Analysis of Munc18c and Syntaxin4 Function During Tumour Cell Invasion

by

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Guelph, Ontario, Canada

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ABSTRACT

ANALYSIS OF MUNC18C AND SYNTAXIN4 FUNCTION DURING TUMOUR CELL INVASION

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University of Guelph, 2015

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Tumour cell invasion through the ECM (extracellular matrix) involves the precise localization of proteins required for ECM proteolysis and cell migration. Membrane trafficking events, mediated by SNAREs (Soluble NSF Attachment Protein Receptors), have been implicated in these cellular processes. Previous studies on SNAREs indicate that Syntaxin4 is involved in the formation of invadopodia (specialized degradative structures formed during tumour cell invasion) in MDA-MB-231 cells; however, it remains unclear how Syntaxin4 function is regulated during tumour cell invasion. Munc18c is a known regulator of Syntaxin4 activity, and a potential role for Munc18c in invadopodium-based ECM degradation and cell migration has been identified. Here, biochemical and microscopic analyses revealed an association between Munc18c and Syntaxin4. Munc18c knockdown perturbed invadopodium formation and cell migration. Expression of a truncated form of Syntaxin4 designed to perturb Syntaxin4-Munc18c interactions had similar effects. These results suggest Munc18c facilitates Syntaxin4 function during tumour cell invasion.
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Last but not least, to my parents and family, I could not have done this without you – the most important tools to succeed were provided by you.
Declaration of Work Performed

Together, Ranko Savic and Megan Brasher performed ligations and subsequently verified the creation of the following expression constructs: N-terminal Syntaxin4-GFP and untagged human Munc18c. Dr. Marc Coppolino created the G418 kill-curve necessary for the selection and maintenance of the stable cell lines described in this thesis. The author performed all other experiments.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>α-SNAP</td>
<td>N-ethylmaleimide-sensitive factor attachment protein alpha</td>
</tr>
<tr>
<td>ARP</td>
<td>Actin-related protein</td>
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<tr>
<td>BCS</td>
<td>Bovine Calf Serum</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CD44</td>
<td>Cluster of differentiation 44</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese Hamster Ovary</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<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>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FL</td>
<td>Full Length</td>
</tr>
<tr>
<td>G418</td>
<td>Geneticin</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose transporter type 4</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>HEK-293</td>
<td>Human embryonic kidney cells 293</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin type G</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>kDa</td>
<td>kiloDaltons</td>
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<tr>
<td>M</td>
<td>Mouse</td>
</tr>
<tr>
<td>miRNA</td>
<td>Micro ribonucleic acid</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloprotease</td>
</tr>
<tr>
<td>MT1-MMP</td>
<td>Membrane Type 1 – Matrix Metalloprotease</td>
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<tr>
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<td>Mammalian uncoordinated-18</td>
</tr>
<tr>
<td>NA</td>
<td>Numerical Aperture</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-terminal</td>
<td>Amino terminal</td>
</tr>
<tr>
<td>N-WASP</td>
<td>Neuronal Wiskott-Aldrich syndrome protein</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PLL</td>
<td>Poly-L-Lysine</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>Rb</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Rab</td>
<td>Ras-related in brain</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>Ras</td>
<td>Rat sarcoma</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>Ribonucleic acid interference</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Sec1</td>
<td>Protein transport protein SEC1</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>STX4</td>
<td>Syntaxin 4</td>
</tr>
<tr>
<td>siRNA</td>
<td>Short interfering ribonucleic acid</td>
</tr>
<tr>
<td>SNAP</td>
<td>Synaptosomal-associated protein</td>
</tr>
<tr>
<td>SNARE</td>
<td>Soluble N-ethylmaleimide-sensitive factor attachment protein receptor</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffered saline with Tween-20 added</td>
</tr>
<tr>
<td>Tris</td>
<td>2-Amino-2(hydroxymethyl)-1,3-propanediol</td>
</tr>
<tr>
<td>TX-100</td>
<td>Triton X-100</td>
</tr>
<tr>
<td>VAMP</td>
<td>Vesicle-associated membrane protein</td>
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1.0 – Introduction

1.1 – Overview

Cellular invasion and migration are fundamental to the homeostasis of multicellular organisms. They are integral parts of many physiological processes including embryogenesis and the maintenance of tissue architecture. During embryonic development, cells must remodel the ECM and migrate to their intended destination in order to form specialized tissues and organs (Moissoglu & Schwartz, 2012). During vertebrate organogenesis, movement through and breakdown of the ECM by epithelial cells of the lung and kidney are important for the expansion of these tissues (Andrew & Ewald, 2010; Lu, Sternlicht, & Werb, 2006). Cellular invasion also plays an important role in wound healing and immune cell surveillance. Mesenchymal stem cells must migrate to the damaged area in order to fill in a wound, and subsequently leukocytes, in response to external stimuli, extravasate through the vascular basement membrane to mitigate foreign material within the wounded area (Huttenlocher & Horwitz, 2011).

Similar cellular processes that mediate cellular invasion in normal physiological processes are also attributed to the progression of pathological disorders such as cancer (Friedl & Alexander, 2011). Metastatic spread of cancer is mediated, in part, by tumour cell invasion through the ECM. Tumour cell invasion through the ECM barrier can be defined as a multistep process comprising cell adhesion, ECM proteolysis and cell migration (Bravo-Cordero, Hodgson, & Condeelis, 2012). To move through the ECM, cells first utilize surface receptors to engage with ECM components. Cells also secrete proteases to degrade the ECM and ultimately migrate through it. The molecular mechanisms that regulate the function of these processes are currently an important area
of research due to their significance to cancer management and treatment. An important area of study that has advanced our understanding of the molecular machinery used by cells during tumour cell invasion is invadopodia. These subcellular structures are actin-driven cell membrane protrusions utilizing both cell surface ECM receptors and proteolytic enzymes for their function and formation. Invadopodia have been studied in cancer cells in a variety of microenvironments in vitro (Artym et al., 2015; Tolde, Rösel, Veselý, Folk, & Brábek, 2010), and evidence from in vivo studies supports their relevance and role in the dissemination of tumour cell populations (Clark et al., 2008; Leong et al., 2014; Lohmer, Kelley, Hagedorn, & Sherwood, 2014).

Membrane trafficking of proteins to invadopodia is required for their formation and function during tumour cell invasion. The molecular mechanisms controlling the trafficking of these proteins are an active area of study. Key players of intracellular membrane trafficking events are SNAREs. These proteins function to localize and fuse vesicles with target membranes. Work has started to highlight an important role for SNARE-mediated trafficking in invadopodium formation and tumour cell invasion; however, the mechanism that controls SNARE function in this context is not well understood. Research into this area may lead to a better understanding of the formation of invadopodia and the mechanisms by which cells can become invasive. Furthermore, by defining the mechanism of SNARE regulation in this process, the development of therapies that could reduce ECM degradation and invasion by tumour cells may be possible.
1.2 – Tumour Cell Invasion

A simple model of metastasis describes the escape of a tumour cell from a primary tumour and movement through the ECM, ultimately leading to its dissemination to distant sites within the body (Beaty & Condeelis, 2014). The degradation of the ECM by invading carcinoma cells is a good example of this (Hanahan & Weinberg, 2011). Carcinomas restricted to the epithelium may be considered benign. However, the acquisition of an invasive phenotype by cells of the tumour would lead to the breaching of the basement membrane, and individual or groups of cancer cells may then begin to invade underlying ECM and nearby lymphatic or vascular vessels (Yamaguchi, 2012) (Figure 1). Once these cells arrive within the lumen of these vessels, they can travel within the blood or lymph to other areas of the body and potentially establish metastases. Invasion into the ECM by carcinoma cells also releases growth and survival factors that have been sequestered by the ECM (Talmadge & Fidler, 2010). Taken together, tumour cell invasion involves alterations in the molecular mechanisms that control cell adhesion, proteolytic degradation of the ECM, and cell migration.

1.3 – Cellular contact and adhesion with the extracellular matrix

The ECM is an important interlocking mesh composed of fibrous proteins and glycoproteins acting as a supportive scaffold and physical barrier to maintain tissue organization (Ridley et al., 2003). Examples of ECM components include: collagen, fibronectin and vitronectin (Oskarsson, 2013). In multicellular organisms, contact between the cell and ECM provides a crucial biochemical bridge between the extracellular and intracellular environments allowing modulation of cellular behaviour. Cells make contact with the ECM through a variety of specialized structures that mediate
Figure 1: Schematic representation of metastasis
In the epithelium, cells of a primary tumour develop an invasive phenotype and invade the basement membrane with the aid of invadopodia. Invasive cells can then migrate through the stromal tissue to intravasate a nearby blood vessel, travel to secondary sites, and potentially form metastases.
homeostasis by conferring anchorage, motility, and signal transduction (Adams, 2002). Anchorage of the cell to the ECM can be provided by mechanically supportive contacts such as hemidesmosomes, which link the cytoskeleton of epithelial cells to the basement membrane through the engagement of specialized adhesion receptors (Borradori & Sonnenberg, 1999). Contractile contacts, such as focal adhesions, mediate cellular movement by utilizing the cells actomyosin motor system to provide force against the rigid ECM via cytoskeletal remodelling (Zamir et al., 2000). Protrusive contacts, such as filopodia, are actin-containing extensions of the plasma membrane at the leading edge of migrating cells, which facilitate extension into the ECM (Mattila & Lappalainen, 2008). Collectively, cell-matrix contacts are dynamic and specialized structures that allow sampling of the environment around the cell body.

