabbit antibody against SEPT2 (Sigma; 1:5000) and a secondary anti-rabbit antibody (LiCor) conjugated to infrared dye was diluted in PBS containing 2% BSA, 0.1% Tween-20, and 0.025% sodium azide, and sequentially incubated on the membrane before scanning with the Odyssey imaging system (LI-COR).

In Fig. 5, equal volumes of canine kidney cytosol samples were resolved on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. Membranes were blocked in 5% dried milk (w/v) in PBS containing 0.1% Tween-20. Rabbit antibody against SEPT2 (Sigma; 1:5000), mouse anti-actin (Sigma, 1:5000), and anti-rabbit or – mouse secondary antibodies (LiCor) conjugated to infrared dyes were diluted in PBS containing 2% BSA, 0.1% Tween-20, and 0.025% sodium azide, and sequentially
incubated on each membrane before scanning with the Odyssey imaging system (LI-COR).

**Image analysis and quantifications**

Image analysis was performed with the Slidebook 6.0 software (Intelligent Imaging Innovations) unless otherwise noted. In Fig. 1B, the peripheral lamellar regions of cells were masked by segmentation of F-actin fluorescence and within these regions, the sum of SEPT2 fluorescence intensity per \( \mu m^2 \) was quantified in each cell. In Fig. 2, the percent of dextran particles with SEPT2 was calculated by creating dextran and SEPT2 masks using fluorescence intensity segmentations. SEPT2 and dextran masks were subtracted to create a new artificial channel with masks that corresponds to all dextran fluorescence that overlaps with SEPT2. The particles of this overlay channel were automatically counted and their number was divided by the total number of dextran particles to derive the percentage of dextran that colocalizes with SEPT2. The same approach was used to derive the percentage of Rab5- and Rab7-mCherry compartments with SEPT2 in peripheral lamellae (Fig. S1) and the percentage of dextran particles with Rab5, Rab7 and LAMP1 (Fig. 3 and 4); LAMP1 background fluorescence was subtracted by Gaussian smoothing. In Fig. 2B-D, time-lapse movies of PM-mCherry of identical capture intervals (5 s) and duration (8 min) were analyzed after applying the 2-D Laplacian of Gaussian function, which improves the clarity of membrane vacuoles and tubules. The formation of nascent macropinosomes from membrane vesicles was tracked manually and clusters of three or more macropinosomes per cell were scored (Fig. 3B). Macropinosome lifetime was quantified by measuring the time that it took for a nascent macropinosome to disappear or merge with another membrane vacuole or tubule (Fig. 3C). The number of nascent macropinosomes that fused with another membrane compartment was calculated as percentage of total macropinosomes
tracked. Number of fusion events per min was the inverse of the time that it took for a nascent macropinosome to undergo a fusion event (Fig. 3D). In Fig. 3E, 2 µm-long line masks were drawn along the hemifused membrane contact of two macropinocytic vacuoles and along their non-contacting perimeters. The intensity profiles of each line scan was exported and plotted in Excel. In Fig. 3F-G and S2, the number and size of dextran particles were quantified in Image J (NIH) by generating masks of dextran fluorescence intensity, which were gated by size (0.2 – 20 µm²) as previously done by others (Wang et al., 2014). The rates of lysosomal delivery of dextran corresponded to the slope of the lines derived from a least squares linear regression fit to the 5, 10 and 30 min points of the plot in Fig. 4C. In vitro fusion (Fig. 5) was quantified by generating fluorescence segmentation masks that correspond to FITC- and TR-dextran-containing macropinosomes/endosomes. The masks were subsequently subtracted from each other to identify macropinosomes/endosomes with overlapping fluorescence. The number of these new masks was automatically calculated and divided by the total number of macropinosomes/endosomes, which was calculated by subtracting the number of masks with FITC/TR overlap from the total number of FITC- and TR-labeled masks.

**Statistical analyses**

All datasets were analyzed using the Shapiro and Levene tests, respectively, for normality and equal variance in the R software, and an unpaired student’s *t* test used to derive *p* values (95% confidence intervals) in the Excel software. All values represent the mean ± SEM.
Results and Discussion

Septins associate preferentially with maturing macropinosomes in a PI(3,5)P2-dependent manner

We previously identified a network of septins fibers in the leading lamellae of migrating MDCK epithelia (Dolat et al., 2014b). This network is interwoven with actin stress fibers, but it is reduced upon mild detergent extraction, indicating that a subset of septins is membrane-bound (Fig. 1, A and B). Given that membrane ruffling activity is high in cellular lamellae, we imaged the localization of septins in MDCK cells that stably express PM-mCherry (Corbett-Nelson et al., 2006), a plasmalemmal marker that marks membrane ruffles, macropinocytic vacuoles and vesicular-tubular endosomes (Fig. S1, A and B). We stained for endogenous SEPT2 as a proxy for the lamellar network of septin fibers, which consist of SEPT2/6/7/9 (Dolat et al., 2014b). SEPT2 was largely absent from membrane ruffles, but localized at the contact sites and membranes of PM-mCherry-labeled vacuoles (Fig. 1, C and D). Consistent with septin association with macropinosomes, SEPT2 localized to the periphery and in between compartments that contained PM-mCherry and dextran, a fluid phase cargo marker that enters cells by macropinocytosis (Fig. 1E). Time-lapse microscopy of PM-mCherry and SEPT2-GFP showed that SEPT2 accumulates at the fusion sites of macropinosomes (Fig. 1F, Video 1); SEPT2 was present on 87% ± 12% of macropinosome contact sites (n = 11 cells).

