Cell migration is regulated by mitochondria and endoplasmic reticulum morphology.

Redaet Daniel

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Department of Biochemistry, Microbiology and Immunology
Faculty of Medicine
University of Ottawa

Abstract:

Cell migration is essential for homeostasis and the development of metastases. We hypothesize that cell migration is regulated by mitochondria and endoplasmic reticulum morphology. Using live cell microscopy, we found that mitochondria specifically migrate into the biochemically dense leading edge of the cell interacting with focal adhesions as well. At the leading edge the mitochondria are visibly shorter and less tubular than the perinuclear area. This is related to the elevated levels of fission events per minute in the leading edge and elevated levels of fusion events per minute in the trailing edge. We observe that mitochondria migrate along microtubules and simultaneously interact with the ER. When the ER is sheet-like the mitochondria are longer and tubular and when the ER is tubular the mitochondria are shorter and punctate. This change in ER and mitochondria morphology changes the cell's ability to migrate. CLIMP63 cells have more sporadic turns, take longer to make turns, have shorter distances travelled and shorter displacements. To determine whether mitochondria dynamics play a role we examined these cell migration parameters in the presence of OPA1 and Drp1. This allowed us to conclude that the ER morphology is responsible for the distance and displacement the cell travels while the mitochondria is responsible for the angles the cell turns. When the ER is sheet-like the cells will be travel shorter total distances and displacements and when the cell has longer mitochondria it will be sporadic turns and take longer to make these turns.

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Table of Contents

List of Figures and Tables

Abbreviations

A

ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
Arp2/3	Actin related protein 2/3
AMPK	AMP activated protein kinase
ATF4/6	Activating transcription factor 4
Abi	Abelson interacting protein

B

Bip	Binding immunoglobulin protein
BCL2	B-cell lymphoma 2

C

Cdc42	Cell division cycle 42
CD	CLIMP63+Drp1
CSCC	Cutaneous squamous cell carcinoma

D

Drp1	Dynamic related protein 1
DNA	Deoxyribonucleic acid

E

ER	Endoplasmic reticulum
EC1	Enzyme commission 1
Ena-VASP	Ena- Vasodilator stimulated phosphoprotein
ECM	Extracellular matrix
EC	Endothelial cell
eIF2α	Eukaryotic initiation factor 2
ERMCs	ER mitochondria contact sites
ERMD	ER mitochondrial division

F

FAK	Focal adhesion kinase
$FADH_2$	Flavin adenine dinucleotide
FAPs	Focal adhesion proteins
FH	Fumarate hydratase
FA	Focal adhesions

G

GADD34	Growth arrest and DNA damage protein 34
GMF	Glia maturation factor

H

HIF1α	Hypoxia inducible factor 1
HCC	Hepatocellular carcinoma

I

IMM	Inner mitochondrial membrane
IRE1α	Inositol-required transmembrane kinase/endonuclease
IP3R	Inositol triphosphate receptor

J

JAMs	Junctional adhesion molecules

L

LE	Leading edge

M

MAPK	Mitogen activated protein kinase
MMPs	Matrix metalloproteinases
MLCK	Myosin light chain kinase
Mfn1/2	Mitofusion 1 and 2
MCU	Mitochondria calcium uniporter
MPC1/2	Mitochondria pyruvate carrier 1 and 2
MOMP	Mitochondrial outer membrane permeabilisation
MPT	Mitochondrial permeability transition
mtDNA	Mitochondrial DNA

N

N-WASP	Neuronal Wiskott-Aldrich Syndrome
NPF	Nucleation promoting factors
Nap125	Nck associated protein 1
NADH	Nicotinamide adnine dinucleotide
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NFE2	Nuclear factor erythroid-2

O

OPA	Optic Atrophy 1
OMM	Outer mitochondrial membrane

P

PTB	Phosphotyrosine-binding
Ptdlns(4)P_2	Phosphatidylinositol 4,5-bisphosphate
PACS2	Phorphofurin acidic cluster sorting proteins 2
PKM	Pyruvate kinase
PC3	Human prostate cancer cell
PERK	Protein kinase-like ER kinase

R

RhoA	Ras homolog family member A
Rac1	Ras related C3 botulinum toxin substrate 1
ROS	Reactive oxygen species
RO	RTN4A+OPA

S

SMC	Smooth muscle cells
Sra-1	Steroid receptor RNA activator 1
SH3	Src homology 3
shRNAs	short hairpin RNAs
SDHB	Succinate dehydrogenase complex iron sulfur subunit B

T

TFAM	Mitochondrial transcription factor A
TE	Trailing edge

U

UPR	Unfolded protein response

V

VEGF	Vascular endothelial growth factor

W

WAVE	WASP-family verprolin-homologous protein
WH2	WASP homology 2

X

XBP1	X-box binding protein 1

#

2DG	2-deoxy-D-glucose

Chapter 1: Introduction

1.1 Cell migration

Cell migration is an essential function needed to maintain proper organization of multicellular organisms and intracellular processes that require migration for individualized function, such as wound healing, metastasis and immune cell migration in response to infection (Trepat *et al* 2012). There are two types of migration: single cell migration and collective cell migration (Trepat *et al* 2012). For cells to migrate efficiently they rely on the communication and cooperation of essential migration aspects such as lamellipodia/pseudopodia formation, polarization, focal adhesions, calcium and rear retraction (Trepat *et al* 2012). Several proteins are involved in the communication between these components that have been identified as the driving force for cell migration. Additionally, these proteins rely on interactions at the cellular level highlighting the involvement of organelles such as the mitochondria and ER.

1.1.1 Collective cell migration

Collective cell migration occurs when a collection of at least 2 cells retain their cell-cell junctions and migrate together through a 2D extracellular matrix or through a 3D interstitial tissue scaffold (Ilina and Friedl, 2009). It is widely seen during development, wound healing and tissue regeneration (Trepat *et al* 2012). It has also been linked to metastasis in epithelial cancers (Trepat *et al* 2012). In collective cell migration, cells are able to interact with each other chemically and mechanically within their group providing them with additional migratory mechanisms such as maintaining tissue cohesiveness and organization, sending mechanical signals through cell-cell junctions, and protecting metastatic clusters from immune response (Trepat *et al* 2012).

Collective cell migration relies on similar components and follows the same principles of single cell migration however they rely on cell-cell and cell-matrix adhesions and their cross communication as seen in Figure 1.1 (Trepat *et al* 2012). In collective cell migration, the cell-cell junctions remain coupled at the leading edge, lateral ends and inside the collective cell group while following the same steps seen in single cell migration (Ilina and Friedl, 2009). The four components within cell-cell adhesions needed for migration are adherens junctions, tight junctions, demosomes and gap junctions (Cavey and Lecuit, 2009). Adheren junctions are responsible for the creation and maintenance of cell-cell adhesions, stabilization of actin cytoskeleton, and transcriptional regulation (Cavey and Lecuit, 2009). Adheren junctions are formed by the interaction between transmembrane glycoproteins or the cadherin family (Trepat *et al* 2012). The transpairing of the EC1 domain in cadherins of adjacent cells allows for the proper conformational change in adheren junctions to mediate cell-cell adhesion (Trepat *et al* 2012). Tight junctions are transmembrane proteins found at the tip of adheren junctions where they play a role as an intramembranous fence which separates protein content of the apical cell membrane to the basolateral cell membrane, or as the hydrophobic barriers involved in ion, protein and fluid transport between the epithelial and endothelial layers (Trepat *et al* 2012). There are three types of tight junction transmembrane proteins: occludins, claudins and the IgG-like family of junctional adhesion molecules (JAMs) (Trepat *et al* 2012). The distribution of these proteins alters the size, strength and transport specificity of the junctions (Trepat *et al* 2012). Demosomes are junctions which connect the intermediate filaments from adjacent cells (Garrod and Chidgey, 2008). They are most commonly found in tissues that experience high volumes of mechanical force like the muscle (Trepat *et al* 2012). As for gap junctions they are