Cell adhesion and interaction with the ECM is primarily mediated through the integrin family of receptors (Berrier & Yamada, 2007). Integrins are transmembrane glycoproteins, which perform their function as heterodimers of α and β subunits. Each subunit comes together to bind ECM substrates located in the pericellular environment and this transmits intracellular signals through the cytoplasmic domains. Briefly, cell adhesion begins with the binding of integrins to ECM substrates resulting in the recruitment of other integrins and signalling and scaffolding proteins that mediate cytoskeletal rearrangements. This focal accumulation of proteins is termed a focal adhesion, and as the cell spreads, more focal adhesions take shape, leading to a fully spread and adherent cell (Figure 2).
Figure 2: Schematic diagram of cell adhesion
Adhesion to the ECM is mediated by integrins. Upon binding to an ECM substrate, integrins recruit cytoskeletal, scaffolding and signalling proteins. Focal sites of attachment form, which can mature into focal adhesions. Focal adhesions are stabilized by F-actin fibres, leading to a fully spread and adherent cell.
1.4 – Cell Migration

Cell migration is a multistep process that occurs in response to external stimuli and involves the repetition of the following steps: 1) membrane extension at the leading edge, 2) cell adhesion to the ECM, 3) contraction of the cell body, and 4) detachment at the cell rear (Ridley et al., 2003). Initially, membrane protrusions such as lamellipodia at the leading edge of the cell take shape and sample the environment beyond the cell body (Figure 3). Regulators of actin polymerization promote the formation of these protrusions. Once an actin containing protrusion has been produced, adhesive contacts must form to prevent retraction of the newly formed membrane protrusion. This is facilitated by focal adhesions, which ultimately link the actin cytoskeleton to the ECM. The next step in cell migration involves the contraction of the cell body. This is primarily mediated through actomyosin-based forces directed against the ECM. Concomitantly, adhesions that were initially produced at the leading edge reach the rear of the cell as the cell body translocates. These older adhesions disassemble in response to actomyosin contraction or protease-directed degradation. Taken together, actomyosin contraction and adhesion disassembly at the rear promotes cell body translocation.

1.5 – Extracellular matrix proteolysis

Dissolution of the ECM is an important prerequisite for tumour cell invasion (Hanahan & Weinberg, 2011). This process has been shown to be largely dependent on the engagement of MMPs with ECM substrates (Egeblad & Werb, 2002; Hojilla, Wood, & Khokha, 2008). Furthermore, invadopodia rely on MMPs to facilitate their maturation (Clark & Weaver, 2008; Linder, 2007). The human MMP family of proteins comprises 23 members that are zinc dependent-endopeptidases with broad substrate specificity
Figure 3: Schematic diagram of two-dimensional cell migration
Cells adherent to ECM establish focal adhesions. Migration begins with membrane protrusions such as lamellipodia that extend in the direction of movement. New focal adhesions form at the leading edge and actomyosin contraction causes the cell body to translocate while older focal adhesions at the cell rear disassemble.
(Jackson, Nebert, & Vasiliou, 2010). Two further divisions categorize these proteins as soluble or membrane-anchored. The membrane-anchored MMPs contain either a transmembrane domain or a glycosylphosphatidylinositol anchor and the soluble MMPs are secreted by cells into the ECM environment (Llano et al., 1999; Sohail et al., 2008; Takino, Sato, Shinagawa, & Seiki, 1995). Both types are translated as inactive zymogens, therefore requiring cleavage of their intrinsic pro-domains to be proteolytically active. The activity of MMPs has been shown to be closely linked to the invasiveness of several aggressive cancers such as melanoma, breast, ovarian, and colorectal (Decock et al., 2008; Seftor et al., 2001; Sodek, Ringuette, & Brown, 2007; Zucker et al., 1999). Tumour progression in these cancers has also been shown to correlate specifically with the enzymatic activity of MT1-MMP (Sabeh, Shimizu-Hirota, & Weiss, 2009). Moreover, invadopodium formation has been shown to be mediated by MT1-MMP (Artym, Zhang, Seillier-Moiseiwitsch, Yamada, & Mueller, 2006). Over-expression of MT1-MMP in moderately invasive melanoma cells was shown to increase matrix degradation and invadopodium activity (Revach & Geiger, 2014). Other studies have demonstrated that over-expression of MT1-MMP in different cancer cell types promoted invasion using in vitro and in vivo models (Hotary et al., 2003; Sabeh, 2004). In addition, the knockdown of MT1-MMP in invasive breast cancer cell lines reduced the ability of cells to invade ECM substrates in vitro (Jiang et al., 2006). MT1-MMP is important to the degradation of ECM components utilized by invasive carcinomas, and much evidence points to a role for MT1-MMP in invadopodium-based tumour cell invasion.
1.6 – Invadopodia Formation

Cellular degradation and migration through the ECM is important for cellular processes involving tissue remodelling, including wound healing, embryogenesis, and immune cell surveillance (Daley, Peters, & Larsen, 2008; Lu, Takai, Weaver, & Werb, 2011; Vicente-Manzanares, 2005). Actin-driven invadosomes are protrusive contacts that facilitate focal ECM degradation and cell motility through the use of proteolytic enzymes (Linder, Wiesner, & Himmel, 2011; Murphy & Courtneidge, 2011). Podosomes refer to these structures in normal cells, such as macrophages and dendritic cells (Burns, 2001; Linder, Hufner, Wintergerst, & Aepfelbacher, 2000), whereas in cancerous cells they are referred to as invadopodia (Gimona, Buccione, Courtneidge, & Linder, 2008). The partial degradation of ECM components is crucial for tumour cell invasion and it is the employment of invadopodia with their localized proteolytic machinery that helps to initiate metastasis (Blouw, Seals, Pass, Díaz, & Courtneidge, 2008; Clark et al., 2008).

In 1989, the term ‘invadopodia’ was first used to describe the phenomenon of overlapping microfilament-containing membrane protrusions and localized sites of substratum degradation in Rous sarcoma virus transformed fibroblasts (Chen, 1989). More accurately, these sub-cellular structures are now described to be located predominantly on the ventral surface and situated below the nucleus of an invasive carcinoma cell (Artym et al., 2015). Furthermore, these protrusions can be observed in vitro, using confocal microscopy techniques, and have been observed to overlap with sites of proteolytic degradation and F-actin punctae when aggressive cancer cells are cultured on various ECM substrates, such as fluorescently conjugated gelatin or fibronectin (Hoshino et al., 2013; Oser et al., 2009).
The multitude of proteins that converge at invadopodia is indicative of a complex and highly regulated means of cellular invasion. Research into these sub-cellular structures has discerned 4 main subgroups of proteins involved in their formation and activity. The first group consists of cell motility and protrusion-inducing machinery, which includes regulators of F-actin polymerization and branching. Examples of these are ARP2/3, N-WASP, coflin, cortactin, and dynamin (Baldassarre, 2002; Oser et al., 2009; Yamaguchi et al., 2005). Adhesion proteins constitute the second group, categorized based on the maintenance of cell-ECM contact. These adhesion molecules are exemplified by integrins and CD44 (Kajita et al., 2001; Wolf et al., 2007). The third group consists of signalling proteins from the tyrosine kinase family and Ras-related GTPases, which ultimately regulate membrane trafficking and dynamics (Neel et al., 2012; Tehrani, Tomasevic, Weed, Sakowicz, & Cooper, 2007). The final group is made up of the proteolytic machinery that facilitates ECM degradation, including members of the MMP family, separase, and urokinase-type plasminogen activators system (Artym, 2002; Monsky et al., 1994; Revach & Geiger, 2014).

During the formation of invadopodia (Figure 4), cells adhere to the ECM through interactions with ventral surface integrins. Concomitantly, growth factors such as epidermal growth factor and platelet derived growth factor trigger a signalling cascade that ultimately leads to the recruitment of actin modifying proteins to sites of forming invadopodia (Mader et al., 2011; Yamaguchi et al., 2005). Next, cortactin associates with N-WASP and the ARP2/3 complex to mediate F-actin assembly and branching, resulting in a protrusion of the plasma membrane, driven by the force of actin filament formation. Subsequently, membrane trafficking of MT1-MMP to invadopodia facilitates membrane
Figure 4: Simplified model of invadopodium formation
Cells initially establish adhesions through integrins and stimulate signalling pathways through growth factor receptor tyrosine kinases (RTKs). F-actin formation and branching by N-WASP and cortactin establishes 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, initiating invadopodia disassembly and subsequent cellular migration through the degraded extracellular matrix.
protrusion into the basement membrane as the invadopodium matures (Clark, Whigham, Yarbrough, & Weaver, 2007; Steffen et al., 2008). MT1-MMP is theorized to invoke a positive, feed forward mechanism through the release of extracellular growth factors from the ECM (Díaz, Yuen, Iizuka, Higashiyama, & Courtneidge, 2013) and the activation of MMP-2, which can also activate growth factor receptors (Dean et al., 2007; Sato et al., 1994). Mature invadopodia are established once ECM degradation has occurred and are relatively long-lived structures persisting for hours. Their disassembly has been suggested to begin with the clearance of F-actin and cortactin leaving behind an MT1-MMP containing invadopodium that is still capable of localized ECM degradation (Artym et al., 2006).

