Analysis of the Regulation of Syntaxin4 and VAMP2 During Invadopodium Formation and Extracellular Matrix Invasion in Tumor Cells

by

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ABSTRACT

ANALYSIS OF THE REGULATION OF SYNTAXIN4 AND VAMP2 DURING INVADOPODIUM FORMATION AND EXTRACELLULAR MATRIX INVASION IN TUMOR CELLS

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Tumor cell invasion involves targeted localization of proteins required for interactions with the extracellular matrix and proteolysis. The localization of many proteins during these cell-extracellular matrix interactions relies on membrane trafficking mediated in part by SNAREs. A few SNARE proteins are involved in the formation of invasive structures called invadopodia; however, it is unclear how these SNARE proteins are regulated during tumor cell invasion. Munc18c is known to regulate the plasma membrane SNARE Stx4, and here it is shown that Munc18c is required for Stx4-mediated invadopodium formation and cell invasion. Biochemical and microscopic analysis revealed a physical association between Munc18c and Stx4, which was later shown to occur through residues 1 through 29 (N-term), or 1 through 15 (1-15) of Stx4. Invadopodium formation, gelatin degradation, cell invasion and cell surface levels of MT1-MMP and EGFR were found to be reliant on interaction between Syntaxin4 and Munc18c, as expression of residues 1 through 29 or 1 through 15 of Stx4 led to decreases in these processes. Munc18c function was found to contribute to SNARE complex formation between Stx4, SNAP23 and
VAMP2, which led us to investigate VAMP2’s role in invadopodium formation. Knockdown and inhibition of endogenous VAMP2-containing SNARE complexes led to decreased invadopodium formation, gelatin degradation, cell migration and cell invasion. Cdc42 has been characterized as a regulator of VAMP2 function and was previously found to interact with residues 1 through 28 (1-28) of VAMP2. Expression of VAMP2-1-28 led to a decrease in invadopodium formation and gelatin degradation, suggesting that regulation of VAMP2 by Cdc42 is required for cell invasion. Overall, these findings highlight the importance of SNARE regulation during tumor cell invasion.
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<tr>
<td>ADAM</td>
<td>A disintegrin and metalloprotease</td>
</tr>
<tr>
<td>BCS</td>
<td>Bovine calf serum</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BM</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>BoNT</td>
<td>Botulinum toxin</td>
</tr>
<tr>
<td>Cdc</td>
<td>Cell division control</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CopI</td>
<td>Coat protomer 1</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOC</td>
<td>Deoxycholate</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-signal-related kinase</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FL</td>
<td>Full length</td>
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<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>G418</td>
<td>Geneticin</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescence protein</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose transporter type 4</td>
</tr>
<tr>
<td>GRB2</td>
<td>Growth-factor-receptor bound 2</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanosine triphosphate hydrolase</td>
</tr>
<tr>
<td>Habc</td>
<td>Domain with anti-parallel α-helices labelled a, b, and c</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin type G</td>
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<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>kDa</td>
<td>kiloDaltons</td>
</tr>
<tr>
<td>M</td>
<td>Mouse</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen-activated protein kinase kinase</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloprotease</td>
</tr>
<tr>
<td>MT1-MMP</td>
<td>Matrix type 1- Matrix metalloprotease</td>
</tr>
<tr>
<td>Munc18</td>
<td>Mammalian uncoordinated-18</td>
</tr>
<tr>
<td>NA</td>
<td>Numerical Aperture</td>
</tr>
<tr>
<td>Nck</td>
<td>Non-catalytic region of tyrosine kinase adaptor protein</td>
</tr>
<tr>
<td>NSF</td>
<td>N-ethylmaleimide sensitive fusion protein</td>
</tr>
<tr>
<td>NP40</td>
<td>Nonylphenoxypolyethoxylethanol</td>
</tr>
<tr>
<td>N-term</td>
<td>N-terminal amino</td>
</tr>
<tr>
<td>N-WASP</td>
<td>Neuronal Wiskott-Aldrich syndrome protein</td>
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<tr>
<td>ORF</td>
<td>Open reading frame</td>
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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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PAK  p21 activated kinase
PBS  Phosphate buffered saline
PCR  Polymerase chain reaction
PDGF  Platelet-derived growth factor
PFA  Paraformaldehyde
PG  Proteoglycan
PI3K  Phosphatidylinositol 3-kinase
PKC  Protein kinase C
PLL  Poly-l-lysine
PMA  Phorbol-12-myristate-13-acetate
PVDF  Polyvinylidene fluoride
Rb  Rabbit
Rac  Ras-related C3 botulinum toxin substrate
Ras  Rat sarcoma
RNA  Ribonucleic acid
RNAi  Ribonucleic acid interference
SDS  Sodium dodecyl sulfate
Sec  Secretory
Sec1  Protein transport protein SEC1
S.E.M.  Standard error of the mean
SH  Src homology domain
SNAP23  Synaptosomal-associated protein 23
Stx4  Syntaxin4
Stx4-1-15  Residues 1 through 15 of Stx4
Stx4-15-29  Residues 15 through 29 of Stx4
Stx4-1-29  Residues 1 through 29 of Stx4
siRNA  Short interfering ribonucleic acid
SNAP  Synaptosomal-associated protein
SNARE  Soluble N-ethylmaleimide-sensitive factor attachment protein
Receptor
Src  Avian sarcoma
T567E  Mutation of residue 567 from a threonine to a glutamic acid
TBST  Tris buffered saline with Tween-20
TKS4  Tyrosine kinase substrate with four SH3 domains
TKS5  Tyrosine kinase substrate with five SH3 domains
Tris  2-Amino-2-(hydroxymethyl)-1,3-propanediol
TX-100  Triton X-100
WASP  Wiskott-aldrich syndrome protein
VAMP  Vesicle-associated membrane protein
VAMP2-1-28  Residues 1 through 28 of VAMP2
1 Introduction

1.1 Overview

Cell invasion and migration are crucial components to the homeostasis of multicellular organisms and are integral parts of key physiological processes including embryogenesis, and the maintenance of tissue architecture (Moissoglu and Schwartz, 2006). The ability of cells to invade and migrate through their surroundings is not only important for development, but can be a primary contributor to the progression of disease (Ridley et al., 2003). When cell migration and cell invasion are not properly regulated, diseases and disorders can arise, examples of which include multiple sclerosis and cancer (Vicente-Manzanares et al., 2005).

The extracellular matrix (ECM) is a network composed of proteins and carbohydrates that provide biochemical and structural support to cells. Cellular adhesion to the ECM provides a physical connection between the cytoskeleton and the ECM, allowing organization and maintenance of cell and tissue architecture (Rozario and DeSimone, 2010). It is possible for cells to move within an ECM, but this typically requires degradation of the ECM by proteases, allowing cells to migrate through the ECM barrier. This process is a well-known part of gastrulation and is required in cellular processes like intestinal morphogenesis in vertebrates (Bonnans et al., 2014a). The same process is also utilized by some tumor cells during tumor metastasis, in which a primary tumor establishes a secondary tumor at another site in the body (Sonbol, 2018). Tumor cell invasion through the ECM barrier is a multistep process consisting of cell adhesion, ECM proteolysis and cell migration (Bravo-Cordero et al., 2013). The molecular mechanisms
regulating cell invasion and migration are a key area of research in order to develop new therapeutics for cancer. One mechanism that some tumor cells use to degrade the surrounding ECM involves the formation of invadopodia. These subcellular structures are actin-driven cell membrane protrusions, in which cell ECM receptors and proteolytic enzymes support their formation and ECM-modifying activities. Invadopodia have not only been studied in vitro (Artym et al., 2015; Ondrej et al., 2010), but there is growing evidence for their formation and function in vivo (Clark et al., 2009; Lohmer et al., 2014).

In order for invadopodia to form, the necessary proteins need to be trafficked to the plasma membrane. The molecular mechanisms controlling the trafficking of these proteins is an active area of study and has been found to involve SNAREs. SNAREs are involved in a variety of membrane trafficking events, and function to localize and fuse vesicles with their target membranes (Hong, 2005). Previous studies have highlighted that SNAREs are required for the trafficking of the key protease, membrane-type 1 matrix metalloproteinase (MT1-MMP), to invadopodia, as part of invadopodia formation and cell invasion (Kean et al., 2009; Williams and Coppolino, 2014; Williams et al., 2014a). The mechanisms that regulate SNARE function in this context are not well understood; however, research surrounding this topic may lead to increased understanding of invadopodium formation, and tumor cell invasion. By understanding the mechanism of SNARE regulation during tumor cell invasion, the development of therapies that could reduce ECM degradation and invasion by tumor cells could be developed, leading to decreased metastasis.
1.2 The Extracellular Matrix

The ECM is the non-cellular material present within all organs and tissues. It is secreted and organized by cells, provides cells with physical support, plays a central role in tissue architecture and contributes to biochemical signaling (Frantz et al., 2010). The ECM is composed of water, protein and polysaccharides, and each tissue contains an ECM that is unique, which alters its biochemical and biophysical interaction with cells (Harburger and Calderwood, 2009). Cellular adhesion to the ECM is mediated by ECM receptors, like integrins (Schmidt and Friedl, 2010), regulates cytoskeletal coupling to the ECM, and is required for cell migration. The ECM is a highly dynamic structure that needs to be constantly remodeled and the molecular components of the ECM are subjected to a variety of post-translational modifications. Morphological organization and physical function of the ECM occurs by binding of growth factors to cell surface receptors to conduct signal transduction (Frantz et al., 2010). The types of ECMs and their structure will be discussed in the next section, illustrating its importance in tumor cell invasion.

1.2.1 Extracellular Matrix Structure

The ECM is composed of two classes of macromolecules, proteoglycans and fibrous proteins (Järveläinen et al., 2009) and can be broken up into two types: basement membrane (BM) and interstitial matrix (Bonnans et al., 2014b). The interstitial matrix surrounds cells and is composed mainly of collagen I and fibronectin, which provide structural scaffolding for tissues. The BM is more compact than interstitial matrix and is composed of collagen IV, laminins, and proteoglycans (PGs). Proteoglycans fill up most of the extracellular cellular space and have a wide variety of functions, such as a role in
glomerular filtration and wound healing (Ghatak et al., 2015; Järveläinen et al., 2009). Proteoglycans are composed of glycosaminoglycan (GAG) chains covalently linked to a specific protein core, and are classified according to their core proteins, localization and GAG composition. The three main families of proteoglycans are small leucine rich proteoglycans, modular proteoglycans and cell-surface proteoglycans. The GAG chains on the protein core are unbranched polysaccharide chains that can be divided into sulfated and non-sulfated GAGs. These molecules are hydrophilic and enable matrices that can withstand high compressive forces (Schaefer and Schaefer, 2010). Some examples of proteoglycans are glycosaminoglycan hydrogel, hyaluronic acid, perlecan and aggrecan (Frantz et al., 2010). Fibrous proteins are also ECM components and include collagen, elastins, fibronectin and laminins. Collagen is the most abundant fibrous protein, and is required for tensile strength, regulation of cellular adhesion and to support chemotaxis and migration (Rozario and DeSimone, 2010). Elastin is another fibrous protein that provides recoil to tissues that need to undergo repeated stretch and are covered by glycoprotein microfibrils, which help elastin maintain its integrity (Wise and Weiss, 2009). Fibronectin is involved in directing the organization of interstitial ECM and has a crucial role in mediating cell attachment and function (Smith et al., 2007).

There are a variety of different types of ECM, including normal, aged, and tumor. Normal ECM contains collagen I, fibronectin, elastin and proteoglycans, like hyaluronic acid and aggrecan. The ECM in normal tissues is composed of a meshwork of type I and type III collagens, elastin and fibronectin that form a relaxed network of fibers and are
surrounded by PGs. The relaxed nature of collagen and elastin fibers allow for healthy ECM to resist a wide range of tensile stresses, allowing the ECM to be highly dynamic. Tissue homeostasis is mediated by secretion of matrix metalloproteinases (MMPs) and the controlled activity of other enzymes. As tissue ages, the levels of proteins that connect cells together, such as cadherins, decrease, causing gaps between epithelial cells. Older tissue is characterized by the thinning of the BM and has elevated levels of fibronectin and MMPs and a decrease in PGs. Tumors generally have a stiffened ECM, and an increase in collagen types I, III and IV, fibronectin, elastin and PGs. MMPs that are secreted and activated by tumor cells remodel the BM surrounding the tumor, and release and activate growth factors. By releasing these growth factors vascular permeability is enhanced, causing new vessel growth, and increased interstitial tissue pressure (Bonnans et al., 2014a). Tumor associated ECM stiffening, reciprocal ECM resistance induced by resident tumor cells, and cell-generated contractility causes a positive feedback loop for tumor growth and survival, which could eventually lead to invasion and metastasis (Butcher et al., 2009; Levental et al., 2009).

1.2.2 Extracellular Matrix Degradation by MMPs

The ECM provides biochemical and structural support to migrating cells; however, it also acts as a physical barrier that deters migration. To move through the ECM, cells utilize proteases to degrade it (Lohmer et al., 2014). MMPs are a family of zinc-dependent endopeptidases that play a crucial role in tissue remodeling, organ development (Page-McCaw et al., 2007), and in diseases such as cancer (Egeblad and Werb, 2002; Hanahan and Weinberg, 2011). There are 23 MMPs expressed in humans which have a conserved
structure consisting of a pro-peptide domain, catalytic domain and a hemopexin-like C-terminal domain. MMPs are first enzymatically inactive and removal of the pro-domain allows protease activity (Sternlicht and Werb, 2001). MMPs were historically thought to primarily degrade structural components of the ECM; however, they also promote cancer progression by cleaving a diverse group of substrates, including growth-factor-binding proteins, growth factor precursors, receptor tyrosine kinases, as well as cell-adhesion molecules (Egeblad and Werb, 2002). Another family of proteins involved in ECM degradation are the a disintegrin and metalloproteinases (ADAM) proteins, which are involved in fertilization, development, as well as cancer (Edwards et al., 2009). Most ADAMs are membrane anchored and function in the pericellular space and only half of them exhibit proteolytic activity (Edwards et al., 2009). The substrates for ADAM metalloproteases are similar to MMPs, and include cell adhesion molecules, ECM proteins, and growth factors. ADAMs have also been shown to play a role in the transactivation of EGFR, and have been implicated in shedding of six out of the seven known EGFR ligands (Giebeler and Zigrino, 2016).

Members of the MMP family are multifunctional zinc-dependent endopeptidases that can degrade a variety of ECM components. Most MMPs are secreted, but 6 out of the 25 are membrane anchored. The expression of MMPs has been linked to highly aggressive cancers, such as melanoma, ovarian and breast (Sato et al., 2005; Seftor et al., 2001; Sodek et al., 2007; Zucker et al., 1999). MT1-MMP is membrane anchored, has been shown to promote tumor growth and local invasion (Hotary et al., 2003) and is key for the high infiltration capacity of glioblastoma (Annabi et al., 2009). MT1-MMP is also
enriched at invadopodia (Poincloux et al., 2009). MT1-MMP is converted catalytically to an active enzyme by proteolytic cleavage by furin in the trans-Golgi network before it arrives at the plasma membrane. MT1-MMP is able to degrade the ECM through proteolytic cleavage of collagen I, II and III, laminins 1 and 5, fibronectin, vitronectin, and can also activate MMP2 to indirectly degrade the ECM (Itoh, 2006). Once MT1-MMP is trafficked to the plasma membrane through a Rab-8 dependent pathway, it mediates the proteolytic degradation of ECM proteins during cell invasion (Bravo-Cordero et al., 2007). MT1-MMP proteolytic activity can be halted through internalization, which occurs through clathrin-mediated and caveolin-mediated recycling pathways (Gálvez et al., 2004; Remacle et al., 2003). MT1-MMP can also be recycled back to the plasma membrane (Wang et al., 2004), which was found to occur in a vesicle-associated membrane protein 7 (VAMP7)-dependent manner (Williams and Coppolino, 2011a).

Apart from needing to be cleaved by furin for enzymatic activity, MT1-MMP can also be regulated by phosphorylation, which affects its function and trafficking (Williams and Coppolino, 2011a). MT1-MMP has 3 phosphorylation sites on its cytoplasmic tail (Sato et al., 2005), and experiments expressing phosphorylation mutants showed that recycling of MT1-MMP is phosphorylation dependent (Williams and Coppolino, 2011a). Mutation of threonine 567 to a glutamic acid (T567E) mimicked a permanently phosphorylated version of MT1-MMP. Experiments expressing this mutated version of MT1-MMP led to increased cell invasion and migration; however, internalization and recycling of MT1-MMP-T567E was normal. This work highlighted the importance of MT1-MMP phosphorylation for its recycling and its role during cell invasion.
1.3 Cell Invasion

ECM remodeling is important for many physiological processes and is tightly regulated, especially in developmental processes like epithelial branching morphogenesis, stem cell maintenance and differentiation, as well as skeletal development. Cell invasion is also important for many physiological processes, such as embryogenesis and wound healing (Murphy and Courtneidge, 2011). When ECM remodeling proteins are deregulated, diseases such as cancer can manifest, leading to abnormal cell invasion. Tumor cells utilize similar mechanisms to breakdown their surrounding ECM in order to migrate; however, these events are regulated differently compared to normal cells (Vicente-Manzanares et al., 2005). Cell invasion and metastasis can be summarized in a few steps: invasion of the basement membrane, detachment of cells from the primary tumor, intravasation, extravasation, arrest and growth at a secondary location (Figure 1.1) (Miki et al., 2005). The next section will focus on our current understanding of cell invasion, which will be described in two main steps: cell adhesion and migration through the ECM.
Figure 1.1 – Cell-ECM interactions during metastasis.

Cell invasion begins by the primary tumor degrading the surrounding ECM, intravasating into the blood stream or lymphatic system, extravasating, arresting at another location and continuing growth.
1.3.1 Cell Adhesion

Cellular adhesion to the ECM is required for a variety of cellular processes, and involves five groups of adhesion molecules (Harjunpää et al., 2019). These five groups of adhesion molecules are known as integrins, selectins, cadherins, and members of the immunoglobulin superfamily including nectins and mucins. Previous findings have highlighted an important role of integrin mediated signaling during tumor cell invasion and cell adhesion and will therefore be discussed more in depth (Harjunpää et al., 2019; Williams and Coppolino, 2014).

Integrins are large heterodimers that consist of $\alpha$- and $\beta$- chains that interact to form an intact receptor in the plasma membrane (Figure 1.2). Integrins bind a wide variety of ligands in the extracellular matrix, on other cells, and soluble proteins. Both $\alpha$- and $\beta$- integrins have a single pass hydrophobic transmembrane domain, a large N-terminal extracellular domain, and a short C-terminal cytoplasmic domain (Harjunpää et al., 2019). The extracellular domains of the $\alpha$- and $\beta$- subunits together form a ligand-binding domain that recognizes ECM substrates (Campbell and Humphries, 2011). The intracellular cytoplasmic domain then transmits intracellular signals through the recruitment of integrin-binding proteins, adaptor proteins, and signaling proteins as a part of focal adhesions. Integrin signaling can be bidirectional, meaning it can be either outside-in or inside-out. Outside-in signaling results from a ligand binding to the extracellular domain, causing a conformational change that exposes the cytoplasmic tail binding sites. Inside-out signaling occurs from a signal inside the cell, involving the integrin cytoplasmic domains, resulting in a conformational change exposing the ligand binding site (Campbell
and Humphries, 2011). Scaffolding proteins bind to the integrin cytoplasmic tails and actin filaments, resulting in the anchoring of the cell cytoskeletal network to the ECM (Campbell and Humphries, 2011). Signaling proteins, including the Src-family protein tyrosine kinases (Harburger and Calderwood, 2009), are recruited to activated integrins and influence cellular processes such as gene expression, cell survival, and cytoskeleton remodeling (Moissoglu and Schwartz, 2006; Ridley et al., 2003).

Intracellular signaling can be regulated by integrins. Integrin (α and β) binding to ligands in the ECM activates focal adhesion kinase (FAK), which binds multiple signaling proteins, including Src and growth-factor-receptor-bound protein 2 (GRB2). FAK autophosphorylation allows it to bind GRB2 and activates RAS. FAK activation also promotes Src-dependent phosphorylation of SHC, leading to GRB2 recruitment and RAS activation. Activation of RAS recruits RAF to the cytoplasmic membrane where it is activated by Src, leading to mitogen-activated protein kinase kinase (MEK) and extracellular-signal-regulated kinase (ERK) activation. RAS also activates phosphatidylinositol 3-kinase (PI3K) and RAF. Activated Src leads to RAC activation, which in conjunction with active CDC42, can regulate many biochemical pathways, including p21-activated kinase (PAK). PAK is able to activate RAF’s kinase activity. MEK can phosphorylate and activate ERK, leading to transcriptional activity, and alterations in integrin affinity for ligands. Activation of these signaling molecules through integrin mediated signaling results in cytoskeletal alterations, contraction, gene transcription and integrin modulation (Hood and Cheresh, 2002).
Integrin signaling induces numerous signaling pathways to control cell migration and invasion. Integrin binding to ligands in the ECM activates downstream signaling pathways, including FAK and PKC. Activating these molecules can lead to alteration of cytoskeletal components, contraction, gene transcription and integrin modulation. Figure adapted from Hood and Cheresh, 2002.

Figure 1.2 – Intracellular signaling by integrins
1.3.2 Cell Migration

Cellular migration is important in many physiological cell processes, such as morphogenesis during embryonic development, the renewal of skin and intestine in adults, as well as tissue repair (Ridley et al., 2003). Cell migration also contributes to pathological processes, including cancer (Bravo-Cordero et al., 2013). Cell migration can be broken down into four key steps, the first of which is membrane extension at the leading edge of the cell, then cell adhesion with the ECM, contraction of the cellular body, and lastly, detachment at the rear of the cell (Figure 1.3). Initial response of a cell to a migration-promoting agent causes the cell to extend protrusions in the direction of migration. These protrusions are known as lamellipodia and are driven by actin polymerization and stabilized by adhering to the ECM. At the leading edge of the cell, actin containing protrusions are produced, and adhesive contacts must form to prevent retraction of the newly created membrane protrusion. This is facilitated by focal adhesions, which link the cytoskeleton to the ECM. Actomyosin-based forces that are directed against the ECM then lead to contraction of the cell body. Adhesions that were initially produced at the leading edge of the cell eventually reach the rear end as the cell body translocates forward. The older focal adhesions are then disassembled in response to actomyosin contraction or protease-mediated degradation (Ridley et al., 2003).
Figure 1.3 - Two-dimensional cell migration.

