Septins in Cancer and Metastasis

by

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Abstract

Septins are GTP-binding cytoskeletal proteins that play a role in cancer cell migration. Their role in mesenchymal cell migration has been studied but not in amoeboid migration. Here, I show that in the highly invasive melanoma cell line, A375m2, loss of all isoforms of SEPT9 promotes switching from the amoeboid round to the less invasive mesenchymal elongated phenotype. It also causes the loss of RhoA-induced stress fibers while increasing the formation of Rac1-induced lamellipodia. Knocking out only the long isoforms of SEPT9 (v1-v3) brought about a similar effect on the amoeboid phenotype in 3D but in 2D caused a change in the types of stress fibers from ventral to dorsal and transverse arcs. Through immunostaining, I show that septins are localized at the back of the amoeboid migrating cell. This study thus shows the potential role of SEPT9 in amoeboid migration and thus in cancer metastasis.
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List of Abbreviations

ARHGEF18: Septin-associated Rho guanine nucleotide exchange factor, also abbreviated as SA-RhoGEF
CRISPR/Cas9: Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated 9
DH: Dbl homology
ECM: Extracellular Matrix
FHOD1: formin homology 2 domain containing protein 1
GAP: GTPase-accelerating proteins
GDP: Guanosine diphosphate
GDI: guanosine nucleotide dissociation inhibitor
GEF: Guanine Exchange Factor
GFP: Green fluorescent protein
GTP: Guanosine triphosphate
GTPase: Guanosine triphosphate hydrolase
JNK: c-Jun-N-terminal kinase
MLC2: myosin light chain 2
MMP: Matrix Metalloprotease
NG2: chondroitin sulfate proteoglycan
PH: Pleckstrin homology
PKCα: Protein kinase C alpha
RhoA: Ras homolog gene family, member A
ROCK: Rho-associated protein kinase
RBD: Rho binding domain
SDS-PAGE: Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
siRNA: Small interfering ribonucleic acid
SUE: Septin unique element
SEPT: Septin
Chapter 1
Introduction

1.1 Metastasis – a multistage process

Metastasis remains the leading cause of death by cancer, since it exhibits resistance to many available therapies. It is defined as one of the hallmarks of cancer, amongst others: sustained proliferative signaling, growth suppressor evasion, resistance to apoptosis, replicative immortality, and angiogenesis (Hanahan and Weinberg 2011). Metastasis is a complex multistage process in which tumor cells disseminate to other locations in the body, giving rise to secondary tumors. The scientific community has described events of metastasis as follows: loss of cell-to-cell contact, cell migration and invasion through the extracellular matrix, cellular intravasation into blood vessels or lymph vessels, followed by extravasation in secondary sites and colonization of new tumors. The metastatic abilities of tumors are influenced greatly by their microenvironments which include inflammatory cells and the extracellular matrix (Rodriguez-Hernandez et al. 2016).

1.2 Types of cell migration

For metastasis to occur, cancer cells can infiltrate their neighboring matrices either as individual cells, denoted as individual cell migration, or in sheets of cells, denoted collective cell migration. In many tumors, both single and collective cell migration occur (Friedl and Wolf 2003). While collective cell movement in cancer permits entry into the lymphatic system and aids metastasis, it is usually the slowest as it involves sheets of cells maintaining cell-cell adhesions for long periods of time. In most cases, it is individual cell movements that are necessary for tumor cells
Cells migrating individually can adopt (i) a mesenchymal form of migration or (ii) an amoeboid form of migration. Mesenchymal cell migration occurs when cells exhibit strong adhesions to their extracellular matrix and migrate in a fibroblast-like elongated method. These cells move by means of filopodia, thin hair-like filamentous actin structures, and lamellipodia, ruffle-like filamentous actin structures. Cells migrating by this approach utilize matrix metalloprotease enzymes (MMP) to digest their way through the extracellular matrix (ECM). On the other hand, cells migrating by an amoeboid mode exhibit weaker interactions with their surrounding ECM and migrate faster in a rounded morphology. They have low MMP activity and instead utilize the contraction of their cortically-arranged actomyosin ring to squeeze themselves through the small spaces with the ECM (Sun and Zaman 2017). Cells from hematopoietic tumors such as leukemia as well as small-cell lung carcinoma (SCLC) and melanoma have been shown to utilize the amoeboid mode. On the other hand, epithelial tumors such as breast and colon carcinoma, as well as sarcomas and glioblastomas exhibit the mesenchymal type (Friedl and Wolf 2003) (Figure 1).
1.3 The Rho Family of small GTPases

The Rho family of small GTPases (~21kDa) belongs to the Ras superfamily of proteins and plays important roles in actin cytoskeleton, cell polarity, cell migration, invasion and metastasis. Rho proteins function by acting as molecular switches where they cycle between the active GTP-bound state and the inactive GDP-bound state.

There are three classes of regulators of Rho GTPases: guanine nucleotide exchange factor (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs). GEFs catalyze the activation of Rho GTPases by facilitating the exchange of GDP to GTP, which allows Rho proteins to activate downstream effector proteins. To turn the switch off, GAPs catalyze the hydrolysis of GTP to GDP, rendering the Rho protein inactive. GDIs function...
by binding to GDP bound Rho, extracting them from membranes and solubilizing them in the cytosol, preventing their diffusion to other sites where they get activated by GEFs.

In humans, there are 20 Rho GTPase genes. The best three characterized members of the family are Rac1, Cdc42 and RhoA, which are highly conserved amongst eukaryotic species. The family of Rho GTPases have been implicated in several diseases including neurodegenerative disease, cancer as well as other diverse diseases (Boettner and Van Aelst 2002).

1.4 Rho GTPases and cell migration

The best three characterized members of the Rho family are Rac1, Cdc42 and RhoA, each have a unique effect on the actin cytoskeleton and consequently the mode of cell migration. Both Rac1 and Cdc42 play an important role in mesenchymal migration through inducing lamellipodia and filopodia formation, respectively. RhoA, on the other hand, promotes amoeboid migration, and stimulates the formation of stress fibers or cortical actomyosin filaments depending on the properties of the matrix surrounding the cells. In amoeboid migration, active RhoA brings about its effects by stimulating the activation of Rho kinases ROCK1/2 which further promote actomyosin contractility by decreasing myosin phosphatase activity and thereby promoting MLC2-phosphorylation and activation.

Both Rho-ROCK signaling and actomyosin contractility have been implicated in metastasis (Rodriguez-Hernandez et al. 2016). Several studies have reported deregulation of the Rho/ROCK signaling pathway in different cancers and its correlation to the different metastasis stages and disease progression. Inhibiting ROCK signaling reduces invasion potential in both *in vitro* and *in vivo* experiments in different tumor models, signifying ROCK inhibitors as potential anti-metastatic agents.(Liu et al. 2009; Patel et al. 2014; Sadok et al. 2015)
As mentioned above, Rho proteins are activated by GEFs. The last decade has witnessed significant progress in the characterization of RhoGEFs and their cellular functions. The human genome encodes nearly 80 RhoGEFs classified into two families: Dbl family and the DOCK family. The Dbl family, whose members activate RhoA, is characterized by a Dbl homology catalytic domain (DH) followed by an adjacent pleckstrin homology (PH) domain, which is responsible for intracellular targeting of the DH domain. The DH-PH domain is the minimal structure required for Rho GTPase activation (Jaiswal, Dvorsky, and Ahmadian 2013).

Studies have uncovered the roles of several GEFs in cell migration and the switch between mesenchymal and amoeboid migration, indicating their importance in cancer cell migration and metastasis. The following GEFs have all been implicated in promoting amoeboid morphology: GEFH1, Net1, LARG1 for RhoA, and DOCK10 for Cdc42. On the other hand, the GEFs DOCK3 and Asef2 for Rac1 have been shown to promote mesenchymal morphology. (Goicoechea, Awadia, and Garcia-Mata 2014).