1.7 – Intracellular membrane trafficking

Eukaryotic plasma membranes provide a physical barrier between a cell and its extracellular environment, and regulate the transport of molecules to and from this environment in order to maintain homeostasis inside the cell. Membranes are also important inside the cell: each organelle within the cell (for example, the Golgi complex) is surrounded by a lipid bilayer, which, similar to the plasma membrane, is involved in regulation of transport into and out of each organelle.

Cell migration and invasion require the precise localization of adhesion and signalling proteins. Membrane trafficking coordinates the transport and localization of these proteins from different membrane compartments. The transport of vesicles between membranes can be broadly defined (Figure 5). In summary, vesicle formation involves the budding off of vesicles from the donor membrane. Vesicle movement is the transportation of vesicles along microtubules or actin filaments through the action of
motor proteins. Vesicle tethering and docking mediates the correct targeting of vesicles and brings donor and target membranes in close proximity. Ultimately, this leads to the process by which lipid bilayers merge, therefore delivering the contents of the vesicle to the target compartment or extracellular environment. SNAREs are proteins that mediate membrane fusion events and the purpose of the studies described in this thesis is to examine the regulation of SNAREs during cellular invasion.

1.8 – SNARE structure and function

Thirty-six SNAREs have been identified in humans (Jahn & Scheller, 2006). SNAREs assemble into complexes containing a 4-helix bundle formed by their SNARE motifs. Initially, SNARE proteins were classified according to the membrane they reside in, the target or vesicle. Elucidation of the 3D structures of SNARE complexes subsequently revealed the presence of a central ionic layer in the α-helix bundle containing three glutamine (Q) residues and one arginine (R). This led to the reclassification of SNARE proteins as either R- or Q-SNAREs depending on which residue they contribute to the ionic layer (Kloeppe, Kienle, & Fasshauer, 2007). The 4-helix bundle of the SNARE complex contains one member of each of the four SNARE subtypes, referred to as R, Qa, Qb, and Qc.

Within cells, SNAREs can be functionally described under three general protein types. Syntaxins, a family of Q-SNARE proteins, are found to be localized to different membrane compartments and function in several trafficking pathways (Teng, Wang, & Tang, 2001). Syntaxin4 is predominantly found at the plasma membrane in many cell types (Band et al., 2002), and in adipocytes it is involved in the fusion of GLUT4 storage
Intracellular trafficking allows transport of cargo contained in vesicles to other membranous compartments within the cell. The biosynthetic pathway shuttles material synthesized in the ER to the Golgi complex for processing and is subsequently secreted to the plasma membrane. Recycling begins with endocytosis at the plasma membrane and trafficking to an early endosomal compartment. Some endocytosed material is then delivered to late endosomes and then lysosomes for degradation. Another endocytic route brings material back to the plasma membrane from the early endosome directly or through a recycling endosome.

**Figure 5: Overview of membrane trafficking pathways**

Intracellular trafficking allows transport of cargo contained in vesicles to other membranous compartments within the cell. The biosynthetic pathway shuttles material synthesized in the ER to the Golgi complex for processing and is subsequently secreted to the plasma membrane. Recycling begins with endocytosis at the plasma membrane and trafficking to an early endosomal compartment. Some endocytosed material is then delivered to late endosomes and then lysosomes for degradation. Another endocytic route brings material back to the plasma membrane from the early endosome directly or through a recycling endosome.
vesicles with the plasma membrane in response to insulin signalling (Tellam, Macaulay, McIntosh, & Hewish, 1997). The cytoplasmic portion of Syntaxin4 contains a conserved SNARE motif, and a regulatory H\textsubscript{abc} domain consisting of three anti-parallel helices at its N-terminus (Ungar & Hughson, 2003). The H\textsubscript{abc} domain is able to regulate SNARE complex formation by conferring an open or closed conformation. The open conformation allows Syntaxin4 to interact with cognate partners. The closed conformation prevents SNARE complex formation (Dulubova et al., 2007). The second type of SNAREs, synaptosome-associated proteins (SNAP), has two Q-SNARE domains. For example, the ubiquitously expressed, SNAP23 interacts with Syntaxin4 during GLUT4 vesicle fusion with the plasma membrane in adipocytes (Widberg, 2003). Separated by a flexible linker region, these proteins are membrane anchored by post-translational palmitoylation of cysteine residues in this linker region (Puri & Roche, 2006). Finally, vesicle of associated membrane proteins (VAMP) are members of the R-SNARE family, and are comprised of a C-terminal transmembrane domain, a SNARE motif, and a variable N-terminal domain. (Jahn & Scheller, 2006). Multiple VAMP isoforms have been identified, with roles in a wide range of cellular trafficking pathways.

SNARE complex formation can occur when a vesicle membrane is apposed to a target membrane (Figure 6). Once the SNAREs are in contact with each other, they form a \textit{trans}-SNARE complex when SNARE motifs form a coiled-coil. The energy released from this complex formation helps overcome the energy barrier to merge the two membranes, promoting membrane fusion and leaving a cis-SNARE complex (Hong, 2005). The disassembly of cis-SNARE complexes does not occur spontaneously and is regulated by the cytosolic proteins NSF (N-ethylmaleimide sensitive fusion protein) and
Figure 6: SNARE-mediated membrane fusion
SNARE complex assembly and vesicle docking occurs as the SNARE motif of an R-SNARE (blue) forms a complex with two other SNARE motifs from a SNAP family member (yellow) and one from Syntaxin (red). Trans-complex formation results from the zippering of the four SNARE motifs. Membrane fusion results in a cis-complex.
α-SNAP (soluble NSF attachment protein alpha). The adaptor protein, α-SNAP, binds to the cis-SNARE complex and recruits NSF, which through its ATPase activity separates the complex. The SNARE proteins are recycled through the retrograde transport of R-SNAREs and separation of Q-SNAREs.

1.9 – SNARE-mediated trafficking during cell migration and invasion

Studies of SNARE-mediated trafficking and integrin localization have shown the relevance of SNARE function during cell migration and cell spreading. Expression of a dominant negative form of NSF (E329Q-NSF) has been observed to impair disassembly of SNARE complexes (Coppolino et al., 2001), thereby reducing the amount of free SNAREs available to mediate membrane fusion. Expression of this construct impaired cell migration in CHO cells (Tayeb et al., 2005). It was then determined that inhibition of specific SNARE-mediated trafficking pathways perturbed trafficking of α5β1 and impeded cell spreading, but not cell adhesion, when CHO cells were seeded on fibronectin (Skalski & Coppolino, 2005).

SNARE-mediated membrane trafficking is also important for the delivery of MMPs to the plasma membrane and during invadopodium formation. Previous work has demonstrated that in the invasive cell line, HT-1080, inhibition of Syntaxin13, SNAP23 and VAMP3 perturbed the trafficking of MT1-MMP and the secretion of MMP2 and MMP9, but did not alter cell migration (Kean et al., 2009). Other studies have shown the importance of SNARE-mediate trafficking of MT1-MMP to the plasma membrane in invasive carcinoma cells (Miyata et al., 2004; Steffen et al., 2008). Using the MDA-MB-231 cell line as a model system for the study of invadopodia, SNAREs have been identified to have a role in invadopodium formation (Williams & Coppolino, 2014). The
SNARE complex responsible for the delivery of MT1-MMP to invadopodia was elucidated to be dependent on the Q-SNAREs SNAP23 and Syntaxin4, and the R-SNARE VAMP7 (Williams, McNeilly, & Coppolino, 2014). Moreover, inhibition of these SNAREs perturbed invadopodium formation. Taken together, specific SNARE-mediated trafficking pathways have been identified which are important for the localization of key proteins involved in cellular invasion.

1.10 – Regulation of SNARE Complex Formation

Although it is known that SNAREs are central mediators of membrane fusion, how the formation of SNARE complexes is regulated during cell invasion is not completely understood. The control of SNARE complex formation has been shown to involve regulatory non-SNARE proteins and post-translational modifications (Hong, 2005; Tomes, 2015). Both of these factors have been implemented in the restriction of SNARE assembly, catalysis of SNARE complex assembly, or the maintenance of SNAREs in an active or inactive conformation. Some of the ways that SNARE complex formation can be regulated by these factors are briefly described below.

The regulation of SNARE complex formation can be controlled by the phosphorylation of SNAREs. For example, SNAP23 is phosphorylated by SNAK kinase, resulting in a lack of association with Syntaxin4 at the plasma membrane (Cabaniols, Ravichandran, & Roche, 1999). SNAP23 has also been shown to be phosphorylated by protein kinase C, resulting in reduced binding to Syntaxin4 (Polgár, Lane, Chung, Houng, & Reed, 2003). Syntaxin4 can be serine/threonine phosphorylated in vitro by casein kinase 2 (Risinger & Bennett, 2002), protein kinase C (Chung, Polgár, & Reed, 2000), and protein kinase A (Foster et al., 1998) and these phosphorylation events reduce
binding to cognate SNAREs. Phosphorylation of Syntaxin4 by Rab3d-kinase was also found to decrease binding to SNAP23 (Pombo et al., 2001). Previous work has made the observation that serine/threonine phosphorylation of Syntaxin4 was decreased during invadopodium formation, suggesting that Syntaxin4 dephosphorylation was regulated during trafficking of MT1-MMP to the invadopodial plasma membrane (Williams et al., 2014). Taken together, phosphorylation of SNARE proteins is a means of regulating cognate SNARE binding and therefore subsequent vesicle-target membrane fusion.