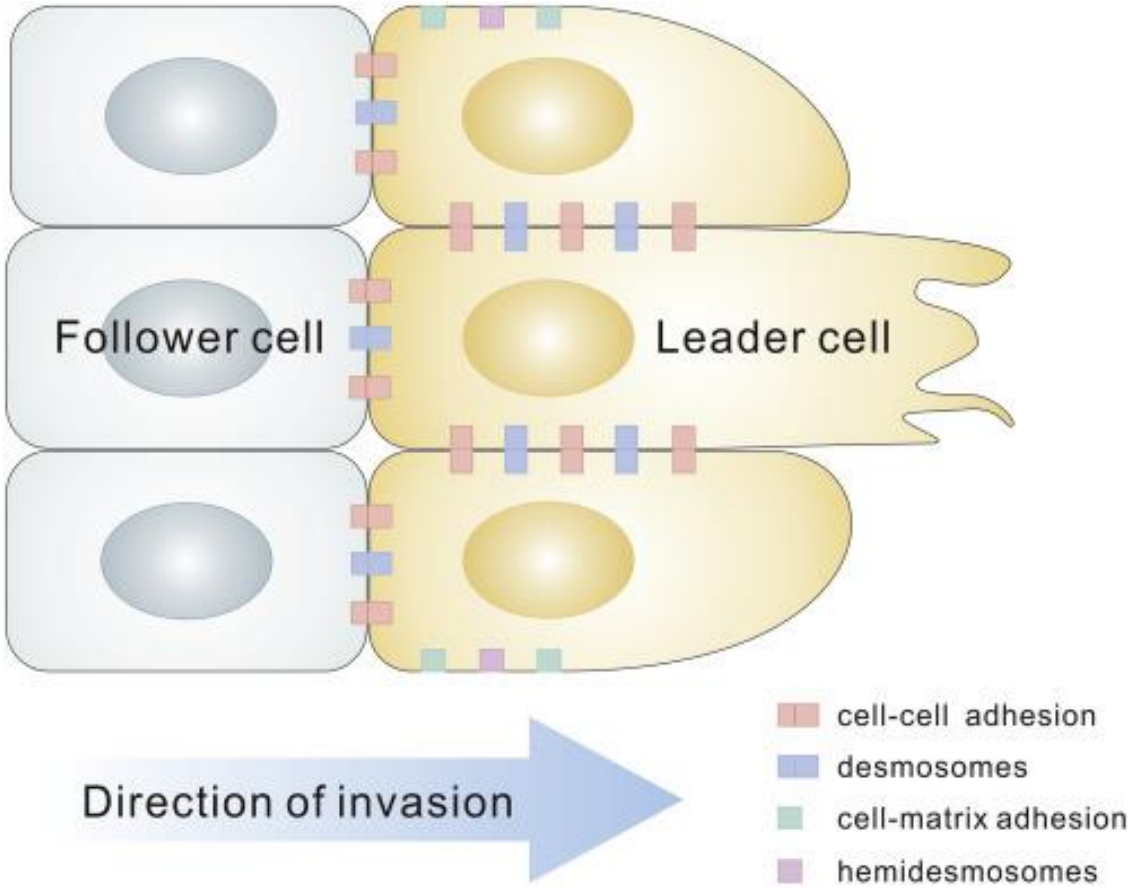

Follower cell
Leader cell
Direction of invasion
cell-cell adhesion
desmosomes
cell-matrix adhesion
hemidesmosomes

Figure 1.1: Collective cell migration relies on follower and leader cells. Leader cells determine the direction of migration and release stimuli that follower cells respond to. Adapted from Chen *et al* 2019.

transmembrane channels which connect the cytoplasm of neighboring cells (Trepat *et al* 2012). Each neighboring cell contributes to the junction by providing half a channel called connexins (Trepat *et al* 2012). These connexins are composed of four transmembrane domains which are connected to two extracellular loops that regulate cell-cell recognition and docking (Trepat *et al* 2012). The connexins are oriented in a cylindrical pattern to create a hollow centre that can be used for transport of molecules smaller than 1kDa (Trepat *et al* 2012).

Cell-matrix interactions seen in collective cell migration are similar interactions seen in single cell migration that will be discussed later. These interactions rely on the presence of actin-rich protrusions, the development of force and focalized proteolysis (Ilina and Friedl, 2009). The actin rich protrusions, commonly known as lamellipodia or filopodia, extend towards an area that has high concentrations of chemoattractants, growth factors and other extracellular ligands (Ilina and Friedl, 2009). Once the protrusions in the leader cells sense the environment, they start cell attachment to adjacent structures and mature adheren junctions that contain E-cadherin (Ilina and Friedl, 2009). This is necessary for the cell-cell contact involved in collective migration in order to later allow protrusions to be mediated and cell-cell junctions to be remodelled (Ilina and Friedl, 2009). A key factor in cell migration are focal adhesions. These are the structures that rely on integrin to connect the extracellular matrix to the actin cytoskeleton of the cell (Ilina and Friedl, 2009). Specifically in collective cell migration, β1 integrins cluster at cell-matrix interaction sites and create a traction force at the leading edge of the cell (Ilina and Friedl, 2009). This force is necessary for the cells to increase their migration speed (Ilina and Friedl, 2009). A major difference in collective migration verses single is that collective cell migration is more space-consuming. In order for there to be enough space for the entire cell sheet to migrate the local matrix must be degraded and the paths must be widened (Ilina and Friedl, 2009). This

process is done using matrix metalloproteinases (MMPs) 1 and 2 which are found to localize in the leading edge of the cells (Ilina and Friedl, 2009).

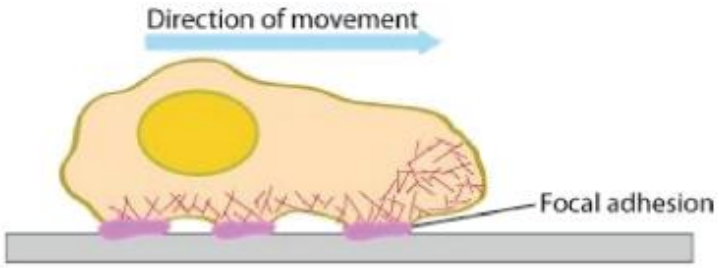
Polarization plays a key role within cell migration. The role of polarization in single cell migration will be discussed later in this section. As for the role of polarization in collective cell migration, it has been deemed as an advantage for cells using this method of migration (Trepat *et al* 2012). Each cell within the group has a specific task/role which is determined by the different patterns of expression according to its position in the group (Trepat *et al* 2012). The simplest example of this is the front-rear polarization where the group of front cells guides the larger set of cells known as the followers (Trepat *et al* 2012). These leader cells tend to have a mesenchymal-like phenotype, a relatively loose cell-cell adhesion, amplified expression of cell-matrix adhesion proteins and actin filaments and microtubule polarized remodelling (Omelchenko *et al* 2003). In angiogenesis, follower cells tend to be nonproliferative and have high levels of VEGF receptor family expression (Hellstrom *et al* 2007). Leader cells are established by the Notch signalling pathway which relies of VEGFR2 activation to upregulate and release Nothc ligand D114 to the leader cells, differentiating them from follower cells (Hellstrom *et al* 2007). Front-rear polarization migration is as follows: the leader cells reach their filopodia which search, lead and create tractions (Trepat *et al* 2012).

In the context of wound healing, collective cell migration heavily relies on group communication. Cells encompassed around the wound, migrate together over a matrix rich in fibrin and fibronectin (Martin and Parkhurst 2004). Epithelial cells require the two modes of collective migration during re-epithelization during wound migration (Martin and Parkhurst 2004). These modes require extensive cooperation between neighbouring cells to release signals (Martin and Parkhurst 2004). These signals allow the first mode to create an assembly of a

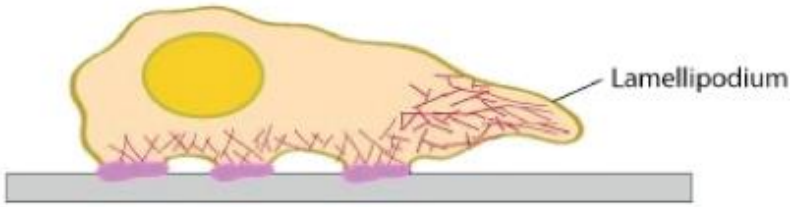
supracellular actin cable to create a contraction force that causes the actin cable to efficiently close the wound (Martin and Parkhurst 2004). The second mode that also requires the cooperation and signalling of neighboring cells is to allow extension of lamellipodia and pseudopodia into the wounded area (Redd *et al* 2004). Collective migration is also seen to be greatly involved in angiogenesis as a mediator during wound healing (Trepat *et al* 2012). When growth factors are signalled, endothelial cells respond by upregulating integrin at the tips of newly forming capillaries so that they can collectively migrate through the tissue (Trepat *et al* 2012).