One specific GEF, Septin Associated-RhoGEF, also known as p114RhoGEF or ARHGEF18, is a RhoA GEF that belongs to the Dbl family of GEFs and was initially discovered and named due to its association with N-terminus of SEPT9 through its C-terminus (Nagata and Inagaki 2005). An expression profiling study of ARHGEF18 in specimens from patients with non-small cell lung cancer has correlated its expression with lymph node metastasis, and not with patients' age, gender, tumor size, differentiation, or stage (Song et al. 2013). Another study showed data indicating that depletion of ARHGEF18 inhibits amoeboid migration (Terry et al. 2012). ARHGEF18 has been implicated with Rho/ROCK signaling in cell motility and lumen formation during tubulogenesis, a fundamental developmental process for epithelial organs (Kim et al. 2015).
1.5 Discovery of Septins

Septins are a family of GTP-binding proteins that assemble into filaments, and are considered the fourth component of the cytoskeleton. They belong to the GTPase family of P-loop nucleotide triphosphatases. They play a role in many cellular processes where they ensure membrane stability, act as diffusion barriers as well as molecular scaffolds for recruiting components of signaling pathways. They were initially discovered during a genetic screen for mutations related to cell division of *Saccharomyces cerevisiae*. Mutations in any of the four septin genes CDC3, CDC10, CDC11 and CDC12 prevented cytokinesis, resulting in large multi-nucleated cells (Hartwell 1971). Septins were later discovered in most eukaryotes but not in plants (Onishi and Pringle 2016). The number of septin genes differs greatly amongst species, for instance *Caenorhabditis elegans* has only two (UNC-59 and UNC-61) while humans have thirteen (SEPT1–SEPT12, SEPT14). This complexity is increased in mammals due to alternative promoters and splicing, generating many different isoforms (McIlhatton et al. 2001) thus it is challenging to completely understand the biology of these proteins.

In humans the 13 septin genes are categorized, based on sequence similarity and structure, into four subgroups: SEPT2 group (SEPT1, 2, 4, 5); SEPT6 group (SEPT6, 8, 10, 11, 14); SEPT7 group (SEPT7); and SEPT3 group (SEPT3, 9, 12). In HeLa cells, for example, septin filaments are composed from the repeated assembly of the octameric units 9-7-6-2-2-6-7-9. Members within the same family may substitute for each other, generating different possibilities for filaments depending on cell type (Mostowy and Cossart 2012).
1.6 Septin structure

Mammalian septins have a common structure composed of a central GTP-binding domain, a polybasic region that binds phosphoinositides (Zhang et al. 1999) at the N-terminal side and a Septin Unique Element (SUE) at the C-terminal side (Pan, Malmberg, and Momany 2007) (Figure 2). Their protein conformation is such that the GTP-domain is facing one side, called G-interface, and both the carboxy- and amino-terminal ends are on the other side forming the NC-interface. Different septin members may have variable N- and C-termini. The C-terminus may also contain up to three coil-coil domains depending on the septin group. These domains are essential for the interaction with other septins or substrates (Versele and Thorner 2005; Casamayor and Snyder 2003).

<table>
<thead>
<tr>
<th>N-terminus (Pro-rich)</th>
<th>Polybasic region</th>
<th>GTP-binding domain</th>
<th>SUE</th>
<th>C-terminus (Coil-coil Domain)</th>
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<td>SEPT2 group (SEPT1, 2, 4 &amp; 5)</td>
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<td>SEPT3 group (SEPT3, 9 &amp; 12)</td>
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<td>SEPT6 group (SEPT6, 8, 10, 11 &amp; 14)</td>
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<td>SEPT7 group (SEPT7)</td>
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**Figure 2 | Septin structure**

Mammalian septins are composed of a variable N-terminal region, a polybasic region, a GTP-binding domain, a Septin Unique Element (SUE) and a variable C-terminus. Septin members are classified into subgroups based on their sequence similarities. All except SEPT3 subgroup bear the coil-coil domain within the C-terminus.
SEPT9 in particular is described as the most complex member of the family as it exhibits extensive alternative splicing. The human genome assembly GRCCg37 (Genome Reference Consortium assembly h37, February 2009) (ensembl.org) has described that the SEPT9 locus may generate up to 47 different transcripts, 34 of which encode distinct proteins. Experimentally, 7 coding transcripts have been described for SEPT9 (SEPT9_i1 – SEPT9_i7). Several studies have allocated different functions and distributions to the different isoforms. Sept9_i1, _i2 and _i3 are very similar but with different promoter start sites. On the other hand, Sept9_i4 - _i7 lack much of the N-terminal region (Connolly et al. 2014).

Septins bring about their cellular functions by forming apolar complexes that further come together to form rings, rods and filaments. As the number of septins varies amongst different species, these complexes vary in components. For instance, C. elegans have only two septins and thus the complexes are composed of these two. In Drosophila, septins exist as hexameric complexes made of duplicate copies of Pnut, Sep1 and Sep2 (Field et al. 1996; Oegema et al. 1998) whereas in S. cerevisiae as octamers of duplicates of Cdc3, Cdc10, Cdc11 and Cdc12 in the following order: Cdc11–Cdc12–Cdc3–Cdc10–Cdc10–Cdc3–Cdc12–Cdc11. The terminal position for Cdc11 could also be replaced by Shs1, another septin member in S. cerevisiae (Bertin et al. 2008).

X-ray crystallography for mammalian septins expressed in E. coli showed that septins exhibit a mirror symmetry where they form their complexes in the order of SEPT7-SEPT6-SEPT2-SEPT2-SEPT6-SEPT7. The crystal structure revealed that septin monomers interact with each other by alternating NC- and G- interfaces (Sirajuddin et al. 2007). The G interface is responsible for the GTPase activity that is essential for GTP hydrolysis, which regulates the septin-septin interaction. Members of SEPT6 group, however, lack a threonine residue that causes them to be
conformationally locked in the GTP-bound state (Sirajuddin et al. 2009). Septins bring about their functions by forming complexes such as rods, rings and filaments. In Hela cells, SEPT9 occupies a terminal position in octameric complexes as SEPT9-SEPT7-SEPT6-SEPT2-SEPT2-SEPT6-SEPT7-SEPT9 (Kim et al. 2011) (Figure 3).

Figure 3 | Septin monomers and polymers
(A) Septin monomers exhibit an NC-interface composed of the N and C termini, as well as a G-interface that is made of the GTP-binding domain. Septin monomers interact with each other either through their NC-interface or G-interface (B) Septins were seen to polymerize into hexamers, with SEPT7 occupying the terminal position (C) Crystal structure of septin hexamer adopted from (Sirajuddin et al. 2007) (D) Septins may also polymerize into octamers, where SEPT9 occupies the terminal position.
1.7 The different functions of Septins

Septins bring about their functions by polymerizing into rods and filaments. At the cellular level, septins are often found in structures with micron-scale membrane curvature (Bridges et al. 2016) such as the cytokinetic furrow in dividing cells (Maddox et al. 2007; Estey et al. 2010), at the base of dendritic spines in neurons (Tada et al. 2007), the base of phagocytic cups (Huang et al. 2008), the annulus of sperm tails (Kwitny, Klaus, and Hunnicutt 2010) and the base of primary cilia. (Hu et al. 2010).

The best-studied model for septins is cytokinesis in *S. cerevisiae*, where they form a ring at the site of cell division from which the new bud emerges (Longtine and Bi 2003). Here they act as macromolecular scaffolds facilitating protein-protein interactions, where they recruit over 100 proteins including bud-specific machinery and actin cytoskeleton regulators (Gladfelter, Pringle, and Lew 2001). The septin ring at the bud is also responsible for the localization of Myo1, the only type II Myosin in *S. cerevisiae*, which is an integral part of actomyosin ring (Bi et al. 1998; Lippincott and Li 1998; Balasubramanian, Bi, and Glotzer 2004). This ring at the mother-bud neck also acts as a diffusion barrier controlling the proteins that diffuse between the mother and the bud (Takizawa et al. 2000).

In mammalian cells, septins also act as scaffolds during cytokinesis, where SEPT2 directly binds non-muscle myosin II in CHO cells. This interaction is essential for the phosphorylation of the regulatory light chains of non-muscle myosin II, which is important for the stability of the cleavage furrow. On the other hand, in interphase, SEPT2 is important for the formation of stress fibers. This shows the importance of septins’ role as macromolecular scaffolds for myosin II activation (Joo, Surka, and Trimble 2007).
Septins also play a role in cortical rigidity of cells. For instance, in spermatozoa septins constitute a ring-like structure at the annulus, the region separating the tail from the rest of the spermatozoa. In SEPT4 knockout mice, the annulus is absent and the tail is bent or breaks as the spermatozoa develop and become motile (Ihara et al. 2005). SEPT12 was also shown to localize to the annulus (Steels et al. 2007) and its mutation causes a bent tail and reduced motility of sperm in mice, which is a phenotype similar to that seen in infertile males carrying a similar mutation: SEPT12(D197N)(Kuo et al. 2015).

Another example of the importance of septins for cortical rigidity is in amoeboid migrating T-cells. Tooely et al showed that septins exhibit a fibrous array through the midzone of the T-cells, and that this is perpendicular to the axis of cell migration. Depletion of SEPT7 resulted in a more protrusive body with elongated tails and with extensive blebbing indicating that the flexibility of the membrane is increased (Tooley et al. 2009).