SNARE complex formation can also be regulated through interactions with non-SNARE proteins. For example, the neuronal protein Snapin complexes with SNAP25 in order to enhance binding to cognate SNAREs and therefore facilitate exocytosis (Pan, Tian, & Sheng, 2009). Other neuronal proteins, synaptophysin and tomosyn, also regulate vesicle fusion by controlling SNARE interactions. Synaptophysin binds to the R-SNARE synaptobrevin preventing any binding with SNAP25, and tomosyn binds with SNAP25 to prevent synaptobrevin binding (Yelamanchili et al., 2005). Another neuronal protein, Munc18a, has been shown to bind to isolated Syntaxin1 in the closed conformation as well as its cognate SNARE trans-complex (Dulubova et al., 2007; Schollmeier, Krause, Kreye, Malsam, & Söllner, 2011). This protein is hypothesized to regulate membrane fusion in neuronal cells either by inhibiting Syntaxin1 from forming complexes or by enhancing membrane fusion – its exact function remains unclear. It has also been shown that in pancreatic beta cells gelsolin binds Syntaxin4, and this interaction prevents SNARE complex formation. Perturbation of this interaction by glucose uptake facilitates insulin exocytosis (Kalwat, Wiseman, Luo, Wang, & Thurmond, 2012).
1.11 – Munc18c Function as a Sec1/Munc18 protein

Sec1/Munc18 (SM) proteins were first identified through a genetic screen of uncoordinated mutants of the nematode Caenorhabditis elegans (Brenner, 1974) and orthologs in mammals were subsequently identified, defining three isoforms: Munc18-a, -b and -c (Hata, Slaughter, & Südhof, 1993). SM proteins are 60-70 kDa proteins, found both in the cytosol and associated with membranes via interaction with their cognate SNAREs. Studies in neuronal tissue identified Syntaxin as a major binding partner of SM proteins (Pevsner, Hsu, & Scheller, 1994). Structural data shows that the overall fold of SM proteins is highly conserved between different species, supporting the notion of a common function (Bracher & Weissenhorn, 2002; Hu, Latham, Gee, James, & Martin, 2007; Misura, Scheller, & Weis, 2000). The structure includes three domains that fold together to form a large cavity on one side, and a groove on the other. SM proteins have been shown to interact with syntaxin by at least one of three ways (Figure 7): through binding to the closed conformation, the N-terminal peptide domain, and the 4-helical SNARE complex (Südhof & Rothman, 2009; Yu et al., 2013).

SM proteins show a similar loss-of-function phenotype as that of SNAREs and are essential for every pathway of intracellular vesicle fusion (Burgoyne et al., 2009; Carr & Rizo, 2010; Toonen & Verhage, 2007). SM proteins have previously been viewed as regulators of SNARE function, but current findings suggest that SM proteins also cooperate with SNARE complexes to mediate membrane fusion. Munc18a is able to bind to closed Syntaxin1, as well as the trans-SNARE complex (Dulubova et al., 2007; Schollmeier et al., 2011). The same observation has been made with Munc18c and its high affinity binding partner, Syntaxin4 (Yu et al., 2013). Munc18a and Munc18c are not
SM proteins (purple) have been shown to interact with their cognate syntaxin (red) by three ways: full-length closed conformation of the syntaxin, the trans-SNARE complex, and the N-terminal domain of Syntaxin.
functionally interchangeable, suggesting there is a conserved mechanism of action between isoforms though differences lie in cognate SNARE recognition. As a result, the current model for SM protein function is that the Q-SNARE Syntaxin adopts a closed conformation and is sequestered from cognate SNAREs by an SM protein (Baker et al., 2015). When the vesicle comes into the proximity of the target membrane, the SM protein-bound Q-SNARE assembles with the R-SNARE and then the other Q-SNAREs. The SM protein then repositions to enhance trans-SNARE complex formation, therefore promoting membrane fusion.

Munc18c has broad tissue distribution (McIntosh, 1995) and has been implicated in several exocytic pathways. It has been found to influence secretion in neutrophils (Brochetta et al., 2008), exocytosis in platelets (Schraw et al., 2004), and the sustained phase of insulin secretion in adipocytes (Oh & Thurmond, 2009). Physiologically, Munc18c has been shown to be crucial for the exocytosis of the glucose transporter, GLUT4, during glucose homeostasis (Jewell et al., 2011). In response to insulin signalling, GLUT4 storage vesicle fusion requires Syntaxin4 and SNAP23 as the Q-SNAREs, VAMP2 as the R-SNARE, and Munc18c as the cognate SM protein (Brandie et al., 2008). Homozygous knockout of Munc18c is embryonic lethal in mice; however, heterozygous mice are viable, exhibiting a large decrease in insulin-stimulated GLUT4 plasma membrane integration compared to wild-type mice (Oh, Spurlin, Pessin, & Thurmond, 2005). Furthermore, disruption of the interaction between endogenous Munc18c and Syntaxin4 was also found to cause a decrease in the fusion of GLUT4-containing vesicles with the plasma membrane and thus glucose uptake (Thurmond, Kanzaki, Khan, & Pessin, 2000).
1.12 – Experimental Objectives

The observed functional importance of SNARE complex regulation on membrane trafficking events in other contexts suggests that it may be contributing to membrane trafficking events that support tumour cell invasion. Recent research has contributed to a model wherein cellular invasion and invadopodium formation are dependent on the SNARE-mediated trafficking of key proteins that facilitate invasion through the ECM (Kean et al., 2009; Williams et al., 2014; Williams & Coppolino, 2014). Syntaxin4 has been identified as one of the SNARE proteins responsible for the trafficking of MT1-MMP to the plasma membrane (Miyata et al., 2004), including sites of invadopodium formation in MDA-MB-231 cells. The ubiquitous expression of Munc18c in tissues suggests that it may be involved in regulating Syntaxin4 in cells forming invadopodia. The purpose of this study is to characterize the relationship between Syntaxin4 and Munc18c during tumour cell invasion in MDA-MB-231 cells. The hypothesis of this study is thus: Munc18c function facilitates invasion of the ECM by MDA-MB-231 cells.

To elucidate the function of Munc18c, the aims of this thesis are as follows:

**Aim 1:** Characterize the interaction between Syntaxin4 and Munc18c during cellular invasion.

This will be achieved by using co-immunoprecipitation experiments to monitor the association between endogenous Munc18c and Syntaxin4. Confocal microscopy will be used to assess the localization of these proteins during invadopodium formation.

**Aim 2:** Examine the role of Munc18c during cellular invasion.

Inhibition of Munc18c function will be carried out using RNAi-mediated knockdown and through the expression of a truncated version of Syntaxin4. Truncated
Syntaxin4 is predicted to act as a competitive inhibitor for Munc18c and Syntaxin4 interactions. Munc18c overexpression will be used to assess the effect of excess Munc18c within cells. Using these approaches, the role of Syntaxin4-Munc18c interactions in cell migration, invasion and invadopodium formation will be examined.
2.0 – Materials and Methods

2.1 – Materials

2.1.1: 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 (Abcam: ab3644, ab78738, ab290, ab175238); mouse anti-Munc18c (Santa Cruz Biotechnology: sc-373813); mouse anti-Syntaxin4, (BD Biosciences: 610439); mouse anti-actin (Pierce: MA5-15739); mouse anti-β1 integrin (Developmental Hybridoma Studies Bank: P4C10). 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). Anti-fade fluorescent mounting medium was obtained from DAKO, Inc. (Burlington, ON).

2.1.2: cDNA constructs

The pEGFP-N1-Syntaxin4-FL construct was described previously (Williams et al., 2014). The N-terminal sequence of Syntaxin4 was PCR amplified from pEGFP-N1-Syntaxin4-FL, and the amplicon was cloned into pEGFP-N1 using XhoI and KpnI to create the plasmid encoding N-terminal Syntaxin4-GFP. Mouse Munc18c with an N-terminal V5-epitope tag in pDEST40 was a generous gift from William S. Trimble (Hospital for Sick Children, Toronto). The untagged ORF was PCR amplified and the amplicon was cloned into pEGFP-C1 using EcoRI and BamHI to create the plasmid encoding GFP-Munc18c. Human Munc18c cDNA in pCMV-Sp6 was purchased from
Dharmacon (MHS6278-202759106). The ORF sequence was PCR amplified, and the amplicon was cloned into pcDNA3.1(-) using XhoI and BamHI to create the plasmid encoding untagged human Munc18c. The following oligonucleotides were used as primers: Forward N-terminal Syntaxin4 (5’ – TGACGGTAAATGGCCCGCTGGCATTATG – 3’), Reverse N-terminal Syntaxin4 (5’ - TTTATCATTGGTACCGGGTGACCACCAGCGCG – 3’), Forward human Munc18c (5’ – TATTTATACCGAGCAGAGATGGGCGCGC – 3’), Reverse human Munc18c (5’ – AATGTATTAGGATCCCATATTAGTAAGATCTCTAAACCCTC – 3’), Forward Mouse Munc18c (5’ – GTCTCGAAGCTTCGATGGCGGCCTATC – 3’), and Reverse Mouse Munc18c (5’ – CGAACCGCGGATCCTCTCCTAGATCAACCACCTTTG – 3’).