A) Cell migration is initiated by actin-dependent protrusions of the cell’s leading edge, which is composed of structures called lamellipodia and filopodia. These protrusions contain actin filaments, with elongating barbed ends pointing towards the plasma membrane. B) As the cell moves, the plasma membrane sticks to the surface at the leading edge. C) The nucleus and the cell body are pushed forward through intracellular contraction force that is mediated by stress fibers. D) Retraction occurs at the rear of the cell and pulls the cell forward.
1.4 Invadopodium Formation

The first demonstration of invadopodia was in 1980 by David-Pfeuty and Singer, in which chicken embryo fibroblasts were transformed with v-src resulting in re-localization of cytoskeletal proteins, such as vinculin and α-actinin, from focal adhesions to form circular puncta (David-Pfeuty and Singer, 1980). In 1985, it was shown that these vinculin and α-actinin rich protrusions at the ventral membrane contained actin and tyrosine phosphorylated proteins and were sites of cell-ECM adhesion (Tarone et al., 1985). The same year it was determined that at these protrusions the ECM was degraded (Chen et al., 1985), and these v-Src enriched sites were podosomes. Due to their degradative nature these structures were called invadopodia (Chen, 1989), and were eventually found in human cancer cell lines. Invadopodia were defined to be membrane protrusions that extend into the ECM to provide localized degradation in tumor cells. Similar structures have been found in normal cell types, known as podosomes; however, they are shorter lived compared to invadopodia (Murphy and Courtneidge, 2011). This section will highlight the four steps of invadopodium formation, which are initiation, assembly, maturation, and disassembly (Figure 1.4).

1.4.1 Invadopodium Initiation and Assembly

Invadopodia are initiated by stimulation with growth factors, such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) (Miki et al., 2005; Varon et al., 2006; Yamaguchi and Condeelis, 2007). These growth factors stimulate downstream signaling pathways, and for example include Src and protein kinase C (PKC), resulting in F-actin nucleation. Src is able to turnover focal adhesions, then binds
and phosphorylates Tyr kinase substrate with five SH3 domains (TKS5), PI3K and PKC (Block et al., 2008; Murphy and Courtneidge, 2011). Lastly, TKS5 colocalizes with cortactin at invadopodial precursors (Oser et al., 2009). Cortactin modulates the actin cytoskeleton in association with Arp2/3 and neural Wiskott-Aldrich syndrome protein (N-WASP) (Artym et al., 2011) and regulates the secretion of MMPs at invadopodia (Clark and Weaver, 2008). Cortactin binding of N-WASP, facilitates N-WASP-mediated activation of Arp2/3, and recruitment of NCK1 to invadopodia (Oser et al., 2009). Cortactin interacts with coflin, preventing actin severing. Once coflin is phosphorylated, it is released and causes an increase in free barbed ends and actin polymerization at invadopodia (Oser et al., 2009). TKS5 interacts with NCK1, NCK2, GRB2 and N-WASP (Oikawa et al., 2008; Stylli et al., 2009). Src phosphorylation of TKS5 results in a direct interaction of TKS5 with the NCK1/2 SH2 domain, recruitment of NCK to invadopodia and stimulation of actin assembly (Murphy and Courtneidge, 2011). TKS5 can also stimulate actin assembly by binding GRB2. TKS5 proteins regulate actin assembly through association of TKS5’s SH3 domain with N-WASP, which is thought to be dependent on NCK binding. GRB2 SH3 domains bind N-WASP, promoting actin nucleation and coordinated recruitment and binding of NCK and GRB2 to N-WASP (Shen et al., 2007).

1.4.2 Invadopodia Maturation

Invadopodia maturation following TKS5 and cortactin recruitment is not well characterized. Stabilization of invadopodia has been shown by the dephosphorylation of cortactin, resulting in cortactin binding to coflin and blocking F-actin severing activity (Oser et al., 2009). Maturation of invadopodia is achieved through the proteolytic ability
of MMPs, and the localization of MT1-MMP at invadopodia is a key step in ECM degradation (Murphy and Courtneidge, 2011). The localization of these proteases partially coordinated by cortactin, Tyrosine kinase substrate with four SH3 domains (TKS4) and β1 integrin. Invadopodia are considered mature upon MMP delivery, and invadopodia can remain for hours by dephosphorylation of cortactin, which blocks the severing ability of cofilin, enabling invadopodium formation stabilization (Miki et al., 2005; Oser et al., 2009). Cortactin expression has also been shown to affect MT1-MMP localization at the plasma membrane and MMP2 and MMP9 secretion. It is possible that cortactin has a role in regulating trafficking from the trans-Golgi network to invadopodia, or in the tethering of vesicles to sites of invadopodia (Clark et al., 2007). Integrins have also been shown to play a role in invadopodia stabilization and maturation through their interaction with MT1-MMP. β1 integrin clustering with MT1-MMP at the plasma membrane leads to decreased endocytosis of MT1-MMP, increasing MT1-MMP cell surface amounts (Foster et al., 1998); however, this has not been shown at invadopodia.

1.4.3 Invadopodia Disassembly

Invadopodia are relatively long-lived structures and can be maintained for hours, and there is much to be learned about their disassembly and turn over. Research suggests that the F-actin cytoskeleton is disassembled and the core structures remain, including MT1-MMP and cortactin (Murphy and Courtneidge, 2011). It is known that Tyr phosphorylation of TKS5 is crucial for formation of invadopodia, and Tyr phosphorylation of cortactin promotes invadopodium turnover (Dorfleutner et al., 2008). Phosphatases may play a key role in invadopodium turnover, and an example of this is Protein Tyr
phosphatase-Ɛ, which has a positive role in osteoclasts through the dephosphorylation and subsequent activation of Src. The turnover of invadopodia has also been shown to be dependent on proteases, such as the Ser protease calpain that cleaves talin and WASP, resulting in podosome turnover (Aran et al., 2011); however, this mechanism has not been studied in invadopodia.
Figure 1.4 - The steps of invadopodium formation.

Cells initially establish adhesion with the ECM through integrins and stimulate signaling pathways through growth factor receptor tyrosine kinases (RTKs). Assembly begins by F-actin formation and branching by N-WASP and cortactin establishing protrusions of the cell membrane into the extracellular matrix. Trafficking of MT1-MMP facilitates invadopodium maturation through degradation of extracellular matrix components. F-actin core disassembly begins through changes to actin modifying proteins, which initiates invadopodia disassembly by the recycling of invadopodial proteins. This leads to subsequent cellular migration through the degraded extracellular matrix.
1.5 Membrane Trafficking

Cell invasion and migration require the precise localization of proteins. In order for these proteins to be delivered at various endosomal compartments and plasma membrane locations, their trafficking must be coordinated. Soluble NSF attachment protein receptors (SNAREs) play a key role in these trafficking events and this section will review SNARE structure and function, SNARE’s roles in cell migration and invasion, as well as how SNAREs are regulated.

1.5.1 SNARE Structure and Function

SNAREs function in an extensive number of membrane trafficking and fusion pathways and 38 SNAREs have been identified in humans. SNAREs can be classified as a vesicle (v-) or target (t-) membrane SNARE; however, they are more commonly classified as either R- or Q-SNAREs, based on conserved arginine or glutamine residues located in their SNARE motif, respectively (Figure 1.5). Most R-SNAREs are located on vesicles, while Q-SNAREs are generally found on target membranes, such as the plasma membrane and endosomes (Jahn and Scheller, 2006).
SNAREs can be divided into two subtypes, target-SNAREs (t-SNARE) or vesicle-SNAREs (v-SNAREs), based on their location. t-SNAREs are located on target membranes, which includes the plasma membrane, and are also known as Q-SNAREs. Examples of t-SNAREs include Syntaxin4, Syntaxin13, SNAP23, and SNAP23. v-SNAREs are located on vesicles and are also known as R-SNAREs. Examples of v-SNAREs include VAMP2, VAMP3, VAMP7 and VAMP8. SNAREs that were studied in this work are indicated with an asterisk.
SNAREs can be divided into three general types, and an overview of their structure can be seen in Figure 1.6. Syntaxins, classified as Q-SNAREs, are localized to a variety of membrane compartments in several trafficking pathways (Yu Hsuan Teng et al., 2001). Syntaxin4 (Stx4) is mostly found at the plasma membrane (Chen and Scheller, 2001) and has a demonstrated role in the fusion of glucose transporter type 4 (GLUT4) vesicles with the plasma membrane during insulin signaling (Brandie et al., 2008). The second type of SNAREs are synaptosome-associated proteins (SNAP), which have two SNARE domains and belong to the family of Q-SNAREs. SNAP proteins have a flexible linker region and are membrane anchored by palmitoylation. Lastly, vesicle-associated membrane proteins (VAMP) are members of the R-SNARE family and contain a C-terminal membrane domain, a SNARE motif and a N-terminal domain (Hong, 2005). As Stx4 and VAMP2 are the main focus of this research, the specifics of their structures will be further discussed.

SNAREs are generally small proteins that range from 100-300 amino acids in length, and at their core they contain an evolutionally conserved SNARE motif. Most SNAREs also contain a hydrophobic C-terminal transmembrane domain that assists in anchoring the SNARE in the membrane. Another important domain of SNAREs is their N-terminal regulatory region (Chen and Scheller, 2001; Hong, 2005; Hong and Lev, 2014). For example, residues 1-29 of Syntaxin4 are required for regulation by Munc18c (Latham et al., 2006), while residues 1-28 of VAMP2 bind to Cdc42 (Nevins and Thurmond, 2005). Most SNAREs also have an extended N-terminal domain, containing coiled-coil regions, as well as additional structural features. Syntaxin SNAREs have three-
helical bundles, preceded by an N-terminal domain (Fasshauer, 2003) and are known as $H_a$, $H_b$, and $H_c$ domains. These $H_a$, $H_b$, and $H_c$ domains can fold back to interact with the C-terminal SNARE motif, generating a closed conformation (Dietrich et al., 2003; Fasshauer, 2003; Misura et al., 2000), that needs to be opened by regulators before or after the assembly of the SNARE complex. Longin domains are found at the N-terminal region of v-SNAREs, like VAMP7. These longin domains can also adopt a folded back or closed conformation in which the longin domain binds to the C-terminal SNARE domain (Tochio et al., 2001). Interestingly, VAMP2 does not contain this longin domain; however, its N-terminus is known to be subjected to regulation by Cdc42 (Nevins and Thurmond, 2005).
Figure 1.6 - SNARE Structure

Most SNAREs structure consists of an N-terminal region, a SNARE domain, and a transmembrane domain. Other SNAREs, including SNAP23 and SNAP25, contain an N-terminal region, and two SNARE domains connected by a linker region. Some SNAREs have varying N-terminal domains. For example, SNAREs like Syntaxin4 and Syntaxin13 contain an N-terminal domain, as well as Ha, Hb, and Hc domains. VAMP7 also has a different N-terminal domain, which is known as a longin domain. Figure adapted from Hong, 2005.
SNAREs function in membrane fusion by bringing vesicles in close proximity to target membranes, and this occurs in a few steps (Figure 1.7). Vesicles are positioned at the region of the target compartment where Q-SNAREs are located. Tethering factors act over a longer distance than SNAREs and interact with both the vesicle and target compartment to facilitate subsequent pairing of the R-SNARE with its appropriate Q-SNAREs (Gillingham and Munro, 2003; Whyte and Munro, 2002). This allows the R- and Q-SNAREs on opposing membranes to interact, forming a trans-SNARE complex (Chen and Scheller, 2001; Jahn et al., 2003). Before complex formation, SNARE domains are monomeric and unstructured (Jahn and Scheller, 2006). During the formation of the trans-SNARE complex, the SNARE motifs become highly organized into a four-helical bundle, which releases energy to overcome the energy barrier for membrane apposition (Hong, 2005). After fusion, the complex becomes a cis-SNARE complex and is now located on the target compartment membrane. Disassembly of this complex requires a considerable amount of energy, which is provided by N-ethylmaleimide-sensitive factor (NSF) and the adaptor protein α-SNAPs (soluble N-ethylmaleimide-sensitive factor attachment protein). α-SNAPs binds to the SNARE complex and recruits NSF, and uses its ATPase activity to separate the complex (Jahn and Scheller, 2006). After complex disassembly, the R-SNARE can be recycled to another compartment by retrograde transport, while the Q-SNAREs are re-organized into cognate Q-SNAREs (Hong, 2005).
Figure 1.7 - SNARE-mediated membrane fusion.

SNARE complex assembly and vesicle docking as the SNARE motif of an R-SNARE (or v-SNARE, in blue) forms a complex with two other SNARE motifs from a SNAP family member (in purple) and one from Syntaxin (red). Trans-SNARE complex formation results from zippering of the four SNARE motifs. All SNAREs are located on the same target membrane, forming a cis-SNARE complex. Cargo and membrane are delivered to the appropriate membrane.
1.5.2 The Importance of SNAREs During Cell Migration and Invasion

Previous findings have highlighted an important role for SNAREs in cell migration, invadopodium formation, and cellular invasion. In 2009 it was shown that the SNARE complex consisting of VAMP3, Syntaxin13 and SNAP23 are involved in secretion of MMPs, and degradation of the ECM in vitro (Kean et al., 2009). The functional roles of SNAREs during cell invasion were determined by expressing mutant forms of SNAREs in HT-1080 fibrosarcoma cells, which are capable of forming invadopodia in vitro. These mutant forms of VAMP3 and Syntaxin13 lacked their transmembrane domains, leaving them as cytosolic proteins. This allows them to bind endogenous SNAREs competitively and inhibit membrane fusion events. Using these constructs illustrated that VAMP3 and SNAP23 are necessary for MMP2 and MMP9 secretion as determined by gelatin zymography, and similar results were seen for MT1-MMP using flow cytometry. Cells expressing the cytoplasmic constructs were also plated onto fluorescently labeled gelatin to view matrix degradation and it was determined that function of SNAP23, Syntaxin13 and VAMP3 containing SNARE complexes are required for matrix degradation (Kean et al., 2009).

The SNARE complex consisting of SNAP23, Syntaxin4 and VAMP7 was also investigated, and results revealed its involvement in matrix degradation (Williams et al., 2014a). Previous studies had showed that VAMP7 was necessary for MT1-MMP recycling, and blocking VAMP7 function decreased cell migration and invasion (Williams and Coppolino, 2011a). In order to determine if Syntaxin4, SNAP23 and VAMP7 were required for invadopodium formation, cytoplasmic constructs as described above were
used to block endogenous SNARE complex formation. MDA-MB-231 cells are a well-studied model for metastatic breast cancer and are used to analyze invadopodium-based invasion in vitro. Cells that expressed SNAP23Δ9, Stx4-Cyto or VAMP7-Cyto had decreased invadopodium formation compared to expression of their full-length counterparts. Cell invasion was also decreased in the cells expressing these constructs, highlighting a role for these SNAREs in invadopodium formation and cell invasion (Williams et al., 2014a).

The formation and maturation of invadopodia was found to be dependent on the SNAREs SNAP23 and Syntaxin13 (Williams and Coppolino, 2014). The epidermal growth factor receptor (EGFR), Src and β1 integrin were found to associate in a manner that was dependent on the function of SNAP23 and Syntaxin13, leading to phosphorylation of EGFR on Tyr845. The interaction of SNAP23 and Syntaxin13 was found to be regulated by β1 integrin, and β1 integrin knockdown decreased invadopodia mediated ECM degradation and cell invasion. These results pointed to a mechanism in which activation of β1 integrin and SNARE-mediated trafficking of Src is necessary for early stages of invadopodium formation (Williams and Coppolino, 2014).

Vesicular trafficking is essential for a number of cellular processes that include membrane fusion, such as fertilization, cell division, maintenance of subcellular compartments, and neurotransmitter release (R. Hepp and K. Langley, 2001). SNAREs have also been shown to contribute to cancer malignance through their role in chemoresistance. For example, in human ovarian cancer cells, Stx6 and VAMP4 were co-localized with a P-type ATPase and contributed to the secretory vesicular transport of
cisplatin from the Golgi to the plasma membrane, promoting cell growth (Meng and Wang, 2015). SNAREs are also involved in mediating inflammation involved in cancer development (Misura et al., 2000). VAMP2 and VAMP7 have been found to play a role in the release of pro-inflammatory cytokines in neutrophils. These cytokines are then able to induce MMP expression, leading to increased invasion and metastasis (Stow et al., 2006). Clinical studies have also shown that human tumour tissues express VAMP, syntaxins and SNAP-25 and certain SNARE isoforms were found to be associated with the development of tumors. For example, in normal parathyroid tissue, SNAP-25 and Stx1 are not expressed, but in parathyroid carcinoma samples both SNAREs were upregulated, and mutated versions of both SNAREs were found (Lu et al., 2008).

1.6 Regulation of SNAREs

The regulation of SNAREs occurs at a variety of levels and is required for the precise delivery and recycling of cellular material. First, two target (Q-) SNAREs interact prior to forming a complete trans-SNARE complex with the necessary vesicle (R-) SNARE. This preformation between target membrane SNAREs allows for a more stable and complete complex (Snyder et al., 2006). This sequential interaction provides regulators of SNAREs both spatial and temporal locations to enhance or inhibit SNARE interactions. SNARE proteins are uniquely distributed in different cellular compartments, allowing only SNAREs close to the site of fusion to be activated. Regulation of SNAREs ensures that SNARE complexes form only at sites of fusion, and this is achieved through the interaction of SNAREs and regulatory proteins. Examples of regulatory proteins include Munc18 and synaptotagmin, and in some cases these can prevent vesicles from fusing to the
membrane even after docking (Gerst, 2003). Phosphorylation of SNAREs can also prevent premature SNARE complex formation when SNAREs are not at appropriate locations of membrane fusion (Snyder et al., 2006). The next two sections aim to discuss what is known about two SNARE regulators, Munc18c and Cdc42.

1.6.1 Regulation of Syntaxin4 by Munc18c

Stx4 is a plasma membrane SNARE that contains an N-terminal H_{abc} domain, a flexible linker region, a SNARE motif and a N-terminal membrane anchor. When Stx4 is free of other SNAREs, the linker region and most of its SNARE motif is able to fold back onto its H_{abc} domain, blocking the SNARE motif and preventing binding to SNAP23. It is unknown how Munc18 proteins function, but it is possible that they bind to Syntaxins when they are in a closed conformation and prevent other SNAREs from binding (Dulubova et al., 1999).

Munc18c belongs to the Sec1/Munc18-like (SM) protein family, which are linked to membrane fusion through their association with SNARE proteins. SM proteins are 60-70 kDa polypeptides that are highly conserved (Rizo and Südhof, 2002), and fold into an arch-shaped clasp (Jahn et al., 2003). The first SM protein discovered was Uncoordinated18 (UNC18) in C. elegans (Brenner, 1974), and an orthologue was later identified to be involved in the yeast secretory pathway (Novick et al., 1980). UNC18's mammalian orthologue is Munc18-1, which binds to syntaxin1 (Hata et al., 1993). SM proteins play a key role in membrane fusion, and mutations in SM proteins completely block fusion, while loss-of-function mutations lead to a mostly lethal phenotype. SM proteins usually bind to syntaxins, and this is conserved from C. elegans, to yeast and...
humans. In Munc18-1 knockout mice, syntaxin1 levels were reduced by 70%, indicating physiological relevance of this interaction (Toonen and Verhage, 2003).

The interaction of Munc18c and Syntaxin4 has been previously shown to regulate exocytic trafficking of GLUT4 in muscle and fat cells (Tamori et al., 1998a; Widberg et al., 2003), exocytosis in pancreatic acinar cells (Gaisano et al., 2004) and cytokine transport in macrophages (Pagan et al., 2003). In 2008, Latham et al. cloned and purified residues 1 through 29 of Syntaxin4, as well as full length Munc18c. The purified proteins were used to identify regions of Syntaxin4 that bound to Munc18c in vitro and in vivo. Not only did Latham et al. determine that Munc18c could interact with residues 1 through 29 of Syntaxin4, they found that mutants of Syntaxin4 that did not contain it's N-terminus were unable to bind to Munc18c, highlighting the importance of this N-terminal region (Latham et al., 2006). Key residues for binding were then predicted based on yeast orthologs. Mutation of the hydrophobic Phe119 of Munc18c to a negatively charged glutamic acid caused a decrease in the amount of Syntaxin4 binding to Munc18c. This was believed to have occurred due to the disruption of the hydrophobic pocket of Munc18c in which Syntaxin4 binds. Mutation of Syntaxin4 Leu8 to a positively charged lysine caused a 70% reduction of binding to Munc18c. Also, the expression of Stx4 1-29 led to an inhibition of Munc18c binding to full length Stx4 by 30%, indicating this peptide could be used as a competitive inhibitor. Lastly, it was found that Munc18c could bind to the SNARE complex consisting of Syntaxin4, VAMP2 and SNAP23, but not to the SNARE complex if Syntaxin4 was not included. Munc18c was found to be required for SNARE complex formation between Syntaxin4, VAMP2, and SNAP23 by allowing Munc18c and Syntaxin4 to interact
by incubating them together on affinity beads. This captured complex was then incubated with purified SNAP23 and VAMP2, causing an increase in SNARE complex formation (Latham et al., 2006). Overall, Munc18c’s function is important in SNARE complex formation; however, this regulation has yet to be studied in the context of the SNARE-mediated trafficking that is associated with cancer cell invasion.

1.6.2 Regulation of VAMP2 by Cdc42

Cdc42 is a known regulator of vesicle transport events through its activation of the WASp-Arp2/3 complex during regulation of actin structures. Cdc42 binds to WASp, which relieves WASp autoinhibition, allowing WASp to activate the Arp2/3 complex, leading to actin branching. Actin is important for vesicle trafficking due to its ability to facilitate membrane deformation to drive vesicle formation and fusion (Hong, 2005). Actin is also required for microfilament tracks for motor proteins that transport and generate the force required for vesicle transport. Cdc42 not only participates in membrane dynamics at the cell cortex, but it localizes to the Golgi where it binds coat protomer I (COPI) (Harris and Tepass, 2010), an important component of the machinery involved in the formation of intracellular transport vesicles.