Septins also play a role in membrane flexibility by directly binding to negatively-charged phospholipids such as PIP2. They do so using a polybasic region at the N-terminal side. SEPT4 for instance has been shown to bind to PIP2 (Zhang et al. 1999). Septins also promote membrane tubulation from PIP liposomes in vitro (Tanaka-Takiguchi, Kinoshita, and Takiguchi 2009).

Another role for septins is in retracting the cell cortex. When T cells were placed in a hypotonic solution, both control and SEPT7-knockout cells swelled at the same rate, but the SEPT7-depleted cells recovered slower during the regulatory volume decrease phase. (Gilden et al. 2012).
In hippocampal neurons, SEPT7 has been shown to be critical for dendrite development during neuronal maturation since its overexpression resulted in increased dendritic branching whereas its knockdown resulted in reduced dendrites and more immature protrusions (Tada et al. 2007).

Septins have also been seen in primary cilia, which are immotile hair-like structures protruding from the membrane and are considered to be important sensory organelles that act as cellular antennas to transmit extracellular cues into the cell. They are sites for the regulation of several developmental signaling pathways such as non-canonical Wnt and Sonic hedgehog pathways (Sasai and Briscoe 2012). SEPT2 was seen to localize as a ring at the base of the primary cilia in IMCD3 cells and was suggested to play a role in the compartmentalization of the cilia, as its depletion resulted in mislocalization of ciliary membrane proteins (Hu et al. 2010). Mutations in SEPT2 or SEPT7 in Xenopus embryos resulted in ciliogenesis defects (Kim et al. 2010). Ghossoub et al., on the other hand, showed that SEPT2, SEPT7, and SEPT9 localize to the axoneme of primary cilia of Retinal Pigment Epithelium (RPE) cells (Ghossoub et al. 2011). Thus it appears that despite the discrepancy in localizing septins in primary cilia, they play a significant role in ciliogenesis development and function (Palander, El-Zeiry, and Trimble 2017).

1.8 Septins - Implication in cancer

In most cancers, septins are overexpressed, although downregulation of septins occurs in some tumors. Septins have documented roles in mitosis as well as during interphase (Pous, Klipfel, and Baillet 2016). In mitosis for instance, Estey et al. showed that after Cdk-1 mediated phosphorylation, long isoforms of SEPT9 are substrates for Pin1, both of which are needed for cytokinesis (Estey et al. 2013). Many oncogenes have been shown to be controlled by Pin1 (Zhou and Lu 2016). Another role for them in mitosis is their contribution to finalize
chromosome congression and segregation in anaphase. They do so by recruiting Centromere-associated protein E (CENP-E), which plays an important role in mitosis (Spiliotis, Kinoshita, and Nelson 2005). Septins also interact with anillin to promote ingression of the intercellular bridge, elongation and narrowing, all steps of intracellular bridge maturation. This primes the bridge for the assembly of the ESCRT III complex at the abscission site (Renshaw et al. 2014).

Septins also play a role in chrysotile fiber-induced tumorigenesis. Exposure to chrysotile fibers have been shown responsible for mesothelioma, lung cancer, and asbestosis. Cells exposed to the fibers had cytokinesis failure that was brought about by SEPT2 overexpression as well as by the mislocalization of anillin and SEPT9. This resulted in aneuploidy, centrosome amplification, and multipolar mitoses (Cortez et al. 2016).

Apart from their role in cell division and mitosis, septins were noted to contribute to tumorigenesis during interphase. For instance, SEPT4i_2, also known as ARTS (endoplasmic reticulum aminopeptidase 1), is a pro-apoptotic agent that, when released in the cytosol from mitochondria, binds XIAP (X-linked inhibitor of apoptosis protein), an anti-apoptotic protein, thereby relieving its inhibition of the caspases and promoting apoptosis (Edison et al. 2012). In acute lymphoid leukemias and in lymphomas, SEPT4_i2 was shown to be reduced in levels, explaining why these tumor cells are more resistant to cell death (Reingewertz et al. 2011; Garcia-Fernandez et al. 2010).

Several lines of research have shown that SEPT9 in particular plays a role in tumorigenesis. For instance, SEPT9 is implicated in the mis-regulation of the Epidermal Growth Factor Receptor (EGFR) where it binds to CIN85 preventing it from binding to the ubiquitin ligase Cbl. This prevents Cbl’s action of ubiquitin-dependant EGFR degradation, thus increasing their levels on
the membrane’s surface and contributing to sustaining proliferative signaling, a known hallmark of several tumors (Diesenberg et al. 2015; Hanahan and Weinberg 2011). Moreover, the quantification of the hypermethylation patterns of the SEPT9 gene in circulating DNA in colorectal cancer patients has been used as a plasma biomarker for detection of the tumor (Rasmussen et al. 2017).

SEPT9, and in particular SEPT9_i1, has been associated with poor prognosis of many cancers including prostate cancer (Gilad et al. 2015) as well as to resistance to many chemotherapeutic agents (Pous, Klipfel, and Baillet 2016; Amir and Mabjeesh 2007). SEPT9_i1 was also seen to be overexpressed during interphase (G1 and S) in breast cancer where it binds and prevents the degradation of the c-Jun-N-terminal kinase (JNK), thus promoting tumor cell proliferation (Gonzalez et al. 2009). It is also upregulated in head and neck cancers and associated with poor prognosis (Stanbery et al. 2010). SEPT9_v4* (currently SEPT9_v5) was also seen to be upregulated in ovarian tumors (Burrows et al. 2003). On the other hand, SEPT9_v3 is downregulated in nearly half of breast cancer patients (Connolly et al. 2011).

Several studies have also shown the role of septins in general, and SEPT9 in particular, in cell migration. They have been seen to interact closely with other components of the cytoskeleton including F-actin. Kinoshita et al showed that septins expressed with adaptor proteins bundle together along F-actin stress fibers. Loss of these stress fibers in the presence of latrunculin B resulted in the septins forming rings instead of filaments (Kinoshita et al. 2002). Septins also contribute in controlling actin filament dynamics where in migrating cells they bind and stabilize transverse arcs and radial stress fibers of lamellipodia (Dolat, Hu, and Spiliotis 2014). Shanker et al showed that the depletion of SEPT9 in several metastatic cancer cells allowed the reversal of the epithelial-mesenchymal transition and reduced cell spreading, migration and invasion.
Moreover, Füchtbauer et al. showed that knocking out SEPT9 in mouse embryonic fibroblasts causes them to migrate more slowly than wild-type cells (Fuchtbauer et al. 2011). SEPT9_i4 in particular has been shown to be involved in controlling the direction of migration in MCF7 breast cancer cells (Chacko et al. 2005).

1.9 Rationale

Several lines of evidence implicate septins in the regulation of actomyosin contractility, which is governed by the Rho/ROCK signaling pathway and important in amoeboid migration: (1) Septins associate with actin stress fibers in interphase cells and with the actomyosin ring at the cleavage furrow during cytokinesis - both of which are RhoA mediated cytoskeletal structures. Depletion of SEPT9 (Surka, Tsang, and Trimble 2002) causes loss of stress fibers in interphase cells and a failure of cytokinesis in dividing cells, indicating its importance for RhoA-mediated effects on the actin cytoskeleton. (2) SEPT2 is a direct binding partner of non-muscle myosin II, the form of myosin used by migrating cells. Depletion of SEPT2, or inhibition of its interaction with myosin, results in a decrease in myosin phosphorylation and activation (Joo, Surka, and Trimble 2007). (3) Cytokinesis studies show that loss of SEPT9 arrests the cell at the last step of cytokinesis, abscission (Estey et al. 2010); a phenotype that also appears with the ROCK inhibitor Y27632.

Accordingly, I wanted to investigate the potential role of septins in amoeboid migration which is governed mostly by RhoA. In this thesis, I explore how septins and specifically SEPT9 contribute to the activation of RhoA via ARHGEF18 and the mesenchymal to amoeboid transitions.
Chapter 2
Materials and Methods

2.1 Antibodies and Small Molecules

Antibodies used for western blotting and imaging are:

\(\alpha\)-SEPT2 (rabbit polyclonal, produced in-house); \(\alpha\)-SEPT5 (rabbit polyclonal, produced in-house), \(\alpha\)-SEPT7 (rabbit polyclonal, Proteintech); \(\alpha\)-SEPT9 (rabbit polyclonal, produced in-house); \(\alpha\)-SEPT11 (rabbit polyclonal, produced in-house); \(\alpha\)-Glyceraldehyde 3-Phosphate Dehydrogenase (mouse monoclonal; Millipore), \(\alpha\)-Flag M2 (mouse monoclonal, Sigma Aldrich), \(\alpha\)-c-Myc (Rabbits monoclonal, Abcam).

Y-27632 ROCK inhibitor was used at 10µM and Blebbistatin Myosin II inhibitor (Sigma Aldrich) was used at 20µM.