1.1.2 Single cell migration

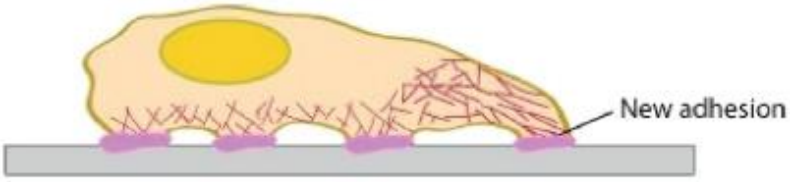
Single cell migration is predominantly seen in fibroblasts, fish or amphibian keratocytes and amoeboid locomotion as seen in leukocytes (Trepat *et al* 2012). Mammalian fibroblasts follow a single cell migration pathway which follows a locomotion cycle seen in Figure 1.2 (Alberts *et al* 2007). In a locomotion cycle, a defined leading and trailing edge are formed (Alberts *et al* 2007). The leading edge is defined as the protrusion created in the direction of migration and the trailing edge is defined as the protrusion which retracts during migration (Alberts *et al* 2007). As a cell migrates, actin polymerization pushes the plasma membrane forward creating protrusive structures such as filopodia and lamellipodia which become our leading edges (Alberts *et al* 2007). The leading edge will then unbind its focal adhesion from the substrate on the surface and extend forward to bind to a new substrate (Alberts *et al* 2007). This creates a contractile force within the cell that is relieved by the trailing edge retracting (Alberts *et al* 2007). The trailing edge focal adhesions will unbind from its substrate and bind with a new substrate as well, relieving this force and this process will continue until the cell reaches its destination or



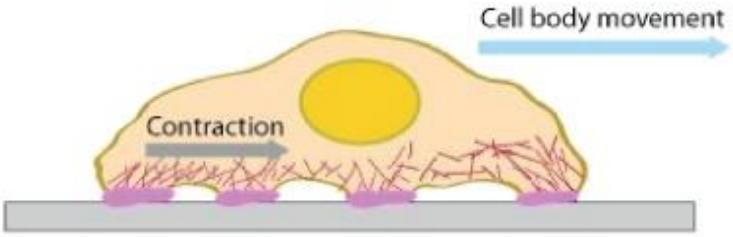
1. Extension



2. Adhesion



3. Translocation



4. De-adhesion

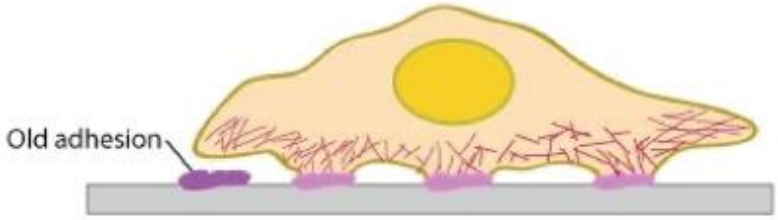


Figure 1.2: Single cell migration has a defined trailing and leading edge. Cells travelling in single cell migration, have a defined leading edge protrude from the cell body (1) to bind to a new adhesion (2). This creates a contractile force (3) which is relieved when the trailing edge unbinds with the focal adhesion and retract (4). Adapted from Ladoux and Nicolas 2012.

undergoes other cellular processes like division or death (Alberts *et al* 2007). Throughout single cell migration several components communicate with one another to facilitate migration, such as focal adhesions assembly and disassembly, actin polymerization, polarization and protrusion formation, extension and retraction which are discussed further in later sections. It has been observed that cells using single cell migration have altered migration abilities when in a 2D or 3D environment (Trepat *et al* 2012). Fibroblasts in 2D cell culture tend to have large lamellipodia and filopodia while in 3D tend to be smaller and fewer in number (Trepat *et al* 2012). Additionally, in 2D environments fibroblasts have extensive adhesion points to the substrate making the lamellipodia very broad, flat and thin (Trepat *et al* 2012). While in a 3D matrix fibroblasts have a mesenchymal motile morphology (Trepat *et al* 2012).

1.1.2.1 Lamellipodia and filopodia

During cell migration there are several protrusions formed such as lamellipodia and filopodia. Lamellipodia are thin, sheet-like membrane based protrusions (Tang and Gerlach, 2017). Lamellipodia formation is regulated by the assembly and disassembly of local actin filaments (Tang and Gerlach, 2017). Actin filament assembly in the lamellipodia are found in two patterns which are branching and elongation (Tang and Gerlach, 2017). This promotes the actin mesh formation seen in cell protrusions (Tang and Gerlach, 2017). Actin depolymerization and debranching happens during migration to allow dynamic remodelling of the actin network and the constant extension and retraction of lamellipodia (Tang and Gerlach, 2017). Arp2/3 complex, an actin nucleating complex, mediates actin filament branching, by binding to the mother filament causing the growth of a daughter filament at a 70 degree angle (Tang and Gerlach, 2017). Arp2/3 activity is regulated by neuronal Wiskott-Aldrich Syndrome (N-WASP) and WASP-family verprolin-homologous protein (WAVE) (Tang and Gerlach, 2017). Once growth

factor receptors are bound with their ligand and cell adhesions occurs, Rho GTPases, Cdc42 and Rac1 bind to the GTPase domains of N-WASP/WAVE, activating them and causing actin filament branching by the Arp2/3 complex (Tang and Gerlach, 2017). Additionally, active Rac can interact with lamellipodin, a scaffold protein which adds to actin filament extension, to bind to the WAVE complex, thereby bringing Rac and the WAVE complex closer together (Ridley 2015). On the other hand, filopodia are long spikes that are also based on actin however filopodia don't have the Arp2/3 complex and nearly none of the capping proteins that lamellipodia have (Davies 2013). Filopodia rely on fascin, which crosslinks filaments into bundles. They begin their growth through the development of 'Λ' shape which is enriched with filopodia proteins such as Ena-VASP and fascin (Davies 2013). Ena-VASP regulates the actin network geometry found in filopodia as the protrusion forms (Bear *et al* 2002). Ena-VASP interacts with the barbed end of the actin filaments, allowing the filopodia to be protected from lamellipodia capping proteins and continuing its filamentous elongation (Bear *et al* 2002). Fascin on the other hand provides the stiffness of the filopodial bundles thereby allowing the filopodia to extend past the leading edge without fear of being bent or damaged (Vignjevic *et al* 2006). Actin filaments then elongate towards the tip while simultaneously clustering with each other (Davies 2013). This clustering and cross-linking strengthens the assembly of the filopodia and makes them more resistant to bending so that is can continue to support an extension of the plasma membrane (Davies 2013). Filopodia or lamellipodia formation is based on which capping protein is available in high concentrations; high concentration of Ena-VASP supports the formation of filopodia (Davies 2013).

1.1.2.2 Focal adhesion

Focal adhesions are points of interaction between the cell and the substrate on the surface during migration (Burridge *et al* 1997). They are traction points and signalling centres used throughout cell migration (Trepat *et al* 2012). When serving as traction points, they release forces to the substrate that initiates actin polymerization in order to create leading edge protrusions (Burridge *et al* 1997). The release of traction points can be inefficient in some cell lines, therefore establishing an optimum strength of attachment to allow the proper release has been observed (Burridge *et al* 1997). As signalling centres, they regulate actin polymerization and the activity of myosin II by RhoA (Burridge *et al* 1997). Focal adhesions components have been broken down in to four categories: 1) ECM components such as, fibronectin, laminin, vitronectin and collagen, 2) transmembrane proteins such as integrins, 3) structural proteins which act as focal adhesion stabilizers, and 4) signalling proteins (Trepat *et al* 2012). Each of these components are heavily involved in the assembly and disassembly of focal adhesions. They are commonly known to disassemble and reassemble throughout the cell during migration, this is not limited to the trailing edge. The most heavily studied protein in adhesion interaction is integrin (Burridge *et al* 1997). Integrin is a transmembrane receptor that links actin via a set of molecules that are involved in adhesion formation such as, talin, vinculin and α-actinin (Figure 1.3) (Burridge *et al* 1997). The binding of talin to integrin β subunit, activates integrin allowing the cytoplasmic domain of integrin β subunits to the actin filaments (Critchley and Gingras 2008). The interaction between talin and integrin increases the affinity of integrin for extracellular matrix proteins and promotes focal adhesions assembly (Critchley and Gingras 2008). Vinculin is activated once it undergoes a conformational change (Humphries *et al* 2007). Once vinculin is activated and in an open conformation it can bind with talin, causing integrin to cluster and