In 2004, Nevins and Thurmond investigated Cdc42 as a potential regulator of VAMP2 function in pancreatic beta cells. Cdc42 was found to associate with VAMP2 by immunoprecipitation (Nevins and Thurmond, 2005). To determine where Cdc42 binds VAMP2, residues 1 through 28, and residues 1 through 56 of VAMP2’s N-terminus were cloned with a GFP tag. CHO-K1 cells were transfected with these N-terminal constructs, and GFP was immunoprecipitated. Immunoprecipitates were probed for Cdc42, and both
constructs were shown to interact with Cdc42, indicating that Cdc42 interacts with residues 1 through 28 of VAMP2’s N-terminus (Nevins and Thurmond, 2005).

VAMP2 is enriched on synaptic vesicles, and is involved in synaptic exocytosis (Hanson et al., 1997) and insulin-stimulated translocation of GLUT4 in adipocytes (Martin et al., 1998). VAMP2 has also been found to co-localize with β1 integrin. siRNA mediated knockdown of VAMP2 decreased cell surface β1 integrin levels without reducing total β1 integrin expression (Hasan and Hu, 2010). VAMP2 knockdown also caused a decrease in cell migration. Altogether, this research highlighted the role of VAMP2 mediated trafficking of β1 integrin, and cell migration (Hasan and Hu, 2010); however, how VAMP2 is regulated during invadopodium formation is unknown. Previous research has shown that Cdc42 acts as a regulator of VAMP2 during insulin exocytosis, and this mechanism remains to be studied during invadopodium formation.

1.7 Rationale and Thesis Objectives

SNARE-mediated trafficking is known to contribute to focal adhesion formation, cell spreading, cell migration, cell invasion, and invadopodium formation (Kean et al., 2009; Tayeb et al., 2005; Williams and Coppolino, 2011a; Williams et al., 2014b). However, little is known about how SNAREs proteins are regulated during these processes. I hypothesize that the regulation of Syntaxin4 and VAMP2 is required for invadopodium formation and extracellular matrix invasion in tumor cells. Research into this area will increase our knowledge of how membrane trafficking is regulated during tumor cell invasion and will enhance our understanding of the metastatic cascade.
The focus of this research is to investigate the regulation of Stx4 and VAMP2 function by Munc18c and Cdc42, respectively, during invadopodium formation and tumor cell invasion. This is being conducted to better understand the mechanisms by which invadopodial proteins are delivered to invadopodia, and where they function to degrade the ECM as tumor cells remodel their local microenvironment.

This thesis has the following objectives:

**Objective 1:** Examine the role of Munc18c regulation on Syntaxin4 during invadopodium formation.

Munc18c will be inhibited by expression of residues 1 through 29 of Stx4 in MDA-MB-231 and HT-1080 cells. The effect of Munc18c regulation on Stx4 during tumor cell invasion will be assessed using techniques that include invadopodium formation assays, gelatin degradation assays, cell invasion assays, co-immunoprecipitation of SNAREs and cell surface levels of invadopodial proteins.

**Objective 2:** Determine if the Stx4/Munc18c interaction can be disrupted using a smaller N-terminal peptide of Stx4.

Stx4’s N-terminus will be split into two sections and expressed in MDA-MB-231 cells to determine if Munc18c can interact with a small portion of Stx4. These smaller portions of Stx4 will then be used to assess their effects on invadopodium formation, gelatin degradation, cell invasion, and trafficking of MT1-MMP and EGFR to the cell surface.
**Objective 3:** Examine the role of VAMP2 during invadopodium formation and ECM invasion.

Inhibition of VAMP2 will be carried out using RNAi-mediated knockdown and through expression of VAMP2-Cyto. The role of VAMP2 will be investigated through invadopodium formation assays, and cell migration and invasion assays. Expression of VAMP2-1-28 will also be used as a competitive inhibitor to determine if Cdc42 regulates VAMP2 during invadopodium formation.
2 Materials and Methods

2.1 Reagents

Reagents and chemicals were purchased from either Fisher-Scientific Ltd. (Nepean, ON) or Sigma-Aldrich Co. (St. Louis, MO, USA), unless otherwise indicated. Primary antibodies were purchased from the following suppliers: Rabbit anti-MMP14, mouse anti-MMP14, rabbit anti-GFP, rabbit anti-Munc18c, rabbit anti-SNAP23 (Abcam: ab3644, ab78738, ab290, ab175238, ab4114); mouse anti-Munc18c, rabbit anti-EGFR (Santa Cruz Biotechnology: sc-373813, sc-03); mouse anti-Syntaxin4, (BD Biosciences: 610439); mouse anti-actin (Pierce: MA5-15739); mouse anti-β1 integrin, mouse anti-β tubulin, mouse anti-GAPDH (Developmental Hybridoma Studies Bank: P4C10, E7, DSHB-hGAPDH-2G7); rabbit anti-VAMP2, rabbit anti-Cdc42, rabbit anti-phospho-ERK1/2, mouse anti-ERK1/2 (Cell Signaling Technology: 13508, 2466, 8544S, 4696S); rabbit anti-FLAG (Millipore-Sigma: F3165). All fluorescently labelled secondary antibodies, Hoechst 33342, and AlexaFluor647-conjugated phalloidin were purchased from Life Technologies (Mississauga, ON). HRP conjugated secondary antibodies were purchased from Bio-Rad (Mississauga, ON). shRNA to VAMP2 was purchased from Millipore Sigma. MT1-MMP inhibitors MAB3329 and NSC 405020 were purchased from Millipore Sigma and Tocris Bioscience, respectively. Cdc42 inhibitor ML141 was purchased from Sigma-Aldrich Co. Antifade fluorescent mounting medium was obtained from DAKO, Inc. (Burlington, ON).
2.2 cDNA Constructs

Stx-FL is a construct of wild-type Stx4 with GFP fused to the C terminus. The GFP moiety is lumenal or extracellular when at the plasma membrane. The Stx-FL construct was cloned from a syntaxin4-myc-myc-His plasmid purchased from Addgene (plasmid 12377) into pEGFP-N1 using Xhol and KpnI. The following oligonucleotides were used as primers: forward Stx-FL (5’- TTCACCTCGAGATGCGGGACAGGACCCAC-3’) and reverse Stx-FL (5’- TTCAGGTACCTCAACCCTGATCGACGCAATG-3’). Stx-FL 3xFLAG was PCR-amplified from pEGFP-N1-Stx-FL (above) and cloned into pcDNA3.1 3xFLAG using HindIII and KpnI. The following oligonucleotides were used as primers: forward Stx-FL 3xFLAG (5’- TTCACAAGCTTATGCGGGACAGGACCCAC-3’) and the same reverse primer as used for GFP-Stx4-FL (above). N-terminal peptide from Stx4 was PCR-amplified from pEGFP-N1- Stx4-FL (above) and cloned into pEGFP-N1 using Xhol and KpnI to create the Stx4 N-terminal peptide fused to GFP (GFP– Stx4–N-term). The following oligonucleotides were used as primers: forward Stx4 N-terminal (5’- TGACGGTAAATGGCCCGCCTGGCATTATG-3’) and reverse Stx4 N-terminal (5’- TTTATCATGGCTACGGGTGCACCACCAGCGCG-3’). Expression of Stx4-FL and Stx-FL were compared with endogenous Stx4 by immunofluorescence microscopy. Expression of Stx4-FL 3xFLAG in parental and Stx-FL stable cells was determined by immunofluorescence microscopy. The N-terminal peptide from Stx3 was PCR amplified from the syntaxin3-myc-myc-His plasmid purchased from Addgene.
(plasmid 12372) and cloned into pEGFP-N1 using HindIII and KpnI to create the plasmid Stx3 N-terminal peptide fused to GFP. The following oligonucleotides were used as primers: forward Stx3 N-terminal (5’-TTCACAAGCTTATGAAGGACCGTCTGGAGCAGCTGAAGG-3’) and reverse Stx3 N-terminal (5’-TTCACGCGTACCCGTGGTTGTAGCAATCTCAACCG-3’). 1-15 and 15-29 from Stx4 was purchased through ThermoFisher (Mississauga, ON) GeneArt subcloning and express cloning service. The two segments of Stx4 were PCR-amplified and cloned into pEGFP-N1 using BamHI and HindII to create the Stx4-1-15 and -15-29 peptides fused to GFP (Stx4-1-15 and Stx4-15-29). The following oligonucleotides were used as primers: forward Stx4-1-15 (5’-TGACGGTAAATGGCCCGCCTGGCATTATG), reverse Stx4-1-15 (5’-TTCACGAATCCTTACTTGTACAGCTCGTCCATGCCGAGA-3’), forward Stx4-15-29 (5’-TTCACGGATCCATGTCGGACGAAGAGGACAAGGAGCGGG-3’) and the same reverse primer as Stx4-1-15. VAMP2 cDNA was purchased from Genscript (Piscataway, NJ) and subcloned into pEGFP-N1 using BamHI and HindIII. The following oligonucleotides were used as primers: forward VAMP2 full length (VAMP2-FL) (5’-TTCACAAGCTTATGTCGGCTACCGCTGCCAC-3’) and VAMP2-FL reverse (5’-TTTCACGATCCATCATCATCATCGTGGCTACCAGC-3’). VAMP2-Cyto was PCR-amplified from the Genscript cDNA and cloned into pEGFP-N1 using BamHI and HindIII. The following oligonucleotides were used as primers: forward VAMP2-Cyto was the same primer as VAMP2-FL, and VAMP2-Cyto reverse (5’ TTTACACGGATCCCCATGCTGATTTCTCAGC-3’). VAMP2 1-28 was PCR-amplified from the Genscript cDNA and cloned into pEGFP-N1 using BamHI and HindIII.
The following oligonucleotides were used as primers: forward VAMP2 1-28 was the same as VAMP2-FL, VAMP2-1-28 reverse: (5’-TTCACGGATCCACTGGTAAGATTTGGAGGAGGTGCAGGG-3’). The cloning of VAMP7-FL, VAMP7-Cyto, MT1-MMP wildtype, and MT1-MMP T567E are described elsewhere (Williams and Coppolino, 2011a). MT1-MMP wildtype 3xFLAG, MT1-MMP T567E 3xFLAG, VAMP2-FL 3xFLAG, VAMP2-Cyto 3xFLAG, VAMP7-FL 3xFLAG, and VAMP7-Cyto 3xFLAG were all subcloned from pEGFP-N1 into pcDNA3.1 3xFLAG using BamHI and HindIII restriction enzymes.

2.3 Cell Culture

MDA-MB-231 and HT-1080 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with 10% BCS. Stable cell lines derived from MDA-MB-231 cells were cultured in selection media comprising DMEM supplemented with 10% BCS and 200 μg/ml G418 (Wisent). Growth conditions were kept at 37°C with humidity and a 5% CO₂, 21% O₂ atmosphere. Cells were lifted using 5 mM EDTA/PBS (pH 7.4).

2.4 Transfections

Cells were transfected using jetPRIME Polyplus (VWR International) according to manufacturer protocol. All transiently transfected constructs were expressed for at least 24 hours. Cells were transfected with 50 nM siRNA and underwent knockdown for 48 hours. Co-transfections were performed using a 1:10 molar ratio of marker pEGFP-N1 plasmid to siRNA for a total of 48 hours.
2.5 Generation of stable cell lines

In a 10 cm tissue culture plate, cells were transfected with either Stx4-FL pEGFP-N1, Stx4 N-terminal pEGFP-N1, Stx4 1-15 pEGFP-N1, Stx4 15-29 pEGFP-N1 or pEGFP-N1 alone. After 24 hours, transfected cells and non-transfected cells were lifted in selection media and split at a ratio of 1:4 into one 15 cm plate. Once distinct colonies had formed and all cells in the control non-transfected plate died, 18 separate colonies were lifted using a P200 pipet tip and seeded onto 24-well plates. Once confluent, each colony-derived population of cells was split into a 6-well dish and western blot analysis was used to confirm expression. The cell lines that indicated the amount of expression compared to transient transfected cells were propagated. One cell line of each treatment was used in experiments.

2.6 Cell Migration Assay

Boyden transwell migration assays were performed as previously described (Williams et al., 2014a). Tissue culture inserts with an 8-µm pore diameter (Corning) in 24-well plates were coated with 20 µg/mL fibronectin/PBS on the membrane. Both transfected cells and stable cell lines were serum starved for 24 hours and subsequently counted using a haemocytometer. In serum-free media, containing 0.1% BSA and Penicillin-Streptomycin, 20,000 cells were added to the top chamber. Cells were allowed to migrate for 20 hours towards the lower chamber containing the above medium, supplemented with 10% BCS. The bottom of the membrane was fixed in 4% PFA/PBS for 20 minutes, washed with 150 mM glycine/PBS for 10 minutes, stained with Hoechst, and mounted slides. Ten fields of cells per membrane were counted, using fluorescence
microscopy. For transient transfections, the data is represented as the number of transfected cells that migrated to the bottom of the membrane divided by the number of transfected cells that remained on top. For stable cell lines, the data is presented as the number of cells that migrated to the bottom of the chamber divided by the number of parental MDA-MB-231 cells that migrated to the bottom of the chamber.

2.7 Cell Invasion Assay

Cell culture inserts were prepared as described previously (Brasher et al., 2017). Briefly, the bottom of transwells inserts (8 μm pore diameter, Corning Inc.) were coated with 20 μg/ml fibronectin/PBS (Sigma-Aldrich) and the tops with 0.125 mg/ml Matrigel (BD Biosciences). Parental and stable cells were serum-starved for 24 hours, lifted, seeded into chambers, and allowed to invade for 24 hours. The cells that invaded towards the lower chamber (10% BCS/0.1% BSA in DMEM) were fixed in 4% paraformaldehyde, stained with Hoescht, and counted. Cells that did not invade were removed with a cotton swab prior to fixation of the sample. Ten fields of cells per membrane were counted per treatment.

2.8 Immunoprecipitation

Antibody was coupled to 25 μl of Protein-G Magnetic beads (Biorad) overnight at 4°C in PBS/0.02%Tween on an end-over-end rotator. Cells were grown to 80% confluency or seeded onto coated tissue culture plates at 60% confluency. Cells were lysed in situ with cold lysis buffer comprising 1% NP40, 10% glycerol, 0.5% NaDOC, 137 mM NaCl, 20 mM Tris-HCl pH 8.0, 10 mM NaF, 10 mM Na₃P₂O₇, 0.2 mM Na₃VO₄ and protease inhibitor cocktail. Lysate was incubated with antibody bound beads overnight at
4°C on an end-over-end rotator, washed 3 times with cold PBS, and eluted with 2.5X SDS-PAGE loading buffer. The resultant beads-antibody-antigen complex was subsequently heated to 95°C for 5 to 20 minutes. Proteins were separated using SDS-PAGE and analyzed using Western immunoblotting. Alternatively, lysate was incubated with antibody-bound Sepharose beads overnight at 4 °C and washed three times with cold lysis buffer. Bound proteins were eluted using 2.5x Laemmli loading buffer heated to 100 °C. Proteins were separated using SDS-PAGE and analyzed by immunoblotting.

2.9 Cell Surface Protein Labeling

Cells were plated onto 0.2% gelatin (as described under ‘invadopodium formation assay’) for 4 hours. Cells were then washed with cold PBS once and incubated with 0.5 mg/ml Sulfo-NHS-SS-Biotin (APExBIO) dissolved in 10 mM boric acid and 150 mM NaCl (pH 8.0) at 4°C with occasional agitations. Plates were then washed with 15 mM glycine/PBS and lysed (as described under ‘Immunoprecipitation’).

2.10 Immunoblotting

Whole cell protein and immunoprecipitation samples were electrophoresed through a polyacrylamide gel and transferred onto a PVDF membrane with a 0.45 µm pore diameter (EMD Millipore). Membranes were blocked in either 5% skim milk or 5% BSA in TBST and probed with primary antibody (diluted 1:1000 in TBST). HRP-conjugated secondary antibodies (diluted 1:300 in blocking solution) were used to detect bound primary antibodies using an enhanced chemiluminescence kit (Millipore).
2.11 Invadopodium Formation Assay

Invadopodium formation was performed as previously described (Artym et al., 2009). Glass coverslips were coated with 50 µg/mL PLL/PBS, followed by crosslinking with 0.5% glutaraldehyde/PBS. Coverslips were then inverted onto 70 µL of AlexaFluor594 labelled gelatin. The coated coverslips were then incubated with 5 mg/mL NaBH₃/PBS and subsequently washed 10 times with PBS. Tissue culture plates were coated similarly; the exception being plates were coated with 0.2% unlabeled gelatin/PBS. Cells were plated at 60% confluency for 4 hours, fixed, stained for F-actin and mounted. Cells were viewed using confocal microscopy, and if spots of F-actin puncta were seen overlapping spots of invadopodium formation (black spots in the gelatin), the cell was scored as forming invadopodia. Fifty cells per treatment were counted and scored.

2.12 Gelatin Degradation Assay

Glass coverslips were coated as under ‘Invadopodium Formation’. Cells were plated at 40% confluency for 24 hours, fixed, stained for F-actin, and mounted. Fifty cells were counted and scored as either full degradation (large amount of gelatin degradation under the cell, +1 point), partial degradation (degradation that looks similar to a 4 hour time point, +0.5 points) or no degradation (0 points).

2.13 Immunofluorescence microscopy

Cells were either grown on glass coverslips overnight or seeded onto 0.2% gelatin coated coverslips (as described under invadopodium formation). Cells were fixed in 4% PFA/PBS for 20 minutes then washed in 150 mM glycine/PBS for 15 minutes at room
temperature or overnight at 4°C with gentle agitation. Cells were permeabilized in 0.1% TX-100/PBS for 10 minutes and then blocked in 5% BSA in PBS prior to staining with primary and secondary antibody. Coverslips were mounted onto glass microscope slides using DAKO fluorescent mounting medium. Samples analyzed by confocal microscopy were imaged through a 63X (NA 1.4) oil immersion lens using a Leica DM-IRE2 inverted 31 microscope with a Leica TCS SP2 scanning head (Leica, Heidelberg, Germany). Images were captured using Leica confocal software. All images were processed and analyzed using ImageJ software (NIH, Bethesda, MD, USA).

### 2.14 Mice and Tumor Experiments

All animal studies and procedures were performed in accordance with protocols approved by the Institutional Animal Care Committee at the BC Cancer Research Centre and University of British Columbia (Vancouver, BC, Canada) under approved animal study protocol A18-0132. All experiments and data analysis involving mice models were performed by collaborators, except for the creation and initial screening of stable cell lines.

1x10^6 MDA-MB-231 cells were injected subcutaneously into the left 4th mammary fat pad in a 50 µL suspension consisting of 50% normal saline and 50% matrigel. To monitor primary tumor growth, tumors were measured 3x/week using digital calipers and tumor volumes were calculated using the modified ellipsoid formula, \((L \times W^2) \times \pi/6\) (L, length; W, width) as previously described (Lou et al., 2011). To monitor survival a surrogate threshold was used and survival events occurred once tumors reached a size
of 400 mm³. Tumor growth and animal weights continued to be monitored until animals became moribund necessitating euthanasia for the entire group.

2.15 Immunohistochemical and Histochemical Staining of Tissues

Two hours before euthanasia mice were injected intraperitoneally (i.p.) with a saline solution containing 60 mg/kg pimonidazole (HypoxyProbe). One hour prior to euthanasia, mice were injected i.p. with a saline solution containing 1500 mg/kg bromodeoxyuridine (Sigma). Formalin fixed paraffin-embedded tissue sections were deparaffinised and rehydrated gradually by incubations in ethanol baths containing decreasing concentrations of ethanol (Chafe et al., 2019; Mcdonald et al., 2019). Sections were stained histochemically with hematoxylin and eosin or immunohistochemically with vimentin (BD Biosciences #550513; 1:200). Metastatic burden was calculated as a percentage of vimentin positive area/total tissue section area on whole sections (Acharyya et al., 2012).

2.16 Statistical Analysis

The percent of controls for three experimental replicates is shown, with error bars representing the standard deviation. The vertical bar for each treatment indicates the mean, in all graphs, unless otherwise indicated. For all experiments, each experimental group was compared with its respective control, vehicle, or wild-type treatment by Student’s t test or ANOVA, with a statistical significance threshold of \( p = 0.05 \). Post hoc analysis from significant ANOVA tests were done using a Tukey’s honestly significant difference test. Treatments that differed significantly from the control \( (p < 0.05) \) are indicated by an
*asterisk in the figures. All statistical analysis was done using Microsoft Excel. Graphs were prepared using GraphPad Prism version 7.0 (GraphPad Software, La Jolla, CA).
3 The Interaction of Munc18c and Syntaxin4 Facilitates Invadopodium Formation and Extracellular Invasion of Tumor Cells


3.1 Summary

Tumor cell invasion involves targeted localization of proteins required for interactions with the extracellular matrix and for proteolysis. The localization of many proteins during these cell–extracellular matrix interactions relies on membrane trafficking mediated in part by SNAREs. The SNARE protein syntaxin4 (Stx4) is involved in the formation of invasive structures called invadopodia; however, it is unclear how Stx4 function is regulated during tumor cell invasion. Munc18c is known to regulate Stx4 activity, and here we show that Munc18c is required for Stx4-mediated invadopodium formation and cell invasion. Biochemical and microscopic analyses revealed a physical association between Munc18c and Stx4, which was enhanced during invadopodium formation, and that a reduction in Munc18c expression decreases invadopodium formation. We also found that an N-terminal Stx4-derived peptide (residues 1 through 29) associates with Munc18c and inhibits endogenous interactions of Stx4 with synaptosome-associated protein 23 (SNAP23) and vesicle-associated membrane protein 2 (VAMP2). Furthermore, expression of the Stx4 N-terminal peptide (Stx4 1-29) decreased invadopodium formation and cell invasion in vitro. Of note, cells expressing the Stx4 N-terminal peptide exhibited impaired trafficking of membrane type 1 matrix metalloproteinase (MT1-MMP) and EGF receptor (EGFR) to the cell surface during invadopodium formation. Our findings implicate Munc18c as a regulator of Stx4-mediated trafficking of MT1-MMP and EGFR, advancing our understanding of the role of SNARE function in the localization of proteins that drive tumor cell invasion.
3.2 Results

3.2.1 Stx4 and Munc18c association is enhanced during invadopodium formation

Previous studies have shown that Stx4 and Munc18c interact and that Munc18c plays an important role during Stx4-mediated exocytosis through this interaction (20, 29). To test whether Munc18c and Stx4 associate in MDA-MB-231 cells, co-immunoprecipitation experiments were performed. Cells were lysed in situ, and analysis of Munc18c immunoprecipitates revealed co-immunoprecipitation of Stx4 (Fig. 3.1A). Immunoprecipitates of Munc18c were also probed for syntaxin3 (Stx3), but co-immunoprecipitation of Stx3 was not observed (Fig. 3.1B). Consistent with the co-immunoprecipitation of Stx4 and Munc18c, confocal immunofluorescence microscopy revealed that Stx4 and Munc18c have a partly overlapping distribution near the ventral membrane of cells, whereas Stx3 and Munc18c do not (Fig. 3.1C). To assess the association of Stx4 and Munc18c during invadopodium formation, cells were seeded onto gelatin (an ECM analogue) or a non-ECM substrate (poly-L-lysine, PLL) as a control. Cells were lysed in situ, and analysis of Munc18c immunoprecipitates determined that the amount of Stx4 associated with Munc18c was increased in samples plated on gelatin when normalized to the amount of Munc18c immunoprecipitated in each sample (Fig. 3.1D). An increase of 21.4% ± 2.3% in the amount of Stx4 co-immunoprecipitated with Munc18c was observed when cells were seeded onto PLL relative to unlifted cells, and an increase of 52.0% ± 3.7% was observed during invadopodium formation on gelatin (Fig. 3.1E). These results suggest that Munc18c and Stx4 associate in a manner that increased significantly during invadopodium formation.
**Figure 3.1** - Analysis of Stx4 and Munc18c association in MDA-MB-231 cells.