To examine whether SEPT2 associates with early or late stage macropinosomes, we performed a pulse-chase experiment with fluorescent dextran. SEPT2 co-localization with dextran peaked after 5-10 min of internalization and diminished after 20-30 min (Fig. 2A), when dextran accumulates in the lysosome (see Fig. 4C). Given that early macropinosomes contain Rab5 while maturing macropinosomes become enriched with Rab7 before fusing with lysosomes (Feliciano et al., 2011; Kerr et al., 2006; Kerr et al.,
we analyzed SEPT2 localization in cells that expressed Rab5- and Rab7-mCherry (Fig. S1, C-E). In peripheral lamellae, SEPT2 was present on 64% ± 4% of Rab7-positive compartments compared to 21% ± 2% of Rab5 (Fig. S1E). SEPT2 was also present on 72 ± 4% (n = 15 cells) of peripheral vacuoles labeled with the PI(3,5)P2-binding ML1Nxx2-GFP probe (Li et al., 2013), indicating that septins associate with mature PI(3,5)P2-positive macropinosomes (Fig. 2B). Treatment of MDCK cells with YM201636, an inhibitor of the FYVE finger containing phosphoinositide kinase (PIKfyve) that converts PI(3)P to PI(3,5)P2 (Jefferies et al., 2008), reduced the intensity of the lamellar septin network and SEPT2 localization on dextran-containing macropinosomes (Fig. 2, C-E). Liposome floatation assays showed that SEPT2/6/7 associates preferentially with PI(3,5)P2-containing membranes (Fig. 2, F and G). In contrast to previous results with giant unilamellar vesicles (GUVs) (Tanaka-Takiguchi et al., 2009), SEPT2/6/7 did not exhibit significant binding to PI(4,5)P2. Given that septin-lipid binding is sensitive to membrane curvature (Bridges et al., 2016), this discrepancy may arise from differences in the diameter of liposomes, which is 50-to-200-fold smaller than GUVs. Our results nevertheless indicate that septins associate preferentially with mature Rab7- and PI(3,5)P2-positive macropinosomes.

Septin depletion impedes the maturation and turnover of macropinosomes, and reduces macropinosome fusion events

We sought to determine how septins affect macropinosome formation and dynamics by imaging PM-mCherry in live MDCK cells, which were treated with control or SEPT2 shRNAs that deplete SEPT2, but also SEPT6, 7 and 9 (Dolat et al., 2014b). In control cells, membrane ruffles gave rise to multiple macropinosomes, which fused into single vacuoles or dissipated within seconds of formation (Fig. 3A, Video 3). In SEPT2-
depleted cells, membrane ruffling and macropinosome formation were not affected. Strikingly, however, SEPT2 knock-down resulted in clusters of numerous macropinosomes (Fig. 3A, Video 3). Clusters of three or more macropinosomes were present in four-fold more cells upon SEPT2 depletion and the average lifetime of nascent macropinosomes increased by 1.7-fold (Fig. 3, B and C). Notably, several macropinosomes appeared docked with their membranes adjoining laterally (Fig. 3E).

In agreement with a slower macropinosome turnover, dextran uptake assays showed a significant increase in the number and size of dextran particles in SEPT2-depleted cells (Fig. 3, F and G). Notably, this increase in number and size persisted after 30 and 60 min of dextran internalization, indicating a defect in the macropinocytic traffic of dextran (Fig. 3, F and G). Similar results were obtained with a different SEPT2 shRNA (Fig. S2, A and B) and in human fibrosarcoma HT1080 cells (Fig. S2, C and D), which have intense membrane ruffling activity due to the expression of an oncogenic mutant of N-ras (Paterson et al., 1987). Expression of an shRNA-resistant SEPT2-YFP rescued the phenotype, reversing the number and size of macropinosomes to control levels (Fig. S2B).

Because membrane tubulation and fusion events underlie the size reduction of maturing macropinosomes, we analyzed macropinosome membrane dynamics in MDCK-PM-mCherry cells. Septin depletion did not alter the percentage (~40%) of macropinosomes that formed membrane tubules and had no effect on their detachment (Fig. 3H, Video 4). Membrane fusion, however, was severely affected. The percentage of nascent macropinosomes that fused with another macropinosome was reduced from 74% to 25% (n = 9 cells). Moreover, the frequency of fusion events between nascent macropinosomes and tubulo-vesicular endosomes decreased markedly (Fig. 3D). Thus,
Septins are required for the membrane fusion events underlying the shrinkage and turnover of macropinosomes.