2.2 Constructs

For transfection experiments, the following constructs within the pcDNA3.1 plasmid were used:

Flag-SEPT2, Flag-SEPT6, Flag-SEPT7, Flag-SEPT9v3, Flag-RhoA. Myc-ARHGEF18 was a kind gift from Dr. Nagata, Aichi Human Service Center, Japan. PH-PLC-delta in EGFP backbone was kindly gifted from Dr. Sergio Grinstein, University of Toronto, Canada.

For siRNA-mediated knockdown experiments, siRNA for control and Septin 9 were GCAGCGACCATGAGTATCA and GCACGATATTGAGAGAAAA, respectively.
To generate SEPT9 long isoform CRISPR/Cas9, the guide RNA sequence, CCTCTTGGGGGCTCGTCGGTGG, was generated to an exon common to SEPT9_v1-4 using a sequence for an online CRISPR design tool developed by the Zheng lab (http://crispr.mit.edu/).
2.3 Cell culture and Transfections

For the RhoA activation experiments in the presence/absence of the Septin complex, Tet-ON HeLa were used for the transfections. For other experiments A375m2 human metastatic melanoma cells were used. The A375m2 cells were obtained from American Type Culture Collection (ATCC) and maintained in DMEM medium containing 10% FBS.

For transient transfections, the cells were seeded at 60% confluency in 12-well plates and grown in DMEM medium containing 10% FBS overnight and transfected the next day using Jet Prime® Transfection reagent (Polyplus Transfection) according to manufacturer protocol. 75µl of Jet prime buffer was pipetted into an eppendorf tube and then 0.8ug of the plasmid was added. The mixture was vortexed for 10s and spun down. 2.4µl of the Jet prime reagent was then added, vortexed for 10s, spun down and the mixture incubated for 10 minutes. It was then added drop-wise onto the cells.

2.4 RhoA-GTP assays

To quantify RhoA-GTP in the presence and absence of septins, two assays were used: RhoA activation Biochemical assay and the GLISA® RhoA activation assay kit from Cytoskeleton. The RhoA Activation assay utilizes Rhotekin RBD Agarose beads to selectively isolate and pull-down the active form of Rho from the sample of interest. The precipitated GTP-RhoA is then detected by western blot using an anti-RhoA specific monoclonal antibody. The GLISA RhoA assay uses a similar principle where the RhoA GTP-binding protein is linked to the wells of a 96 well plate. Active, GTP-bound Rho in cell lysates when added to the wells bind to the protein while inactive GDP-bound Rho is removed during washing steps. The bound active RhoA is then
detected with the help of RhoA specific antibody and a HRP-linked secondary antibody using HRP reagents and a visible light plate reader (Molecular Devices VersaMax 190).

Tet-ON HeLa cells were seeded in 12-well plates at 60% confluency and transfected the following day as mentioned above. The next day cells were washed twice in cold PBS, harvested by scrapping and transferred into a 1.5-ml Eppendorf tube. The cells were spun down at 1500 rpm for 10 minutes. The supernatant was then discarded and the cell pellet lysed in ice-cold lysis buffer supplemented with 1x anti-protease cocktail, by pipetting up and down and vortexing. Immediately, cell lysate was clarified by centrifugation at 10,000 x g, 4°C for 1 min.

For the RhoA activation assay Biochem kit from Cytoskeleton, immediately after, 10% of the lysate was added to an Eppendorf with Rhotekin-RBD beads on ice and incubated on a rocker at 4°C for 1 h. The beads were then pelleted by centrifugation at 5,000 x g at 4°C for 1 min. 90% of the supernatant was then removed and the beads washed with 500µl Wash Buffer and again centrifuged at 5,000 x g at 4°C for 3 min. The supernatant was then removed and the beads were eluted in 2x Laemmli sample buffer and the sample boiled at 95°C for 5 min and then analyzed by SDS-PAGE and Western blot.

For the GLISA® RhoA activation assay kit, 60µl of cell lysate were mixed with 60µl ice-cold binding buffer in a 1.5-ml Eppendorf tube placed on ice. GLISA strips were kept on ice and reconstituted with 100µl ice-cold distilled water. The water was then removed by flicking the strips and 50µl of the Lysate-Binding buffer mixture was added. Immediately The plate bearing the GLISA strips was kept on a cold orbital microplate shaker (400 rpm) at 4°C for 30 min after which the lysate was discarded and washed twice with 200µl Wash buffer at room temperature. 200µl of Antigen Presenting Buffer was then added and incubated at room temperature for 2
minutes and then flicked out. The strips were again washed with the Wash buffer three times. 50µl of anti-RhoA primary antibody (prepared by dilution in the Antibody dilution buffer at a ratio of 1:250) were added to the strips and the plate left to incubate on an orbital shaker (400rpm) at room temperature for 45 min. The solution was then flicked out and washed three times with wash buffer. 50µl of the HRP-conjugated secondary antibody was then added, which was previously prepared by diluting it in Antibody dilution Buffer at a ratio of 1:62.5. The plate was again left to incubate on an orbital shaker (400rpm) at room temperature for 45 min. The solution was then washed three times with 200µl wash buffer. 50µl of a mixture of the HRP detection reagent A and B was then added and the plate incubated at 37°C for 10-15 min. The signal was then read at 490 nm using a microplate spectrophotometer.

2.5 Visualizing cells in 3D

A375m2 melanoma cells were visualized in both 3D gels made of matrigel or collagen. For Matrigel, 8-well Labtek culture plates were coated with 30µl ice-cold matrigel and left to gel for 30 min at 37°C. The cells were trypsinized, counted and an aliquot of 30,000 cells was added to an Eppendorf tube with 150µl ice-cold matrigel placed on ice. Immediately, the cells with the matrigel were added onto the pre-coated well and left again to gel for 30 min at 37°C. 100µl of DMEM medium containing 10% FBS was then added on top. The cells were visualized the next day on an epifluorescence microscope (Leica) or a spinning-disc confocal microscope (Olympus).

3D collagen gels were also used to visualize A375m2 cells and in 3D immunostaining experiments. To a cold Eppendorf tube, 168µl Bovine Collagen Solution, Type I, 3 mg/ml (PureCol) and 30µl of 10x Hank’s buffer Salt Solution with Phenol red (Sigma Aldrich) was added. 0.1M NaOH was added dropwise until color changed from yellow to red bringing the pH
of collagen solution to pH 7.2-7.6. In another Eppendorf tube, 30,000 cells were added and cells were pelleted down at 2000rpm for 5 min. After the supernatant was removed, 30µl of FBS were used to resuspend the cells and then all were transferred onto the collagen solution. Around 25µl of ice-cold sterile distilled water was used to bring the final volume to 300µl. The whole content was then transferred to a well of the 8-well Labtek culture plates and the collagen gel left to polymerize for 2 hours at 37°C. 150µl of DMEM medium containing 10% FBS was then added on top and the cells visualized the next day.

2.6 Immunofluorescence

2-Dimensional Staining

For F-actin staining in 2D, cells were grown on coverslips and the next day fixed with 4% paraformaldehyde for 15 min at room temperature. Coverslips were then rinsed with PBS twice and then permeabilized by 0.1% Triton X-100 made in PBS solution for 10 min at room temperature and then rinsed again with PBS. Coverslips were then incubated in blocking solution (5% horse serum in PBS) for 20 min, and then incubated with Alexa Fluor™ 488 Phalloidin at a 1:500 dilution for 30 min. Finally, the coverslips were stained with DAPI stain at 1:10,000 in PBS for 2 min, rinsed 3 times in PBS, and mounted with the fluorescent mounting medium (Dako) on microscopic glass and sealed with transparent nail polish.

For septin staining in 2D, the same procedure was followed until the blocking solution step, after which the coverslips were incubated with the primary antibody diluted in PBS for 1 hour, rinsed three times with PBS and then incubated with Alexa Fluor™-conjugated secondary antibodies at a 1:500 dilution for 1 hour. As mentioned above, coverslips were finally stained with Hoechst and mounted on microscopic glass.
Slides were visualized on an epifluorescence microscope (Leica DMIRE2) or a spinning-disc confocal microscope (Olympus IX81). Image analysis was performed with the software Volocity (Perkin Elmer).

3-Dimensional Staining
A375m2 cells were seeded in collagen gels in the 8-well Labtek culture plates as described. The next day, cells were washed three times with room temperature PBS and were then fixed with Zinc-buffered formalin for 20 min. Cells were then washed with TBS-T three times and then incubated with 200µl block solution (5% FBS in TBS-T) for 20 min followed by an overnight incubation with 200µl primary antibody (diluted 1:100 in block solution). The next day, the wells were washed 3 times with TBS-T and then incubated for 2 hrs with Flourescently-labelled secondary antibody (diluted 1:500 in TBS-T). The wells were then peeled off and the collagen gels mounted with VictaShield mounting media with DAPI), covered with a microscopic glass and sealed with transparent nail polish.