A) Munc18c immunoprecipitates were probed for Munc18c (arrow) and Stx4 (arrow). B+A, beads plus antibody; B+L, beads plus lysate; B+L+IgG, beads plus lysate plus unrelated IgG; IP, immunoprecipitation; Ms, mouse. B) Munc18c immunoprecipitates were probed for Munc18c, Stx4 (arrow), and Stx3 (arrow). C) Cells were grown for 4 hours on glass coverslips, fixed, permeabilized, and stained for either Munc18c and Stx4 or Munc18c and Sx3. A representative confocal section from the ventral region of a cell is shown. Scale bars = 10 μm. D) Analysis of Stx4 and Munc18c association during invadopodium formation (IVF). Cells were seeded onto coverslips coated with PLL or gelatin, incubated for 4 hours, lysed, and analyzed by immunoprecipitation/Western blotting. Munc18c immunoprecipitates were probed for Munc18c and Stx4 (arrow). E) Quantification of Stx4 co-immunoprecipitated with Munc18c. Percent of controls are from
three independent experiments ± S.D. Asterisks denote values significantly different from control unlifted cells (*, p < 0.05). All data represent three or more biological replicates with at least three technical replicates.
3.2.2 Knockdown of Munc18c impairs invadopodium formation

Involvement of Munc18c at invadopodia was supported by microscopic analysis of the localization of Munc18c during invadopodium formation. Munc18c was observed, along with Stx4, at invadopodia marked by β1 integrin and focal points of degradation in labeled gelatin (Fig. 3.2A). To test the requirement for Munc18c function in invadopodium formation, cells were transfected with a pool of siRNA targeting human Munc18c or nonspecific control siRNA. Knockdown of Munc18c was seen after 48 h, when Munc18c protein levels were reduced by 47.5% ± 2.8%. Protein levels of β1 integrin and MT1-MMP were unaffected (0.05% ± 0.08% and 0.08% ± 0.04%, respectively) (Fig. 3.2, B and C). Invadopodium formation was subsequently analyzed, and the number of cells forming invadopodia was reduced by 56.2% ± 4.0% in samples with decreased levels of Munc18c expression relative to controls (Fig. 3.2, D and E).
Figure 3.2 - RNAi-mediated knockdown of Munc18c impairs invadopodium formation. A) distribution of Munc18c during the formation of invadopodia. Cells were analyzed by confocal microscopy after being plated on Alexa Fluor 594–labeled gelatin for 4 hours, fixed, permeabilized, and stained as indicated: β1 integrin and Stx4 (cyan, white arrows), Munc18c (green, yellow arrows). Arrows pointing to dark spots in the gelatin indicate spots where Munc18c was shown to co-localize with either Stx4 or β1 integrin at sites of gelatin degradation that correspond to invadopodia formation. The overlay images have arrows indicating signal overlap of Munc18c and Stx4 or β1 integrin at sites of
invadopodium formation, which can be seen in the previous images. B) cells were transfected with siRNA targeting Munc18c or nonspecific control siRNA, lysed, and analyzed for Munc18c by SDS-PAGE/Western blotting. C) Quantification of Munc18c knockdown normalized to actin. D) Cells with F-actin puncta overlying dark spots of gelatin degradation were counted as cells forming invadopodia. Shown are percentages of cells forming invadopodia, normalized to control (GFP-transfected) cells. E) Invadopodium-based degradation of Alexa Fluor 594 gelatin by cells co-transfected with GFP and control siRNA or Munc18c siRNA. Cells were transfected for 44 hours, seeded onto gelatin for 4 hours, and then fixed, permeabilized, stained for F-actin, and analyzed by confocal microscopy. Percent of controls are from three independent experiments ± S.D. Asterisks denotes values significantly different from control cells (*, p < 0.05). Scale bars = 10μm. All data represent three or more biological replicates with at least three technical replicates.
3.2.3 A Stx4 N-terminal peptide associates with Munc18c and inhibits cognate SNARE binding with endogenous Stx4

Previous work has shown that the N-terminal 29 amino acids of Stx4 are required for binding to Munc18c in vitro and in vivo (Latham et al., 2006). In vitro pulldown experiments showed that the presence of this polypeptide reduced the degree of association between Munc18c and Stx4, suggesting that this N-terminal domain can act as a competitive inhibitor of Munc18c and Stx4 interactions (Latham et al., 2006). We hypothesized that an exogenously expressed peptide corresponding to the N-terminal 29 amino acids of Stx4 would bind to endogenous Munc18c and therefore impair normal Munc18c-dependent SNARE complex formation involving Stx4. A GFP-tagged construct encoding the N-terminal 29 amino acids of Stx4 (Stx4–N-term) was used to derive a stable cell line from MDA-MB-231 cells. Stable cell lines expressing GFP or Stx4-FL were also generated. Co-immunoprecipitations were done using GFP cells and Stx4–N-term cells lysed in situ. GFP immunoprecipitates from Stx4-N-term cells were found to contain a GFP-tagged protein with a molecular mass of 31 kDa, as predicted for the Stx4–N-term construct. These immunoprecipitates also contained Munc18c, consistent with the association of Munc18c and the N-terminal peptide construct (Fig. 3.3A). To assess whether the Stx4–N-term construct was able to compete with full-length Stx4 for binding to Munc18c, parental MDA-MB-231 cells and Stx4–N-term stable cells were transiently transfected with Stx4-FL-3xFLAG. Cells were lysed 24 hours after transfection, and FLAG was immunoprecipitated (Fig. 3.3B). In Stx4–N-term stable cells, a 62.45% ± 6.65% decrease in the amount of Munc18c co-immunoprecipitated compared with parental cells was observed (Fig. 3.3C). To determine whether expression of Stx4–N-term inhibits
endogenous Stx4 from forming cognate SNARE complexes, we immunoprecipitated SNAP23 from cells stably expressing Stx4–N-term and observed nearly undetectable amounts of Stx4 associated with SNAP23 compared with cells expressing Stx4-FL or non-transfected MDA-MB-231 control cells (Fig. 3.4A,B). VAMP2 was also immunoprecipitated from Stx4-FL and Stx4–N-term stable cells, and co-immunoprecipitation of endogenous Stx4 was assessed (Fig. 3.4C). Stx4–N-term stable cells showed a decrease of 93.25% ± 24.11% in the amount of Stx4 that was co-immunoprecipitated with VAMP2 compared with Stx4-FL cells (Fig. 3.4D).
Figure 3.3 - Association of Stx4 N-terminal peptide reduces endogenous Munc18c binding to Stx4-FL 3xFLAG.

A) Parental MDA-MB-231 cells, GFP expressing stable cells, and Stx4-N-term-expressing stable cells were lysed, and GFP was immunoprecipitated. Immunoprecipitates (IP) were probed for Munc18c (arrow). B+A, beads plus antibody; FLAG IgG, beads plus mouse anti-FLAG antibody plus Stx4-N-term peptide lysate; Rabbit IgG, rabbit IgG plus Stx4-N-term lysate. B) parental and stable cells expressing Stx4–N-term were transiently transfected with Stx4-FL 3xFLAG for 24 hours and lysed, and FLAG was immunoprecipitated. Immunoprecipitates were probed for Munc18c and FLAG (arrow). B+L, beads plus lysate from parental cells. C) Densitometry of the amount of Munc18c co-immunoprecipitated relative to Stx4-FL 3xFLAG as shown in B. All data are presented as percent of control ± S.D. The asterisk denotes values significantly different from control (parental cells) (*, p < 0.05). All data represent three or more biological replicates with at least three technical replicates.
**Figure 3.4** - Association of Stx4 N-terminal peptide with Munc18c inhibits Stx4 – SNAP23 and Stx4 – VAMP2 interaction.
A) Parental MDA-MB-231 cells, and stable cells expressing either Stx4-FL or Stx4-N-term were lysed, and SNAP23 was immunoprecipitated. Immunoprecipitates (IP) were probed for Stx4 (arrow) and SNAP23. B+A, beads plus antibody; B+L, beads plus lysate from parental cells. B) Densitometry of the amount of Stx4 co-immunoprecipitated relative to SNAP23 as shown in A. C) Parental MDA-MB-231 cells and stable cells expressing either Stx4-FL or Stx4-N-term were lysed, and VAMP2 was immunoprecipitated. Immunoprecipitates were probed for Stx4 and VAMP2. D) Densitometry of the amount of Stx4 co-immunoprecipitated relative to VAMP2 as shown in C. All data are presented as percent of control S.D. Asterisks denote values significantly different from control (parental cells) (*, p < 0.05). All data represent three or more biological replicates with at least three technical replicates.
3.2.4 Expression of Stx4 N-terminal peptide impairs invadopodium formation and gelatin degradation

Having observed inhibition of Stx4-SNAP23 complex formation caused by expression of Stx4–N-term, the effect of transient expression of this construct on invadopodium formation was examined. Overexpression of Stx4–N-term reduced the number of cells forming invadopodia by 65.1% ± 1.3% (Fig. 3.5, A and B). Transfection of cells with the N-terminus of Stx3 did not alter invadopodium formation (Fig. 3.5B). To determine whether expression of the Stx4 N-term and Munc18c knockdown were affecting the same pathway during invadopodium formation, we co-transfected cells with Stx4–N-term and Munc18c siRNA. Co-transfection did not cause a significantly different change in invadopodium formation compared with transfections with either the Stx4–N-term or Munc18c siRNA alone (Fig. 3.5B). ECM degradation was examined by transfecting cells with GFP, Stx4-FL, or Stx4–N-term for 24 hours and then plating onto fluorescent gelatin for 24 hours. Relative to GFP-expressing cells, no significant change in gelatin degradation was seen for Stx4-FL-expressing cells. Stx4–N-term–expressing cells exhibited a 62.2% ± 2.0% decrease in gelatin degradation compared with Stx4-FL expressing cells (Fig. 3.5C). Stable cell lines were also used to assess invadopodium formation, and similar results were found. Relative to parental MDA-MB-231 cells, no significant change in invadopodium formation was observed for GFP or Stx4-FL cell lines. The Stx4–N-term cell line displayed a 50.7% ± 5.4% decrease in invadopodium formation (Fig. 3.6, A and B). In gelatin degradation assays, no significant difference was observed between GFP or Stx4-FL cells. Stx4–N-term cells displayed a 74.4% ± 0.8% decrease in gelatin degradation compared with Stx4-FL control cells (Fig. 3.6C).
Figure 3.5 - Stx4 N-terminal peptide impairs invadopodium formation and gelatin degradation.

A) Invadopodium-based degradation of gelatin by cells transfected with GFP (Control), Stx4-FL, and Stx4–N-term. Cells were transfected for 20 hours, seeded onto fluorescent gelatin for 4 hours, and then fixed, permeabilized, stained for F-actin, and analyzed by confocal microscopy. Scale bars = 10 μm. B) Quantification of invadopodium formation. Cells were transfected with the following: GFP alone, Stx4–FL, Stx4–N-term, Stx3–N-terminal peptide, GFP + control siRNA, GFP + Munc18c siRNA, Stx4–FL + siRNA Control, Stx4–FL + siRNA Munc18c, Stx4–N-term + siRNA control, and Stx4–N-term + Munc18c siRNA. Cells were transfected for 44 hours and then seeded onto fluorescent gelatin and processed as in A. Cells with F-actin puncta overlying dark spots of gelatin degradation
were counted as cells forming invadopodia. Percentages of cells forming invadopodia, normalized to GFP alone, were determined by counting 50 cells/sample. C) Cells expressing either GFP, Stx4-FL, or Stx4 -N-term were transfected for 24 hours, seeded onto fluorescent gelatin for 24 hours, and then fixed. Parental cells and cells expressing GFP were analyzed for dark areas of degradation and scored as described under “Experimental procedures.” All data are presented as percent of control ± S.D. Asterisks denote values significantly different from control (*, p < 0.05). All data represent three or more biological replicates with at least three technical replicates.
Figure 3.6 - Stable cell lines expressing Stx4 N-terminal peptide have reduced invadopodium formation and gelatin degradation.

A) Parental MDA-MB231 cells and stable cell lines expressing GFP, Stx4-FL, or Stx4–N-term were seeded onto Alexa Fluor 594-labeled gelatin and incubated for 4 hours prior to fixation, staining for F-actin, and analysis by confocal microscopy. Scale bars = 10μm.

B) Quantification of invadopodium formation. Cells with F-actin puncta overlying dark spots of gelatin degradation were counted as cells forming invadopodia. Percentages of cells forming invadopodia are shown from three independent experiments in which 100 cells/sample were counted and normalized to parental MDA-MB-231 cells.

C) Gelatin Degradation (% of Control)
Quantification of gelatin degradation. Parental, GFP, Stx4-FL, and Stx4–N-term stable cells were plated onto fluorescent gelatin for 24 hours and then fixed. Cells were analyzed for dark areas of degradation and scored as described under “Experimental Procedures.” Percentages of cells degrading gelatin are shown from three independent experiments in which 50 cells/sample were analyzed. All data are presented as percent of control S.D. Asterisks denote values significantly different from control (*, p < 0.05). All data represent three or more biological replicates with at least three technical replicates.
3.2.5 Expression of the Stx4 N-terminal peptide inhibits cellular invasion and trafficking of MT1-MMP and EGFR to the cell surface

Stx4 and SNAP23 have been shown previously to contribute to trafficking of MT1-MMP in MDA-MB-231 cells (Williams et al., 2014a). Given the observed reduction in Stx4–SNAP23 and Stx4–VAMP2 interactions in cells expressing Stx4–N-term (Fig. 3.4B), we analyzed the trafficking of key regulators required for invadopodium formation and ECM invasion. The amount of MT1-MMP, EGFR, and β1 integrin at the plasma membrane during invadopodium formation was assessed by biotinylating cell surface proteins (Fig. 3.7A,B). It was found that MT1-MMP, EGFR, and β1 integrin cell surface levels on Stx4-FL stable cells were reduced by 41.2% ± 0.1% and 24.7% ± 0.1%, respectively, compared with parental cells (Fig. 3.7B). Cell-surface MT1-MMP was further reduced by 40.0% ± 0.2%, and cell surface EGFR was further reduced by 48.1% ± 0.1% in cells stably expressing Stx4–N-term compared with those expressing Stx4-FL. The levels of β1 integrin at the cell surface, although variable to some extent, were not found to be significantly different in any of the cell lines. Given that invadopodium formation, gelatin degradation, and trafficking of MT1-MMP and EGFR were decreased in cells stably expressing Stx4–N-term, the ability of these cells to invade was examined using Matrigel-based invasion assays. Cells expressing Stx4–N-term exhibited a 77.0% ± 2.9% reduction in invasion compared with cells expressing Stx4-FL (Fig. 3.7C). No significant difference was found between parental MDA-MB-231 cells, GFP cells, and Stx4-FL cells.
Figure 3.7 - Stx4 N-terminal peptide expression reduces cell surface levels of MT1-MMP and EGFR as well as cellular invasion.

A) Parental MDA-MB-231 cells and stable cell lines expressing GFP, Stx4-FL, or Stx4-N-term were plated onto gelatin for 4 hours, exposed to biotin, and then lysed and analyzed by precipitation with streptavidin beads. Cell surface protein levels of β1 integrin, MT1-MMP, EGFR, and β-tubulin were assessed by Western blotting. Parental biotin-ve indicates cells exposed to buffer only. B) Densitometric analysis of the amount of β1 integrin, EGFR, and MT1-MMP in streptavidin precipitations, as in A, relative to control. C) parental, GFP, Stx4-FL, and Stx4-N-term stable cells were serum-starved for 24 hours, seeded onto transwell membranes coated with Matrigel or uncoated membranes (control), and allowed to invade for 20 hours. Percentages of cells invading are shown from experiments in which at least 10 fields of view were counted per treatment. All data represent percent of control ± S.D. Asterisks denote values significantly different from control (*, p < 0.05). All data represent three or more biological replicates with at least three technical replicates.
3.2.6 Inhibition of expression and function of Munc18c shows a conserved phenotype between tumor cell lines

To determine whether similar phenotypes are seen in other invasive cell lines, the effect of siRNA knockdown and inhibition of Munc18c on invadopodium formation and gelatin degradation was performed in HT-1080 fibrosarcoma cells. Immunoprecipitation of Munc18c from HT-1080 cells revealed co-immunoprecipitation of Stx4 but not Stx3 (Fig. 3.8A). HT-1080 cells were then transfected with a pool of siRNA targeting human Munc18c or nonspecific control siRNA (Fig. 3.8B), and clear knockdown of Munc18c expression was observed after 48 h (Fig. 3.8B, C). Invadopodium formation was then analyzed, and the number of cells forming invadopodia was reduced by 73.0% ± 10.5% in samples with reduced Munc18c expression (Fig. 3.8D). Consistent with this observation, expression of Stx4–N-term caused a decrease (44.79% ± 14.06%) in invadopodium formation compared with cells expressing Stx4-FL (Fig. 3.8E). Inhibiting Munc18c through expression of Stx4–N-term in HT-1080 cells also decreased gelatin degradation by 57.76% ± 5.00% compared with Stx4-FL control cells (Fig. 3.8F).
**Figure 3.8** - Inhibition of expression and function of Munc18c shows a conserved phenotype in HT-1080 cells.

A) Munc18c immunoprecipitates (IP) were probed for Munc18c and Stx4. B+A, beads plus antibody; B+L, beads plus lysate; B+L+IgG, beads plus lysate plus unrelated IgG. B) HT-1080 cells were transfected with siRNA targeting Munc18c or nonspecific control siRNA, lysed, and analyzed for Munc18c by SDS-PAGE/Western blotting. C) quantification of Munc18c knockdown normalized to GAPDH. D) Quantification of...
invadopodium formation. Cells were transfected with siRNA targeting Munc18c or nonspecific control siRNA for 44 hours and then plated onto fluorescent gelatin for 4 hours. Cells with F-actin puncta overlying dark spots of gelatin degradation were counted as cells forming invadopodia. Percentages of cells forming invadopodia are shown from three independent experiments in which 50 cells/sample were counted and normalized to parental MDA-MB231 cells. E) Invadopodium-based degradation of Alexa Fluor 594 gelatin by cells transfected with GFP (control), Stx4-FL, or Stx4–N-term. Cells were transfected for 20 hours, seeded onto fluorescent gelatin for 4 hours, and then fixed, permeabilized, stained for F-actin, and analyzed by confocal microscopy. Percentages of cells forming invadopodia are shown from three independent experiments in which 50 cells/sample were counted and normalized to GFP-expressing cells. F) Cells were transfected with GFP (control), Stx4-FL, or Stx4–N-term for 24 hours, plated onto fluorescent gelatin for 24 hours, and then fixed. Cells were analyzed for dark areas of degradation and scored as described under “Gelatin degradation assay.” Percentages of cells degrading gelatin are presented as the mean ± S.D. from three independent experiments in which 50 cells/sample were analyzed. Asterisks denote values significantly different from control (*, p < 0.05). All treatments are a percentage of GFP alone, which was used as the control group. All data represent three or more biological replicates with at least three technical replicates.
3.3 Discussion

SNARE-mediated trafficking is important for several aspects of tumor cell invasion (Kean et al., 2009; Steffen et al., 2008; Williams and Coppolino, 2014; Williams et al., 2014b), including invadopodium formation (Chen and Scheller, 2001; Steffen et al., 2008), cell migration (Day et al., 2011; Proux-Gillard, 2005; Riggs et al., 2012; Tayeb et al., 2005; Veale et al., 2011), and MMP-directed ECM degradation (Miyata et al., 2004). While SNAREs have been shown to play important roles in these processes, there remains much to be understood about how SNARE function is regulated during tumor cell invasion. Previous research has described the SM protein Munc18c as both a negative (Tamori et al., 1998b; Thurmond et al., 1998) and positive regulator of its binding partner Stx4 (Latham et al., 2006; Oh et al., 2005a; Thurmond et al., 2000). Here, we demonstrate that Munc18c contributes to invadopodium formation and cell invasion, in MDA-MB-231 and HT-1080 cells, by facilitating Stx4-mediated trafficking. Stx4 mediated membrane traffic is required for delivery of MT1-MMP and EGFR to the plasma membrane and can thus support modulation of ECM interactions during tumor cell invasion. SM proteins form interactions with their cognate Syntaxins, and play an important role in regulating membrane fusion (Carr and Rizo, 2010). Munc18c and Stx4 have been shown to associate in some cell lines (e.g. 3T3-L1 adipocytes) (Thurmond et al., 2000; Yu et al., 2013), however; their interaction has not been well studied in cancer cell lines. Our findings suggest that the interaction of Munc18c and Stx4 is a novel and important point of regulation in the control of MT1-MMP-mediated ECM degradation by invasive tumor cells.
The observed distribution of Stx4 in MDA-MB-231 cells is consistent with its function at the plasma membrane and possibly on membrane-bound compartments that contribute to the transport of material to and from the cell surface (Fig. 1B). Analyses of the distributions of Munc18c and Stx4 revealed that these two proteins partly co-localize in cells at rest or forming invadopodia. Co-immunoprecipitation of Munc18c and Stx4 was enhanced in cells forming invadopodia, compared to non-invading cells, without an obvious change in the co-localization of the proteins. This suggests that Munc18c and Stx4 might interact in a manner that increases in affinity during invadopodium formation.