**Septins regulate fluid phase cargo traffic and delivery to the lysosome**

Given that macropinosomes transport fluid phase cargo from the plasma membrane to the lysosome, we sought to determine whether septins affect the endocytic trafficking and lysosomal delivery of extracellular dextran. Using a pulse-chase approach, MDCK cells were pulse-labeled with fluorescent dextran and its localization with respect to Rab5, Rab7 and the lysosomal associated membrane protein 1 (LAMP1) was analyzed after 5, 10 and 30 min, respectively. Septin depletion did not affect dextran colocalization with Rab5 and Rab7 (Fig. S2, E-H). However, the percentage of dextran particles that colocalized with LAMP1 decreased by >60% and this defect was rescued by shRNA-resistant SEPT2-YFP (Fig. 4, A and B). These data suggest that septins are required for the delivery of dextran to lysosomes, but do not affect dextran traffic to Rab5- and Rab7-positive endosomes or the recruitment of Rab5/Rab7 to dextran-containing macropinosomes.

To further test whether septins regulate the lysosomal delivery of fluid phase cargo, we analyzed kinetically the accumulation of dextran in lysosomes. Dextran colocalization with LAMP1 was quantified after 5, 15 and 30 min of internalization under conditions of septin depletion and over-expression. Between 5 and 30 min of internalization, SEPT2 depletion decreased the rate of lysosomal delivery from 2.34% to 1.02% of total dextran per min (Fig. 4C). Notably, the percentage of dextran colocalizing with LAMP1 in SEPT2-depleted cells did not change between 30 and 60 min of internalization (45 ± 6% vs. 50 ± 4%), which was indicative of a block in the traffic of fluid phase cargo.
Next, we tested the effects of SEPT2 over-expression using stable MDCK cell lines that express SEPT2-YFP at low and high levels (Fig. 4D). In the cells that express SEPT2-YFP at low levels, dextran delivery to the lysosome was similar to cells treated with control shRNAs (Fig. 4E). High levels of SEPT2-YFP expression, however, increased the efficiency of dextran delivery to the lysosome (Fig. 4E). Thus, lysosomal delivery of fluid phase cargo is dependent on the expression levels of SEPT2.

**Septins promote fusion of macropinosomes with endosomes/lysosomes.**

To test whether septins regulate lysosomal delivery of fluid phase cargo by directly modulating membrane fusion, we performed dual color dextran mixing assays in live cells and in vitro using a reconstitution assay of endosomal fusion (Barysch et al., 2010).

In MDCK cells, we tested how septin knock-down affects the delivery of FITC-dextran into a cohort of endosomes/lysosomes that were pre-loaded with TexasRed (TR)-dextran. After a 5 min pulse/15 min chase with TR-dextran, we performed 5 min pulse/15 min chase with FITC-dextran to assess its macropinocytic traffic into TR-dextran/LAMP1-positive lysosomes. Septin depletion decreased FITC-dextran entry into lysosomes with TR-dextran by 50% (Fig. 5, A and B).

Next, we performed a cell-free assay of macropinosome/endosome fusion using post-nuclear extracts from two MDCK cell populations, which were labeled separately with TR- and FITC-dextran; fusion was induced with canine kidney cytosol and ATP and assayed microscopically by quantifying the fraction of endosomes with both TR- and FITC-dextran (Fig. 5, C and D). Addition of a SEPT2 function-blocking antibody or immunodepletion of SEPT2 from the cytosol abrogated fusion (Fig. 5, C-F). Surprisingly, recombinant SEPT2/6/7 and ATP induced fusion in the absence of cytosol (Fig. 5G). Of
note, addition of recombinant SEPT2/6/7 to the cytosol did not increase fusion, possibly
due to saturation by cytosolic septins. Cytochalasin D, an actin depolymerizing
compound, had no effect on the endosome fusion induced by SEPT2/6/7 in the absence
of cytosol and did not alter fusion in the presence of cytosol (Fig. 5, D and G). Similarly,
fusion was not altered by the microtubule depolymerizing drug nocodazole. Thus,
septins can directly induce membrane fusion independently of F-actin and microtubules.

Taken together, our results show that septins promote the membrane fusion
events that underlie the maturation of macropinosomes and the delivery of fluid phase
cargo to lysosomes. This is the first evidence for a direct role of septins in clathrin-
independent endocytosis and the fusion of endomembranes. In the context of
exocytosis, septins interact with SNARE proteins and the exocyst complex (Beites et al.,
1999; Hsu et al., 1998; Ito et al., 2009; Taniguchi et al., 2007). The SNARE and vesicle
tethering complexes that mediate the fusion events of the macropinocytic pathway are
largely unknown. The exocyst Sec6/8 complex localizes to the endosomes of MDCKs
(Oztan et al., 2007), but septins did not colocalize with Sec6/8 or Exo70 at the contact
sites of macropinosomes/endosomes (Fig. S3, C-E). Septin knock-down also did not
affect the localization of Vps39 (Fig. S3, F and G), a component of the HOPS vesicle-
tethering complex (Balderhaar and Ungermann, 2013). Because septin depletion
increases dramatically the fraction of docked macropinosomes/endosomes (Fig. 2),
septins appear to play a distinct role in membrane fusion without affecting membrane
tethering and/or docking. Notably, this septin role is coupled to PI(3,5)P2, a low
abundance phosphoinositide that is specifically involved in the fusion of mature
macropinosomes/endosomes with lysosomes (Kerr et al., 2006; McCartney et al., 2014).
Thus, similar to the evolutionarily related Rab GTPases, septin GTPases function as
phosphoinositide effectors that catalyze SNARE-mediated fusion. Future studies will
determine whether this function involves the stabilization of specific SNARE complexes and the induction or sensing of membrane curvature, which promotes lipid mixing (Martens and McMahon, 2008).