2.7 SDS PAGE & Western Blot
Cells were lysed in SDS-loading buffer (20% v/v glycerol, 1% v/v β- mercaptoethanol, 2% w/v SDS, 65 mM Tris-HCl, pH 6.8, 0.001% Bromophenol Blue), boiled for 5 min and spun down. The samples were then loaded on SDS-polyacrylamide gels (4% stacking, 10% resolving), and ran at 120V until the dye front reaches the bottom of the gel. Samples were then transferred onto PVDF membranes at 400mA for 1.5hrs. PVDF membranes, which were previously pre-activated by incubating 10s in methanol, were then incubated with blocking solution (5% non-fat milk in
Tris-buffered saline-Tween (TBS-T) solution) for 1 hr and then incubated with the primary antibody diluted in TBS-T overnight at 4°C. The membrane was then washed with TBS-T for 5 min on a shaker 3 times and then incubated with the respective secondary antibody conjugated to horse radish peroxidase (HRP) at 1:3000 dilution in TBS-T for 1 hr on a shaker. Membranes were then washed again in TBS-T for 3 times and then treated with enhanced chemiluminescence (ECL) reagent and visualized using the LICOR® Odyssey Fc imaging system. Any quantification was performed by the Image Studio Lite software.

For preparation of cell lysate from cells in 3D collagen gels, sterile Collagenase/Dispase (Sigma Aldrich) diluted with PBS to 1 mg/ml was first added to the collagen gels and incubated for 30 min at 37°C. The mixture was then diluted in PBS and spun down at 2000rpm for 5min. The supernatant was then removed and the cell pellet lysed in SDS-loading buffer, boiled for 5 min and spun down.
Chapter 3
Results

3.1 Septins increase the GEF activity of ARHGEF18

Nagata et al (Nagata and Inagaki 2005) as well as previous work from the lab (unpublished data) have shown that the C-terminus of ARHGEF18 binds directly to the N-terminus of SEPT9. Unpublished data from the lab also indicate the involvement of septins in the Rho/ROCK signaling pathway as septins significantly increase the ARHGEF18 activation of RhoA. To start with, I was interested in quantifying the ARHGEF18-mediated RhoA-activation level in the presence and absence of septins. To address this subject, I first ensured equimolar expression of SEPT2, SEPT6, SEPT7, SEPT9_v3, RhoA and ARHGEF18 by western blotting 24hrs following transfecting them in Tet-On HeLa cells. (Figure 4) (All constructs bear a Flag-tag except ARHGEF18, which bears a myc tag).
Figure 4 | Optimization of expression of Septin complex with RhoA and ARHGEF18.
Separate immunoblot images (A for Flag tag, B for Myc tag) showing the equal expression of septins (Flag tagged), RhoA (Flag tagged) and ARHGEF18 (Myc tagged) 24 hrs after their transfection into Tet-On HeLa cells. The same samples were used in both immunoblots. Single transfections of septins and Myc-ARHGEF18 are also shown for reference.
To quantify the ARHGEF18-mediated RhoA-activation in presence and absence of septins, I used the RhoA-GTP pulldown assay with the Rhotekin-beads (Cytoskeleton). Figure 5A shows the RhoA-GTP levels in the presence of septins and Myc-ARHGEF18 as well as total RhoA to confirm equal levels of expression of RhoA. Controls included RhoA alone, and the complex without the septins or ARHGEF18. ImageStudio software was used to quantify the bands following densitometry, and the RhoA-GTP activation for n=3 was calculated and graphed as a fold increase over controls using Microsoft Excel (Figure 5B). I found that in the presence of septins and ARHGEF18, RhoA is activated 12.5-fold compared to only a 6-fold increase with ARHGEF18 alone. As a confirmatory experiment, the G-LISA Small GTPase Activation Assays for RhoA-GTP (Cytoskeleton) was also used (n=1). As positive control, I used RhoA expressed with DHPH (the constitutively activated truncation of ARHGEF18) as well as constitutively active RhoA (CA-RhoA) alone. With the GLISA assay, RhoA was activated ~9-fold in presence of septins and ARHGEF18, compared to 2.5-fold with ARHGEF18 alone. It is noteworthy that with the DHPH, RhoA was activated 10.4-folds which is very close to that in presence of septins and ARHGEF18 (Figure 5C).
Figure 5 | Septins augment ARHGEF18-mediated RhoA activation
(A) Septin complex increases ARHGEF18-mediated activation of RhoA, determined by RhoA-GTP Rhotekin pulldown assay. (B) Quantification of ARHGEF18-mediated RhoA activation in presence and absence of septins by RhoA-GTP pulldowns (n=3) and (C) by GLISA immunoassay (n=1). RhoA-DHPH was used as a positive control where DHPH is the activated truncated version of ARHGEF18. cA-RhoA (constitutively active RhoA) was also used as a positive control. (ARHGEF18 is abbreviated as GEF18 in fig B and C).
3.2 Septins are expressed in melanoma cells that undergo amoeboid migration

The involvement of Septins in RhoA activation supports the possibility of their potential role in RhoA-based round amoeboid migration. Accordingly, I decided to investigate the effect of septins on the highly invasive melanoma cell line, A375m2 for my studies as it resembles an endogenous system with high Rho/ROCK activity and is a perfect model for amoeboid migration. Initially, I characterized the septin profile of the A375m2 and checked for the expression of ARHGEF18. As positive controls, I used Tet-On HeLa cells as well as Mouse brain lysate. GAPDH was used as a loading control (Figure 6). The A375m2 cells express SEPT2, SEPT11, SEPT7 and two versions of SEPT9, the long isoform, and the short isoform Sept9_v5 (Sept9_v4*). In addition to septins, these cells strongly express ARHGEF18 as well as RhoA (Results not shown).

Figure 6 | Expression profile of Septins in A375m2 cells.
Expression of SEPT2, SEPT5, SEPT7, SEPT9 and SEPT11 in A375m2. TON HeLa and mouse brain lysate are used as positive controls. GAPDH is used as a loading control.
3.3 Knockdown of SEPT9 eliminates stress fibers and increases lamellipodia

High RhoA activity is seen in cells grown in 2D as stress fibers, whereas high Rac1 activity is seen as lamellipodia. Accordingly, I decided to visualize the F-actin structures in these cells grown in 2D. Since SEPT9 was shown by Nagata et al (Nagata and Inagaki 2005) to be the binding partner to ARHGEF18, which activates RhoA, I also wanted to investigate the effect of loss of SEPT9 on the F-actin filaments in these cells. Figure 7A shows that wild type A375m2 cells exhibit strong stress fibers in 2D. These stress fibers are lost with the knockdown of SEPT9 (siRNA – all isoforms) similar to the inhibition of ROCK with the ROCK inhibitor Y-27632. In fact, these stress fibers were replaced by lamellipodia in both cases, indicating the increase in activity of Rac1, a known phenomenon of counter balance where Rac1’s activity increases upon the reduction of RhoA’s activity (Moorman et al. 1999). Figure 7B shows the immunoblot confirming the loss of SEPT9 by siRNA after 72hrs with GAPDH used as a loading control. These results implicate SEPT9 in the RhoA/ROCK pathway as essential in amoeboid migration.
Figure 7 | Effect of loss of SEPT9 on morphology of A375m2 in 2D
(A) Loss of SEPT9 in A375m2 cells results in the loss of stress fibers (RhoA-mediated actin structures) and generation of lamellipodia (Rac1-mediated actin structures) similar to the use of ROCK inhibitor (Y-27632). Cells are stained for F-actin. (B) Immunoblot showing the loss of SEPT9 by siRNA (72hrs). GAPDH was used as a loading control.
3.4 Knockdown of SEPT9 causes cells to elongate in 3D gels

As amoeboid migration requires a 3D environment, I initially chose to perform these studies in matrigel, in which 95% of the A375m2 cells are round (Sahai and Marshall 2003). The cells were seeded in the 3D matrigels and then visualized by light microscopy the next day. Figure 8A shows the wild-type A375m2 cells in matrigel. Next I investigated the effect of loss of SEPT9 on amoeboid morphology. For positive controls, ROCK inhibitor (Y27632) and Myosin II inhibitor (Blebbistatin) were used. Figure 8A also shows that loss of SEPT9 (by siRNA for all isoforms) shifts the morphology of the cells from amoeboid (round) to mesenchymal (elongated), similar to the ROCK and Myosin II inhibitors, confirming the involvement of SEPT9 in RhoA-ROCK-Actomyosin pathway. For statistical purposes, the degree of roundness was measured for 200 cells in each group by measuring the perimeter of the cell. This quantification was performed by the roundness indicator in ImageJ software (Figure 8B) where a score of 1 was given for a completely round cell and a score of zero for a completely elongated cell. The average roundness for the wild-type A375m2 was 0.9 whereas the average roundness for SEPT9 knockdown was 0.72, for ROCK inhibitor Y-27632 compound 0.63 and for Myosin inhibitor Blebbistatin 0.55. The roundness of SEPT9 knockdown as well as ROCK and Myosin inhibitor were all significantly different from wild-type A375m2.
Figure 8 | Effect of loss of SEPT9 on morphology of A375m2 in 3D.