Knockdown of Munc18c was performed using a commercially available pool of 3 siRNA duplexes targeting human Munc18c. Munc18c and control siRNA were purchased from Santa Cruz Biotechnologies (SC-42312 and SC-37007).

2.1.3: Cell Culture

MDA-MB-231 and HEK-293 cells were 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 5% BCS, 1 mg/mL G418 (BioShop) and Penicillin-Streptomycin (Life Technologies). Growth conditions were kept at 37°C with humidity and a 5% CO2 atmosphere. Cells were lifted by using 5 mM EDTA/PBS, pH 7.4. For all experiments, cells were used between passage number 5 and 20. All cells were passaged at most, 24 hours before each experiment.
2.1.4: Transfections

Cells were transfected using jetPRIME Polyplus (VWR International) as per the manufacturer’s protocol. All transiently transfected constructs were expressed for a total of 24 hours. Cells were transfected with 50 nM siRNA and underwent knockdown for 48 hours. Co-transfections were performed using the manufacturer’s recommended amount of marker pEGFP-C1 plasmid in addition to 50 nM siRNA for a total of 48 hours.

2.2 – Methods

2.2.1: Creation of Stable Cell Lines

In a 10 cm tissue culture plate, cells were transfected with either pcDNA3.1(-)-Munc18c, pEGFP-N1-full length Syntaxin4, or pEGFP-N1-Nterminal Syntaxin4. 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 highest level of expression were propagated.

2.2.2: Cell Migration Assay

Boyden transwell migration assays were performed as previously described (Williams et al., 2014). Tissue culture inserts with an 8-µm pore diameter (Corning) in 24-well plates were coated with 20 µg/mL fibronectin/PBS on the bottom of the membrane. Both transfected cells and stable cell lines were serum starved for 2 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 6 hours towards the lower chamber containing the above medium, supplemented with 10% FBS. The top and 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 on coverslips. 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.

2.2.3: 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% gluteraldehyde/PBS. Coverslips were then inverted onto 70 µL of AlexaFluor-594 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% unlabelled gelatin/PBS.

2.2.4: Immunoprecipitation

Antibody was coupled to 450 µg of Protein-G Dynabeads (Invitrogen) 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 Na$_2$P$_4$O$_7$, 0.2 mM Na$_3$VO$_4$ and protease inhibitor cocktail. Lysate was incubated with antibody bound beads for 1 hour at 4°C on an end-over-end rotator, washed 3 times with cold PBS, and eluted with 2.5X SDS-PAGE loading buffer containing 700 mM β-mercaptoethanol. The resultant beads-antibody-antigen complex was subsequently heated to 70°C for 15 minutes. Proteins were separated using SDS-PAGE, and analyzed using Western immunoblotting.

2.2.5: 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 powder or 5% BSA in TBST and probed with primary antibody (diluted 1:1000 in TBST). HRP-conjugated secondary antibodies (diluted 1:7500 in blocking solution) were used to detect bound primary antibodies using an enhanced chemiluminescence kit (Bio-Rad).

2.2.6: 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 10 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 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
microscope with a Leica TCS SP2 scanning head (Leica, Heidelberg, Germany). Images were captured using Leica confocal software. Phase-contrast and epifluorescence images of HEK-293 cells were acquired using a Nikon Eclipse Ti-S inverted microscope through a 40X (NA 0.6) lens (Melville, NY, USA). Images were captured using Nikon imaging software. All images were processed and analyzed using ImageJ software (NIH, Bethesda, MD, USA).

2.2.7: Statistical Analysis

The mean of three independent experiments is shown (unless indicated otherwise), where error bars represent the standard error of the mean. For all treatments, the experimental group was compared to the respective control group by Student’s t-test, where the statistical significance threshold was p=0.05. An asterisk in figures represented a treatment that was significantly different from the control treatment (p<0.05). Microsoft Excel was used to perform statistical analyses.
3.0 Results

3.1 Syntaxin4 associates with Munc18c in MDA-MB-231 cells

Previous studies have shown that Syntaxin4 and Munc18c are strong interacting partners and the latter plays an important role during Syntaxin4-mediated exocytosis through this interaction (Latham et al., 2006; Yu et al., 2013). To test if Munc18c and Syntaxin4 are associating in MDA-MB-231 cells, co-immunoprecipitation experiments were utilized. Cells were lysed in situ and Munc18c was immunoprecipitated. Eluents were subjected to SDS-PAGE and Western blot analyses using the same Munc18c antibody for immunoprecipitation in addition to a Syntaxin4 antibody. The results revealed successful immunoprecipitation of Munc18c, as indicated by the enrichment of protein in the immunoprecipitation at the predicted molecular weight of ~67 kDa (Figure 8A). Moreover, there was a significant reduction of Munc18c in the output lane relative to the input control. Successful co-immunoprecipitation of Syntaxin4 was also observed in Munc18c immunoprecipitates compared to immunoprecipitation control samples.

To further evaluate the association of Munc18c and Syntaxin4, confocal immunofluorescence microscopy was utilized to assess intracellular localization. Since Syntaxin4 is a plasma membrane SNARE (Torres, Funk, Zegers, & Beest, 2011), images of the ventral plasma membrane were analyzed in comparison to the midcell region of cells grown on glass coverslips. Image analyses revealed that Munc18c and Syntaxin4 were predominantly localized at the ventral plasma membrane relative to the midcell region (Figure 8B). At the ventral surface, strong co-localization was seen at the edges of lamellipodia. Moreover, co-localization across the ventral focal plane was incomplete.
Figure 8: Analysis of Syntaxin4 and Munc18c association in MDA-MB-231 cells. Munc18c and Syntaxin4 association was analyzed via immunoprecipitation/SDS-PAGE/Western blot and confocal microscopy. (A) Munc18c immunoprecipitates were probed for Munc18c and Syntaxin4. (B) Cells were grown overnight on glass coverslips fixed, permeabilized, and stained for Rb α Munc18c and M α Syntaxin4. To visualize the ventral and midcell regions of cells, the confocal z-plane was changed and the observed signal was centered in the image. White arrows indicate areas of strong co-localization. Scale bar, 10 µm.
3.2 Syntaxin4 and Munc18c association is enhanced during invadopodium formation

Syntaxin4 has been shown to play an important role in the trafficking of MT1-MMP to sites of invadopodia formation in MDA-MB-231 cells (Williams et al., 2014). Since Syntaxin4 and Munc18c appeared to be associating in MDA-MB-231 cells, changes to this association were examined in the context of invadopodium formation. Cells were seeded onto a non-ECM substrate (PLL) or an ECM substrate (gelatin) for 4 hours to induce invadopodium formation. Cells were lysed in situ and Munc18c was immunoprecipitated. Eluents were subjected to SDS-PAGE and Western blot analyses. In all treatments, to normalize the amount of Syntaxin4 associating with immunoprecipitated Munc18c, a second antibody specific to Munc18c was used for immunoblotting. An increase of about 20%, relative to unlifted cells, in the amount of Syntaxin4 co-immunoprecipitated with Munc18c was observed when cells were seeded onto PLL (Figure 9). An increase of about 50%, relative to unlifted cells, in the amount of Syntaxin4 co-immunoprecipitated with Munc18c was observed during invadopodium formation on gelatin.

3.3 Analysis of Munc18c localization relative to invadopodium components

The increase in Syntaxin4 co-immunoprecipitated with Munc18c during invadopodium formation suggested that there was an important role for the association of Munc18c and Syntaxin4 during this process. To evaluate this role, confocal immunofluorescence microscopy was utilized to assess the localization of Munc18c relative to proteins typically associated with invadopodium formation and function. Cells were seeded onto coverslips coated with a thin AlexaFluor-594-labelled gelatin matrix and incubated for 4 hours prior to fixation and staining. Use of a thin fluorescently
Figure 9: Syntaxin4 and Munc18c association increases during invadopodium formation.

Cells were seeded onto PLL and gelatin (IVF; invadopodium formation) coated plates for 4 hours, lysed and analyzed by immunoprecipitation/SDS-PAGE/Western blot. (A) Munc18c immunoprecipitates were probed for Munc18c and Syntaxin4. (B) Quantification of the amount Syntaxin4 co-immunoprecipitated with Munc18c normalized to unlifted cells. Means are from three independent experiments, +/- S.E.M. Asterisks denote values significantly different from control unlifted cells (p<0.05).
labelled gelatin matrix allowed detection of invadopodia-forming cells within a heterogeneous population of cells, in addition to analysis of proteins that localize to focal points of gelatin degradation. In parallel with Munc18c staining, cells were stained for F-actin, β1 integrin, MT1-MMP, and Syntaxin4 (Figure 10). Confocal microscopy of the ventral surface revealed that Munc18c was distributed across the focal plane. Black spots of gelatin degradation that overlaid with F-actin, MT1-MMP and Syntaxin4 at the centre had Munc18c localized to the edges of degradation. β1 integrin and Munc18c partly co-localized to the edges of some black spots of gelatin degradation. Munc18c was also observed to co-localize with F-actin, MT1-MMP, Syntaxin4, and β1 integrin in areas other than focal degradation sites. However, co-localization of Munc18c with the above proteins was not complete across the focal plane.