Previous studies have shown that inhibition or RNAi-mediated knockdown of Munc18c impairs Stx4-mediated exocytic events, such as GLUT4 translocation (Jewell et al., 2011; Oh and Thurmond, 2009). Here, RNAi-mediated knockdown of Munc18c was observed to significantly inhibit invadopodium formation in MDA-MB-231 and HT-1080 cells, similarly to that observed when Stx4 was knocked down by siRNA (Williams et al., 2014a). Together, the observations support a model wherein Stx4-mediated trafficking to the plasma membrane facilitates the delivery of proteins required for invadopodium formation in a manner that is regulated, in part, by Munc18c. How Munc18c functions to regulate Stx4 function is not clearly understood. Studies have demonstrated that Munc18c binds to the N-terminus of Stx4, which may place Stx4 into a more open conformation that facilitates SNARE complex assembly (Latham et al., 2006). Consistent with this, the binding of Munc18c to Stx4 has been found to involve Phe119 of Munc18c, and accelerate the formation of SNARE complexes (Latham et al., 2006). There is evidence that Munc18c can associate with other SNAREs, but may only do so when
interacting with the N-terminal domain of Stx4 (Latham et al., 2006; Peng and Gallwitz, 2004). Therefore, it is reasonable to predict that overexpression of the N-terminal 29 amino acids of Stx4 could compete for binding of Stx4 to Munc18c, and it has been shown that this polypeptide can reduce the interaction of recombinant Munc18c and Stx4 in pull-down experiments (Latham et al., 2006). Here, we expressed a GFP-tagged version of a Stx4 N-terminal peptide in MDA-MB-231 cells and observed perturbations to Stx4-SNAP23 interaction, Stx4-VAMP2 interaction, MT1-MMP and EGFR trafficking, invadopodium formation and cell invasion. Our observations of reduced interaction between SNAP23 and Stx4, as well as Stx4 and VAMP2, resulting from expression of the Stx4 N-terminal peptide are consistent with previous findings that the Munc18c-Stx4 interaction promotes complex formation involving Stx4, VAMP2 and SNAP23 (Latham et al., 2006). Furthermore, experiments performed in HT-1080 cells revealed that regulation of Stx4 by Munc18c is conserved between two invasive cancer cell lines.

Previously, it has been demonstrated that SNAREs are involved in the trafficking of key invadopodial proteins to the cell surface, including EGFR (Williams and Coppolino, 2014) and MT1-MMP (Williams et al., 2014a). Here, we find that the regulation of Stx4 by Munc18c is required for MT1-MMP and EGFR trafficking to the cell surface, and disruption of this contributes to decreased invadopodium formation and cell invasion. This is consistent with a role for Stx4 in invadopodium formation and cell invasion, as previously determined by siRNA knockdown of Stx4 (Williams et al., 2014b). It has also been shown that a significant decrease in EGFR levels at the cell surface results when SNAP23 and Syntaxin13 function are impaired (Williams and Coppolino, 2014), which is
also consistent with our findings. Decreases in MT1-MMP and EGFR at the cell surface were observed in cells stably expressing Stx4-FL, but these changes did not correlate with alteration to invadopodium formation or cell invasion.

Over the past decade it has become clear that trafficking of MMPs and adhesion molecules has an essential role in the remodeling of the ECM during tumor cell invasion, facilitating tumor cell movement through interstitial tissues. The model that has emerged involves formation of invadopodia, and subsequent matrix degradation by invasive tumor cells, through coordinated cytoskeletal rearrangements and membrane trafficking. While membrane traffic mediated by SNAREs, exocyst complexes, and other components of the membrane fusion machinery is crucial to tumor cell invasion (Poincloux et al., 2009), the direct roles that regulators of SNARE function have in cell invasion remain to be defined. Our findings implicate the interaction of Stx4 and Munc18c in the trafficking of MT1-MMP and EGFR, and this advances our understanding of the SNARE-mediated mechanisms that coordinate the localization of proteins that drive tumor cell invasion.
4 Trafficking of MT1-MMP During Tumor Cell Invasion is Mediated by the SNARE Complex SNAP23, Syntaxin4 and VAMP2 and regulated by Munc18c
4.1 Summary

Trafficking of proteins to invadopodia is necessary for their formation and function during tumor cell invasion (Poincloux et al., 2009). Intracellular, vesicular transport of cellular cargo is dependent on SNAREs (Chen and Scheller, 2001), and has been shown to play a role in invadopodia formation, including trafficking of MT1-MMP (Kean et al., 2009; Williams et al., 2014a) and EGFR to the cell surface (Brasher et al., 2017). The molecular mechanisms by which SNARE activity is regulated represent important features of cargo trafficking that could be targeted to interfere with invadopodium formation and degradation of ECM. Regulation of the plasma membrane SNARE Syntaxin4 by Munc18c has been shown to facilitate trafficking of MT1-MMP and EGFR to the cell surface during invadopodium formation, and is thus an attractive target for potential mitigation of metastatic progression (Brasher et al., 2017). Munc18c binds to the N-terminal region of Stx4 and regulates vesicle trafficking involving Stx4-containing complexes. While the peptide construct containing amino acids 1-29 of Stx4 can interfere with Stx4-Munc18c interaction, it has also been shown that the eighth leucine of Stx4 is required for Stx4 binding of Munc18c in vitro (Latham et al., 2006). This prompted examination of smaller fragments of the Stx4 N-terminus region for effects on Stx4-Munc18c binding, Stx4 function, and cell-ECM interactions. Here, I report that MDA-MB-231 cells, expressing residues 1-15 of the Stx4 N-terminus (Stx4-1-15), have perturbed trafficking of MT1-MMP and EGFR, reduced invadopodia formation, and lowered invasive capacity in vitro. Cells expressing Stx4-N-term also led to increased survival and reduced metastatic burden in a mouse xenograft model. These findings suggest that targeting the
interaction of Stx4 with Munc18c can dramatically alter metastatic behavior of cancer cells
in vivo, and that targeting this interaction warrants further investigation for therapeutic
potential.

4.2 Results

4.2.1 Expression of an N-terminal fragment of Stx4 (a.a. 1-15) impairs formation
of endogenous Stx4-containing SNARE complexes

Having previously observed inhibition of invadopodium formation and gelatin
degradation in cells expressing Stx4-N-term (Brasher et al., 2017), shorter segments of
this peptide were tested to determine which portion of the 29 amino acid long N-terminal
segment is required for Munc18c/Stx4 binding. Latham et al., reported that the binding of
Munc18c to Stx4 required leucine 8 of Stx4 (Latham et al., 2006). Therefore, we cloned
residues 1-15 and 15-29 of Stx4 separately into pEGFP-N1 to determine their effect on
invadopodium formation and gelatin degradation. As in chapter 3, MDA-MB-231 cells
were used due to their ability to form invadopodia in vitro. Stable cells lines were
generated expressing Stx4-1-15 and Stx4-15-29, and previously generated cell lines were
used as controls (Brasher et al., 2017). GFP was immunoprecipitated from these cells
and immunoprecipitates were probed for Munc18c. A band of 28 kDa was seen in GFP
blots corresponding to Stx4-1-15. Munc18c was co-immunoprecipitated, but not in the
control sample (cells expressing GFP alone) indicating Munc18c can interact with Stx4-
1-15 (Figure 4.1A). Previous research has shown that expression of Stx4-N-term causes
a decrease in SNARE complex formation involving Stx4, SNAP23 and VAMP2 (Brasher
et al., 2017). Here, we examined SNAP23 immunoprecipitates from cells stably
expressing Stx4-1-15 (Figure 4.1B,C) and found that these cells had a 103.1% ± 12.22%
decrease in the amount of Stx4 associated with SNAP23, compared to cells expressing Stx4-FL. Consistent with previous findings, cells expressing Stx4-N-term also had a significant decrease in the amount of Stx4 associating with SNAP23 (Brasher et al., 2017). This effect was not observed in cells expressing Stx4-15-29.
Figure 4.1 - Association of Stx4-1-15 with Munc18c inhibits Stx4–SNAP23 interaction.

A) Parental MDA-MB-231 cells, and cell lines stably expressing GFP, GFP-Stx4-1-15 or GFP-Stx4-15-29 were lysed, and GFP was immunoprecipitated. Immunoprecipitates (IP) were probed for Munc18c. B+α, beads plus antibody, B+L, beads plus GFP lysate. B) Parental MDA-MB-231 cells, and stable cells expressing either Stx4-FL, Stx4–N-term, Stx4-1-15, or Stx4-15-29 were lysed, and SNAP23 was immunoprecipitated. Immunoprecipitates (IP) were probed for Stx4 and SNAP23. B+α, beads plus antibody; B+L, beads plus lysate from parental cells. C) Densitometry of the amount of Stx4 co-immunoprecipitated relative to SNAP23 as shown in A. (*, p < 0.05). All data represent three or more biological replicates with at least three technical replicates.
4.2.2 Transient expression of Stx4-1-15 and Stx4-15-29 impairs invadopodium formation and gelatin degradation

It was previously observed that expression of Stx4-N-term in MDA-MB-231 cells inhibited invadopodium formation and gelatin degradation (Brasher et al., 2017). Here, MDA-MB-231 cells were transiently transfected with GFP, Stx4-FL, Stx4-N-term, Stx4-1-15 or Stx4-15-29 for 24 hours, then plated onto fluorescently coated gelatin coverslips for 4 hours to study invadopodium formation (Figure 4.2A,B). Relative to GFP-expressing cells, no significant change in invadopodium formation was seen for cells expressing Stx4-FL. Transfection of cells with Stx4-N-term or Stx4-1-15 led to an 82.46% ± 1.53% and 66.83 ± 2.88% decrease in invadopodium formation, respectively. Cells transfected with Stx4-15-29 showed a 40.79% ± 4.72% decrease in invadopodium formation, compared to control cells. ECM degradation was examined by transfecting cells as above, and plating them onto fluorescent gelatin for 24 hours (Figure 4.2C). Relative to GFP expressing cells, no significant change in gelatin degradation was seen for Stx4-FL expressing cells. Cells expressing either Stx4-N-term, or Stx4-1-15 had a decrease in gelatin degradation by 54.34% ± 1.15% and 51.31% ± 0.58%, respectively. A 10.40% ± 5.50% decrease in gelatin degradation in cells expressing Stx4-15-29 was also seen, compared to control cells.
**Figure 4.2** - Transient Stx4 1-15 and Stx4 15-29 peptide expression impairs invadopodium formation and gelatin degradation.

A) Invadopodium-based degradation of gelatin by cells transfected with GFP (Control), Stx4-FL, Stx4–N-term, Stx4-1-15 and Stx4-15-29. Cells were transfected for 20 hours, seeded onto fluorescent gelatin for 4 hours, and then fixed, permeabilized, stained for F-actin, and analyzed by confocal microscopy. Scale bars = 10 μm. B) Quantification of invadopodium formation. Cells with F-actin puncta overlying dark spots of gelatin degradation were counted as cells forming invadopodia. Percentages of cells forming invadopodia, normalized to GFP alone, were determined by counting 50 cells/sample. C) The above cells were seeded onto fluorescent gelatin for 24 hours, and then fixed. Parental cells and cells expressing GFP were analyzed for dark areas of degradation and scored as described under “Experimental procedures.” All data are presented as percent of control ± S.D. Asterisks denote values significantly different from control (*, p < 0.05). All data represent three or more biological replicates with at least three technical replicates.
4.2.3 Expression of Stx4-1-15 impairs invadopodium formation and gelatin degradation

The finding of decreased invadopodium formation in cells transiently expressing Stx4-15-29 was unexpected, given that expression of Stx4-15-29 did not affect SNARE complex formation (Figure 4.1). Stable cells lines were therefore used for additional analyses of invadopodium formation and gelatin degradation assays. MDA-MB-231 parental, GFP (empty vector), Stx4-FL, Stx4-N-term, Stx4-1-15 and Stx4-15-29 stable cells were plated onto fluorescent gelatin for 4 or 24 hours, to study invadopodium formation and gelatin degradation assays, respectively (Figure 4.3). Interestingly, only Stx4-N-term and Stx4-1-15 expressing cells were observed to have reduced invadopodium formation and gelatin degradation. Stx4-N-term expressing cells showed an 85.8% ± 13.0% decrease in invadopodium formation and a 76.8% ± 10.6% decrease in gelatin degradation, compared to Stx4-FL cells. Stx4-1-15 cells had an 89.51% ± 17.30% decrease in invadopodium formation and an 83.51% ± 2.37% decrease in gelatin degradation, compared to control. There was no significant decrease in invadopodium formation or gelatin degradation in stable cells expressing Stx4-15-29.
Figure 4.3 - Stx4 1-15 peptide expression impairs invadopodium formation and gelatin degradation.

A) Invadopodium-based degradation of gelatin by cells transfected with GFP (Control), Stx4-FL, Stx4–N-term, Stx4-1-15 and Stx4-15-29. Cells were seeded onto fluorescent gelatin for 4 hours, and then fixed, permeabilized, stained for F-actin, and analyzed by confocal microscopy. Scale bars = 10 μm. B) Quantification of invadopodium formation. Cells were seeded onto fluorescent gelatin and processed as in A. Cells with F-actin puncta overlying dark spots of gelatin degradation were counted as cells forming invadopodia. Percentages of cells forming invadopodia, normalized to GFP alone, were determined by counting 50 cells/sample. C) The above cells were seeded onto fluorescent gelatin for 24 hours, and then fixed. Parental cells and cells expressing GFP were analyzed for dark areas of degradation and scored as described under “Experimental procedures.” All data are presented as percent of control ± S.D. Asterisks denote values significantly different from control (*, p < 0.05). All data represent three or more biological replicates with at least three technical replicates.
4.2.4 Expression of Stx4-1-15 inhibits cellular migration, invasion and trafficking of MT1-MMP to the cell surface

It has previously been demonstrated that Stx4, SNAP23 and VAMP2 contribute to trafficking of MT1-MMP in MDA-MB-231 cells (Brasher et al., 2017; Williams et al., 2014a). Since a decreased interaction between Stx4 and SNAP23 was seen in cells expressing Stx4-1-15, we analyzed the trafficking of key invadopodial proteins during invadopodium formation. The amount of MT1-MMP, EGFR and β1 integrin was assessed by biotinylating cell surface proteins while cells are plated onto gelatin for 4 hours (Figure 4.4A, B). The amount of MT1-MMP and EGFR cell surface protein levels were decreased by 57.53% ± 22.29% and 96.45% ± 3.86%, respectively, in cells expressing Stx4 1-15, compared to control. Stx4-FL cells also showed a significant decrease in MT1-MMP levels by 33.41% ± 18.73%, which was seen previously (Brasher et al., 2017). Given that invadopodium formation, gelatin degradation, and trafficking of MT1-MMP and EGFR protein levels were decreased in cells stably expressing Stx4-1-15, the ability of these cells to migrate and invade was examined using Boyden chamber assays and Matrigel-based invasion assays (Figure 4.4C, D). Cells expressing Stx4-1-15 had a 66% ± 4.31% and 70% ± 11.66% decrease in cell migration and invasion, respectively. No significant difference was found between parental MDA-MB-231 cells, GFP cells, Stx4-FL cells or Stx4-15-29 cells.
Figure 4.4 - Expression of Stx4-1-15 decreases cell surface levels of MT1-MMP and EGFR, as well as cell migration and invasion.

A) Parental MDA-MB-231 cells and stable cell lines expressing Stx4-FL, Stx4-N-term, Stx4-1-15 and Stx4-15-29 were plated onto gelatin for 4 hours, exposed to biotin, and then lysed and analyzed by precipitation with streptavidin beads. Cell surface levels of $\beta_1$ integrin, MT1-MMP, and EGFR were assessed by Western blotting. GAPDH was used to assess equal protein loading. B) Densitometric analysis of the amounts of $\beta_1$ integrin, EGFR, MT1-MMP in streptavidin precipitates, as in A, relative to control. C) The above cells were serum-starved for 24 hours, seeded onto uncoated membranes, and allowed to invade for 20 hours. Percentages of cells migrating are shown from experiments in which at least 10 fields of view were counted per treatment. D) The above cells were serum-starved for 24 hours, seeded onto coated membranes, and allowed to invade for 24 hours. Percentages of cells invading are shown from experiments in which at least 10 fields of view were counted per treatment. All data represent percent of control ± S.D. Asterisks denote values significantly different from controls (*, $p < 0.05$). All data represent three or more biological replicates with at least three technical replicates.
4.2.5 Expression of MT1-MMP-T567E increases invadopodium formation in cells expressing Stx4-N-term or Stx4-1-15 peptides

To determine if invadopodium formation and gelatin degradation could be rescued in cells expressing Stx4-N-term or Stx4-1-15, cells were transfected with either MT1-MMP-3xFLAG or MT1-MMP-T567E-3xFLAG and plated onto fluorescent gelatin for 4 and 24 hours, respectively. Previous research has shown that overexpression of MT1-MMP-T567E increases cellular migration and invasion, possibly due to altered trafficking of the enzyme or altered biochemical signaling as a result of the phospho-mimetic mutation (Williams and Coppolino, 2011b). Here, all cells expressing MT1-MMP-T567E had a significant increase in invadopodium formation and gelatin degradation (Figure 4.5). Stx4-N-term cells expressing MT1-MMP-T567E-3xFLAG showed a 36.86% ± 10.07% increase in invadopodium formation, and a 18.42% ± 5.23% increase in gelatin degradation, when compared to cells expressing Stx4-N-term and MT1-MMP-3xFLAG. Stx4-1-15 peptide cells expressing MT1-MMP-T567E-3xFLAG had a 12.79% ± 4.19% increase in invadopodium formation, and a 23.23% ± 8.07% in gelatin degradation compared to cells expressing Stx4-1-15 and MT1-MMP-3xFLAG. These observations suggest that MT1-MMP-T567E expression can increase invadopodium formation when Munc18c function in inhibited, possibly by MT1-MMP-mediated activation of ERK (Williams and Coppolino, 2011a), which will be discussed in chapter 6.
**Figure 4.5** - Expression of MT1-MMP-T567E partially rescues invadopodium formation and gelatin degradation in cells expressing Stx4-N-term or Stx4 1-15.

A) Parental MDA-MB-231 cells and stable cells expressing Stx4-FL, Stx4-N-term, Stx4-1-15 and Stx4-15-29 were transfected with either MT1-MMP 3xFLAG or MT1-MMP T567E-3xFLAG for 20 hours, plated onto gelatin for 4 h, then fixed, permeabilized, stained for F-actin and analyzed by confocal microscopy. B) The above cells were transfected with either MT1-MMP-3xFLAG or MT1-MMP-T567E-3xFLAG for 20 hours, plated onto gelatin for 4 h, then fixed, permeabilized, stained for F-actin and analyzed by confocal microscopy. All data are presented as percent of control ± S.D. C) The above cells were plated onto gelatin for 24 h, then fixed, permeabilized, stained for F-actin and analyzed by confocal microscopy. All data are presented as percent of control ± S.D. Asterisks denote values significantly different from control (*, p < 0.05). All data represent three or more biological replicates with at least three technical replicates.
4.2.6 Inhibition of MT1-MMP further reduces invadopodium formation and gelatin degradation in cells expressing Stx4-N-term and Stx4-1-15

The preceding results clearly implicate SNARE-mediated trafficking of MT1-MMP in invadopodium-based ECM degradation in our system. To assess the degree to which these processes are dependent upon MT1-MMP’s catalytic activity, we treated cells with the MT1-MMP inhibitor, NSC405020, along with the MT1-MMP-blocking antibody, MAB3329. Previous findings have shown that under these conditions ECM degradation was abolished (Kumar et al., 2018). Following treatment with the combination of MSC405020 and MT1-MMP blocking antibody, MDA-MB-231 parental, Stx4-FL, Stx4-N-term, Stx4-1-15 and Stx4-15-29 stable cells all showed a significant decrease in invadopodium formation, as well as gelatin degradation compared to control cells (Figure 4.6). Confocal images of Stx4-1-15 and Stx4-15-29 were not included in the above figure due to their phenotypes strongly resembling Stx4-N-term or Stx4-FL, respectively.
Figure 4.6 - Inhibition of MT1-MMP decreases invadopodium formation in cells expressing Stx4-N-term or Stx4-1-15.

A) Parental MDA-MB-231 cells and stable cells expressing Stx4-FL, Stx4-N-term, Stx4-1-15 and Stx4-15-29 were treated with either DMSO (Control) or MAB3329 and NSC405020 and plated onto gelatin for 4 h, then fixed, permeabilized, stained for F-actin and analyzed by confocal microscopy. All data are presented as percent of control ± S.D.

B) The above cells were plated onto gelatin for 24 h, then fixed, permeabilized, stained for F-actin and analyzed by confocal microscopy. All data are presented as percent of control ± S.D.
4.2.7 The expression of Stx4-N-term decreases breast tumor cell invasion and metastasis

The results above indicate that interfering with Stx4-Munc18c interaction, by expressing Stx4-N-term or Stx4-N-term-1-15, impairs MT1-MMP-dependent invadopodia formation and gelatin degradation in MDA-MB-231 cells. To assess whether this has impact on metastatic potential in vivo, we orthotopically implanted MDA-MB-231 Stx4-FL, Stx4-N-term and Stx4-Cyto variants into immunocompromised mice. Tumours form MDA-MB-231 cells expressing Stx4 cytoplasmic domain (Stx4-Cyto), or the N-terminal 29 amino acids of Stx4 (Stx4-N-term), showed delayed growth and these mice had extended survival compared to mice bearing tumours from MDA-MB-231 cells expressing full-length Stx4 (Stx4-FL) (Figure 4.7A, B). Weighing lungs upon necropsy revealed that lungs removed from both the Stx4-N-term and Stx4-Cyto tumour bearing mice weighed significantly less than Stx4-FL tumour-bearing mice (Figure 4.7C). To assess whether this was due to differences in metastatic burden, lung sections were stained with hematoxylin and eosin and vimentin (Acharyya et al., 2012) (Figure 4.7D). Mice bearing Stx4-FL tumours had substantive metastatic growth in the lungs. In contrast, expression of Stx4-Cyto or Stx4-N-term nearly abrogated the metastatic capacity of the MDA-MB-231 tumours, reducing metastatic burden by 98.7% and 97.3%, respectively (Figure 4.7E). Together, with our in vitro findings, the data suggest that interfering with the Stx4-Munc18c interaction delays tumor progression.
Figure 4.7 - Expression of truncated Stx4 constructs reduces metastasis in a mouse xenograft model.