Oncogenic proteins of the Ras family of GTPases are long known to up-regulate membrane ruffling and macropinocytosis, which in turn increases nutrient uptake to support the metabolic needs of growing cancers (Kimmelman, 2015; White, 2013). Moreover, lysosomal delivery of fluid phase cargo (e.g., amino acids) is critical for the activation of signaling pathways that regulate cell growth and division (Commissio et al., 2013; Settembre et al., 2013). Septins are over-expressed in many cancers, but their functions in cancer are poorly understood (Connolly et al., 2011a; Dolat et al., 2014a). Because SEPT2 alone does not induce fusion in vitro (Fig. 5G) and SEPT2-YFP over-expression does not alter the expression levels of SEPT6/7 (data not shown), we posit that over-expression of a single septin (e.g., SEPT2) may promote the formation of membrane-bound SEPT2/6/7 polymers, which could functionally support the elevated macropinocytic activity of cancer cells. Future studies may explore how this novel function of septins might be therapeutically targetable.
Figure 1 - Dolat et al
**Figure 7.1. Septins localize to macropinocytic vacuoles and tubulo-vesicular endosomes, and their contact/fusion sites.** (A) Confocal images of MDCKs stained for SEPT2 and F-actin with or without pre-extraction with 0.1% Triton-X100 prior to fixation. (B) Bar graph shows the sum intensity of SEPT2 per cell area (n = 15). (C-D) 3D-rendered confocal images of MDCK-PM-mCherry cells stained for endogenous SEPT2. Arrows point to SEPT2 at the contact sites between macropinocytic vacuoles (C) and tubulo-vesicular endosomes (D). (E) MDCK-PM-mCherry cells were incubated with FITC-dextran and stained for SEPT2. Images show SEPT2 at the periphery and in between (arrow) macropinosomes/endosomes. (F-G) MDCK-PM-mCherry cells were transfected with SEPT2-GFP and imaged live with wide-field deconvolution microscopy. Still frames show SEPT2 accumulation (arrows) at the site of fusion between two macropinosomes (F) and the contacting membranes of a macropinosome and a tubular endosome (G).
Figure 2 - Dolat et al.
Figure 7.2. Septins associate preferentially with maturing macropinosomes in a PI(3,5)P2-dependent manner. (A) MDCKs were pulsed with TR-dextran for 5 min, chased for the indicated times, stained for SEPT2 and imaged with confocal microscopy. Bar graph shows the percent of dextran-containing macropinosomes/endosomes with SEPT2 (n = 12 cells). (B) Recombinant SEPT2/6/7 was mixed with liposomes of the indicated phosphoinositides and centrifuged on a sucrose gradient. Coomassie-stained gels show equal volumes from the top (liposome-bound SEPT2/6/7; B) and bottom (unbound SEPT2/6/7; U) fractions. (C) Maximum intensity projections of confocal images of MDCKs, which were incubated with DMSO (control) or YM201636 (800 nM) for 2 h, and treated with TR-dextran for 10 min before fixing and staining for SEPT2. Insets show outlined areas in higher magnification. (D) Bar graph shows the fraction of TR-dextran puncta (>0.2 µm²) with SEPT2 (n = 20 cells).
A. Time-lapse images of cells with and without SEPT2 shRNA. Controls show normal cell behavior, while SEPT2 shRNA affects cellular structure and function.

B. Bar graph showing the percentage of cells with clusters after treatment with control or SEPT2 shRNA. SEPT2 shRNA decreases the percentage of cells with clusters.

C. Mean lifetime of clusters in control and SEPT2 shRNA conditions. SEPT2 shRNA significantly increases the mean lifetime of clusters.

D. Fusion events per minute for control and SEPT2 shRNA conditions. SEPT2 shRNA reduces fusion events.

E. Graph showing intensity (AU) vs. distance (μm) for different treatments. SEPT2 shRNA alters the intensity profile compared to control.

F. Bar graph showing the number of macropinosomes per cell after treatment with control or SEPT2 shRNA. SEPT2 shRNA significantly increases the number of macropinosomes.

G. Bar graph showing the macropinosome size (μm²) after treatment with control or SEPT2 shRNA. SEPT2 shRNA increases the size of macropinosomes.