3.4 Munc18c knockdown impairs invadopodium formation

Previous work has shown that knockdown of Syntaxin4 inhibits invadopodium formation in MDA-MB-231 cells (Williams et al., 2014). Since Munc18c and Syntaxin4 are associating in these cells, we sought to test whether Munc18c knockdown produced a similar phenotype. Cells were transfected with a pool of siRNA targeting human Munc18c or a non-specific control siRNA. Optimal knockdown of Munc18c was seen after 48 hours, as determined by SDS-PAGE and Western blot analyses (data not shown). Relative to cells transfected with control siRNA, Munc18c levels were found to be reduced by approximately 50% (Figure 11). Munc18c knockdown had no observed effects on β1 integrin, Syntaxin4, and MT1-MMP protein levels. Invadopodium formation was subsequently analyzed to evaluate the effect of reduced Munc18c expression after 48 hours. Cells co-transfected with pEGFP-C1 and siRNA were
Figure 10: Subcellular distribution of Munc18c during the formation of invadopodia

Cells were analyzed by confocal microscopy after being plated on AlexaFluor-594-labeled gelatin for 4 hours, fixed, permeabilized, and stained for markers of invadopodia as indicated: F-actin, β1 integrin, MT1-MMP, and Syntaxin4 (cyan; white arrow). Cells were also stained for Munc18c (green; orange arrow). Dark spots in the red field indicate sites of gelatin degradation corresponding to invadopodia. Scale bar, 10 µm.
Figure 11: RNAi-mediated knockdown of Munc18c

Cells were transfected with siRNA targeting Munc18c or non-specific control siRNA. (A-B) Cells were lysed, and lysate was analyzed to assess knockdown of Munc18c via SDS-PAGE/Western blot. (C) Quantification of the amount of Munc18c protein present after knockdown normalized to actin. Means are representative of three independent experiments; +/- S.E.M. Asterisks denote values significantly different from control. (p<0.05).
incubated for 44 hours, and subsequently seeded onto coverslips coated with AlexaFluor-594 labelled gelatin. After 4 hours of invadopodium formation, cells were fixed and stained for F- actin or MT1-MMP (Figure 12). A GFP-positive cell that had F-actin punctae overlying black spots of degradation was counted as a cell that was forming invadopodia. Results indicated that knockdown of Munc18c reduced the number of GFP-positive cells forming invadopodia by approximately 50% relative to control transfections.

### 3.5 Expression of N-terminal Syntaxin4-GFP impairs invadopodium formation

Previous work has shown that the N-terminal 29 amino acids of Syntaxin4 facilitates binding to Munc18c (Latham et al., 2006). *In vitro* pull-down experiments showed that the presence of this small polypeptide reduced the association of Munc18c and Syntaxin4, suggesting this N-terminal domain can act as a competitive inhibitor of Munc18c and Syntaxin4 interactions. We therefore examined the effect of a GFP-tagged N-terminal domain of Syntaxin4 (Figure 12) on invadopodium formation. Cells transfected with either N-terminal Syntaxin4-GFP or GFP alone were subjected to invadopodium formation assays (Figure 13). Overexpression of N-terminal Syntaxin4-GFP reduced the amount of cells forming invadopodia by approximately 80% relative to cells transfected with control GFP.

To assess the biochemical effects of N-terminal Syntaxin4-GFP, co-immunoprecipitation experiments were utilized to probe for changes in the amount of Syntaxin4 associated with Munc18c. Cells were lysed *in situ* and eluents of Munc18c immunoprecipitates from cells that were either non-transfected, GFP transfected, or N-terminal Syntaxin4-GFP transfected were analyzed via SDS-PAGE and Western blot
Figure 12: RNAi-mediated knockdown of Munc18c impairs invadopodium formation.

(A) Invadopodium-based degradation of AlexaFluor-594-gelatin by cells co-transfected with GFP and control siRNA or Munc18c siRNA Cells transfected for 44 hours, seeded onto gelatin for 4 hours and then fixed, permeabilized, stained for MT1-MMP and analyzed by confocal microscopy. (B) Cells with F-actin punctae overlying dark spots of gelatin degradation were counted as cells forming invadopodia. Percentages of cells forming invadopodia, normalized to control (GFP-transfected) cells, are presented as the mean +/- S.E.M. from four independent experiments in which 50 cells per sample were counted. Asterisks denote values significantly different from control (p<0.05). Bar, 10 µm.
**Figure 13: Segmental architecture of N-terminal Syntaxin4-GFP.**
The segmental architecture of Syntaxin4 (red) comprises: an N-terminal domain, H_{abc} domain, SNARE motif, and membrane anchor. To create N-terminal Syntaxin4-GFP, the N-terminal domain (red stripes) of Syntaxin4 was fused to the N-terminus of GFP (green).
Figure 13: N-terminal Syntaxin4-GFP impairs invadopodium formation.

(A) Invadopodia-based degradation of gelatin by cells transfected with GFP (control) and N-terminal Syntaxin4–GFP. Cells transfected for 20 hours, seeded onto gelatin for 4 hours, and then fixed, permeabilized, stained for F-actin and analyzed by confocal microscopy. (B) Cells with F-actin punctae overlying dark areas of gelatin degradation were counted as cells forming invadopodia. Percentages of cells forming invadopodia are presented as the mean +/- S.E.M. from four independent experiments, in which 50 cells per sample were counted. Asterisks denote values significantly different from control (p<0.05). Bar, 10 µm.
(Figure ). The amount of Syntaxin4 co-immunoprecipitated with Munc18c did not change relative to control samples. No co-immunoprecipitation of GFP alone or N-terminal Syntaxin4-GFP was detected.

3.6 Munc18c knockdown and expression of N-terminal Syntaxin4-GFP impair cell migration

Since invadopodium formation was reduced by Munc18c knockdown or the expression of N-terminal Syntaxin4-GFP, we examined the effects of these two treatments on cell migration using transwell migration assays. Compared to control treatments, Munc18c knockdown or expression of N-terminal Syntaxin4-GFP inhibited cell migration by approximately 60% and 50%, respectively (Figure 14).

3.7 Creation of stable cell lines expressing either Munc18c, Syntaxin4-FL-GFP or N-terminal Syntaxin4-GFP

Previous work has shown that the overexpression of Munc18c can inhibit vesicle fusion events that require Syntaxin4 (Tamori et al., 1998). However, other work has also shown that overexpression of Munc18c had no significant effect on Syntaxin4-mediated exocytosis (Yu et al., 2013). To examine the effects of Munc18c overexpression in MDA-MB-231 cells, transient transfections were first employed using GFP-tagged and untagged mouse Munc18c constructs. Fluorescence microscopy and western blot analyses showed that transient transfections yielded no expression of the aforementioned Munc18c constructs in MDA-MB-231 cells (data not shown). To confirm that the lack of expression was not due to a problem with the expression vector, the GFP-Munc18c construct was transfected into HEK-293 cells. Fluorescence microscopy showed that HEK-293 cells were expressing GFP-Munc18c (Figure 15).

Next, the creation of a cell line stably expressing untagged human Munc18c was
Figure 15: N-terminal Syntaxin4-GFP does not alter co-immunoprecipitation of Syntaxin4 with Munc18c.

Cells were either untransfected (UT) or transfected with GFP or N-terminal Syntaxin4-GFP for 24 hours before lysing and analysis by immunoprecipitation/SDS-PAGE/Western blot. Munc18c immunoprecipitates were first probed for Munc18c and Syntaxin4. Membranes were stripped and reprobed for GFP; two exposure times are shown to minimize antibody light chain over-saturation in the immunoprecipitation lanes.
Figure 14: Munc18c knockdown or overexpression of N-terminal Syntaxin4 impairs cell migration.

Transfected cells were lifted, counted and subjected to transwell migration assays. (A) Cells were transfected with GFP (control) or N-terminal Syntaxin4–GFP for 18 hours. (B) Cells were co-transfected with GFP and control siRNA or Munc18c siRNA for 38 hours. Means are +/- S.E.M from three independent experiments. Asterisks denote values significantly different from control (p<0.05).
Figure 15: Expression of GFP-Munc18c in HEK-293 cells.
Cells were transfected and incubated for 20 hours prior to imaging with fluorescence microscopy. (A) Phase-contrast and GFP fluorescence overlay of HEK293 cells on tissue culture plate. (B) Zoom-in of GFP fluorescence. Scale bar, 10 µm.
undertaken to selectively pressure cells into expressing Munc18c. Cell lines overexpressing full length Syntaxin4-GFP and N-terminal Syntaxin4-GFP were also created for use in future experiments in addition to confirming previous results from transient transfections. After selection and propagation of G418-resistant colonies, SDS-PAGE and Western blot analyses were performed to assess expression of the proteins of interest (Figure 16). Probing for Munc18c revealed no increase in Munc18c expression relative to parental MDA-MB-231 cells. Probing for Syntaxin4 revealed protein bands at the predicted molecular weights of ~63 kDa (full length Syntaxin4-GFP) and ~34 kDa (endogenous Syntaxin4) in the full-length Syntaxin4-GFP cell line. A GFP antibody was used to probe for N-terminal Syntaxin4-GFP, yielding a band at the predicted molecular weight of ~31 kDa in the N-terminal Syntaxin4-GFP cell line. No observed effects on MT1-MMP expression were seen in any of the cell lines.
Figure 16: Expression of Munc18c, GFP-Syntaxin4-full length or GFP-Syntaxin4-N-terminus in stable cell lines.
Stable cell lines were generated to express untagged Munc18c, Syntaxin4-FL-GFP and N-terminal Syntaxin4 – GFP. Parental MDA-MB-231 cells were lysed in conjunction with stable cell lines and lysates were analyzed via SDS-PAGE/Western blot.
3.8 Evaluating invadopodium formation and cell migration by stable cell lines

The stable cell lines created were subjected to invadopodium formation assays and transwell migration assays. Cell lines were seeded onto coverslips coated with AlexaFluor-594-labelled gelatin and incubated for 4 hours prior to fixation and staining for F-actin (Figure 17). Relative to parental MDA-MB-231 cells, no significant change in invadopodium formation was seen for Munc18c and full-length Syntaxin4-GFP cell lines (Figure 18). The N-terminal Syntaxin4-GFP cell line displayed approximately a 60% decrease in invadopodium formation. Transwell migration assays revealed no significant change for Munc18c and full-length Syntaxin4-GFP cell lines, but the N-terminal Syntaxin4 cell line showed approximately a 60% decrease in cell migration.
Figure 17: Analysis of invadopodium formation by stable cell lines.
Parental MDA-MB-231 cells or stable cell lines expressing untagged Munc18c, Syntaxin4-FL-GFP or N-terminal Syntaxin4–GFP were seeded onto AlexaFluor-594-labeled gelatin and incubated for 4 hours prior to fixation, staining for F-actin and analysis by confocal microscopy. Scale bar, 10 µm.
Figure 18: Quantification of invadopodium formation and cell migration by stable cell lines.