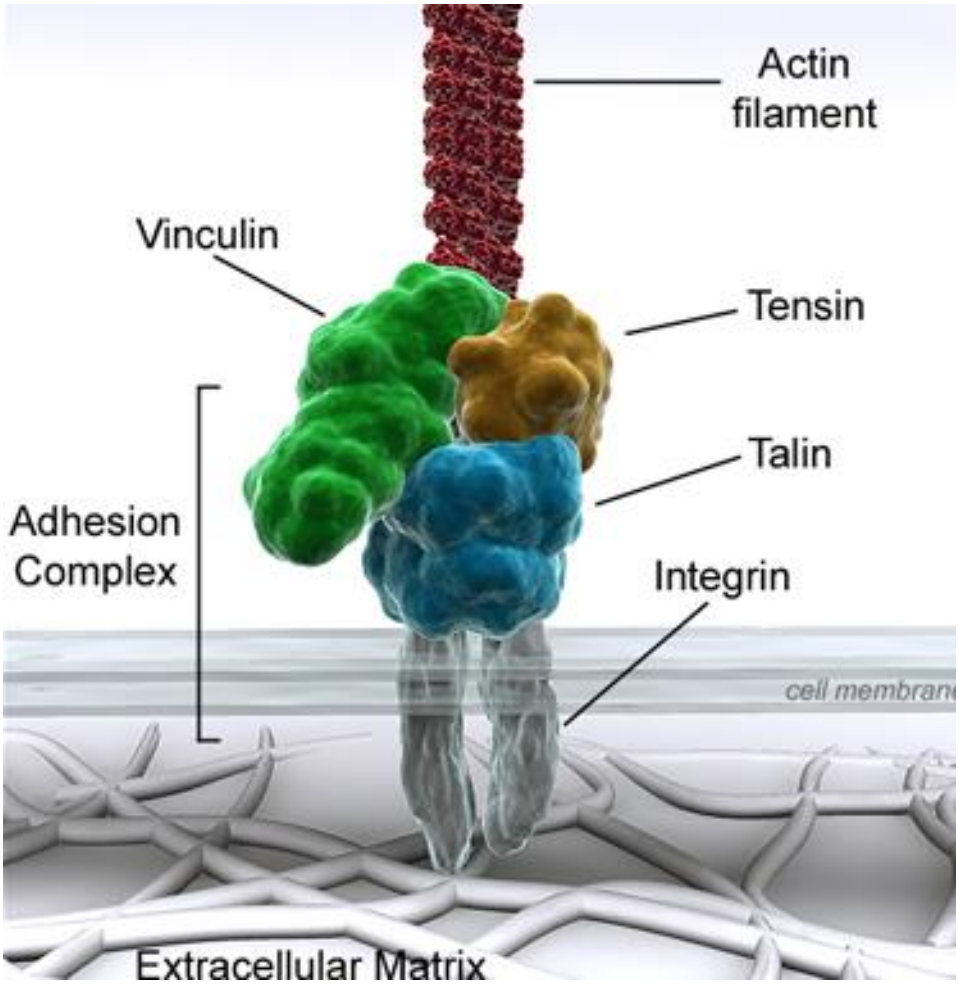

Actin
filament
Vinculin
Tensin
Talin
Adhesion
Complex
Integrin
cell membran
Extracellular Matrix

Figure 1.3: Focal adhesions are composed of integrin binding to talin and vinculin. The integrin is responsible for binding the complex to the extracellular matrix, while talin and vinculin works as linking factors for the complex to connect to actin filaments. Adapted from Dash.

enlarges focal adhesions (Humphries *et al* 2007). Additionally, the interaction between vinculin and actin acts as the linking point of the focal adhesion core to the actin cytoskeleton (Humphries *et al* 2007). Newly formed adhesions appear in temporal waves as small aggregates (Burridge *et al* 1997). As the cell migrates, they begin to grow in size and intensity (Burridge *et al* 1997).

Integrin has been heavily implicated in focal adhesion assembly. It relies on the signalling of growth factors to mediate this reaction (Carragher *et al* 1999). First integrin is activated by growth factors which bind the integrin β-subunit cytoplasmic domain with the talin phosphotyrosine-binding (PTB) domain (Carragher *et al* 1999). Once activated, integrin binds with the ECM and recruits signaling proteins by the cytoplasmic domain (Carragher *et al* 1999). To complete assembly, RhoA must be activated so that the MLC is phosphorylated to create a tension on actin (Carragher *et al* 1999). This tension needed for cell speed, leads to the clustering of integrin found bound to the ECM (Carragher *et al* 1999). Additionally, the activation of RhoA leads to a conformational change of Ptdlns(4,5)P_2 in vinculin which allows the binding of talin and actin to strengthen the connection between integrins and actin (Carragher *et al* 1999). This integrin clustering also leads to focal adhesion kinase (FAK) phosphorylation, mitogen-activated protein kinase (MAPK) activation, paxillin binding and the creation of a complex which consists of vinculin, FAK, α-actinin, WASP, tensin, Src and zyxin (Carragher *et al* 1999). Several studies have highlighted that the absence of these signalling components, led to altered focal adhesion structure and number which consequently changed cell migration ability (Carragher *et al* 1999). An example being the knockdown of FAK, led to an increase in the number of focal adhesions and reduced cell migration (Carragher *et al* 1999).

The disassembly of these adhesions in the leading edge in response to protrusion or feed components is termed as adhesion turnover (Vicente-Manzanares *et al* 2005). During protrusion

extension, new adhesions that disassemble can either go through adhesion turnover or mature into larger adhesions (Vicente-Manzanares *et al* 2005). Adhesion disassembly occurs through 3 pathways: 1) adhesion release by ECM degradation, 2) adhesion turnover triggered by microtubules and 3) degradation by kinases and proteases (Lauffenburger *et al* 1996). It has been reported that extracellular matrix (ECM) degradation by matrix metalloproteinases (MMPS) trigger adhesion release thereby increasing cell migration (Kaverina *et al* 1999). This is seen in a study where paxillin and vinculin are almost totally absent when smooth muscle cells (SMC) were treated with degraded collagen (Kaverina *et al* 1999). In terms of microtubule involvement in disassembly, the exact mechanism is still unknown but it is believed that the microtubules serve as a track to deliver focal adhesion disassembly proteins (Yue *et al* 2014). It was seen that cells that were treated with nocodazole, a microtubule inhibitor, led to an increase in assembly in focal adhesions (Kaverina *et al* 1999). The opposite is also seen, when the microtubules in these same cells were recovering there was an increase in the disassembly of focal adhesions (Kaverina *et al* 1999). They had examined 61 focal adhesion sites and found that 29 disappeared when the microtubules were recovering from nocodazole treatment (Kaverina *et al* 1999). There were still 32 focal adhesions that remained during the recovery time but they had become smaller in size and the fluorescence intensity had decreased (Kaverina *et al* 1999). One of the proteases involved in focal adhesion disassembly is calpain. Calpain cleaves adhesion components within the trailing edge, by acting on talin which is the linker of actin and integrin (Carragher *et al* 1999). Additionally, we see degraded collagen promotes the cleavage of focal adhesion kinases by calpain (Carragher *et al* 1999).

1.1.2.3 Actin filaments

The formation of filamentous actin by the polymerization of globular actin is essential in cell migration (Devreotes *et al* 2015). This creates oriented filaments that push the leading edge forward, driving cell migration (Devreotes *et al* 2015). Polymerization creates polar filaments, forming a fast polymerized barbed positive end and a slow polymerized pointed negative end (Devreotes *et al* 2015). These actin filments are then regulated by formins and Arp2/3 complex (Devreotes *et al* 2015). Formins are responsible for nucleating and regulating growth of linear actin filaments by stabilizing the actin dimer using their formin homology 2 domain (Devreotes *et al* 2015, Schaks *et al* 2019). In stabilizing the dimer, the formins then recruit actin or profilin-actin complexes (Schaks *et al* 2019). Formins are regulated by RhoA and Cdc42 and require G-actin bound profilin interaction to completely establish actin polymerization (Devreotes *et al* 2015).On the other hand, Arp2/3 nucleate branches of pre-existing actin filaments to create the dendritic actin network that is identified in the leading edge of the cell (Devreotes *et al* 2015). Arp2/3 must be activated by nucleation promoting factors (NPF) of class I to begin this process (Schaks *et al* 2019). Two NPFs must bind their actin monomer binding WH2 region to Arp2/3s complex binding connector and acidic domains (Schaks *et al* 2019). Additionally, Arp2/3's ability to nucleate is dependent on Cdc42 and Rac1 acting on WASP/WAVE containing protein complexes (Devreotes *et al* 2015). Rac1 triggers the disassociation of Abi, Nap125 and Sra-1 from WAVE in order to activate WAVE (Devreotes *et al* 2015). For actin filament elongation, profilin-actin complexes must be present to bind to the proline-rich FH1 domain in formins (Schaks *et al* 2019). When profilin is present, this binding occurs and actin monomers are quickly added to the FH2-capped barbed end and continues this process of elongation (Breitsprecher and Goode, 2013). As for disassembly, this is mediate by the hydrolysis of bound ATP once the actin monomers incorporated in the filaments have aged (Schaks *et al* 2019).

Disassembly occurs towards the pointed end and relies on Arp2/3 complex networks in the filaments to be debranched by proteins in the coronin and GMF family (Schaks *et al* 2019). Binding of these proteins to Arp2/3 creates an open conformation, thereby making it inactive and breaking the branches in the actin network (Sokolova *et al* 2017).

1.1.2.4 Polarization

Another aspect necessary for cell migration is the establishment of spatial asymmetry to allow cells to turn to intracellularly generated forces into net cell body turns (Lauffenburger *et al* 1996). Polarization in a uniform stimulus environment may come from perceived spatial or temporal stimulus gradients caused by nonuniformities or kinetic fluctuations in receptor-ligand binding (Lauffenburger *et al* 1996). Front back polarity seen in leading and trailing edges need actin polymerization to occur, highlighting the importance of proteins such as cortactin to facilitate actin polymerization activation (Devreotes *et al* 2015). Cortactin is an actin nucleating promoting factor found on cortical actin structures that has been used *in vitro* to mark polarity (MacGrath and Koleske 2012). Cortactin has a C-terminal SH3 domain allowing it to bind to several proteins that involve lamellipodial protrusion and directed cell migration (MacGrath and Koleske 2012). Lamellipodia are best known for containing polarized array of actin filaments that rely on Arp2/3 complex (Ammer and Weed 2008). Extensive work has shown that cortactin has an influence on Arp2/3 through the assembly and formation of lamella-associated adhesion structures (Ammer and Weed 2008). Suppression of cortactin reduced the rate of new adhesion formation in lamellipodia and reduced lamellipodial persistence (Ammer and Weed 2008). It is in the NTA domain of cortactin that the Arp3 subunit of Arp2/3 complex binds, stabilizing F actin (Ammer and Weed 2008). Additionally, the NTA domain initiates migration by increasing

lamellipodial persistence and adhesion assembly once Arp2/3 binding and activation occurs (Ammer and Weed 2008).