(A) Tumor growth curves for the indicated cell lines inoculated in the left 4th mammary fat pad; n=10. (B) Mice were weighed and tumour volume measured 5 days post inoculation, and every 2 days following. Survival is shown using Kaplan-Meier plots, with tumour volume (400m$^3$) as surrogate for endpoint. (C) Wet lung mass (g) for individual mice. (D) Lungs were excised, fixed, sectioned, and stained with H&E or with antibodies to vimentin. (E) Metastatic burden was calculated for entire lung sections using Case Viewer to draw ROIs around vimentin-positive clusters, summing the area of the ROIs (µm$^2$), and dividing by the total area (µm$^2$) for the section.
4.3 Discussion

Here, it is shown that MT1-MMP driven invadopodium formation and metastasis are regulated by the interaction of Syntaxin4 and Munc18c. Previous experiments have shown that Munc18c interacts with Stx4 through residues 1-29 of Stx4; however, a smaller portion of Stx4 has never been shown to interact with Munc18c. Using co-immunoprecipitation, Munc18c was found to interact with residues 1-15, but not 15-29, of Stx4. Expression of Stx4-1-15 in MDA-MB-231 cells decreased the formation of Stx4-containing SNARE complexes and perturbed MT1-MMP and EGFR delivery to the plasma membrane, and reduced invadopodium formation and gelatin degradation in vitro. In vivo studies highlighted the importance of the regulation of Stx4 by Munc18c, as expression of Stx4 1-29 reduced the metastatic progression of MDA-MB-231 tumors in mouse xenografts. To my knowledge, this is the first in vivo demonstration of a role for SNARE/Munc18c-regulated trafficking in tumor progression.

SNAREs have been shown to play a key role in invadopodium formation, cell migration, and MMP-mediated ECM degradation (Brasher et al., 2017; Kean et al., 2009; Williams and Coppolino, 2011a, 2014; Williams et al., 2014a); however, there is little information that defines how SNAREs are regulated during these processes. Munc18c has been characterized as both a positive (Latham et al., 2006; Oh et al., 2005b; Thurmond et al., 2000) and negative regulator (Tamori et al., 1998a; Thurmond et al., 1998) of Stx4, and here it is demonstrated that disrupting Stx4-Munc18c interaction decreases Stx4 activity and reduces invadopodium-based tumour cell invasion and metastatic spread. Furthermore, we determined that the function of Stx4 can be disrupted
by ectopic expression of a GFP-tagged peptide fragment comprising the first 15 amino acids of Stx4. This Stx4 fragment exerts its effects by binding Munc18c, and competitively inhibiting the binding of endogenous Stx4, leading to impaired trafficking of MT1-MMP and EGFR. The importance of MT1-MMP trafficking was underscored by the observation that expression of a mutant form of MT1-MMP (MT1-MMP-T567E) increased the in vitro invasive capacity of cells with a deficit in Stx4-mediated trafficking.

Cells that were transiently transfected with Stx4-1-15 or -15-29 showed decreases in invadopodium formation and gelatin degradation. Decreased invadopodium formation in cells expressing Stx4-15-29 was unexpected, as this portion of Stx4’s N-terminus does not contain leucine 8, which has been shown to be required for Munc18c binding (Latham et al., 2006). Previous research has shown that residues 1 through 19 of Stx4 interact with Munc18c’s binding pocket (Hu et al., 2007), meaning that residues 15-19 may play a role in the interaction of Munc18c and Stx4, however this has yet to be investigated directly. The decrease in invadopodium formation in cells expressing Stx4-15-29 was modest compared to Stx4-1-15 or Stx4-N-term expressing cells and cells stably expressing Stx4-15-29 showed no significant decrease in invadopodium formation or gelatin degradation. One possible explanation for this difference could be varying expression levels of Stx4-15-29 in transiently transfected versus stably expressing cells. To determine if cell expression levels are the reason for this difference in invadopodium formation, additional clones of stable Stx4-15-29 cells should be created and subjected to the same assays.
Overexpression of MT1-MMP-T567E in stable cell lines expressing Stx4-N-term or Stx4-1-15 caused an increase in invadopodium formation and gelatin degradation. MT1-MMP is known to be trafficked by the SNARE complex Stx4/SNAP23/VAMP2 during invadopodium formation (Brasher et al., 2017), and recycled in a VAMP7-dependent manner (Williams and Coppolino, 2011b). Since Munc18c function is inhibited in cells expressing Stx4-N-term or Stx4-1-15, SNARE complex formation between Stx4/SNAP23/VAMP2 is decreased. It is unknown how MT1-MMP-T567E expression caused increased invadopodium formation in these cells and experiments studying the activation of downstream signaling pathways involving MT1-MMP should be studied. These experiments, and other possible explanations of the effects of MT1-MMP-T567E on invadopodium formation, will be further explored in chapter 6.

Previous research has examined inhibition of MT1-MMP function by the use of the inhibitor NSC405020, along with the MT1-MMP blocking antibody. When MDA-MB-231 cells were treated with these compounds, a significant decrease in invadopodium formation was seen (Kumar et al., 2018), indicating that invadopodium formation in these cells is driven by the proteolytic ability of MT1-MMP. The same inhibitors were used in cells expressing Stx4-FL, Stx4-N-term, Stx4-1-15 and Stx4-15-29, and all cell lines showed a decrease in invadopodium formation and gelatin degradation. These results highlighted the importance of MT1-MMP’s catalytic activity for invadopodium formation. These results also indicate that cells expressing Stx4-1-15 or Stx4-N-term do traffic some catalytically active MT1-MMP, as invadopodium formation was further decreased when the MT1-MMP inhibitor and blocking antibodies were used.
The ability of cancer cells to form invadopodia has been demonstrated in primary tumor cells that were isolated from patients with invasive tumors (Meirson and Gil-Henn, 2018); however, there is no designated therapeutic treatment to block invadopodial-mediated metastasis (Meirson and Gil-Henn, 2018). SNARE-mediated trafficking of key proteins has been previously found to be involved in tumor cell invasion (Brasher et al., 2017; Kean et al., 2009; Steffen et al., 2008; Williams and Coppolino, 2014; Williams et al., 2014a), which includes invadopodium formation (Brasher et al., 2017; Chen and Scheller, 2001; Poincloux et al., 2009), cell migration (Day et al., 2011; Riggs et al., 2012; Tayeb et al., 2005), and MMP-directed ECM degradation (Miyata et al., 2004). It is clear that SNAREs play a key role in these processes; however, how SNAREs are regulated during tumor cell invasion remains largely unexplored. Many metastasis-directed therapies inhibit cell growth and division, making their clinical application challenging. There are a variety of small molecular inhibitors that are FDA approved for the treatment of cancer in combination with other therapies, and these include inhibitors that target the RAF-MEK-MAPK pathway and matrix metalloprotease inhibitors (Gatzka, 2018). There are no small molecular inhibitors that are able to target SNARE-mediated trafficking of invadopodial proteins in hopes of decreasing invadopodium formation, and overall metastasis. Here, we show the first 15 or 29 residues of Stx4 is able to inhibit Munc18c binding to Stx4, which causes a decrease in invadopodium formation, gelatin degradation, cell invasion, trafficking of MT1-MMP and EGFR to the cell surface, as well as tumor growth and metastasis in vivo, and could be a potential avenue for therapeutic targeting.
VAMP2 Function and Regulation if Required for Invadopodium Formation, Gelatin Degradation, Cell Migration and Cell Invasion
5.1 Summary

Previous experiments have revealed the role of the SNARE complex Stx4-SNAP23-VAMP2 in delivery of MT1-MMP to the cell surface during invadopodium formation (Brasher et al., 2017); however, VAMP2’s role in cell invasion has not been studied directly. VAMP2 has been shown to be important in cell migration in HeLa cells (Hasan and Hu, 2010), and to be regulated by Cdc42 during insulin exocytosis in pancreatic beta cells (Nevins and Thurmond, 2005). Here, we show that VAMP2 expression and function are required for invadopodium formation, and gelatin degradation. Expression of the cytoplasmic domain of VAMP2 caused a decrease in cell invasion and migration by inhibiting formation of endogenous VAMP2-containing SNARE complexes. A VAMP2 N-terminal peptide, consisting of residues 1 through 28 (VAMP2-1-28), was generated and predicted to act as a competitive inhibitor for Cdc42 binding to endogenous VAMP2. Expression of this peptide caused a decrease in invadopodium formation and gelatin degradation, suggesting that the regulation of VAMP2 is required for these invasive processes. Additionally, to determine if VAMP2 and VAMP7 share a common role during invadopodium formation, VAMP2-Cyto and VAMP7-Cyto were co-transfected into stable cells expressing Stx4-N-term or Stx4-1-15. Cells expressing Stx4-N-term or Stx4-1-15, as well as either VAMP2-Cyto or VAMP7-Cyto exhibited further decreases in invadopodium formation, suggesting VAMP2 and VAMP7 do not have completely redundant trafficking roles in the cell. These findings highlight a role for VAMP2 during tumor cell invasion and implicate Cdc42 as a regulator of VAMP2-mediated trafficking during invadopodium formation and gelatin degradation.
5.2 Results

5.2.1 Knockdown of VAMP2 impairs invadopodium formation and gelatin degradation

To determine if VAMP2 expression is required for invadopodium formation, cells were transfected with two different shRNAs to target VAMP2. Knockdown of VAMP2 was seen after 48 hours, when VAMP2 protein levels were reduced by 91.29% ± 4.49% and 81.84% ± 6.43% through expression of VAMP2 shRNA 4 and 5, respectively (Figure 5.1A,B). Protein levels of β tubulin were unaffected. Invadopodium formation was reduced by 78.32% ± 7.37% and 71.39% ± 8.37% in cells expressing VAMP2 shRNA 4 and 5, respectively (Figure 5.1C). The number of cells degrading gelatin was correspondingly reduced by 77.85% ± 19.82% and 80.94% ± 12.82% in cells treated with VAMP2 shRNA 4 and 5, respectively (Figure 5.1D).
Figure 5.1 - Knockdown of VAMP2 decreases invadopodium formation and gelatin degradation.

A) Cells were transfected with shRNA targeting VAMP2 or empty vector, lysed, and analyzed for VAMP2 by SDS-PAGE/Western blotting. B) Quantification of VAMP2 knockdown normalized to tubulin. C) Invadopodium-based degradation of gelatin by parental cells, cells transfected with empty vector (Control), and two different shRNAs targeted to VAMP2. Cells were transfected for 44 hours, seeded onto fluorescent gelatin for 4 hours, and then fixed, permeabilized, stained for F-actin, and analyzed by confocal microscopy. Scale bars = 10 μm. D) Quantification of invadopodium formation. Cells with F-actin puncta overlying dark spots of gelatin degradation were counted as cells forming invadopodia. Percentages of cells forming invadopodia, normalized to empty vector, were determined by counting 50 cells/sample. E) The above cells were seeded onto fluorescent gelatin for 24 h, and then fixed. Cells were analyzed for dark areas of degradation and scored as described under “Experimental procedures.” All data are presented as percent of control ± S.D. Asterisks denote values significantly different from control (*, p < 0.05). All data represent three or more biological replicates with at least three technical replicates.
5.2.2 Expression of VAMP2-Cyto decreases invadopodium formation and gelatin degradation

Previous research has shown that VAMP7 function is required for invadopodium formation (Williams et al., 2014b). To test if VAMP2 function is also required for invadopodium formation, formation of VAMP2 containing SNARE complexes was inhibited by expressing a truncated mutant VAMP2 construct that is predicted to exert dominant-negative effects on the formation of VAMP2-containing SNARE complexes. Similar constructs have been used extensively in other experimental systems to impair membrane trafficking (Hirling et al., 2000; Kean et al., 2009; Skalski et al., 2011; Trimble et al., 2019). SNAREs lacking their transmembrane domain - for example, VAMP2-Cyto and Stx4-Cyto, form SNARE complexes with cognate SNAREs, blocking interactions with endogenous membrane-associated SNARE partners. Here, cells were transfected with either GFP (empty vector), VAMP2-FL or VAMP2-Cyto for 24 hours, then plated onto fluorescently coated coverslips for 4 hours to test for invadopodium formation (Figure 5.2). Overexpression of VAMP2-Cyto reduced the number of cells forming invadopodia by 62.45% ± 6.33%, compared to cells expressing VAMP2-FL. VAMP2-Cyto expressing cells exhibited a 67.04% ± 11.01% decrease in gelatin degradation, compared to VAMP2-FL expressing cells, which supported gelatin degradation to a similar degree as control cells.
Figure 5.2 - Expression of VAMP2 Cyto decreases invadopodium formation and gelatin degradation.
A) Invadopodium-based degradation of gelatin by parental cells, cells transfected with empty vector (GFP control), VAMP2 Cyto or VAMP2 full length. Cells were transfected for 20 hours, seeded onto fluorescent gelatin for 4 hours, and then fixed, permeabilized, stained for F-actin, and analyzed by confocal microscopy. Scale bars = 10 μm. B) Quantification of invadopodium formation. Cells with F-actin puncta overlying dark spots

*
of gelatin degradation were counted as cells forming invadopodia. Percentages of cells forming invadopodia, normalized to empty vector, were determined by counting 50 cells/sample. C) The above cells were seeded onto fluorescent gelatin for 24 h, and then fixed. Parental cells and cells expressing GFP were analyzed for dark areas of degradation and scored as described under “Experimental procedures.” All data are presented as percent of control ± S.D. Asterisks denote values significantly different from control (*, p < 0.05). All data represent three or more biological replicates with at least three technical replicates.
5.2.3 VAMP2 function is required for cell migration and cell invasion

Given that invadopodium formation and gelatin degradation were decreased in cells expressing VAMP2-Cyto, the ability of these cells to migrate and invade was tested using Boyden chamber migration assays and Matrigel-based invasion assays. Cells expressing VAMP2-Cyto exhibited a 48.20% ± 21.79% reduction in migration compared to cells expressing VAMP2-FL. Cells expressing VAMP2-Cyto showed a 48.03% ± 3.61% decrease in cell invasion, compared to cells expressing VAMP2-FL. No significant difference was found between parental MDA-MB-231 cells and GFP-expressing cells, or GFP-expressing cells and VAMP2-FL-expressing cells in terms of cell migration or invasion (Figure 5.3).
Figure 5.3 - Expression of VAMP2-Cyto decreases cell migration and cell invasion. A) Untransfected cells or cells transfected with GFP, VAMP2-Cyto and VAMP2-FL were serum-starved for 24 h, seeded onto transwell membranes and allowed to invade for 20 hours. Percentages of cells invading are shown from experiments in which at least 10 fields of view were counted per treatment. B) The above cells were serum-starved for 24 h, seeded onto transwell membranes coated with Matrigel or uncoated membranes (control) and allowed to invade for 24 h. Percentages of cells invading are shown from experiments in which at least 10 fields of view were counted per treatment. All data represent percent of control ± S.D. Asterisks denote values significantly different from control (*, p < 0.05). All data represent three or more biological replicates with at least three technical replicates.
5.2.4 VAMP2 and Cdc42 localize to sites of invadopodium formation

Previous studies have shown that Cdc42 and VAMP2 interact through VAMP2’s N-terminus, and that Cdc42 plays an important role during VAMP2-mediated insulin exocytosis through this interaction (Nevins and Thurmond, 2005). To determine if VAMP2 and Cdc42 have similar distributions in MDA-MB-231 cells, confocal immunofluorescence microscopy was used. Expression of GFP-VAMP2-FL or GFP-VAMP2-1-28 and staining of Cdc42 showed partial overlapping of these two proteins near the ventral membrane of the cells, whereas GFP alone and Cdc42 did not (Figure 5.4A). The involvement of Cdc42 in invadopodium formation was supported by microscopic analysis of the Cdc42 localization during invadopodium formation assays. Similarly to VAMP2, Cdc42 was observed at invadopodia, marked by F-actin and focal points of degradation in labeled gelatin (Figure 5.4B). To assess the association of Cdc42 and VAMP2 during invadopodium formation, cells transfected with empty vector or VAMP2-FL were seeded onto fluorescent coverslips, fixed, and stained for Cdc42. Cells expressing VAMP2-FL and stained for Cdc42 show overlapping distribution at sites of degradation, supporting their involvement during invadopodium formation.
Figure 5.4 - Analysis of Cdc42 and VAMP2 localization in MDA-MB-231 cells
A) Cells were transfected with either empty vector (GFP), VAMP2-FL or VAMP2-1-28 and grown for 4 hours on glass coverslips, fixed, permeabilized and stained for Cdc42. A representative confocal section from the ventral region of a cell is shown. White arrows point to sites of signal overlap between VAMP2-FL or VAMP2 1-28 and Cdc4. B) Distribution of Cdc42 and VAMP2 during the formation of invadopodia. Cells were analyzed by confocal microscopy after being plated on Alexa Fluor 594-labeled gelatin for 4 hours, fixed, and stained for VAMP2 or Cdc42 and F-actin. Dark spots in the gelatin field indicate sites of gelatin degradation corresponding to invadopodia. Arrows indicate spots of signal overlapping gelatin degradation. White arrows point to spots of Cdc42 or VAMP2 localized to sites of invadopodium formation (black spots). C) Cells were transfected with either empty vector (GFP), or VAMP2-FL and grown on fluorescently labeled coverslips for 4 hours. Cells were fixed, permeabilized and stained for Cdc42 and analyzed by confocal microscopy. Black arrows indicate spots of VAMP2-FL and Cdc42 signal overlap gelatin degradation.
5.2.5 Inhibition of Cdc42 by ML141 decreases invadopodia formation and gelatin degradation

Previous research has shown that inhibition of Cdc42 using the inhibitor ML141 decreases association between Cdc42 and PAK, as well as Bradykinin-induced filopodia formation in 3T3 cells (Surviladze et al., 2010). To test if ML141 was able to inhibit Cdc42 in our system, the phosphorylation of ERK1/2 in cells treated with ML141 was investigated, since ERK1/2 is a downstream target of PAK. Cells treated with 10 μM ML141 showed a 88.46% ± 26.14% decrease in ERK1/2 phosphorylation (Figure 5.5A,B).

To determine if Cdc42-mediated signaling is required for invadopodia formation and gelatin degradation, we tested the effects of ML141 in our system. MDA-MB-231 cells were treated with 10 μM of ML141 or DMSO (control) and plated onto gelatin coated coverslips for 4 or 24 hours to test for invadopodium formation or gelatin degradation, respectively. Compared to DMSO treated control samples, cells treated with ML141 showed an 86.55% ± 3.01% decrease in invadopodium formation, and an 87.01% ± 3.01% decrease in gelatin degradation (Figure 5.5C, D, E).
Figure 5.5 - The Cdc42 inhibitor, ML141, decreases ERK1/2 phosphorylation, invadopodium formation and gelatin degradation.

A) Treatment of ML141 decreases ERK1/2 phosphorylation. Cells were serum starved overnight, and then treated with 10 μM ML141 for 45 minutes. Cells were lysed, and extracted proteins were separated by SDS-PAGE prior to analysis by Western blot for ERK and phospho-ERK. B) Western blots from at least three independent experiments were analyzed by ImageJ from to quantify the decrease in ERK phosphorylation. C) Invadopodium-based degradation of gelatin by cells treated with DMSO or ML141. Cells were treated, seeded onto fluorescent gelatin for 4 hours, and then fixed, permeabilized, stained for F-actin, and analyzed by confocal microscopy. Scale bars = 10 μm. D)
Quantification of invadopodium formation. Cells were treated in C, and cells with F-actin puncta overlying dark spots of gelatin degradation were counted as cells forming invadopodia. E) Quantification of gelatin degradation. Cells were treated as in C. Cells were quantified as in “Experimental procedures.” All data are presented as percent of control ± S.D. Asterisks denote values significantly different from control (*, p < 0.05). All data represent three or more biological replicates with at least three technical replicates.
5.2.6 Expression of VAMP2 N-terminal domain impairs invadopodium formation and gelatin degradation

Previous research has shown that Cdc42 interacts with VAMP2 through its N-terminal domain, consisting of residues 1 through 28. In order to determine if the interaction between VAMP2 and Cdc42 is required for invadopodium formation and gelatin degradation, a peptide containing residues 1 through 28 of VAMP2, tagged with GFP, was expressed in MDA-MB-231 cells for 24 hours. These cells were then plated on fluorescently coated coverslips for 4 hours or 24 hours to monitor invadopodium formation or gelatin degradation, respectively. Compared to cells expressing VAMP2-FL, cells expressing VAMP2 1-28 had a 68.9% ± 19.2% decrease in invadopodium formation (Figure 5.6A, B). Cells expressing VAMP2-1-28 also showed a decrease in gelatin degradation of 79.19% ± 12.40%, compared to cells expressing VAMP2-FL (Figure 5.6C).
Figure 5.6 – Expression of VAMP2 1-28 decreases invadopodium formation and gelatin degradation.