(A) Cells with F-actin punctae overlying dark spots of gelatin degradation were counted as cells forming invadopodia. Percentages of cells forming invadopodia are presented as means +/- S.E.M. from three independent experiments in which 100 cells per sample were counted and normalized to parental MDA-MB-231 cells. (B) Stable cell lines were lifted, counted and subjected to transwell migration assays. Means +/- S.E.M. are presented from three independent experiments. Asterisks denote values significantly different from control (p<0.05).
4.0 Discussion

The field of study pertaining to intracellular trafficking of proteins that are part of the molecular machinery utilized by cells during tumour cell invasion has implications on the establishment of cancer therapies. Previous work has shown the importance of SNARE-mediated trafficking in all aspects of tumour cell invasion including: invadopodium formation, cell migration, cell adhesion, and MMP-directed ECM degradation. Although SNAREs have been identified to play an important role in membrane trafficking events during the above cellular processes, there is no direct knowledge of the role SNARE regulation has during tumour cell invasion. A recent study has demonstrated an important role for the Q-SNARE, Syntaxin4, during tumour cell invasion by MDA-MB-231 cells (Williams et al., 2014). Here, we demonstrate the cognate SM protein of Syntaxin4, Munc18c, contributes to cellular migration and invadopodium formation in these cells. These data may provide insight into the mechanism of SNARE regulation during tumour cell invasion.

SM proteins and their cognate Syntaxins form strong interactions. Furthermore, both of these proteins have been shown to be important components of the membrane fusion machinery (Carr & Rizo, 2010). Munc18c and Syntaxin4 have been well documented to associate in cell lines such as 3T3-L1 adipocytes (Thurmond et al., 2000; Yu et al., 2013), but their association has not been demonstrated in invasive cancer cell lines. Thus, the first step in exploring a possible function for Munc18c during tumour cell invasion was to determine if Syntaxin4 and Munc18c associate in MDA-MB-231 cells in culture. Co-immunoprecipitation experiments indicated that Munc18c and Syntaxin4 associate. Furthermore, co-localization analysis by confocal microscopy suggested the
association observed by co-immunoprecipitation was occurring at the plasma membrane. These findings are consistent with a role for Munc18c in regulating Syntaxin4-mediated fusion events at the plasma membrane. Experiments showing the staining pattern of Munc18c and Syntaxin4, compared to the localization of a plasma membrane marker can be performed to confirm plasma membrane localization, for example using a construct that encodes the acylation motif of Lyn kinase tagged with GFP (Teruel, Blanpied, Shen, Augustine, & Meyer, 1999; Williams & Coppolino, 2014). Additionally, the detection of Syntaxin4 and Munc18c in the mid-cell region, presumably around the nucleus, suggests that these two proteins are also found on the endoplasmic reticulum. Future experiments will have to assess the staining pattern of calnexin, an endoplasmic reticulum-specific protein, relative to Syntaxin4 and Munc18c to confirm this possibility.

The evident association of Munc18c and Syntaxin4 prompted the evaluation of changes to this association in the context of invadopodium formation. Cells seeded onto PLL and gelatin both resulted in a significant increase in the amount of Syntaxin4 associating with Munc18c when compared to unlifted cells. The increase in Munc18c and Syntaxin4 association was greater in cells seeded onto gelatin (invadopodia formation) than those seeded onto PLL, a non-ECM substrate. The increased association when cells were seeded onto PLL suggests that Munc18c and Syntaxin4 may have a putative role during cell spreading and not during integrin-mediated cell attachment. Integrin-mediated cell attachment, and the signalling pathways this stimulates are minimal when cells are seeded onto PLL. Negatively charged cell surface proteins are proposed to mediate the attachment to positively-charged PLL (Mazia, 1975). Future experiments will need to evaluate the co-immunoprecipitates of Syntaxin4 with Munc18c from cells in
suspension, to confirm if their association is a result of cell-substratum interactions. It is predicted that there will be less Syntaxin4 associating with Munc18c in suspended cells relative to unlifted cells, because there will be no cell-substratum interactions to support cell spreading or integrin-mediated signalling.

A previous study has demonstrated that serine/threonine phosphorylation of Syntaxin4 in MDA-MB-231 cells was significantly reduced during invadopodium formation, compared to cells seeded onto PLL (Williams et al., 2014). Another study has shown that phosphorylation of Syntaxin4 and Munc18c inhibits their interaction (Fu, Naren, Gao, Ahmmed, & Malik, 2005). Therefore, it is reasonable to speculate that increased association of Syntaxin4 with Munc18c during invadopodium formation is a result of changes in Syntaxin4 serine/threonine phosphorylation. Future experiments to analyze the phosphorylation state of Munc18c-associated Syntaxin4 and Munc18c will extend our understanding of the association between Munc18c and Syntaxin4 during invadopodium formation in MDA-MB-231 cells.

The increased association of Munc18c and Syntaxin4 during invadopodium formation prompted an analysis of the subcellular localization of Munc18c during this process using confocal microscopy. Syntaxin4, MT1-MMP, and F-actin have been shown to localize to the center of sites of invadopodium formation when invasive cell lines are seeded onto an ECM substrate (Oser et al., 2009; Williams et al., 2014). β1 integrin has been implicated in the initiation of invadopodia (Artym et al., 2015; Destaing et al., 2010; Williams & Coppolino, 2014), in addition to their maturation, as exemplified by integrin containing adhesion rings around focal sites of invadopodium degradation (Branch, Hoshino, & Weaver, 2012). When analyzed under confocal microscopy, Munc18c was
distributed throughout the confocal plane representative of the ventral plasma membrane. The location of the ventral plasma membrane was indicated by the strongest signal given by invadopodium markers. Co-localization of Munc18c and Syntaxin4 observed in unlifted cells was not consistent with localization observed during invadopodium formation. It is plausible the observed co-localization of Munc18c and Syntaxin4 in unlifted cells may be due to Munc18c priming Syntaxin4 for vesicle fusion events. During invadopodium formation, the requirement for Syntaxin4-mediated vesicle fusion is stimulated, resulting in changes to Munc18c localization relative to Syntaxin4. Additionally, given the observed distribution of Munc18c at the cell periphery, it could be suggested that Munc18c function is not exclusive to invadopodium formation, and may therefore be involved in a variety of unidentified Syntaxin4-mediated exocytic events within these cells. When analyzing invadopodia specifically, Munc18c was found at some of the edges of sites of degradation showing stronger co-localization with β1 integrin. This suggests that Munc18c may be involved in regulating Syntaxin4-mediated exocytic events that promote the maturation of invadopodia.