1.1.2.5 Cell migration and calcium regulation

Calcium is an essential chemical element for humans (Tsai *et al* 2015). At the organismic level it composes bone, at the tissue level it regulates membrane potentials and at the cellular level it triggers physiological processes such as cell migration (Tsai *et al* 2015). As previously mentioned, cells migrate through the release of substrates at adhesions at the leading edge, the push of the plasma membrane forward then the release of substrates at adhesions in the trailing edge (Trepat *et al 2012*). For myosin's involvement in this process, small local calcium signals are pulsed from the lamella and lamellipodia to activate myosin light chain kinase (MLCK) (Tsai *et al* 2015). Active MLCK will then phosphorylate myosin light chain which triggers myosin contractions (Tsai *et al* 2015). The small local calcium levels are only required in nanomolar scales as there is a high affinity between calcium-calmodulin complexes and MLCK (Tsai *et al* 2015). This indicates that the leading edge must be free of free calcium so that MLCK can remain inactivate and respond to calcium increases as stimuli to trigger the myosin contractions needed for migration (Tsai *et al* 2015). Moreover, it highlights that there is a gradient of calcium seen through the cell where the leading edge must be at a low concentration and the trailing edge must be at a high concentration (Tsai *et al* 2015). Additionally, blocking calcium influx at the leading edge lead to the disassembly of actin filaments and lamellipodia activity (Prudent *et al* 2016). This was rescued with the treatment of active Rac1, highlighting that calcium and Rho-GTPases may work together throughout cell migration (Prudent *et al* 2016). It is clear that calcium plays a role on several aspects related to cell migration such as myosin activation and Rho-GTPases. It also plays a part on actin filaments which have been previously mentioned as

necessary components within cell migration. As mentioned above, myosin contractions are triggered by calcium and myosin contractions stabilize focal adhesions in the leading edge of a cell (Kaverina *et al* 1999). The contractions provide the traction force needed on the complexes by actin bundles binding to them (Kaverina *et al* 1999). This force then causes the remodeling and stabilization of components in focal adhesions (Kaverina *et al* 1999). Additionally, calpain a protein needed in focal adhesion disassembly is a calcium dependent intracellular protease (Carragher *et al* 1999). Altogether, calcium is extremely necessary to allow cell migration machinery to operate.

1.1.5 Cell migration and mitochondrial ATP

Cell migration is an energy dependent process (Cunniff *et al* 2016). ATP consumption alone by protrusion creation can cause a massive deficit in local ATP that could stop cytoskeletal dynamics and cell migration, highlighting the necessity of mitochondria involvement in cell migration (Cunniff *et al* 2016). Mitochondria provide ATP through a process known as oxidative phosphorylation. This process relies on energy rich molecules, NADH and $FADH_2$, to transfer electrons to oxygen (Berg *et al* 2002). These electrons flow using protein complexes found in the inner mitochondrial membrane to pump protons out of the mitochondrial matrix (Berg *et al* 2002). This results in an uneven distribution of protons, generating a pH gradient and a transmembrane electrical potential creating a proton-motive force (Berg *et al* 2002). Finally, establishing a proton-motive force creates an assembly flow of protons back into the mitochondrial matrix to completely finish synthesizing ATP using the ATP synthase (Berg *et al* 2002). In this last step, as the proton travels through the ATP synthase, ADP found in the matrix will bind with an additional Pi to create ATP (Berg *et al* 2002). This process alone creates 26 out of the 30 molecules of ATP when glucose is completely oxidized to CO_2 and H_2O (Berg *et al*

2002). Many studies look at the inhibition of mitochondrial metabolism. One inhibitor commonly used is oligomycin. Oligomycin is an antibiotic which specifically affects the ATP synthase, as it stops the transport of protons back into the matrix (Bender 2003). As a result, malate and succinate are not oxidized and further electron transport is inhibited (Bender 2003).

As mitochondria are the primary producers of ATP extensive research has been done to understand the role mitochondria play in cell migration. Cunniff *et al* 2016 found that ATP levels were significantly higher in protrusions than the cell body. Additionally, they found ATP:ADP ratio was significantly lower in the pseudopodia than the cell body, suggesting that these protrusions use large amounts of energy for migration purposes (Cunniff *et al* 2016). This brings to light that AMP-activated protein kinase (AMPK) may be involved as well since it acts as a sensor for energy balance (Cunniff *et al* 2016). AMPK is known to be activated when there are elevated levels of ADP and AMP, which Cunniff *et al.* had found in the protrusions. When examining these same pseudopodia that had lower ATP:ADP ratio they found that AMPK activity was significantly enriched (Cunniff *et al* 2016). Moreover, when treating migrating cells with compound C, an AMPK inhibitor, they found mitochondria undergoing fission and immobilization of the leading edge and decreased leading edge dynamics (Cunniff *et al* 2016). Altogether, this study highlights the need of mitochondria and mitochondrial ATP in the leading edge of the cell.

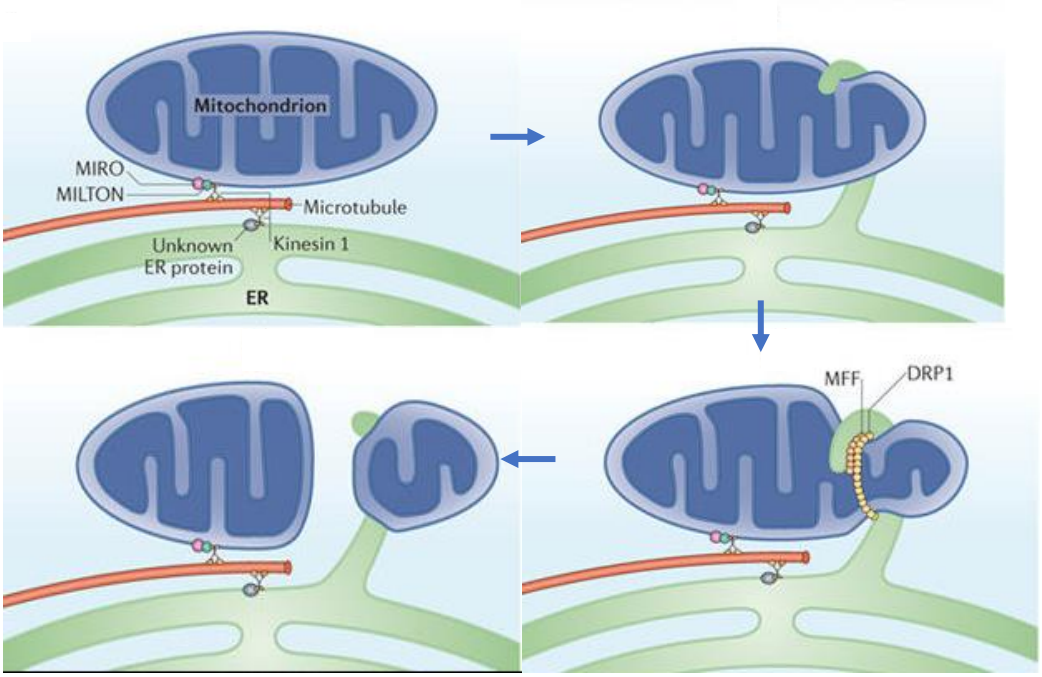
It is worth noting that cancer cells also heavily rely on ATP provided by the mitochondria for metastasis. In cancer cells, ATP interacts with P2 receptors which mediates biological functions in tumours like cellular invasion and proliferation (Liu *et al* 2018). In a study conducted by Liu *et al.* they looked into identifying the role ATP production has on S100A4, a Ca^{2+} binding protein. S100A4 is implicated in biological processes such as cell motility and invasion (Liu *et al*

2018). They had found that in various breast cancer cell lines, ATP treatment upregulated the expression of S100A4 (Liu *et al* 2018). When inhibiting S100A4 using niclosamide, a glucose uptake inhibitor, there was a decrease in cell motility (Liu *et al* 2018). Additionally, they had found that ATP treatment lead to more and longer lamellipodia and filopodia creation which were also reduced when S100A4 was inhibited (Liu *et al* 2018). Taken together, these studies highlight the necessity of mitochondrial involvement in producing ATP to generate protrusions.