(A) Morphological changes of A375m2 in 3D matrigel following the knockdown of SEPT9, from round to elongated. This phenotype is similar to that of ROCK inhibition by Y27632 or Myosin II inhibition by Blebbistatin. (B) The calculated degree of roundness of the aforementioned groups of cells. Roundness was analysed by ImageJ (roundness = 1, elongated = zero) A375m2: 0.73, S9KD 72hrs: 0.61, Y-27632 10µM, Blebbistatin 20µM: 0.55.
3.5 CRISPR-mediated deletion of the long isoforms of SEPT9 disrupts stress fibers

The long isoforms of SEPT9, v1-3, bear the N-terminal region that binds ARHGEF18. Accordingly, I sought to investigate whether the long isoforms of SEPT9 were required, or if the short isoform was sufficient, to maintain the amoeboid morphology. Accordingly, I proceeded with deleting just the long isoforms and investigated whether this would generate a similar phenotype in 3D matrigel. Using Crispr/Cas9 technology, I generated a knockout of the long SEPT9 isoforms in A375m2 (Figure 9A). I also checked the expression levels of SEPT2 as a measure of other Septin components and found by immunoblotting that they were unaffected (Figure 9A). Immunostaining with an antibody specific for the long isoforms of SEPT9 shows a loss of the SEPT9 filaments. Staining for SEPT2 revealed that the existing septin filaments were short stubs rather than long filaments, a feature also seen upon loss of SEPT9 in other cell lines, indicating that SEPT9v5 is unable to form long Septin filaments with other septin members (Figure 9B).

Next I wanted to visualize the stress fibers in the WT A375m2 cells as well as the SEPT9v1-3 KO A375m2 cells. Following staining them with phalloidin on 2D glass coverslips, I noticed that the WT cells exhibited strong ventral stress fibers (Figure 10A). On the other hand, the SEPT9v1-3 KO A375m2 cells lacked those strong ventral stress fibers but exhibited two other types of stress fibers: transverse arcs and dorsal stress fibers (Figure 10B). Previous studies have described how these different types of stress fibers are mediated by different Rho family members (Figure 10C). While the ventral stress fibers are mediated by RhoA, the dorsal stress fibers are mediated by Rac1 (Vallenius 2013). This again confirms the role of the long isoforms of SEPT9 in the RhoA-ROCK-Myosin II pathway as once they are absent RhoA is no longer
activated, and this activity is taken over by Rac1 resulting in the dorsal stress fibers.

Figure 9 | Generation of SEPT9v1-3 KO-A375m2 by Crispr/Cas9 Technology

(A) Immunoblot for SEPT9v1-3 KO-A375m2 stained for SEPT9 (antibody for all isoforms) and SEPT2 showing the loss of the long isoforms of SEPT9. SEPT9v5 and SEPT2 expression levels remain the same. (B) 2D immunostaining of A375m2 and SEPT9v1-3 KO-A375m2 for SEPT9 filaments (antibody for long isoforms only) and SEPT2 filaments showing the disruption of the long filaments for both in the SEPT9v1-3KO cells but not in the WT-A375m2 cells.
Figure 10 | Actin staining showing stress fibers of WT-A375m2 and SEPT9v1-3 KO-A375m2
(A) WT-A375m2 showing strong ventral stress fibers in green (B) SEPT9v1-3 KO-A375m2 showing dorsal stress fibers and transverse arcs (C) A model depicting different types of stress fibers (Vallenius 2013).
3.6 Deletion of SEPT9 long isoforms causes cell rounding

Next I wanted to investigate the 3D morphology of these SEPT9v1-3 KO cells in 3D collagen gels. The cells were seeded in 3D collagen matrices and visualized with light microscopy the next day. The SEPT9v1-3 KO cells were seen to change from the rounded morphology to the elongated one, similar to ROCK inhibition and Myosin II inhibition (Figure 11) again conforming the importance of these long isoforms of SEPT9 in the activation of RhoA/ROCK/Myosin II pathway which is essential in maintaining the amoeboid morphology.

![Figure 11](image)

**Figure 11 | Effect of loss of long isoforms of SEPT9 on A375m2 morphology in 3D collagen gels.** WT A375m2 cells exhibited the rounded morphology, whereas inhibition of ROCK by Y27632 and Myosin II by Blebbistatin resulted in elongated morphology. The SEPT9v1-3KO A375m2 cells also exhibited an elongated morphology similar to ROCK and Myosin II inhibition.
3.7 Septins are localized in the uropod of amoeboid cells

I then examined the intracellular localization of Septin filaments in A375m2 cells grown in 3D gels. Cells migrating by the amoeboid mode are round and appear to lack polarization. However, in order for cells to determine their directionality during movement, they need to establish a form of polarity. Lorentzen et al showed that amoeboid cells, exemplified by A375m2, have an ezrin-rich uropod-like structure that, even though does not protrude from the cell, is enriched for Phosphatidylinositol 4,5-bisphosphate (PIP2) and actomyosin machinery (Lorentzen et al. 2011). Since septins bind to PIP2 and myosin, we speculated that septins will also be enriched at the back of the cell. To identify the uropod, wild-type cells were transfected with PH-PLC delta-GFP, a marker of PIP2, and then seeded on collagen gels and the next day immunostained for SEPT2. Figure 12A shows SEPT2 enrichment at the back of the amoeboid cells while Figure 12B shows PIP2 also at the back of the cell. Figure 12C shows the co-localization of both together confirming the hypothesis that septins are enriched at the back of the cell in those moving by amoeboid migration.

For further work in this project, it would be relevant to characterize proteins expressed in the cells when they are in the 3D gels. To determine if we could isolate and extract these proteins, I harvested cells grown in 3D collagen gels by dissolving the gels using collagenase/dispase. The solution bearing the round cells was then centrifuged and the pelleted cells were harvested and lysed and run in an immunoblot and probed for SEPT2 and SEPT9 (all isoforms) (Figure 12D), demonstrating our success at harvesting proteins from cells grown in matrigel.
Figure 12 | Immunostaining and immunoblots of A375m2 in 3D collagen gels. (A) Immunostaining of A375m2 in 3D collagen gel for SEPT2 in Red (B) Phosphatidylinositol 4,5-bisphosphate (PIP2) following transfection of PH-PLC-delta (Green) in 3D collagen gel (C) Co-localization of SEPT2 and PIP2 in A375m2 at the uropods. (D) Immunoblot of SEPT9 (all isoforms) and SEPT2 from total protein lysate of cells harvested from 3D collagen gels.
Chapter 4
Discussion

Septins have been shown to play a role in a number of processes where they ensure membrane stability, act as diffusion barriers as well as molecular scaffolds for recruiting components of signaling pathways (Mostowy and Cossart 2012). Septins have also been shown to play a role in mesenchymal cell migration where SEPT9 overexpression increases mesenchymal-like motility of renal cells. (Dolat et al. 2014). In addition to mesenchymal migration, amoeboid migration has been described to contribute to tumorigenesis and metastasis, especially in certain types of tumors such as melanoma (Lorentzen et al. 2011). It specifically depends on the activity of RhoA (Sadok et al. 2015). The role of septins in amoeboid migration has not been previously established. Nagata and Inagaki (Nagata and Inagaki 2005) as well as our lab (unpublished data) have shown the role of SEPT9 in activating RhoA, which is essential for amoeboid migration. We therefore hypothesized that septins would contribute to amoeboid migration due it effects in controlling RhoA and tested this possibility in the present thesis.