H. Time-lapse images of macropinosome formation in control and SEPT2 shRNA conditions. SEPT2 shRNA affects the growth and distribution of macropinosomes.
Figure 7.3. Septin depletion decreases macropinocytic fusion events and hinders macropinosome maturation and turnover. (A) Time-lapse frames from widefield deconvolution microscopy show the dynamics of PM-mCherry-labeled macropinosomes in MDCK cells treated with control and SEPT2 shRNAs. (B) Control (n=41) and SEPT2-depleted MDCK-PM-mCherry cells (n=31) were imaged live every 5 s for 8 min. Bar graph shows percent cells with a cluster of three or more macropinosomes formed from a lamellar ruffle. (C - D) Bar graphs show the lifetime (C) and fusion events (D) of nascent macropinosomes (n = 24-27). (E) Quantification of PM-mCherry fluorescence along the lateral hemi-fused contact (1) and non-contacting segments (2, 3) of two macropinosomes from a SEPT2-depleted cell. (F-G) MDCKs were incubated with FITC-dextran for the indicated times. Bar graphs show the number of FITC-dextran-containing macropinosomes/endosomes (F) and their average size (G) per cell (n=18). (H) Still frames show the formation and detachment of PM-mCherry-labeled membrane tubules (arrows) in live MDCKs.
Figure 4 - Dolat et al
**Figure 7.4. Septins regulate the lysosomal delivery of fluid phase cargo.** (A) MDCKs were transfected with GFP-expressing control or SEPT2 shRNAs for 48 h. After a 5 min pulse/15 min chase with TR-dextran, cells were stained for LAMP1. Images show maximum intensity projections of confocal image stacks. (B) Bar graph shows percent of TR-dextran particles that colocalize with LAMP1 (n = 20 cells). (C) Plot shows percent of TR-dextran with LAMP1 in control and SEPT2-depleted MDCKs (n=15), which were pulsed with TR-dextran for 5 min and chased for the indicated times. (D) Lysates from MDCKs that stably express SEPT2-YFP at low and high levels were analyzed by SDS-PAGE and blotted for SEPT2. (E) Plot shows percent TR-dextran with LAMP1 in MDCK cells (n=14) that express SEPT2-YFP at low and high levels. Cells were pulsed with TR-dextran for 5 min and chased for the indicated times.
Figure 5 - Dolat et al
**Figure 7.5.** Septins are required for fusion of macropinosomes/endosomes with lysosomes, and promote fusion directly in an in vitro reconstitution assay. (A) MDCKs were treated with control or SEPT2 siRNA, and two rounds of 5 min pulse/15 min chase were performed with TR- and FITC-dextran, successively. Cells were stained with anti-LAMP1 and imaged with confocal microscopy. Images show maximum intensity projections of TR-dextran, LAMP1 and their areas of overlap (pseudo-colored in red) as well as FITC-dextran (green), and its overlay with the pseudo TR-dextran/LAMP1 channel. (B) Bar graph shows percent TR-dextran/LAMP1 that contains FITC-dextran (n = 18 cells). (C-D) MDCKs were separately incubated with TR- or FITC-labeled dextran for 5 min, and post-nuclear supernatants (PNS) were mixed with canine kidney cytosol with/without ATP, and with ATP plus cytochalasin D (cyto D; 10 µM), nocodazole (10 µM), control or anti-SEPT2 IgG. Images (C) show dextran-containing macropinosomes/endosomes after incubation under the indicated conditions. Bar graph (D) shows the fraction of fused macropinosomes/endosomes with both TR- and FITC-labeled dextran (n = 15 images). (E) Bar graph shows the fraction of fused endosomes after incubation of PNS with ATP and whole or SEPT2 immunodepleted (ΔSEPT2) cytosol (n = 15). (F) Gel shows equal volumes of whole and ΔSEPT2 cytosol blotted for SEPT2 and actin. (G) Bar graphs show the fraction of fused endosomes after incubation of PNS with/without recombinant SEPT2/6/7 in the presence/absence of 10 µM cyto D (n = 15).
E