1.2 Mitochondria

Mitochondria are dynamic organelles involved in a variety of cellular processes (Phillips and Voeltz 2015). It was previously believed that various organelles were near the mitochondria, however as live cell microscopy has advanced more research has come out to show that mitochondria interact/localize with various other organelles (Phillips and Voeltz 2015). One of the key interactions is between mitochondria and the endoplasmic reticulum (ER) (Phillips and Voeltz 2015). The interaction between the mitochondria and ER facilitates mitochondrial fission and fusion (Nicholls and Ferguson 2013). When mitochondria and the ER tubules interact in a fission reaction, the tubules begin the process by squeezing the mitochondria (Figure 1.4A) (Nicholls and Ferguson 2013). Dynamin related protein 1 (Drp1) is then recruited to the contact site on the outer mitochondrial membrane (OMM) (Nicholls and Ferguson 2013). Drp1 cycles to the OMM to create a scission site by forming large homomultimeric complexes (Nicholls and Ferguson 2013). These complexes will circle around the mitochondria in spirals and constrict to complete the division of the mitochondria into its individual daughter mitochondria (Nicholls and Ferguson 2013). On the other spectrum mitochondrial fusion occurs when neighbouring mitochondria bind to one another (Figure 1.4B). Mitochondrial fusion requires the OMM of the two mitochondria to bind which is facilitated by the recruitment of mitofusion 1 and 2 (Mfn1 and

A


Mitochondrion
MIRO
MILTON
Microtubule
Unknown
ER protein
Kinesin 1
ER
MFF
DRP1

B

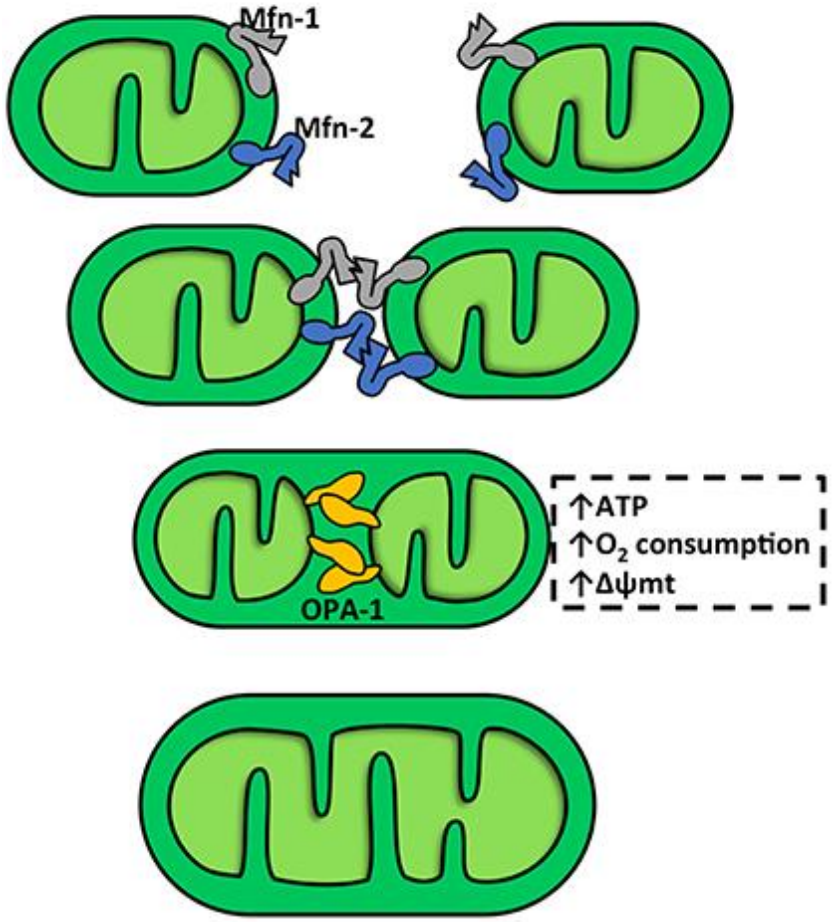

Mfn-1
Mfn-2
OPA-1
↑ATP
↑O_2 consumption
↑Δψmt

Figure 1.4: ER and mitochondria make contact for fission and fusion. A) When the ER and mitochondria make contact Drp1 is recruited which begins the scission process that wraps around the mitochondria until it divides. B) Fusion starts with the OMM of two mitochondria binding through Mfn1 and Mfn2. Once the OMM binds the IMM binding through OPA1 binding finishes the fusion process. Adapted from Rowland *et al* 2012 and Kerkhofs *et al* 2018.

2) (Nicholls and Ferguson 2013). Mfn1 and Mfn2 are found on the OMM where their N- and C-terminals allow for the Mfn proteins on the neighbouring mitochondria to bind to (Nicholls and Ferguson 2013). Once the OMM are fused, the inner mitochondrial membrane (IMM) must fuse to complete the fusion cycle (Nicholls and Ferguson 2013). IMM fusion is facilitated by optic atrophy 1 (OPA1), where OPA1 is found on either end of the IMM and bind to one another (Nicholls and Ferguson 2013). Mitochondrial structure and function correlate with subcellular distribution, meaning mitochondria shape is a factor to its function and distribution throughout the cell (Nicholls and Ferguson 2013). It has been highlighted in several studies that mitochondrial fission and fusion have a role in cell migration.

1.2.1 Cell migration and mitochondrial fission

Studies have shown that mitochondrial fission proteins are necessary for cell migration. A study done by Zhao *et al.* looked at drp1 silencing in the context of breast cancer cell migration. They found when silencing drp1 it reduced CM-induced migration and invasion (Zhao *et al* 2013). Similar results were seen when a drp1 inhibitor was used (Zhao *et al* 2013). Also, less lamellipodia formation was detected in cells that had drp1 silenced (Zhao *et al* 2013).

This was replicated in hepatocellular carcinoma (HCC) cells and similar results were found. When drp1 was knock down in these tumor cells there was a decrease in intrahepatic and lung metastasis (Sun *et al* 2017). On the other hand, when drp1 was overexpressed there was an increase in intrahepatic and lung metastasis (Sun *et al* 2017). Additionally, they identified that mitochondria fission mediated cell migration independent of glycolysis (Sun *et al* 2017). HCC cells rely on glycolytic ATP which they wondered might affect mitochondrial fission through drp1. However, when HCC cells were treated with a glycolysis inhibitor, 2DG, they found that the cells that were overexpressing drp1 were motile (Sun *et al* 2017). Sun *et al.* also found

similar results that drp1 knockdown had decreased lamellipodia formation but enhanced focal adhesions (Sun *et al* 2017). As identified through multiple cell lines in various labs, mitochondrial fission protein drp1 is critically involved in cell migration.

1.2.2 Cell migration and mitochondrial fusion

Mitochondria fusion is also thought to regulate cell migration. When Mfn1 or Mfn2 was overexpressed in MDA-MB-231 and MDA-MB-436 cells migration and invasion was reduced (Leal *et al* 2016). Therefore, when Mfn1 and Mfn2 were silenced there was a 30% increase in cell migration and invasion ability (Leal *et al* 2016). They found that cells that had Mfn1 and Mfn2 silenced were co-transfected with siRNA-insensitive Mfn2 the increase in cell migration was abolished and fragmented mitochondria phenotype was restored (Leal *et al* 2016). As for protrusion formation, Mfn1 overexpression led to less cell spreading and decreased lamellipodia formation (Leal *et al* 2016). Additionally, Mfn1 and Mfn2 silencing lead to an increase in mitochondria accumulation in lamellipodia by 35% (Leal *et al* 2016). Taken together, mitochondrial fusion has the opposite role of fission in which it alters cell migration by decreasing it.

1.2.3 Cell migration and mitochondria calcium

Recent evidence has highlighted that mitochondria Ca^{2+} is essential in cell migration (Prudent *et al* 2016). Cell migration is regulated by external and internal factors such as actin cytoskeleton remodelling and focal adhesion proteins, and mitochondria control intracellular Ca^{2+} levels (Prudent *et al* 2016). Prudent *et al*. identified a relationship between cell migration and mitochondria calcium by examining the mitochondria calcium uniporter (MCU), a pore that allows the transfer of Ca^{2+} through the inner mitochondrial membrane (Mishra *et al* 2017). When

the MCU is silenced there was a decrease in the mitochondria's ability to uptake calcium which led to human breast cancer cells having slower migration speeds in a wound (Prudent *et al* 2016). Similar migration impairment was seen in MCU silenced cells in a serum gradient chamber assay (Prudent *et al* 2016). Additionally, typical polarization at the leading and trailing edge was absent in cells lacking MCU (Prudent *et al* 2016). As mentioned earlier, cell migration relies on protrusion formation which was also altered in the absence of the MCU. Lamellipodia formation and retraction relies on Rac1 and RhoA, which was significantly decreased in short hairpin RNAs (shRNAs) MCU cells (Prudent *et al* 2016). MCU cells had also an increase in phosphorylated myosin light chain residues Thr18 and Ser19 leading to observed cytoskeleton stiffness (Prudent *et al* 2016). The stiffness seen in those cells were found to relate to higher f-actin fiber and actin bundle density (Prudent *et al* 2016). As actin bundles interact with focal adhesion proteins (FAPs) to provide mechanisms for cell migration, there was reason to believe that silenced MCU cells had impairments in FAPs (Prudent *et al* 2016). Examining paxillin, a scaffold protein used to recruit signaling proteins, staining in MCU silenced cells, Prudent *et al.* found that the staining remained consistent throughout their time lapse implying that FAPs dynamics and structure were impaired. Similar results were reported by Tosatto et al. Additionally, the work done by Tosatto *et al*. found that MCU expression lead to an increase in tumor progression. When mice were injected with $MCU^{-/-}$ tumor growth was slower and lymph node infiltration and lung metastasis was dramatically abolished (Tosatto *et al* 2016).These studies suggest that not only does mitochondria provide energy for cell migration but there are underlying aspects of mitochondrial functionality, such as fission/fusion and calcium uptake that play a role in cell migration within cancer cell lines.