4.1 Septins augment ARHGEF18-mediated RhoA activation

SEPT9 has been shown to be a binding partner of ARHGEF18, one of the GEFs that activate RhoA (Nagata and Inagaki 2005). In preliminary results, our lab has found that SEPT9 augments the ARHGEF18-mediated RhoA activation. Accordingly, I first started by quantifying this increase in activation. Through RhoA-GTP pulldown experiments, I was able to show that in presence of the septin complex (SEPT2-SEPT6-SEPT7-SEPT9), ARHGEF18 activates RhoA 12.5-fold compared to ~6-fold without the complex (n=3). Using the GLISA technique, in
presence of the septin complex, ARHGEF18 activates RhoA ~9-fold as opposed to 2.5-fold without the complex. In comparison, RhoA was activated 10.4-fold in presence of the active form of ARHGEF18 (DHPH). The maximum sensitivity of the assay was 17.2-fold above the control for the constitutively active form of RhoA. This data shows that the septin complex has the ability to augment ARHGEF18-mediated RhoA activation at least 2-fold. Several GEFs have been shown to exist in an auto-inhibited conformation and that their activation occurs when this autoinhibition is relieved by binding to another protein (Cherfils and Zeghouf 2013). In a similar manner, we speculate that the septin complex relieves this auto-inhibition on ARHGEF18 by directly binding to the C-terminus of ARHGEF18, allowing the N-terminal DH-PH domain to exchange GTP nucleotides on RhoA.

The role of septins as Rho activators may be conserved, since septins have been shown to be involved in activation of Rho proteins in fission yeast. In that case septins interact with Gef3, a RhoGEF, which then further binds Rho4 GTPase and activates it bringing about the septation process (Wang et al. 2015).

The involvement of ARHGEF18 in tumors have been documented by several studies. In non-small cell lung cancer, ARHGEF18 expression levels correlated with the degree of lymph node metastasis (Song et al. 2013). Depleting ARHGEF18 expression levels was also correlated with reduced tumor cell amoeboid-like migration as well as reduced collective cell migration (Terry et al. 2012). The results discussed herewith showing the role of septins in augmenting ARHGEF18-mediated RhoA activation imply that septins may have a role in amoeboid migration and thereby cancer metastasis.
4.2 Septins and RhoA processes

Several studies have shown that septins play a role in RhoA-mediated processes. For instance, septins have been shown to co-localize with F-actin stress fibers. The disruption of the F-actin stress fibers by cytochalasin D results in the formation of septin rings instead of septin filaments (Kim et al. 2011). Septins are also seen to localize at the cleavage furrow where they are thought to be responsible for the recruitment of many proteins that mediate cytokinesis, a RhoA-dependant process (Maddox et al. 2007; Estey et al. 2010). At the cleavage furrow is also the actomyosin contractile ring that promotes the final steps of daughter cells separation. Both the septin ring and the actomyosin ring interact together through SEPT2 binding to non-muscle myosin II. Inhibition of the SEPT2-Myosin II interaction results in an instability of the ingressed cleavage furrow and a dissociation of Myosin II from ROCK, a downstream effector of RhoA (Joo, Surka, and Trimble 2007). Similarly, we hypothesized that since amoeboid migration depends mostly on RhoA, that septins would have a role in amoeboid migration.

Amoeboid migration is one way by which tumor cells migrate to metastasize and form new secondary tumors. This mode of migration is specifically used by highly metastatic tumors such as melanoma. Cells migrating with amoeboid mode exhibit weak interactions with their surrounding extracellular matrix (ECM), have a low matrix metalloprotease activity and utilize the contraction of their cortically-arranged actomyosin ring to squeeze themselves through the small spaces with the ECM. Unlike mesenchymal migration which depends on Rac1, amoeboid migration depends mostly on RhoA (Sun and Zaman 2017).

To investigate the role of septins in amoeboid migration, I chose the A375m2 cells as they have been shown to exhibit amoeboid morphology, migrate by amoeboid migration in 3D gels and
have a high intrinsic RhoA activity (Sahai and Marshall 2003). The A375m2 cells are highly malignant human melanoma cells that have been isolated after two rounds of selection of their parental line, A375P cells, from the lung metastasis in immunocompromised mice. Initially, I started by characterizing the expressed septin members in the WT-A375m2 cells by expressing one septin member from each septin family: SEPT2 from the SEPT2 group, SEPT6 from the SEPT6 group, SEPT7 from SEPT7 group and SEPT9 from the SEPT3 group, thus have the sufficient septin members to form septin filaments.

Plating these cells on 2D coverglass and staining for F-actin filaments, the ventral stress fibers were very prominent, a sign of high intrinsic levels of RhoA activity. Inhibiting the downstream effector of RhoA, ROCK, by the Y27632 compound showed the loss of the ventral stress fibers and the formation of lamelopodia, an indication of higher Rac1 activity, a known phenomenon of counterbalance where Rac1’s activity increases upon the reduction of RhoA’s activity (Moorman et al. 1999). Similar results were seen with SEPT9 knockdown where the ventral stress fibers were replaced with lamelopodia, indicating the role of septins in activating RhoA, which, upon the loss of SEPT9, caused a counterbalance of Rac1 activity increase.

4.3 The effects of SEPT9 downregulation on amoeboid morphology

The involvement of Septins in RhoA activation provides preliminary evidence of their potential role in RhoA-based amoeboid migration. As amoeboid migration requires a 3D-environment, I decided to perform these studies in matrigel, in which 95% of the A375m2 cells are round (Sahai and Marshall 2003). I investigated the effect of loss of SEPT9 on amoeboid morphology. For positive controls, ROCK inhibitor (Y27632) and Myosin II inhibitor (Blebbistatin) were used. The WT-A375m2 cells exhibited a rounded morphology in the 3D matrigels. This rounded
amoeboid morphology shifted to a mesenchymal, elongated one in the 3D gels for the ROCK inhibitor, Myosin II inhibitor as well as SEPT9 knockdown, confirming the importance of septins, in specific SEPT9, in maintaining the amoeboid morphology and that septins play a role in the RhoA/ROCK/Actomyosin pathway.

In a similar manner, Vaškovičová et al. showed that PKCα (Protein kinase C alpha) activation was important for transforming the morphology of both K2 sarcoma and MDA-MB231 breast cancer cells from the elongated mesenchymal to rounded amoeboid in 3D gels. In A375m2 melanoma cells, PKCα inhibition led to the transition from amoeboid to mesenchymal morphology. When the group further investigated the effect of PKCα on the migration ability of the cancer cell lines, they showed that inhibiting PKCα reduced the invasive potential of these cancer cells.

Pankova et al. (Pankova et al. 2012) also showed that the NG2 chondroitin sulfate proteoglycan was essential for amoeboid morphology in A375m2 cells as siRNA-mediated downregulation of NG2 induced a rounded amoeboid to elongated mesenchymal transition. This downregulation caused a decrease in the invasiveness of these melanoma cells in the 3D collagen gels as well as caused an inactivation of the Rho GTPase.

The results discussed for SEPT9 are similar to those described for PKCα and NG2 implying that in a similar fashion, SEPT9 may play a role in controlling cancer cell invasiveness through the amoeboid pathway.
4.4 The effects of SEPT9 long isoforms knockout on stress fibers

Actin stress fibers in migrating fibroblasts are further classified as ventral stress fibers, dorsal stress fibers and transverse arcs, based on their localization within the cell. The ventral stress fibers are long actin filaments located underneath the nucleus and in the trailing area. They are activated by RhoA whereas the dorsal stress fibers on the other hand are non-contractile long filaments attached between focal adhesions at the leading edge at one end and the transverse arcs at the other end and are activated by Rac1. The transverse arcs are contractile curve-shaped arcs that are parallel to the leading edge and move towards the nucleus (Vallenius 2013).

As the long isoforms of SEPT9, v1-3, bear the N-terminal region which binds ARHGEF18, an activator of RhoA, I sought to investigate whether the long isoforms of SEPT9 were required, or if the short isoform was sufficient for the amoeboid morphology mediated by RhoA activation. Following the generation of SEPT9 v1-3KO A375m2 cells and checking their actin cytoskeleton in 2D, it was found that these cells exhibited dorsal stress fibers and transverse arcs unlike the WT-A375m2 cells which exhibited only ventral stress fibers. These results indicate that indeed the long isoforms of SEPT9 (v1-v3) are critical for activation of RhoA.

FHOD1 (formin homology 2 domain containing protein 1) is a downstream effector of the Rho/ROCK pathway that controls actin. Schulze et al has shown that the formin FHOD1, when activated, stimulates the formation of ventral stress fibers and inhibits the formation of dorsal stress fibers (Schulze et al. 2014). The group has concluded from their work that FHOD1 is essential for the dynamic cell behavior that occurs during cell migration.

I speculate that similarly, the long isoforms of SEPT9 through their N-terminus interaction with ARHGEF18 and activation of RhoA plays a role in controlling the subtypes of F-actin filaments
and thus the dynamics of cell migration.

4.5 The effect of loss of SEPT9 long isoforms on amoeboid morphology

As I had investigated the effect of SEPT9 knockdown on the amoeboid morphology in the A375m2 melanoma cells, I next wanted to check whether the knockout of SEPT9 long isoforms would generate similar results as their 2D results discussed above showed that they are required for RhoA function on actin cytoskeleton. Plating the SEPT9v1-v3KO cells in 3D collagen gels showed that they lose their roundness and exhibit the elongated morphology similar to the ROCK inhibited and Myosin II-inhibited WT cells.