A) Invadopodium-based degradation of gelatin by parental cells, cells transfected with empty vector (GFP control), VAMP2-FL or VAMP2-1-28. Cells were transfected for 20 hours, seeded onto fluorescent gelatin for 4 hours, and then fixed, permeabilized, stained for F-actin, and analyzed by confocal microscopy. Scale bars = 10 μm. B) Quantification of invadopodium formation. Cells with F-actin puncta overlying dark spots of gelatin degradation were counted as cells forming invadopodia. Percentages of cells forming invadopodia, normalized to empty vector, were determined by counting 50 cells/sample. C) The above cells were seeded onto fluorescent gelatin for 24 hours, and then fixed. Parental cells and cells expressing GFP were analyzed for dark areas of degradation and scored as described under “Experimental procedures.” All data are presented as percent of control ± S.D. Asterisks denote values significantly different from control (*, p < 0.05). All data represent three or more biological replicates with at least three technical replicates.
5.2.7 Co-expression of either VAMP2-Cyto or VAMP7-Cyto augments the disruption of invadopodium formation and ECM degradation caused by inhibition of Stx4

Previous studies have shown that SNAP23 and Syntaxin4 can interact with both VAMP2 (Brasher et al., 2017) and VAMP7 (Williams et al., 2014a), and the dependence of these v-SNAREs on Stx4 function for their roles in invadopodium formation has not been studied. To test this, MDA-MB-231 cells stably expressing Stx4-N-term were transiently transfected with the following 3xFLAG tagged constructs to inhibit formation of either VAMP7- or VAMP2-containing SNARE complexes: VAMP7-FL, VAMP7-Cyto, VAMP2-FL or VAMP2-Cyto. All stable cells expressing VAMP2-Cyto (Fig. 5.7A-C) or VAMP7-Cyto (Fig. 5.7D-F) showed a decrease in invadopodium formation, as well as gelatin degradation. Stx4-N-term stable cells transfected with VAMP2-Cyto also showed a decrease in invadopodium formation (18.35% ± 5.67%), compared to Stx4 N-terminal peptide stable cells expressing VAMP2-FL, suggesting that the trafficking of MT1-MMP is facilitated by SNAREs other than Stx4. In gelatin degradation assays, all cell lines expressing VAMP2-Cyto showed a decrease in gelatin degradation (Fig. 5.7C). All stable cells expressing VAMP7-Cyto showed decreased invadopodium formation and gelatin degradation, when compared to stable cells expressing VAMP7-FL (Fig. 5.7E, F). Of note, cells expressing Stx4 N-terminal peptide and VAMP7-Cyto showed a 16.31% ± 3.62% in invadopodium formation, and a decrease of 3.16% ± 0.28% in gelatin degradation. Confocal images of Stx4-1-15 and Stx4-15-29 were not included in the above figure due to their phenotypes closely resembling Stx4-N-term and Stx4-FL, respectively.
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VAMP2 Cyto 3xFLAG
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VAMP7-FL 3xFLAG
VAMP7 Cyto 3xFLAG
Figure 5.7 - Co-expression of VAMP2-Cyto or VAMP7-Cyto with Stx4-N-term or Stx4-1-15 further decreases invadopodium formation and gelatin degradation.

A) Parental MDA-MB-231 cells and stable cells expressing Stx4-FL, Stx4-N-term, Stx4-1-15 and Stx4-15-29 were transfected with either VAMP2-FL or VAMP2-Cyto for 20 hours, plated onto gelatin for 4 hours, then fixed, permeabilized, stained for F-actin and analyzed by confocal microscopy. B) The above cells were plated onto gelatin for 4 hours, then fixed, permeabilized, stained for F-actin and analyzed by confocal microscopy. All data are presented as percent of control ± S.D. C) The above cells were plated onto gelatin for 24 hours, then fixed, permeabilized, stained for F-actin and analyzed by confocal microscopy. All data are presented as percent of control ± S.D. D) Parental MDA-MB-231 cells and stable cells expressing Stx4-FL, Stx4-N-term, Stx4-1-15 and Stx4-15-29 were transfected with either VAMP7-FL or VAMP7-Cyto for 20 hours, plated onto gelatin for 4 hours, then fixed, permeabilized, stained for F-actin and analyzed by confocal microscopy. E) The above cells were plated onto gelatin for 4 hours, then fixed, permeabilized, stained for F-actin and analyzed by confocal microscopy. All data are presented as percent of control ± S.D. F) The above cells were plated onto gelatin for 24 hours, then fixed, permeabilized, stained for F-actin and analyzed by confocal microscopy. All data are presented as percent of control ± S.D. Asterisks denote values significantly different from control (*, p < 0.05). All data represent three or more biological replicates with at least three technical replicates.
5.3 Discussion

Invadopodium formation, cell migration and ECM invasion are part of the metastatic process that relies on trafficking of key proteins in a SNARE-dependent manner. Previous work has illustrated that Stx4, SNAP23 and VAMP2 form a SNARE complex that is regulated by Munc18c, leading to trafficking of MT1-MMP to invadopodium (Brasher et al., 2017). The roles of Stx4 and SNAP23 have been previously explored, and expression and function of both SNAREs is required for invadopodium formation (Williams et al., 2014a). VAMP2 has been shown to be required for β1 integrin trafficking and cell migration in HeLa cells (Hasan and Hu, 2010), but its role during tumor cell invasion was unknown. Here, I show that VAMP2 expression and function is required for invadopodium formation and gelatin degradation. Munc18c is known to regulate Stx4 during tumor cell invasion; however, regulation of other SNAREs had yet to be investigated in this context. The regulation of VAMP2 by Cdc42 had been shown previously in pancreatic beta cells (Nevins and Thurmond, 2005), and Cdc42 has established roles in cancer progression through its regulation of migration/invasion, metastasis, cell proliferation and cell-ECM interactions (Del Mar Maldonado and Dharmawardhane, 2018). In this chapter, I demonstrate that Cdc42’s interaction with VAMP2, through VAMP2’s N-terminus, contributes to invadopodium formation and gelatin degradation in MDA-MB-231 cells. These findings suggest that the interaction of Cdc42 and VAMP2 is an important point of regulation in the process of invadopodium formation in tumor cells.
The observed distribution of VAMP2 and Cdc42 in MDA-MB-231 cells is consistent with their function at the plasma membrane during invadopodium formation. Analyses of the distributions of Cdc42 and VAMP2 revealed that these two proteins partly co-localize in cells at rest or forming invadopodium. Co-immunoprecipitation of VAMP2 and Cdc42 was attempted but was unsuccessful. This experiment may have been unsuccessful due to a lack of suitable antibodies. Future experiments testing different antibodies should be done to confirm the interaction between Cdc42 and VAMP2 in our cell system.

To elucidate the mechanism of VAMP2 regulation by Cdc42, future experiments will involve studying their interaction during invadopodium formation. Previous research has shown an increased interaction between Stx4 and Munc18c during invadopodium formation (Brasher et al., 2017), and it is possible a similar interaction would be seen between VAMP2 and Cdc42, as expression of VAMP2-1-28 decreased invadopodium formation. If Cdc42 acts as a positive regulator of VAMP2 during invadopodium formation, their association should be increased. Further experiments could also include monitoring the effects of expressing VAMP2-1-28 on both cell migration and invasion, in order to determine if the interaction of VAMP2 and Cdc42 is required for other aspects of cell invasion.

Previous studies have shown that inhibition of RNAi-mediated knockdown of Stx4, SNAP23 or VAMP7 impairs invadopodium formation and gelatin degradation. Here, RNAi-mediated knockdown and inhibition of VAMP2, or inhibition of its function using truncated constructs, was observed to inhibit invadopodium formation in MDA-MB-231 cells, similar to that observed when VAMP7 was knocked down by siRNA (Williams et al.,
2014b). Together, the observations support a model wherein VAMP2-mediated trafficking to the plasma membrane facilitates the delivery of proteins required for invadopodium formation. Future experiments could examine the effects of VAMP2 inhibition on MT1-MMP cell surface levels to advance our broader knowledge on MT1-MMP trafficking.

How Cdc42 functions to regulate VAMP2 is not understood. A decrease in invadopodium formation was seen in cells treated with ML141, and in cells expressing VAMP2-1-28, indicating that VAMP2 and Cdc42 are involved in invadopodium formation. Whether the treatment of cells with ML141 affects Cdc42’s regulation of VAMP2 remains to be studied. To further understand this interaction, cells plated on gelatin should be treated with ML141, and then the interaction of VAMP2 and Cdc42 analyzed by co-immunoprecipitation. If treatment of ML141 affects Cdc42’s ability to regulate VAMP2, a decrease in the amount of Cdc42 associating with VAMP2 might be detected. Although it has been shown that Cdc42 binds to VAMP2-1-28 in pancreatic beta cells (Nevins and Thurmond, 2005), it has yet to be demonstrated in our cell system. Future experiments are planned to immunoprecipitate GFP-VAMP2-1-28, and co-immunoprecipitate Cdc42 in order to show that Cdc42 can interact with VAMP2’s N-terminus in MDA-MB-231 cells. As well, experiments to assess cell invasion and cell surface levels of key invadopodial proteins in cells expressing VAMP2-1-28 should be performed to determine if Cdc42 function is required for these processes. Such analyses would provide insight into SNAREs and their regulators, and the mechanisms that surround invadopodium formation.
Cdc42 activates signaling pathways that regulate invadopodium formation and cell invasion. This includes the activation of N-WASP, leading to actin nucleation, and activation of PAK, which leads to activation of ERK. It is unknown if VAMP2 plays a role in any Cdc42 signaling pathway, which could result in downstream activation of either Arp2/3 or ERK. F-actin staining of ML141 treated cells was normal (Figure 5.4), indicating that Cdc42 inhibition by ML141 does not affect Arp2/3 signaling. In order to determine if Cdc42 regulation of VAMP2 is required for ERK signaling, the phosphorylation of ERK in cells expressing VAMP2-1-28 should be investigated using phosphorylation-specific antibodies. The possible signaling mechanisms involving VAMP2 and Cdc42 will be further discussed in chapter 6 and should be topics of future research.

To my knowledge, these findings show the first evidence of VAMP2’s involvement in invadopodium formation, as well as the regulation of VAMP2 by Cdc42 during tumor cell invasion. This work advances our understanding of the SNARE-mediated mechanisms that are involved in invadopodium formation and cell invasion, two key components of metastasis, by highlighting a SNARE complex involving VAMP2 that is required for invadopodium formation.
6 General Discussion and Future Directions

6.1 General Conclusions and Discussion

The results of the current studies show the importance of the regulation of SNARE-mediated trafficking during cell invasion and invadopodium formation. Previous studies have illustrated that SNAREs are required for delivery of key invadopodial proteins, such as EGFR and MT1-MMP (Williams and Coppolino, 2014; Williams et al., 2014b), to the cell surface; however, how SNARE function within these processes is regulated is not understood. Initial studies demonstrated that Munc18c acts as a regulator of Stx4, which was found to be important in invadopodium formation, gelatin degradation, cell invasion and delivery of MT1-MMP and EGFR to the cell surface (Brasher et al., 2017). We have since advanced our understanding of the region of Stx4 that is involved in binding to Munc18c, and determined that over-expression of a peptide containing the first fifteen residues of Stx4 is able to perturb this interaction, and decrease invadopodium formation, cell invasion, cell migration and cell surface levels of MT1-MMP and EGFR. Furthermore, we have shown that VAMP2, which remained uncharacterized in its role during invadopodium formation, is required for invadopodium formation, and is regulated by Cdc42 during cellular invasion.

Previous investigations of SNARE function during invadopodium formation identified the involvement of Stx4, SNAP23, VAMP7, Stx13 and VAMP3 (Kean et al., 2009; Williams et al., 2014b). Inhibition of Stx13/VAMP3/SNAP23 reduced total MT1-MMP at the plasma membrane during phorbol 12-myristate 13-acetate (PMA)-induced exocytosis (Kean et al., 2009), and inhibition of Stx4/SNAP23/VAMP7 was found to
decrease invadopodium formation, gelatin degradation, as well as total MT1-MMP cell surface levels (Williams et al., 2014b). While it was determined that Munc18c regulates the formation of the SNARE complex Stx4/SNAP23/VAMP2 (Brasher et al., 2017), a regulator of the SNARE complex Stx13/SNAP23/VAMP7 has yet to be found, and such a regulator may play an additional role in trafficking of MT1-MMP to the cell surface. It would be of interest to determine if Stx13 is also regulated by a SM protein, and this could be done through the immunoprecipitation of Stx3 and using mass spectrometry to find potential regulators (Gingras et al., 2007). It would be also of interest to determine if inhibition of the Stx4/SNAP23/VAMP2 SNARE complex could shed light onto other SNARE interactions in order to investigate the redundancy of SNAREs during invadopodium formation. This could be investigated by expression of Stx4-N-term and using co-immunoprecipitation to examine the interactions between Stx13/VAMP3 and SNAP23 during invadopodium formation.

6.2 Coordination of Membrane Trafficking and Signaling

In cells expressing Stx4-N-term or Stx4-1-15, invadopodium formation and MT1-MMP cell surface levels were decreased, which is likely due to decreased SNARE complex formation between Stx4/SNAP23/VAMP2. Previous findings have shown that expression of the MT1-MMP mutant T567E increased cell migration in HT-1080 cells (Williams and Coppolino, 2011a), and this construct was over-expressed to determine if invadopodium formation could be increased in cells expressing Stx4-N-term or Stx4-1-15. An increase in invadopodium formation was observed in cells co-expressing Stx4-N-term or Stx4-1-15 and MT1-MMP-T567E. Since SNARE-mediated trafficking is impaired
in these cells through inhibition of Munc18c function, it is understandable that normal levels of invadopodium formation were not seen. Previous experiments expressing MT1-MMP-T567E in HT-1080 cells led to increased phosphorylation of ERK (Williams and Coppolino, 2011b), suggesting that phosphorylation of MT1-MMP leads to ERK activation. Cells expressing MT1-MMP-T567E had increased invadopodium formation, which could be due to increased ERK phosphorylation, as ERK signaling increases cell invasion and migration (see model in Figure 6.1) (Wortzel and Seger, 2011). In order to test if this is the case, ERK phosphorylation levels in cells expressing Stx4-N-term or Stx4-1-15 and MT1-MMP T567E should be tested using a phospho-ERK antibody. If invadopodium formation is increased due to MT1-MMP-T567E-mediated ERK phosphorylation, cells expressing Stx4-N-term or Stx4-1-15 and MT1-MMP-T567E should have increased phospho-ERK, compared to control.
Figure 6.1 - Possible MT1-MMP-T567E Signaling Pathway.

1) Munc18c is inhibited by Stx4-N-term (a), causing a decrease in Stx4/SNAP23 interaction. This decrease of Stx4/SNAP23 interaction perturbs VAMP2 SNARE complex formation (b). Cells expressing Stx4-N-term and MT1-MMP-T567E have increased invadopodium formation, compared to cells expressing Stx4-N-term and MT1-MMP wildtype. A possible mechanism behind increased invadopodium could be the activation of ERK by MT1-MMP-T567E, leading to increased cell invasion and migration.
As stated previously, cells expressing Stx4-N-term or Stx4-1-15 and MT1-MMP-T567E had increased invadopodium formation. This increase in invadopodium formation could be explained by alternate SNARE complexes transporting MT1-MMP to the cell surface. MT1-MMP is known to be trafficked by not only the Stx4/SNAP23/VAMP2 SNARE complex (Brasher et al., 2017), but also VAMP7 containing complexes (Williams and Coppolino, 2011a). In cells expressing Stx4-N-term or Stx4-1-15, SNARE complex formation between Stx4/SNAP23/VAMP2 is decreased; however, VAMP7 function is not dependent on Munc18c. Previous findings from our lab have shown that MT1-MMP T567E is recycled faster than wildtype MT1-MMP, and MT1-MMP recycling occurs in a VAMP7 dependent manner (Williams and Coppolino, 2011a). It is possible that VAMP7 could interact with alternate plasma membrane Q-SNAREs, such as Stx13 and SNAP23, in order to transport invadopodial proteins to the cell surface (Bethani et al., 2007; Xue and Zhang, 2002). It is plausible that in Stx4’s functional absence, VAMP7 is able to compensate and deliver MT1-MMP-T567E to sites of invadopodium formation. Interestingly, cells expressing Stx4-N-term or Stx4-1-15 and MT1-MMP wildtype did not show increased invadopodium formation. These results suggest that ERK phosphorylation by MT1-MMP-T567E is more likely to be responsible for the increase in invadopodium formation in cells expressing Stx4-N-term or Stx4-1-15 and MT1-MMP-T567E, compared to the possibility that VAMP7 is able to compensate the trafficking of MT1-MMP to the cell surface, in Stx4/SNAP23/VAMP2’s functional absence.
6.3 Role of Cdc42 in SNARE-Mediated Cell Invasion

Cdc42 is known to play a role in cell migration and invasion by activating downstream pathways like Arp2/3, leading to actin nucleation, as well as PAK, allowing for ERK activation (Zhang et al., 2019). ML141 is a Cdc42 inhibitor, that causes decreased PAK activation and filopodia formation (Surviladze et al., 2010). Cells treated with ML141 showed decreased ERK1/2 phosphorylation, which is consistent with Cdc42’s ability to activate PAK. To determine if Cdc42 function is required for invadopodium formation, cells were treated with ML141. Treatment of MDA-MB-231 cells with ML141 caused a significant decrease in invadopodium and gelatin degradation; however, F-actin structure was unaffected, as seen by phalloidin staining. These results of normal F-actin staining suggest that ML141 inhibition of Cdc42 does not affect Arp2/3 activation (See model in Figure 6.2). Treatment of cells with ML141 showed a decrease in ERK1/2 phosphorylation, which is downstream from PAK signaling. This suggests that the decrease in invadopodium seen in ML141 treated cells is in part due to a decrease in ERK1/2 phosphorylation. Although previous research has shown that Cdc42 regulates VAMP2, and here it is shown that Cdc42 regulation of VAMP2 is required for invadopodium formation, it is unknown if VAMP2 is involved in Cdc42 mediated signaling of PAK. The possible coordination of Cdc42- and VAMP2-mediated signaling, leading to invadopodium formation, are highlighted in Figure 6.2. It is possible that VAMP2 functions independently of PAK-mediated signaling and regulates invadopodium formation solely by trafficking proteins like EGFR and MT1-MMP to the cell surface (Figure 6.2C). It also is possible that Cdc42 regulates VAMP2, which then activates PAK, leading to cell
invasion or migration (Figure 6.2D). To determine if VAMP2 regulation by Cdc42 affects PAK activation, cells expressing VAMP2-1-28 should be probed for phospho-PAK or phospho-ERK. If Cdc42 regulation of VAMP2 is required for PAK activation, the phosphorylation of PAK or ERK should be decreased in cells expressing VAMP2-1-28. These results highlight the importance of the regulation of VAMP2 by Cdc42 during invadopodium formation, and that Cdc42 function is required for invadopodium formation.
Figure 6.2 - Possible CDC42 Signaling Pathways involving VAMP2

a) Cdc42 is able to activate N-WASP, leading to downstream signaling of the Arp2/3 complex. This causes actin nucleation. Due to visualization of normal actin structure under ML141 treatment, it is believed that this pathway is not affected. b) Cdc42 is able to activate PAK, leading to RAF activation and ERK phosphorylation. This leads to cell invasion and migration. c) Cdc42 is known to interact with VAMP2, and this interaction is required for invadopodium formation, and possibly cell invasion and migration. d) It remains undetermined if Cdc42 activation of VAMP2 leads to PAK activation, or if this signaling is unrelated, as in c).
6.4 Roles of VAMP2 and VAMP7 in Cell Invasion

Our investigations over-expressing Stx4 N-terminal peptide led to observations that Munc18c function promotes SNARE complex formation between Stx4/SNAP23/VAMP2 (Brasher et al., 2017). VAMP2 was already known to interact with Stx4 and SNAP23 (Chen and Scheller, 2001), but its role in invadopodium formation and cell invasion had yet to be explored. This led us to use RNAi and VAMP2-Cyto constructs to study VAMP2's role in these processes. Here, I show that VAMP2 is required for invadopodium formation and cell invasion. Both VAMP2 and VAMP7 are known to interact with Stx4 and SNAP23 (Brasher et al., 2017; Hong, 2005; Williams et al., 2014a), and are involved in trafficking of cellular material to the plasma membrane. VAMP7 is involved in trafficking from the lysosome and late endosomes, and VAMP2 has been found to traffic from sorting endosomes and secretory granules (Hong, 2005). VAMP7 is not only able to bind to Stx4 and SNAP23, but also Stx7, and Stx8, while VAMP2 can also interact with Stx13, Stx1 and SNAP25 (Hong, 2005). VAMP2 and VAMP7 have both been shown to play a role in the inflammation involved in cancer development, as they regulate intracellular trafficking and release of pro-inflammatory cytokines involved in tumorigenesis (Stow et al., 2006). VAMP2 and VAMP7 also have overlapping and independent roles in the nervous system (Hong, 2005). The roles of these SNAREs may provide redundancy and specificity in delivery of cellular material, but their roles in tumor cell invasion are largely unknown. To determine if MT1-MMP is trafficked by multiple SNARE complexes during invadopodium formation and gelatin degradation, stable cells expressing Stx4-N-term or Stx4-1-15 were transiently transfected with either VAMP2- or
VAMP7-Cyto and subjected to invadopodium formation and gelatin degradation assays. When Stx4-N-term or Stx4-1-15 was expressed alongside VAMP2- or VAMP7-Cyto, an even greater decrease was seen in invadopodium formation and gelatin degradation compared to cells expressing Stx4 N-term or Stx4-1-15 and VAMP2/7-FL. A possible mechanism to explain the further decrease in invadopodium formation in cells expressing Stx4-N-term or Stx4-1-15 with VAMP2-Cyto is summarized in Figure 6.3.

In MDA-MB-231 cells (Figure 6.3 part 1), Munc18c is able to interact with Stx4, allowing for SNARE complex formation between Stx4/SNAP23/VAMP2. This allows for the delivery of MT1-MMP and EGFR to invadopodia, promoting invadopodium formation. When Munc18c is inhibited through expression of Stx4-N-term or Stx4-1-15 (Figure 6.3, part 2), interaction between Stx4 and SNAP23 is decreased, preventing VAMP2 from interacting, and results in decreased invadopodium formation. When Munc18c and VAMP2 are both inhibited through the expression of Stx4-N-term or Stx4-1-15 and VAMP2-Cyto (Figure 6.3. part 3), invadopodium formation is further reduced. It is speculated that the further reduction caused by VAMP2-Cyto is due to inhibition of VAMP2 interaction with different plasma membrane Q-SNAREs than Stx4, further reducing MT1-MMP and EGFR delivery (Figure 6.3, part 4). It remains to be investigated further which Q-SNAREs these might be. Overall, the co-expression of Stx4-N-term or Stx4-1-15 and VAMP2-Cyto provides evidence of a separate VAMP2 SNARE complex that is able to deliver MT1-MMP and EGFR to the cell surface, allowing for invadopodium formation.
Figure 6.3- Possible Alternative VAMP2 Trafficking Pathway.
1) During normal cell invasion, MT1-MMP and EGFR are trafficked to sites of invadopodium formation by Stx4, SNAP23 and VAMP2. 2) When Munc18c is inhibited by expression of Stx4-N-term (2a), invadopodium formation is decreased. This is due to decreased Stx4/SNAP23/VAMP2 SNARE complex formation (2b), which leads to decreased MT1-MMP and EGFR delivery to invadopodia. 3) When Munc18c is inhibited by expression of Stx4-N-term (3a), and VAMP2-Cyto 3xFLAG is also expressed (3b), invadopodium formation decreases further. This is possibly due to VAMP2-Cyto perturbing SNARE complexes that involve VAMP2, but not Stx4 and SNAP23. 4) Overall, when Munc18c is inhibited (4a) our results suggest that VAMP2 is able to interact with other Q-SNAREs in order to influence invadopodium formation.
Interestingly, some cells, expressing both Stx4-N-term and VAMP2/7-Cyto, were still able to form invadopodia. Since VAMP2 and VAMP7 both interact with SNAP23 and Stx4, it is therefore possible that VAMP2 and VAMP7 do not have completely redundant roles in the trafficking of cellular material. Future experiments investigating the possible redundancy of these v-SNAREs could include inhibiting either VAMP2 or VAMP7, knocking out the other vesicle SNARE, and examining invadopodium formation. Previous experiments have revealed a role for VAMP7 in MT1-MMP’s recycling to the plasma membrane (Williams and Coppolino, 2011a), therefore it is possible that VAMP7 is required for recycling, whereas VAMP2 is required for initial delivery. Experiments studying the trafficking of MT1-MMP have been previously done in our lab by fluorescently tagging MT1-MMP, and Rab proteins to mark specific endosomal compartments (Williams and Coppolino, 2011b). Co-localization of MT1-MMP with Rab markers can then be visualized at selected time points in cells expressing VAMP2- or VAMP7-Cyto. These timepoint experiments would aid in the understanding of the different roles that VAMP2 or VAMP7 play in MT1-MMP trafficking to invadopodia.