Percent endosomes with SEPT2

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Figures A, B, C, and D show images of cellular structures stained with various markers. Figure E displays a bar graph illustrating the percentage of endosomes with SEPT2.
**Figure 7.6. PM-mCherry and SEPT2 localization with respect to Rab5 and Rab7.** (A-B) MDCK-PM-mCherry cells were transfected with Rab5-GFP (A) and Rab7-GFP (B), fixed and imaged by confocal microscopy. Images show 3D reconstructions from confocal z-stacks. Outlined regions are shown in higher magnification. Arrows point to vesicular endosomes with Rab5 or Rab7 and PM-mCherry. Arrowheads point to tubular endosomes with Rab7 and PM-mCherry. (C-D) Confocal microscopy images show MDCK cells that were transfected with Rab5 or Rab7-mCherry, fixed and stained for endogenous SEPT2. Outlined regions are shown in higher magnification and 3D reconstructions of the numbered endosomes (arrows) are shown in higher magnification. (E) Bar graph shows the percentage of Rab5- and Rab7-mCherry-positive compartments with SEPT2 (n = 17-18 cells).
Figure 7.7. SEPT2 knock-down increases macropinosome size and number, but does not disrupt colocalization of macropinocytic cargo with Rab5 and Rab7. (A) MDCKs were transfected with plasmids that express GFP or mCherry and non-targeting or SEPT2 shRNAs (GFP/shRNA #1, mCherry/shRNA #2) for 48 h. Cells were fixed and stained for endogenous SEPT2. Images show maximum intensity projections of SEPT2 fluorescence from 3D confocal microscopy images; insets show GFP/mCherry fluorescence. (B) MDCKs were treated with control or SEPT2 shRNA # 2, and incubated with FITC-dextran for 10 min. Bar graphs show the number of FITC-dextran-containing macropinosomes/endosomes (puncta >0.2 μm²) and their average size per cell (n = 21 cells). (C) HT1080 cells were transfected with plasmids that express GFP and control or SEPT2 shRNA, and stained for endogenous SEPT2. Images show maximum intensity projections of SEPT2 and GFP (insets) fluorescence. Bar graph shows the quantification of SEPT2 intensity per cell area (n = 20 cells). (D) Control and SEPT2-depleted HT1080 cells were incubated with TR-dextran for 10 min. Bar graphs show the number of TR-dextran-containing macropinosomes/endosomes (puncta >0.2 μm²) and their average size per cell (n = 20 cells). (E – F) MDCKs were transfected with GFP-expressing control or SEPT2 shRNAs for 48 h. Cells were pulsed with TR-dextran for 5 min and stained for endogenous Rab5 (E) and Rab7 (F) after a 5 and 10 min chase, respectively. Images show maximum intensity projections of confocal image stacks. Outlined regions with merged fluorescence are shown in higher magnification. Bar graphs show percent of TR-dextran particles that colocalize with Rab5 (E) and Rab7 (F) (n = 20 cells).
Figure 7.8. SEPT2 does not colocalize with Sec6/8 and Exo70 at the contact sites of macropinosomes/endosomes. (A – C) Images show 3D reconstructions of confocal image stacks of MDCK-PM-mCherry cells stained for endogenous SEPT2 and Exo70 (A), Sec8 (B) or Sec6 (C). Outlined regions are shown in higher magnification (scale bars, 1 μm). Green arrows point to SEPT2 at the sites of contact between PM-mCherry-labeled macropinosomes/endosomes, and blue arrowheads point to the localization of Exo70, Sec8 or Sec6.
CHAPTER 8. Conclusions and future directions

This dissertation focuses on the function of septin GTPases in epithelial cell migration and macropinocytosis. While septins have been shown previously to associate with the actin cytoskeleton and cell membranes, the molecular requirements have remained poorly understood. Moreover, septins are frequently overexpressed in epithelial cancers but the role of septins in tumor cell growth and metastasis is unknown. In this dissertation, I uncovered a mechanism by which septins interact directly with the actin cytoskeleton and promote epithelial migration and invasion. In addition, I discovered that septins associate with macropinosomes in a PI(3,5)P2-dependent manner and regulate the delivery of macropinocytic cargo to endolysosomes, a molecular pathway that promotes tumor cell growth.

Septins in epithelial cell migration

Previous studies have shown that septins exhibit an interdependent relationship with the actin cytoskeleton (Kinoshita et al., 1997), but whether septins bind directly to actin was unknown. In the first part of this thesis, I discovered that septins localize to the contractile transverse actin fibers and their points of connection with radial actin fibers. Septin knock-down abolishes the actin stress fiber network by reducing the number of radial fibers and thinning the transverse actin fibers. Significantly, I found that SEPT9 directly crosslinks actin filaments and interferes with the binding of the myosin motor domain in its low-affinity state and coflin, an actin-severing protein. Thus, septins are bona fide actin cross-linking proteins that may protect nascent actin filaments from depolymerizing forces, such as contractile myosin filaments and actin-severing proteins, during epithelial cell migration.

Because septins are overexpressed frequently in cancers, I assayed for changes in septin expression during epithelia-to-mesenchymal (EMT) transition, a critical step in
tumor cell invasion. I found a significant increase in SEPT9 expression and tested the effects of SEPT9 overexpression on the motile properties of epithelia undergoing EMT. Indeed, SEPT9 overexpression promotes epithelial cell migration in 2D and 3D environments, while SEPT9 knockdown or pharmacological targeting of septins inhibits cell migration. Taken together, my findings indicate that septins promote epithelial cell migration by directly cross-linking actin filaments and regulating the assembly and organization of the contractile actin stress fiber network.

**Future studies for septins in actin filament assembly and organization**

In the leading edge of motile epithelia, the actin stress fiber network is characterized by the assembly radial actin fibers, which are anchored to focal adhesions, and transverse arc stress fibers, which are crosslinked by contractile myosin II filaments. Through the linkage of these two stress fiber networks, forces are transduced to focal adhesions to promote forward translocation of the cell. My data indicate that septins are essential for the assembly of a cohesive stress fiber network and may regulate force transduction during cell migration. To test this hypothesis, the forces exerted upon focal adhesions can be measured experimentally using atomic force microscopy and gel contraction assays, which provide direct and indirect readouts, respectively.