These results indicate that, as hypothesized, the long isoforms of SEPT9 (v1-v3), and not the short one (v5), are needed for activating RhoA and promoting the amoeboid morphology. As the difference between the long and short isoforms is the N-terminal region, and this is the region through which SEPT9 interacts with ARHGEF18 (Nagata and Inagaki 2005), it is thus the N-terminal region of SEPT9 that is essential for promoting the amoeboid morphology.

The long isoforms of SEPT9 have been shown to play a role in cancer progression in several studies. Estey et al showed that the long isoforms of SEPT9 become phosphorylated by CDK1 after which they become substrates for the propyl-isomerase Pin1, and are therefore used to complete cytokinesis (Estey et al. 2013). Many oncogenes and tumor suppressors are also controlled by Pin1 (Zhou and Lu 2016), thus the long isoforms of SEPT9 may participate in cancer cell formation by controlling cell division, which is an important hallmark for tumorigenesis.
c-Jun N-terminal kinases (JNKs) play a role in tumorigenesis and tumor progression by regulating cell proliferation, migration and apoptosis (Dhanasekaran and Reddy 2017). One study showed that a specific version of SEPT9 (SEPT9v1) stabilizes JNK, promoting longer JNK signaling, c-Jun phosphorylation, cyclin D1 expression thus promoting increased cell division (Gonzalez et al. 2009). The increased SEPT9_v1 expression also increases the malignant pathogenesis of certain types of breast tumors (Gonzalez et al. 2007).

The long isoforms of SEPT9 thus play several roles in tumorigenesis as seen in these different studies. The role of these isoforms in maintaining the amoeboid morphology shows that they may also be crucial for amoeboid migration which is an important mechanism of metastasis used in many tumor types.

4.6 The potential role of Septins and their localization in amoeboid A375m2 cells in 3D

Amoeboid cells, exemplified by A375m2, migrate in a rounded mode in 3D matrices. Despite their rounded shape, they establish a form of polarity using an ezrin-rich uropod-like structure that, even though does not protrude from the cell, is enriched for Phosphatidylinositol 4,5-bisphosphate (PIP2), ROCK and actomyosin. This uropod, which is situated at the back of the cell, bears the necessary machinery that acts as a driving force for the cell to move forward (Lorentzen et al. 2011).

Septins, stained for SEPT2, were enriched at the back of the cell as shown in the results section. They were specifically located within the uropod, which were identified by co-expressing a marker for PIP2. We speculate that septins are thus part of this driving machinery.
Several lines of evidence link septins with these components enriched in the uropod - PIP2, ROCK and actomyosin. Septins were previously shown by our lab to bind to membranes enriched with PIP2 (Zhang et al. 1999). In addition, Joo et al showed SEPT2 binds directly to non-muscle myosin II and scaffolds it for its further interaction with its kinases (Joo, Surka, and Trimble 2007). Septins have also been suggested to bind and crosslink with actin filaments to form long fibers as well as curved bundles (Mavrakis et al. 2014; Dolat et al. 2014). Finally, this thesis shows that septins contributes to the activation of RhoA, the upstream regulator of ROCK.

These lines of evidence along with the localization of septins within the uropod support the hypothesis that septins play a role in propagating the cell forward in amoeboid migration.
Chapter 5
Conclusion and Future Directions

In this study, it was shown that septins are important for activating RhoA as well as for the amoeboid morphology of highly invasive melanoma cells. Expressing the septin complex was capable of augmenting the ARHGEF18-mediated RhoA activation. The knockdown of all isoforms of SEPT9 was capable of switching the morphology of the invasive melanoma cells from the amoeboid round to the mesenchymal long phenotype in 3D matrices, and reducing the formation of RhoA-induced stress fibers and increasing the formation of Rac1-induced lamellipodia. Knocking out only the long isoforms of SEPT9 (v1-v3) was capable of bringing about a similar effect on the amoeboid phenotype in 3D but in 2D caused a change in the types of stress fibers from ventral to dorsal and transverse arcs. Through immunostaining experiments, septins were localized at the back of the amoeboid migrating cell, within the uropod that acts as the polarity structure of the cell.

To continue investigating the role of septins in amoeboid migration, we first would like to address the question of how septins activate RhoA signaling through ARHGEF18, and potentially other GEFs. As Nagata et al (Nagata and Inagaki 2005) has previously showed that the C-terminus of ARHGEF18 binds to the N-terminus of SEPT9. It would be important to identify whether direct SEPT9 binding is sufficient to activate the GEF, or if the presence of SEPT9 in septin filaments/octamers contributes to this activation. To determine the importance of filaments, we would use our lab-generated Sept2ΔC mutants that, when transfected with other septin members, forms octamers but cannot form filaments. We would then use this mutant to quantify the ARHGEF18-mediated RhoA activation in presence of septin octamers by the RhoA-
GTP pulldown/GLISA assay. Upon getting comparable activation, we would then test SEPT9 alone, as well as increasingly smaller SEPT9 fragments (of the N-terminus), to identify the minimum region required.

We would also like to determine if other GEFs can also interact with, and be activated by, Septins. To do so, we would screen for RhoA GEFs that co-localize with septins (on stress fibers) and assay for their interaction with SEPT9 by co-immunoprecipitation.

Next we would like to determine the involvement of Septins and ARHGEF18 in the Mesenchymal-to-Amoeboid transition and amoeboid migration. Initially, we would want to know if the knockdown of ARHGEF18 is sufficient to bring about effects similar to those of SEPT9 with regards to the morphology in 3D and the F-actin cytoskeleton in 2D. We are also interested in determining whether the loss of SEPT9v5, which lacks the GEF-interacting region at the N-terminus, generates similar phenotypes to those of SEPT9 long isoforms. If knocking out only SEPT9v5 brings similar phenotypic changes, it would imply that it also plays a role Rho/ROCK/Actomyosin pathway. However, if not, we can conclude that the N-terminus region of SEPT9 is important for the pathway activation.

Another point to investigate would be the effect of loss of SEPT9 on the invasive potential of A375m2 cells. By the use of Boyden chambers, we can assess the percentage of cells travelling down the chamber to reach the bottom as a measure of cell migration potential. Single-cell migration assays would also be used to characterize the invasive potential of the knockout cells where cells will be seeded on collagen gels, and then overlayed with more collagen and later imaged by time-lapse microscopy where single cells would be tracked and the migrated distance quantified.
As the A375m2 cells are derived from the lung after two rounds of metastasis of the parental A375P melanoma cells, we would be interested in knowing if these parental cells have a similar expression profile for the isoforms of SEPT9. With the help of real-time PCR, we quantify the mRNA transcript levels in both cell lines to determine if their changes in metastatic capacity correlated with changes in SEPT9 expression.

As we have shown the importance of SEPT9 in amoeboid morphology, we would be interested in determining its effects on other key players of amoeboid migration. The phosphorylation of MLC2 by the Rho/ROCK signaling leads to activation of myosin II which brings about strong actomyosin contractility (Rodriguez-Hernandez et al. 2016). Sanz-Moreno et al, showed that JAK1-STAT3 co-ordinates with Rho/ROCK signaling in controlling amoeboid migration and proposed that JAK-STAT3 are under the influence of pp-MLC2 which, upon activation, acts in a positive feedback mechanism to further stimulate Rho/ROCK and thereby increasing actomyosin contractility (Sanz-Moreno et al. 2011). Data from our lab also indicate that loss of SEPT2 prevents the phosphorylation of MLC2 (Joo, Surka, and Trimble 2007). Accordingly, we would be interested in comparing the phosphorylation levels of both MLC2 and STAT3 in WT and SEPT9 knockout lines by immunoblots and immunostaining of cells growing in 3D collagen gels.

To further support the results of this study with in-vivo data, we would use fluorescently labelled A375m2 with the knockout of the SEPT9 isoforms of interest (based on the in-vitro results) as well as the parental cell line A375P with overexpression of the same genes/isoforms. These cells would be injected into tails of immunocompromised mice, and the degree of metastasis to their lungs assessed. The cells would also be injected subcutaneously into the lower flanks of the mice and the tumor growth monitored, which would act as a control to confirm that changes in lung
tumour formation are not caused by a general increase in tumorigenesis. At 8-10 weeks, mice would be sacrificed and the xenografts would be assessed for SEPT9 both at the invasive fronts and the center of the tumor.

Together, these results would provide us with both in vitro and in vivo evidence for the roles of SEPT9 in modifying the metastatic potential of cancer cells, and may in the long term provide a new therapeutic target for the control of cancer metastasis.
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