1.3 Microtubules and mitochondria

Among the list of organelles mitochondria make contact with are microtubules (Figure 1.5). Microtubules are made up of alpha and beta-tubulin subunits arranged in a head to tail fashion to create a positive and negative end polarized orientation (Melkov and Abdu, 2018). This polarized orientation is crucial for the directed migration of the cell and the creation of protrusions (Melkov and Abdu, 2018). It was found that in migratory cells, microtubules create centrosomal arrays (Melkov and Abdu, 2018). This causes radial microtubule organization where free positive ends face the cellular membrane (Melkov and Abdu, 2018). When a fibroblast cell migrates, the centrosome localizes towards the migration axis in front of the nucleus to establish a high microtubule density network facing the leading edge (Melkov and Abdu, 2018). To have mitochondria migrate along the microtubules they rely on kinesin (Melkov and Abdu, 2018). Different kinesins are used when transporting mitochondria from the negative end or the positive end. Positive end transport relies on kinesin-1 (Melkov and Abdu, 2018). Kinesin-1 binds and moves along stable microtubules that have been polyglutamylated, acetylated or de-tyrosinated (Melkov and Abdu, 2018). Specifically, for mitochondria the microtubules must be acetylated and then kinesin-1 will bind with both organelles and transport the mitochondria (Barlan and Gelfand, 2010). This binding relies on adaptor proteins, Miro and Milton (Barlan and Gelfand, 2010). Miro, is a mitochondria receptor that relies on intracellular Ca^{2+} levels to stimulate a response, while Milton is an adaptor protein that recruits kinesin to mitochondria based on glucose levels (Barlan and Gelfand, 2017). At normal homeostatic levels Ca^{2+} levels, Miro binds with the tail of kinesin through Milton (Barlan and Gelfand, 2017). At elevated levels of Ca^{2+}, Miro undergoes a conformational change as calcium binds to the two EF-hand domains allowing Miro to bind to kinesin independently of Milton (Barlan and Gelfand, 2017). This stops kinesin from binding to the microtubules which also stops mitochondrial migration (Barlan and Gelfand,

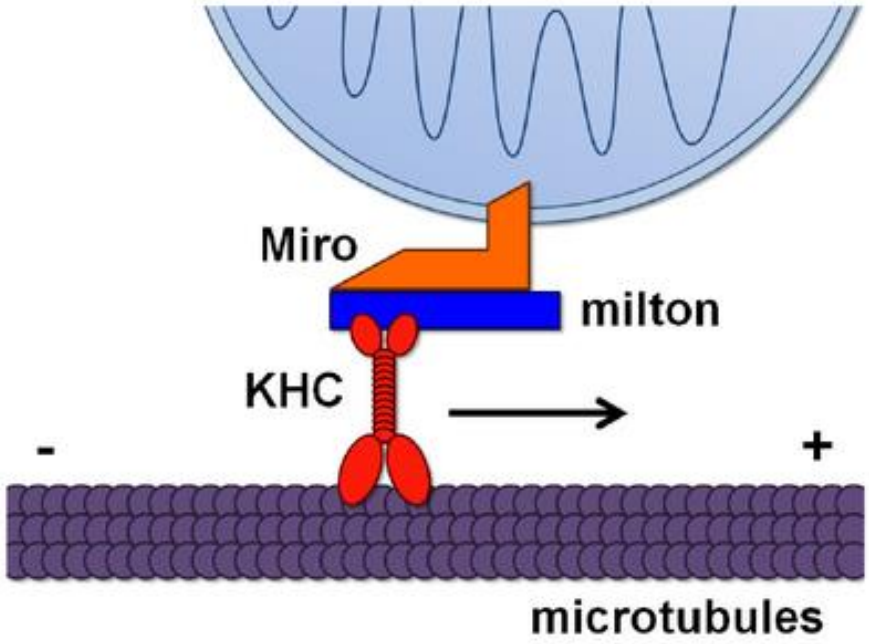

Miro
milton
KHC
-
+
microtubules

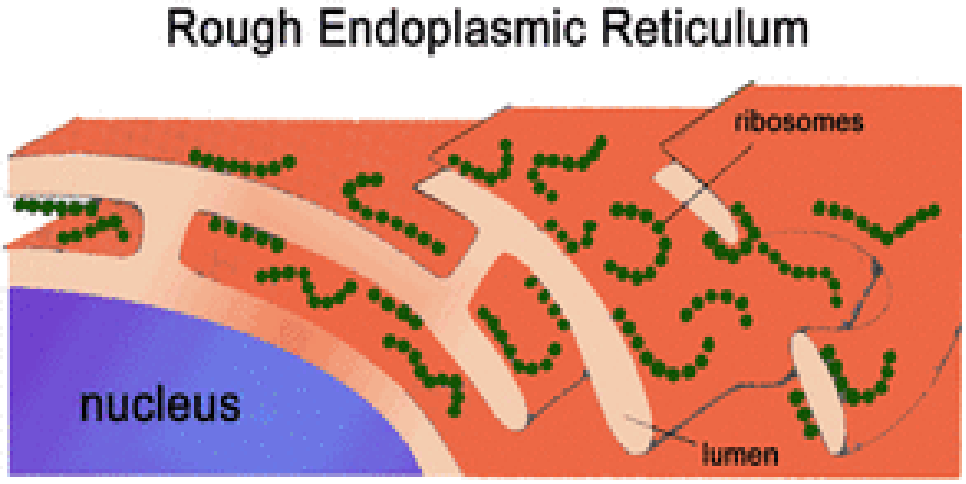
Figure 1.5: Mitochondria contact microtubules in order to migrate. Mitochondria rely on adaptor proteins, Milton and Miro in order to connect with kinesin-1. Kinesin-1 works as the motor to move the mitochondria along the microtubules. Adapted from Course and Wang 2016.

2017). Based off of this it is believed that the mitochondria use microtubules as “highways” to migrate throughout the cell. During their migration, mitochondria will then come in contact with the ER and undergo further biological processes.

1.4 ER

The endoplasmic reticulum (ER) is the largest membrane-bound organelle that is essential in protein synthesis, folding and secretion, and calcium storage and release (Lewis *et al* 2016). The ER has two compositions: the rough (sheet-like) and smooth (tubular) ER (Figure 1.6) (Lewis *et al* 2016). These structures are stabilized by reticulon proteins such as RTN4A and by membrane protein, CLIMP63 (Lewis *et al* 2016). RTN4A is a key factor in establishing the ratio of ER tubules and ER sheets by localizing and stabilizing the curves of ER tubules (Kiseleva *et al* 2007, Lewis *et al* 2016). RTN4A was first identified to play a role in ER tubules in 2000. Voeltz *et al* 2006 found that RTN4A localizes to ER tubules and the overexpression of RTN4A lead to the complete abolishment of ER sheets. It is believed that RTN4A stabilizes ER tubules because it allows for the stabilization of the crosstalk between the tubules (Voeltz *et al* 2006). This state is considered energetically unfavourable, therefore for this tubular structure to occur it heavily relies on the presence of RTN4A (Voeltz *et al* 2006). CLIMP63 stabilizes ER sheets by maintaining the luminal distance between each ER membrane bilayer (Lewis *et al* 2016). It is believed by maintaining the optimal size of the luminal space, CLIMP63 allows luminal chaperones to be accommodated to continue to stabilize the sheet-like structure and have it be packed into minimal space (Shibata *et al* 2010). Sheet-like ER are seen to be covered in ribosomes and are involved in the synthesis, translocation and folding of membrane, luminal and secreted proteins (Lewis *et al* 2016). On the other hand, tubular ER interact with fewer

CLIMP63



RTN4A

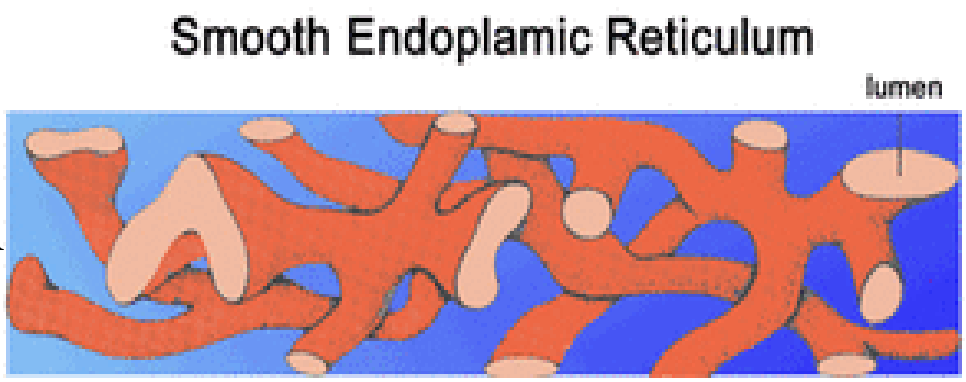


Figure 1.6: The ER has two structures, tubular (smooth ER) or sheet-like (rough ER). CLIMP63 stabilizes the ER sheets through luminal distance while RTN4A stabilizes ER tubules through the stabilization of ER tubular curves. Adapted from *Cell Analogy*.

ribosomes and form membrane contact sites with various organelles such as the mitochondria (Lewis *et al* 2016).