Furthermore, radial actin fibers link up with transverse actin fibers in an orthogonal manner. My preliminary studies in vitro show that recombinant SEPT9 can crosslink and promote the orthogonal organization of purified actin filaments (Appendix 1.1). Actin filaments alone assemble into short linear filaments (Appendix 1.1B). However, when co-polymerized with recombinant SEPT9, actin filaments appear thicker, which suggests that they are crosslinked, and are arranged in a more branched-like organization with orthogonal linkages between filaments (Appendix 1.1C).
More work is necessary to better understand how SEPT9 can promote this geometric arrangement of actin filaments. Because SEPT9 can bind to three different sites on the actin filament, it is possible that SEPT9 promotes orthogonal interactions when bound to a specific site. High-resolution electron microscopy studies can reveal the precise localization of SEPT9 on orthogonal actin filaments and how its position on the actin filament may promote this geometry. Moreover, SEPT9 is a component of the SEPT2-6-7-9 octamer and likely functions within the cell as part of a molecular complex, but how SEPT9 binds to actin when in an octameric filament has not been determined. The SEPT2-6-7 crystal structure shows a hinged region at the SEPT2 interface, which provides flexibility to the filament (Sirajuddin et al., 2007), and could promote the branched and orthogonal arrangement of actin filaments.

In addition, I showed that SEPT6 promotes the recruitment of cortactin to the lamellipodium, a branched network of actin filaments (Hu et al., 2012). Copolymerization of actin with the recombinant SEPT2-6-7 complex results in curved and circular actin filaments (Mavrakis et al., 2014). Interestingly, the Arp2/3 complex preferentially initiates nascent filament assembly on curved actin filaments (Risca et al., 2012). Thus, septins may promote branched actin assembly by favoring geometrically the spatial localization of Arp2/3 activity. Moreover, yeast two-hybrid experiments identified that SEPT6 interacts with the Arp2 of the Arp2/3 complex (Nakahira et al., 2010), but we were unable to identify this interaction in cells or biochemically. Future studies involving co-polymerization of actin with the Arp2/3 complex are necessary to better understand the role of septins in branched actin assembly. Here, testing the effects of recombinant septins in an actin pyrene assay, which provides a fluorescence readout for actin polymerization, can reveal whether septins promote directly actin polymerization.
Future studies for septins in integrin trafficking

The assembly of radial actin fibers is initiated by the engagement of integrin receptors with ECM proteins (Parsons et al., 2010). Cytosolic tails of engaged, or activated, integrin receptors recruit actin nucleation factors that polymerize actin filaments and promote focal adhesion assembly and maturation. Thus far, two integrin trafficking pathways have been identified: fast Rab4-dependent recycling and slow Rab11-dependent recycling (Caswell and Norman, 2006). Significantly, the trafficking of integrins is essential for tumor cell migration and invasion.

Based on previous studies and my preliminary data, I hypothesize that septins may also have a role in integrin trafficking. Notably, Rab11 localizes to the golgi complex and regulates the delivery of exocytic vesicles to the plasma membrane (Chen et al., 1998). Septins localize to post-golgi microtubules and regulate the trafficking of intracellular vesicles to the plasma membrane. Thus, septins may also promote the trafficking of Rab11-dependent integrins to the membrane where they can engage with ECM proteins. Alternatively, my preliminary studies show that septins colocalize with juxtanuclear Rab4-positive vesicles (Appendix 1.2) in Cos7 cells, a renal fibroblast-like cell line. Future studies investigating the role of septins in intracellular trafficking of integrin receptors may unveil a second mechanism by which septins regulate cell migration.

Septins in tumor cell migration and metastasis

In Chapter 5, I tested the effects of septin depletion on epithelial cell migration in 3D collagen matrices, which better recapitulates the physiological environment. Interestingly, septin depletion transitions the cell to a more rounded and protrusive shape and slows significantly cell motility, suggesting that septins may have a role in the mechanism that regulates the mode of tumor cell motility. Future studies using
biomimetic cell-derived matrices derived from fibroblasts, which better mimic the tumor microenvironment, will provide a better context for studying tumor cell migration. In these matrices, tumor cells can transition between three different modes of migration: mesenchymal-like, amoeboid and lobopodial. While the molecular pathways that regulate the transition between these modes is poorly understood, septins may play a role in the spatial organization of the actomyosin cytoskeleton, whose contractile activity regulates focal adhesion dynamics during mesenchymal-like motility and the protrusive behavior in amoeboid and lobopodial motility.

Ultimately, it will be necessary to test how septin expression affects tumor cell motility in vivo using a mouse tumor model, which can be visualized with intravital imaging, and whether pharmacological targeting of septins inhibits metastasis. Collectively, these studies will report on the efficacy septin-targeting therapeutics in cancer.