6.5 Other Possible Regulators of Syntaxin4

Our studies thoroughly investigated the interaction between Stx4’s N-terminus and Munc18c; however, there are other domains of Stx4 that are known to interact with other regulators. An example of Stx4 regulation was established in MIN6 pancreatic beta cells, in which F-actin was found to interact with residues 39 through 112 of Stx4, comprising Stx4’s H₈ and H₉ domains. This region of Stx4 was then used as a competitive inhibitor of F-actin-binding, and the importance of the interaction of Stx4 and F-actin on glucose-
stimulated insulin secretion was studied. Cells expressing Stx4 39-112 had increased insulin release, which suggests that putative disruption of F-actin binding to Stx4 enhanced insulin exocytosis, making F-actin a negative regulator of Stx4 function (Jewell et al., 2008a). To test if F-actin function was required for Stx4 interaction with VAMP2, GST-VAMP2 was linked to Sepharose beads and Stx4 containing lysate was added either in the presence or absence of latrunculin, an F-actin inhibitor that destabilizes actin filaments by sequestering actin monomers (Yarmola et al., 2000). Interestingly, an increase in the amount of Stx4 that interacted with GST-VAMP2 was seen in cells treated with latrunculin. The data suggests F-actin is able to negatively regulate exocytosis by binding to, and reducing Stx4 activity (Jewell et al., 2008a). It would be of interest to determine if expression of this domain of Stx4 can affect the regulation of SNARE-mediated trafficking during invadopodium formation.

Another known regulator of Stx4 is gelsolin, which is an ubiquitously expressed F-actin-severing/capping protein found to play a positive role in insulin secretion (Nelson and Boyd, 1985; Tomas et al., 2006). Gelsolin was found as a potential Stx4 binding partner through a yeast two-hybrid screen, which was then verified in MIN6 β-cell lysates by co-immunoprecipitation (Kalwat et al., 2012). Through the subsequent deletion of Stx4 domains, gelsolin was found to interact with residues 39 through 70 of Stx4, which corresponds to its Ha domain. Introduction of this competitive inhibitory peptide into pancreatic β-cells disrupted endogenous Stx4-gelsolin complexes, as well as insulin stimulated secretion. Apart from our findings relating to regulation of Stx4 by Munc18c through Stx4’s N-terminus, little is known about the other domains of Stx4 and their
relation to invadopodium formation (Kalwat et al., 2012). As discussed above, F-actin is known to interact with Stx4; however, this was not studied in tumor cells. Since gelsolin, F-actin, and Munc18c all bind to similar regions of Stx4 it is possible that they interact during regulation of SNARE-mediated trafficking (Jewell et al., 2008b; Kalwat et al., 2012). It would be informative to examine if Munc18c binding to Stx4 reduces or increases binding with other possible regulators, as our findings suggest that Munc18c increases SNARE complex formation (Brasher et al., 2017). Co-immunoprecipitation experiments in our lab have also shown associations between Syntaxin4 and gelsolin in MDA-MB-231 cells (data not shown). Future experiments assessing gelsolin’s role in invadopodium formation could be conducted by expression of residues 39-70 of Stx4 and observing any changes to invadopodium formation.

6.6 Limitations of the Above Work

A limitation of the completed work is the use of biotinylation to look at cell surface proteins during invadopodium formation, as biotinylation only allows proteins that have an extracellular domain to be analyzed. The localization of a variety of invadopodial proteins could be altered when Munc18c function is inhibited; however, other methods would have to be used to quantify this. One method that could be used is immunofluorescence microscopy to examine the location of invadopodial proteins, such as cortactin or Src, both of which are implicated in invadopodium formation (Williams and Coppolino, 2014). Challenges of this approach include ensuring adequate resolution of these images, and specificity of the primary antibody (Donaldson, 1998). Another technique that could be used would be invadopodia isolation, which involves allowing
cells to form invadopodia, using a glass pipette to remove cell bodies, and analyzing the proteins remaining in the gelatin by Western immunoblotting (Mueller et al., 1992). Since invadopodia are small subcellular structures, isolating them is difficult, and obtaining sufficient quantities of proteins for analysis may be challenging.

Another limitation of the studies described here is the use of two-dimensional substrates in the study of invadopodium formation and cell invasion. When cells are plated on two-dimensional substrates, invadopodium form under the cell and are restricted to growth within these extremely thin matrices. Because of this, invadopodia size is limited and could alter their formation, compared to a three-dimensional environment (Ondrej et al., 2010). As an alternative approach, the three-dimensional tumor-tissue invasion model developed by Puls et al. could be used to model tumor invasion more accurately, compared to a two-dimensional model. This three-dimensional tumor-tissue invasion model uses a custom-designed fabrication system, and standardized oligomeric type I collagen to define and modulate a physiological ECM environment (Puls et al., 2018). This 3D tumor model would allow us to depict more accurately how the regulation of SNAREs contribute to invadopodium formation.

Another limitation of the studies described here is the ability to distinguish the difference between podosomes and invadopodia using immunofluorescence methods. Typically, degradative ventral protrusions formed by normal cells are known as podosomes, whereas invadopodia are found in tumor cells. Invadopodia have been shown to be more stable than podosomes, and invadopodia also protrude further into the ECM (Murphy and Courtneidge, 2011). Tumor cells plated onto a fluorescent ECM
analogue to allow for matrix degradation, along with staining of either TKS5 or F-actin has commonly been used to quantify invadopodium formation (Artym et al., 2009; Kean et al., 2009; Williams and Coppolino, 2014). In this research, invadopodium formation is determined using fluorescent gelatin and F-actin staining; however, it could be said that this is not sufficient to distinguish between invadopodia and podosomes. To help differentiate invadopodia and podosomes, cells could be stained with an additional marker of invadopodia, such as TKS5 or cortactin.

It is important to note that the xenograft in vivo experiments shown in chapter 4 are preliminary, and their interpretation is limited by the fact that both primary tumor growth and lung metastasis were inhibited by the cell’s manipulation. This makes it difficult to know if there was a direct effect on metastasis or if the reduction in metastatic capability was a result of a reduction in tumor growth. Also, only one breast cancer cell line was tested, and this cell line represents only one of the various molecular subtypes of breast cancer, where each molecular subtype might have different mechanisms of invasion. Another important point to note is that cell proliferation could play an important factor in metastasis, as cells that grow faster could have an increased rate of tumor growth and metastasis. All cell lines used were tested by our collaborators to determine if cell proliferation could be a contributing factor; however, no cell line exhibited a significant difference in proliferation rate. Lastly, all mice in a group were euthanized once a single individual tumor became larger than 400 mm$^3$, meaning that mice with smaller tumors were euthanized at the same time. Premature euthanasia of mice could skew metastasis results; however, it should be noted that mice injected with any of the 3 cell lines sustained
primary tumours that grew to similar sizes, but the formation of secondary tumors was dramatically decreased in mice injected with Stx4-N-term or Stx4-Cyto.

6.7 Future Directions

Cortactin’s role in SNARE mediated trafficking during invadopodium has yet to be thoroughly studied in our system. Cortactin is an actin assembly protein that functions to activate and stabilize branched actin assembly by the Arp2/3 complex. Cortactin is present at sites of dynamic actin assembly, including invadopodium during the formation phase (Bryce et al., 2005; Cao et al., 2005; Hill et al., 2006; Patel et al., 1998); however, the exact role of cortactin in this context is not well defined. Studies have shown that cortactin is required for two-dimensional migration and invasion, and overexpression of cortactin can lead to increased cell migration (Bryce et al., 2005; Hill et al., 2006). Aside from its role in migration and invasion, cortactin is involved in the regulation of vesicle trafficking as it has been shown to regulate clathrin-dependent endocytosis and Golgi transport (Cao et al., 2003, 2005). Cortactin has also been shown to promote exosome secretion by controlling trafficking and plasma membrane docking of multivesicular late endosomes with the Arp2/3 complex (Jeannot and Besson, 2017). Cortactin expression is regulated by the microRNA miR-182, which is also known to influence invadopodia formation. miRNAs are endogenous, small, non-coding RNAs that are 20-25 nucleotides in length, and regulate gene expression post-translationally by binding to the 3’ untranslated region of target genes (Svoronos et al., 2016). miR-182 is part of the miR-183/-96/-182 cluster, and is directly involved in tumorigenesis, migration and regulation of cortactin levels (Lei et al., 2014). Experiments have shown that miR-182 expression
decreased cortactin levels, leading to a decrease of invadopodium formation, migration and invasion of lung cancer cells (Li et al., 2018). Due to cortactin’s regulation of vesicle trafficking (Cao et al., 2003, 2005) and MT1-MMP delivery (Rossé et al., 2014), it is reasonable to speculate that cortactin could be associating with the SNAREs that are involved in invadopodium formation, and this could be a logical avenue for future investigation. In order to elucidate cortactin’s role in MT1-MMP trafficking, cortactin could be overexpressed, or knocked-out, in our system. These changes in cortactin’s expression and their effects on SNARE complex formation and MT1-MMP cell surface levels could then be quantified in order to further understand cortactin’s role during cell invasion.

In order to further understand the regulation of SNARE-mediated trafficking during invadopodium formation, the phosphorylation of SNAREs, and regulators thereof should be investigated. Syntaxin4 has 5 known phosphorylation sites (Dephoure et al., 2008; Zhou et al., 2013), but the role of phosphorylation in SNARE complex formation and regulation are not well understood. Previous research has indicated that protein kinase C (PKC) plays a role in Stx4 phosphorylation, and when cells were treated with PKC inhibitors, Stx4 phosphorylation was decreased, and its interaction with SNAP23 was diminished (Chung and Reed, 2000). Experiments from our laboratory have shown that the phosphorylation of Stx4 is decreased during invadopodium formation; however, the amount of SNAP23 interacting with Stx4 increased under these conditions. This suggests that during SNARE complex formation and invadopodium formation Stx4 is not phosphorylated (Williams et al., 2014a). Changes in Stx4 phosphorylation were
determined by immunoprecipitating Stx4 from whole cell lysate and probing for phosphor-Ser/Thr residues. No commercial antibodies exist that specifically recognize phosphorylated Stx4, so an alternative approach, such as LC-MS/MS, could be used to explore further conditions in which Stx4 is phosphorylated (Gerrits and Bodenmiller, 2010). Munc18c has been previously shown to be regulated by phosphorylation, and it can be phosphorylated by PKC (Snyder et al., 2006). Munc18c function during insulin-stimulated translocation of GLUT4 is dependent on the dephosphorylation of Tyr521; however, the phosphatase responsible for this is unknown. Phosphorylation of Tyr521 was studied by expressing a phosphomimetic version of Munc18c, resulting in a decrease in SNARE complex formation (Kioumourtzoglou et al., 2014). Munc18c acts as a positive regulator of Stx4 function during invadopodium formation (Brasher et al., 2017), which is contradictory to findings during GLUT4 trafficking (Jewell et al., 2011). It will therefore be important to investigate the role that Munc18c and Stx4 phosphorylation has in invadopodium formation in more detail. The above study tested SNARE complex formation in 3T3-L1 mouse cells (Jewell et al., 2011), whereas our findings of Munc18c acting as a positive regulator were made in a human cell type, which may explain the difference in findings. Testing phosphomimetic versions of Munc18c in tumor cells would increase our knowledge of how Munc18c phosphorylation is involved in invadopodium formation, and how this affects its interaction with Stx4.

Cells expressing Stx4-N-term showed decreased invadopodium formation; however, some cells were still able to form invadopodia. Cells that were expressing Stx4-N-term and at the same time treated with MT1-MMP inhibitors had nearly undetectable
levels of invadopodium formation. This suggests that a combination of inhibitors and putative treatment with interfering peptides might be a possible treatment to target breast cancer metastasis. After decades of research, the use of inhibitors of matrix metalloproteinases to block cancer metastasis has yielded disappointing results (Meirson and Gil-Henn, 2018). Over 50 MMP inhibitors have failed in clinical trials, even though preclinical data supported the use of these inhibitors as anti-metastasis agents (Vandenbroucke and Libert, 2014). A major issue with MMP inhibitors is their dose-limiting toxicities, making them inefficient in treating metastatic cancer. Here, we show that expression of residues 1 through 29 or 1 through 15 of Syntaxin4 is able to decrease invadopodium formation in vitro, and in vivo data suggests that expression of Stx4-N-term can decrease metastasis in mice in a xenograft model. Primary tumor size in these mice was not affected; however, lung metastasis was decreased, and survival the mice was increased when Stx4 was inhibited.

Cancer recurrence remains a problem for patients, even if initial therapies are successful. Small molecule inhibitors, to target components of cancer cells, have been routinely studied as potential new anti-cancer drugs, but problems with off-target effects often necessitate the need for alternative approaches. A novel class of anti-cancer agents is based on peptides, and utilizes small molecular weight molecules, which are modeled after endogenous proteins, and which can be readily synthesized and modified. Small peptide inhibitors have applications in cancer treatments, as they can provide specificity for tumor tissues, and may decrease chances of tumors developing resistance (Fosgerau and Hoffmann, 2015; J. Boohaker et al., 2012). Peptides are short linear chains of amino
acids that are usually <50 amino acids in length and are designed to bind with high specificity and modulate a protein interaction of interest (Marqus et al.). Here, it is shown that either Stx4-N-term or Stx4-1-15 could potentially be used as peptide inhibitors, due to their ability to reduce metastasis through decreased invadopodium formation by tumor cells. In order to determine if Stx4-N-term or Stx4-1-15 could be used as a peptide inhibitor, their function in other cells should first be tested. For example, Munc18c and Stx4 function has previously been shown to be required for insulin exocytosis (Ramalingam et al., 2014). In order to use Stx4-N-term or Stx4-1-15 as peptides in cancer therapeutics, it would be beneficial to determine their effect on important cellular processes in other cell types, like insulin exocytosis, to see if either peptide causes unwanted effects.

Combination therapy has been a technique used to combat breast cancer for many years, and has shown increased survival rates (Foroodi and Singh, 2006; Holmes, 1996; Fukuda et al., 1999; Zanardi et al., 2015). Since multiple SNARE complexes are able to transport invadopodial proteins (ie. Stx4-SNAP23-VAMP2 (Brasher et al., 2017)/Stx4-SNAP23-VAMP7 (Williams et al., 2014a)/Stx13-SNAP23-VAMP3 (Kean et al., 2009; Williams and Coppolino, 2014)), leading to invadopodium formation, it might be reasonable to target not only SNARE-mediated pathways, but also the modulators of the ECM, or key signaling molecules involved in invadopodium formation. There are a large number of available drugs that target proteins involved in the regulation of invadopodium formation. These inhibitors target a wide range of protein families that include receptor tyrosine kinases, phosphatases, as well as proteases (Meirson and Gil-Henn, 2018).
Several invadopodia-associated genes have been found to be upregulated in breast cancer patients and in order to determine which genes are the most likely to lead to invadopodium formation, RNA microarrays of primary tumors were analyzed. The analysis showed that most genes encoding invadopodium components had an elevated hazard ratio, which meant that overexpression was associated with increased rate of metastasis. Some of the genes that were the most commonly overexpressed in these patients were MMP1, HIF1A, MCL1 and HSP90AA1. All of the genes that were analyzed are recognized druggable targets, and had either FDA approved drugs, or were in preclinical trials (Meirson and Gil-Henn, 2018). For example, MMP1, which is able to degrade several types of collagen, can be inhibited using marimastat or rebimastat (Weiss et al., 2012), and these inhibitors can also target MMP19, MMP2, MMP9, MMP13 and MT1-MMP.

There is an abundance of pre-clinical data that implicates MMP1 and other MMPs as therapeutic targets; however, MMP inhibitors have shown disappointing results (Coussens et al., 2002; Overall and López-Otín, 2002; Vihinen et al., 2005). This could be, in part, due to an incomplete understanding of the trafficking of MMPs during tumor cell invasion. The SNAREs that traffic MT1-MMP to invadopodia have been well-characterized by our group (Brasher et al., 2017; Kean et al., 2009; Williams and Coppolino, 2011a; Williams et al., 2014a); however, there is little information available about the trafficking of other MMPs to invadopodia. As shown above, experiments involving the combination of MT1-MMP inhibitors and Stx4-N-term/Stx4-1-15 expression highlight the importance of examining combinations of therapeutic approaches, as MT1-
MMP (Brasher et al., 2017; Williams and Coppolino, 2011a), and presumably other MMPs, can be trafficked by multiple SNARE-mediated pathways.

6.8 Summary

The research presented here has characterized important binding partners for SNAREs that contribute to MT1-MMP trafficking during tumor cell invasion. An important area of research for the future will be to assess the role of the regulation of these SNAREs in normal cell invasion and pathological conditions other than cancer. Remodeling of the ECM is important in morphogenesis of the lungs, intestine, as well as mammary glands (Sonbol, 2018). As well, abnormal remodeling of the ECM is not just an factor in cancer progression (Hanahan and Weinberg, 2011), but also other pathological conditions, for example, fibrosis and osteoarthritis. Fibrosis is the result of severe tissue injuries that lead to excessive ECM production and deposition without balanced degradation, and this can lead to only organ failure, and also an increased risk of cancer (Bonnans et al., 2014b). Since fibrosis is partly caused by an increase in ECM production it would be interesting to examine if SNARE-mediated trafficking of proteases is altered in these cells, compared to normal cells.

The findings presented in this thesis can be summarized as follows (Figure 6.4): During invadopodium formation, MT1-MMP and EGFR must be trafficked to the plasma membrane, and this is dependent on the SNAREs Stx4, VAMP2 and SNAP23. Complex formation between these SNAREs is facilitated by binding of Munc18c to Stx4, allowing Stx4 to interact with SNAP23 at the plasma membrane. Cdc42 interacts with VAMP2;
however, it is unknown at which stage this occurs, or how this interaction affects SNARE complex formation.

Overall, these studies offer new insight into how SNARE mediated membrane trafficking is regulated during delivery of invadopodial proteins to the cell surface and how this contributes to the progression of metastasis. Regulators of SNARE-mediated trafficking in this context have also been characterized and considered as possible targets for future development therapeutic approaches to combat the progression of malignant tumours.
**Figure 6.4** - Proposed model of SNARE regulation during invadopodium formation.

1) Prior to binding of Munc18c, Stx4 is in a 'closed confirmation'. Cdc42 interacts with VAMP2. 2) Munc18c binds to Stx4, which hinges Stx4 open, allowing Stx4 to bind to SNAP23. 3/4) VAMP2, located on a vesicle, can then form a trans-SNARE complex with Stx4 and SNAP23. 5) Cargo, such as EGFR and MT1-MMP, can then be delivered to invadopodia.
Table 1 - List of inhibitors used to impair membrane trafficking

<table>
<thead>
<tr>
<th>Membrane Trafficking Protein</th>
<th>Localization</th>
<th>Inhibitor(s) Used</th>
<th>Effect of inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syntaxin4/Q-SNARE</td>
<td>Plasma membrane</td>
<td>Syntaxin4-Cyto:</td>
<td>- Forms a non-functional complex with Stx4 cognate SNARE partners</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Transmembrane domain deletion</td>
<td>- Functions as a competitive inhibitor impairing exocytosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Syntaxin4-N-terminal peptide:</td>
<td>- Binds Munc18c to act as a competitive inhibitor of endogenous Munc18c/Stx4 interaction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>First 29 amino acids of Stx4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Syntaxin4 1-15 peptide:</td>
<td>- Binds Munc18c to act as a competitive inhibitor of endogenous Munc18c/Stx4 interaction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>First 15 amino acids of Stx4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Syntaxin4 15-29:</td>
<td>- Used as a control for Stx4 N-terminal studies</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Residues 15-29 of Stx4’s N-terminus</td>
<td></td>
</tr>
</tbody>
</table>
| VAMP2/R-SNARE | Plasma membrane
Synaptic vesicle
Sorting endosome | VAMP2-Cyto:
Transmembrane domain deleted | - Forms a non-functional complex with Stx4 cognate SNARE partners
- Functions as a competitive inhibitor impairing exocytosis |
<table>
<thead>
<tr>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>VAMP2 1-28:</td>
<td>First 28 amino acids of VAMP2</td>
<td>- Binds to Cdc42 to act as a competitive inhibitor of endogenous Cdc42/VAMP2 interaction</td>
</tr>
<tr>
<td>VAMP2 shRNA 4</td>
<td></td>
<td>- Knocks down VAMP2 expression</td>
</tr>
<tr>
<td>VAMP2 shRNA 5</td>
<td></td>
<td>- Knocks down VAMP2 expression</td>
</tr>
</tbody>
</table>
| VAMP7/R-SNARE  | Late endosome         | VAMP7-Cyto:
Transmembrane domain deleted | - Forms a non-functional complex with VAMP7 cognate SNARE partners
- Functions as a competitive inhibitor impairing exocytosis |
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