The potential role of Munc18c during invadopodium formation prompted an analysis of Munc18c inhibition using RNAi-mediated knockdown to reduce Munc18c expression. Previous studies have shown that the depletion or RNAi-mediated knockdown of Munc18c perturbed Syntaxin4-mediated exocytic events, such as GLUT4 translocation (Jewell et al., 2011; Oh & Thurmond, 2009). Also, RNAi-mediated knockdown of its cognate SNARE partner, Syntaxin4, in MDA-MB-231 cells was found to significantly perturb invadopodium formation and cellular invasion, but not cell migration (Williams et al., 2014). In the present study, RNAi-mediated knockdown of
Munc18c was observed to significantly inhibit both invadopodium formation and cell migration, suggesting that the presence of Munc18c facilitates these processes. The inhibition of cell migration is surprising, given the previous study that found no effect on cell migration (Williams et al., 2014). Cell migration requires the precise localization of adhesion complexes and polarized recycling of integrins (Bravo-Cordero et al., 2012). A previous study showed that Munc18c stabilizes Syntaxin4 at the plasma membrane, preventing disorganized intracellular localization of Syntaxin4 (Torres et al., 2011). This study also showed that depletion of Syntaxin4 leads to the intracellular localization of proteins such as β1 integrin. The phenotype observed by Williams et al., 2014 might therefore be due to the cellular compensation for depleted Syntaxin4 by using an alternative SNARE-mediated trafficking pathway during cell migration, but not during invadopodium formation. Torres et al., 2011 also showed that Munc18c depletion did not alter Syntaxin4 localization to the plasma membrane. Therefore, in the present study, the available Syntaxin4 at the plasma membrane in MDA-MB-231 cells may require Munc18c to mediate the membrane fusion of key proteins during invadopodium formation and cell migration. Future experiments will need to verify the intracellular localization of Syntaxin4 and β1 integrin during invadopodium formation in Munc18c depleted cells. It is predicted that there will be no alterations in Syntaxin4 plasma membrane localization and there will be an increased intracellular accumulation of β1 integrin. Taken together, the observations here support a model wherein Syntaxin4-mediated exocytic machinery, and trafficking of proteins required for cell migration and invadopodium formation, is dependent on Munc18c.
A second method to inhibit Munc18c utilized the N-terminal 29 amino acids of Syntaxin4. The N-terminal domain of Syntaxin4 forms facilitates binding between Munc18c and Syntaxin4 (Hu et al., 2007). A previous study demonstrated that the presence of this small polypeptide inhibited recombinant Munc18c and Syntaxin4 interactions using pull-down experiments (Latham et al., 2006). Here, we expressed a GFP-tagged version of this N-terminal domain and observed a significant decrease in both invadopodium formation and cell migration. These results support a role for Munc18c and Syntaxin4 interactions during invadopodium formation and cell migration. Attempts to detect an interaction between Munc18c and N-terminal Syntaxin4-GFP were unsuccessful. These results may be due to the difference in transfection efficiency between N-terminal Syntaxin4-GFP and GFP. Since there was little N-terminal Syntaxin4-GFP detected in the lysate, this could suggest the co-immunoprecipitation experiments were not sensitive enough to detect N-terminal Syntaxin4-GFP. Moreover, the immunoprecipitation conditions and/or the bulkiness of the GFP-tag may be contributing to the lack of N-terminal Syntaxin4-GFP co-immunoprecipitation. Future experiments should employ a FLAG-tagged N-terminal Syntaxin4 – the size and molecular weight of a FLAG-tag is much smaller than GFP. It is predicted that a FLAG-tag will allow for the co-immunoprecipitation of N-terminal Syntaxin4 with Munc18c and will produce a similar level of impairment with respect to invadopodium formation and cell migration.

Munc18c overexpression studies were attempted using a transiently transfected expression construct containing a CMV promoter upstream of the Munc18c ORF. Initial experiments were performed using the mouse Munc18c homolog N-terminally tagged
with GFP. The expression of GFP-Munc18c in MDA-MB-231 cells was not successful as revealed by fluorescence microscopy. The same result was seen using untagged human Munc18c. Interestingly, expression of GFP was observed when HEK-293 cells were transfected with mouse GFP-Munc18c. Future experiments should employ western blot analyses to determine if the observed GFP fluorescence in HEK-293 cells is from GFP-Munc18c and not GFP where Munc18c is removed by proteases.

To selectively pressure MDA-MB-231 cells into overexpressing Munc18c, stable cell lines that were transfected with the untagged Munc18c construct were created. Despite having propagated cells that were resistant to antibiotic, no Munc18c overexpression was observed. These observations suggest that Munc18c expression might be regulated at the post-transcriptional (such as miRNA-mediated inhibition) or post-translational level (such as proteasomal degradation) and future experiments will need to be performed to explain these results. Interestingly, several studies have shown that Syntaxin4 and Munc18c expression are interdependent (Kanda et al., 2005; Torres et al., 2011). However, in the present study, no alteration in Syntaxin4 expression was seen in the Munc18c depleted cells. It is plausible that the expression of Munc18c was not depleted enough to see a significant reduction in Syntaxin4.

Stable cell lines derived from MDA-MB-231 cells were created to overexpress N-terminal Syntaxin4-GFP and Syntaxin4-FL-GFP in order to confirm previous results seen with transient transfections. Interestingly, the cell line expressing N-terminal-Syntaxin4-GFP had impaired invadopodium formation and cell migration relative to control parental MDA-MB-231 cells and Syntaxin4-FL-GFP expressing cells. Further experiments will need to be performed to elucidate how Syntaxin4-N-terminal-GFP is influencing cell
migration and invadopodium formation. Using these stable cell lines, sucrose gradient centrifugation could be used as a tool to assess the intracellular localization of proteins involved in invadopodium formation and cell migration. This technique allows for the detection of proteins accumulated at the plasma membrane and endosomal compartments. For example, N-terminal Syntaxin4-GFP could be interfering with endogenous Munc18c and Syntaxin4 function leading to the accumulation of integrins in an endosomal compartment. The current model of SM protein function is that SM proteins interact with their cognate Syntaxin and then reposition to impart specificity in SNARE complex formation (Baker et al., 2015; Yu et al., 2013). Therefore, if N-terminal Syntaxin4-GFP is interfering with Munc18c and Syntaxin4 interactions within MDA-MB-231 cells then there may be less Syntaxin4 interaction with cognate SNARE partners. VAMP7, SNAP23, and Syntaxin4 are required for the delivery of MT1-MMP to sites of invadopodium formation. Thus, it is plausible that during invadopodium formation Syntaxin4 immunoprecipitates from the cell line expressing N-terminal Syntaxin4-GFP would contain less VAMP7 and SNAP23, relative to parental MDA-MB-231 cells, resulting from N-terminal Syntaxin4-GFP inhibition of SNARE complex formation.

Studies over the past decade have made it clear that trafficking of MMPs and adhesion molecules has an essential role in the remodelling of the extracellular matrix during tumour cell invasion, facilitating tumour cell migration through interstitial tissues. The picture that has emerged is that formation of invadopodia and subsequent matrix degradation in invasive tumour cells rely on the coordination of cytoskeletal and specific membrane trafficking events. Membrane trafficking mediated by SNAREs, exocyst complexes and other components of the membrane fusion machinery have been observed
to function during tumour cell invasion (Poincloux, Lizárraga, & Chavrier, 2009). Nonetheless, a direct role that regulators of SNARE function have on SNARE-mediated trafficking during tumour cell invasion has yet to be defined. To our knowledge, this study is the only direct evidence for a role in SNARE regulation during tumour cell invasion. Previous studies have identified gelsolin, an actin modifying protein, as an interaction partner for Syntaxin4 (Kalwat et al., 2012), and gelsolin has been identified separately to have a role during invadopodium formation (Crowley, Smith, Fang, Takizawa, & Luna, 2009). Whether or not the interaction between gelsolin and Syntaxin4 influences invadopodium formation and cell migration, however, is not known. Thus, the current study furthers our knowledge of the SNARE-mediated mechanisms that coordinate the precise localization of proteins during tumour cell invasion. Our results indicate that regulation of Syntaxin4-mediated events by Munc18c contributes to the formation of invadopodia and migration by MDA-MB-231 cells.
5.0 Summary

The aim of this study was to analyze the function of Munc18c and Syntaxin4 during tumour cell invasion by MDA-MB-231 cells. Using co-immunoprecipitation experiments and confocal microscopy, an association between Munc18c and Syntaxin4 was observed. This association was enhanced when cells were seeded onto non-ECM (PLL) or ECM (gelatin) substrates. These results suggest that the association of Munc18c with Syntaxin4 in these cells is not exclusive to cellular processes regulated by cell-ECM interactions. ECM degradation assays and confocal microscopy indicated Munc18c could be involved in a variety of Syntaxin4-mediated events within MDA-MB-231 cells, including the maturation of invadopodia. Munc18c knockdown was shown to impair invadopodium formation and cell migration. The expression of N-terminal Syntaxin4-GFP, designed to impair Munc18c and Syntaxin4 interactions within cells, reduced both invadopodium formation and cell migration. These results were confirmed in a stable cell line expressing this protein domain of Syntaxin4. Disruption of the interaction between Munc18c and Syntaxin4 by N-terminal Syntaxin4-GFP could not be demonstrated using co-immunoprecipitation experiments, and future experiments will be employed to determine if N-terminal Syntaxin4 fused with a smaller epitope tag is interacting with Munc18c. Taken together, these data suggest Munc18c interacts with Syntaxin4 in MDA-MB-231 cells and this interaction contributes to cell migration and invadopodium formation. Additional biochemical experiments are needed to fully elucidate the effects of Munc18c knockdown and N-terminal Syntaxin4 expression. Together, the findings support a model for Munc18c-mediated tumour cell invasion wherein Munc18c regulates the formation of Syntaxin4-containing SNARE complexes that contribute to the
localization of proteins involved in cell migration, and invadopodium formation (Figure 19). Ultimately, these data contribute to our understanding of possible regulatory mechanisms that control SNARE function during tumour cell invasion. The information provided in this thesis could potentially be used to design cancer therapies that inhibit SNARE regulation by non-SNARE proteins within tumour cells, and by extension metastasis.
Figure 19: Proposed model of Munc18c function during tumour cell invasion
Munc18c and Syntaxin4 interactions impart Syntaxin4 stability and specificity in SNARE complex formation. This process is carried out in the following steps: [1] Munc18c (purple) is initially associated with Syntaxin4 (red). Vesicles that contain proteins facilitating cell migration and invadopodium formation are trafficked to specific sites of the plasma membrane. [2] Munc18c repositions to mediate trans-SNARE complex formation with the cognate R-SNARE (blue) and Qbc SNARE (yellow) and subsequent vesicle fusion (not shown) with target membrane. [3] Munc18c dissociates from the cis-SNARE complex. [4] NSF and α-SNAP separate the cis-SNARE complex.
6.0 References