**Septins in membrane fusion dynamics**

From yeast to mammals, membrane-associated septins are posited to restrict the lateral diffusion of membrane proteins and promote the compartmentalization of the plasma membrane (Caudron and Barral, 2009). Septins also modulate exocytic events at the plasma membrane (Tokhtaeva et al., 2015), but how septins function in endocytic membrane traffic and dynamics is little understood. In Chapter 7, I show that septins associate with macropinosomes in a PI(3,5)P2-dependent manner. Interestingly, PI(3,5)P2 is a low-abundance lipid that localizes exclusively to late endosomes and lysosomes and promotes macropinosome fusion (Kerr et al., 2010). In agreement with this finding, septins colocalize with Rab7-positive late endosomes and control the delivery of macropinosytic cargo to lysosomes. Moreover, reconstitution of vesicle fusion in vitro shows that septin inhibition and immunodepletion blocks vesicle fusion, while the
addition of recombinant septins promotes vesicle fusion. Thus, septins are an essential component of the molecular machinery that regulates membrane fusion.

**Vesicle fusion dynamics**

Membrane fusion relies on the coordinated recruitment of vesicle adaptor proteins, which can recognize specific lipid domains, and the assembly and disassembly of SNARE proteins between two vesicles. In addition, the microtubule and actin cytoskeleton play intricate roles in localizing and docking intracellular vesicles, respectively, to promote vesicle fusion.

In Chapter 7, I reconstituted vesicle fusion in vitro using a crude post-nuclear supernatant that contained fluorescently-labeled endosomes, whose lipid composition and constituent adaptor protein population was unknown. However, I was able to rule out the role of the actin and microtubule cytoskeleton in this assay through pharmacological inhibition. Thus, I hypothesize that septins function mechanistically at the level of SNARE-mediated membrane fusion.

Septins were reported to interact directly with SNAREs at the plasma membrane and inhibit exocytosis (Beites et al., 1999). However, the SNAREs constitute a large family of proteins that localize to specific intracellular membrane compartments. The SNAREs that promote macropinosome fusion with lysosomes are unknown, but syntaxin-7 is important for lysosome fusion and syntaxin-17 regulates autophagosome fusion with lysosomes (Itakura et al., 2012; Jahn and Scheller, 2006).

At a mechanistic level, septins may function at the level of SNARE assembly and/or endosomal membrane organization, which in turn can control SNARE dynamics. To address the first question, biochemical analyses of septin-SNARE interactions and vesicle fusion assays using purified liposomes reconstituted with SNARE proteins will provide a better readout for septin functions during membrane fusion. In this assay,
fluorescently-labeled liposomes containing donor and acceptor SNAREs are mixed together and assayed microscopically. If septins promote membrane fusion through a SNARE-dependent mechanism, the addition of recombinant septins could result in more vesicle fusion by promoting SNARE assembly, which tethers vesicles together, or SNARE disassembly, which drives membrane fusion (Jahn and Scheller, 2006).

Alternatively, septins may function at the level of membrane organization. By compartmentalizing the plasma membrane, septins are required for the organization of PI(4,5)P2-rich domains that organize the Orai1 calcium channel at ER-PM contact sites (Sharma et al., 2013a). Moreover, septins were reported recently to bind preferentially to highly-curved membranes, which are present on the plasma membrane and endomembranes. Interestingly, both lipid domain organization and membrane curvature can regulate the clustering of SNAREs into distinct membrane domains, which is necessary for vesicle docking and fusion. High resolution microscopy, such as super-resolution microscopy, which can provide a lateral resolution of ~ 20 – 100 nm, and electron microscopy can report on the localization of septins with respect to SNAREs on the endosomal membrane. In addition, testing the effects of septin depletion on endolysosomal SNAREs will report on their assembly and clustering into discrete domains on the endomembrane.

Taken together, these experiments could uncover the mechanism by which septins regulate vesicle fusion, and whether septins have a more universal role in the delivery of cargo from other endocytic pathways to the lysosome.
LIST OF REFERENCES


by MLL-SEPT6, or phenotype induced by the loss of Sept4. Molecular and cellular biology. 25:10965-10978.


Appendix 1.1. In vitro actin crosslinking and co-polymerization of actin filaments with SEPT9

(A) Pre-polymerized rhodamine-labeled actin filaments were incubated with or without recombinant full-length (FL) SEPT9. The length of actin filaments and the intensity per length were quantified and plotted.

(B) Polymerization of rhodamine-actin alone

(C) Co-polymerization of rhodamine-actin with recombinant SEPT9-GFP.
Appendix 1.2. SEPT9 colocalization with Rab4

Cos7 cells were fixed and stained for SEPT9 (green) and Rab4 (red). SEPT9 colocalizes with Rab4-positive vesicles. Bottom panel shows higher magnification of the region of interest (ROI).
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Articles:

1) Lee Dolat and Elias T. Spiliotis. Septins promote macropinosome maturation and traffic to the lysosome by facilitating membrane fusion. *In revision.*

Book Chapters:


Honors:

Ruth L Kirschstein NRSA F31 pre-doctoral fellowship. NCI. NIH 2014 – 2016
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