Additionally, the ER is as dynamic as the mitochondria and undergoes ER sliding which is the structural rearrangement along the microtubule cytoskeleton (Rowland and Voeltz 2012). It is believed that ER sliding occurs simultaneously with mitochondrial migration by using the same motor, kinesin-1 and dynein, in order for joined organelles to migrate together even though these organelles are not fused (Rowland and Voeltz 2012). The stability of this migration is believed to be regulated by Miro, an outer mitochondrial membrane protein that is enriched at mitochondria and ER contact sites (Rowland and Voeltz 2012). Miro is linked to dynein through a cytosolic factor, Milton, to facilitate this contact stability during migration (Rowland and Voeltz 2012). Although this hasn't been examined in mammalian cells, the yeast orthologue of Miro, Gem1, when deleted results in the assembly and disassembly of ER-mitochondrial encounter structure complexes which are necessary to maintain ER-mitochondria membrane contact sites (Rowland and Voeltz 2012).

1.4.1 ER and cell migration

ER stress has been predominately linked to altering cell migration. ER stress has been implicated in vascular dysfunction seen in diabetic retinopathy, cancer, obesity, atherosclerosis and ischemia (Paridaens *et al* 2014). Using tunicamycin, an antibiotic that induces ER stress, epithelial sheet migration was impaired and delayed wound healing was observed (Paridaens *et al* 2014). In a study conducted by Saez *et al.* they examined endothelial cell (EC) migration under ER stress. EC migration has three mechanisms: 1) chemotaxis, 2) haptotaxis and 3) mechanotaxis (Saez *et al* 2014). ER stress is found to increase expression of VEGF, a chemoattractant for EC and other EC migration and angiogenesis proteins (Saez *et al* 2014).

Additionally, Sec62, a protein involved in translocation apparatus in the ER membrane has been implicated in tumor cell migration (Greiner *et al* 2011). When using Sec62-siRNA, there was a 13% decrease in PC3 cell invasion and a significant decrease in migratory abilities (Greiner *et al* 2011). These findings remained universal when examining invasion in other tumor cell lines such as fibrosarcoma, glioblastoma and lung carcinoma cells (Greiner *et al* 2011). Put together, this highlights that ER attributes affect cell migration.

1.5 ER and mitochondrial contact site

As previously mentioned, the ER and mitochondria interact at sites called contact sites throughout the cell and the ER plays a role in determining mitochondria shape. ER-mitochondria contact sites have structural variations which exclude the ribosomes (Lewis *et al* 2016). In certain instances, ER-mitochondria contact sites have ER tubules circumscribe nearly around the entire mitochondria (Lewis *et al* 2016). Contact sites are also stable structures that remain undisturbed during cell migration (Lewis *et al* 2016). Nucleoids are complexes of mitochondrial inheritance, mtDNA (Lewis *et al* 2016). TFAM, a nuclear encoded DNA binding protein, is responsible for the packing of mtDNA into nucleoids (Lewis *et al* 2016). To further examine the mitochondria and ER contact sites, Lewis *et al.* monitored nucleoids. TFAM was found to be spaced evenly throughout the mitochondria and were localized adjacent to contact sites between ER tubules and mitochondria (Lewis *et al* 2016). Additionally, a portion of these nucleoids remained stably linked to the contact sites throughout ER remodeling and mitochondria migration (Lewis *et al* 2016). Nucleoids were also found at ER-mitochondrial division (ERMD) sites (Lewis *et al* 2016). This data suggests that mitochondrial DNA replication is found to occur at sites of ER-mitochondrial contact. When examining ERMD and nucleoids, they found that a majority of ERMD events had the presence of POLG2-labeled nucleoids, which was a replisome

involved in mtDNA (Lewis *et al* 2016). Put together this highlights that mitochondrial division that occurs at contact sites with the ER have the presence of mtDNA and its respective mechanisms. When examining nucleoids when ER structure was stabilized with either CLIMP63 or RTN4A, it was found that there were fewer nucleoids and decreased nucleoid fluorescence intensity in cells expressing CLIMP63(Lewis *et al* 2016). When cells were simultaneously expressing CLIMP63 and RTN4A, they found that ER morphology and nucleoid distribution was similar to control cells (Lewis *et al* 2016). Put together this highlights that ER structure plays a role in mtDNA mechanism and their distribution.

Additionally, at these contact sites calcium storage and flux is regulated. The ER is the main store for calcium while the mitochondria accumulates it (Rowland and Voeltz 2012). When basal levels of cytosolic calcium rise so does mitochondrial calcium levels (Rowland and Voeltz 2012). Calcium can be released from the ER into the cytosol where it can be absorbed by the mitochondria more efficiently in response to inositol triphosphate receptor (IP3R) (Rowland and Voeltz 2012). Mfn2 knockout has been found to lead to increase ER-mitochondria contact by increasing the length the ER stretches and increased calcium transfer between the two organelles (Tubbs and Rieusset 2017). Additionally, phorphofurin acidic cluster-sorting proteins 2 (PACS2) has been located at ER-mitochondria interfaces which when depleted disturb mitochondria surface and ER homeostasis resulting in the inhibition of calcium signalling transmissions (Rowland and Voeltz 2012).

There are 3 functions associated with calcium release from the ER to the mitochondria: 1) to provide calcium to mitochondrial membrane proteins that need calcium for their function, 2) mitochondrial division relies on changes in calcium concentration, and 3) apoptosis (Rowland and Voeltz 2012). Calcium influx into the matrix from the IMM requires free calcium that can

only be produced from these sites (Rowland and Voeltz 2012). However, free calcium is unable to move through the IMM easily and must go through the MCU (Rowland and Voeltz 2012). Therefore, free calcium produced by these contact sites are necessary for the influx of calcium through the MCU into the matrix for protein function. As previously mentioned, in yeast cells mitochondria division is regulated by Gem1 (Rowland and Voeltz 2012). In order for Gem1 to initiate fission, it's EF-hand calcium binding domain must colocalize with ER-mitochondria encounter structure complex puncta (Rowland and Voeltz 2012). Local calcium flux stimulates apoptosis when opening the mitochondrial permeability transition pore (Rowland and Voeltz 2012). This results in cytochrome c being released and propagation of caspase cascade (Rowland and Voeltz 2012). IP3R is believed to be the channel that causes the released of calcium stores from the ER to the mitochondrial membrane to begin this reaction (Rowland and Voeltz 2012). It has been seen that deletion of IP3R lead to the resistance of apoptotic stimuli in several cell lines (Rowland and Voeltz 2012).

1.6 Cancer

According to the Canadian Cancer Society, cancer occurs when cells stop listening to the internal regulator systems to control cell growth and division and begin to divide uncontrollably. Overtime this accumulation of cells with altered genes create a lump in the human body called a tumour. According to the Canadian Cancer Society, cancer made up 30% of health related deaths in Canada (Cancer Statistics at a Glance). It is believed that in 2019, an average of 604 Canadians were diagnosed with cancer every day and 225 Canadians died from cancer every day (Cancer Statistics at a Glance). Cancer continues to become more and more prominent in our society. Early detection continues to be the key tool to help cancer patients fight the disease. With the increasing severity of the disease through metastasis, extensive research has been done

to find potential treatments. Through this work the survival rates have increased to 63% of cancer patients surviving for 5 years or more after a cancer diagnosis (Cancer Statistics at a Glance).

There are natural indictors in place to allow cells to be eliminated if there is DNA damage to allow for the proper balance of healthy cells. In cancer cells there are many mutations that inhibit apoptosis. One of the key mutations associated to cancer cells is the gene p53 (Alberts *et al* 2002). This gene determines a cells response to damaged DNA and other stresses to the cells (Alberts *et al* 2002). It is found to be mutated in nearly half of all human cancers (Alberts *et al* 2002). When p53 does not function properly cancer cells evade being recognized with damaged DNA, thereby evading apoptosis (Alberts *et al* 2002). By avoiding apoptosis, these cancer cells are then able to continue to proliferate, therefore causing an accumulation of damaged DNA that can lead to cancer (Alberts *et al* 2002). Proliferation is another aspect where mutations occur in cancer cells. One of the most implicated genes in proliferation is Rb (Alberts *et al* 2002). Rb, a tumour suppressor gene, functions as a brake that inhibits entry into the S phase (Alberts *et al* 2002). To allow the entry of S phase, Rb must unbind from gene regulatory proteins and be exposed for phosphorylation (Alberts *et al* 2002). This allows genes to be available for the progression of the division cycle and stops Rb's inhibitory affcects (Alberts *et al* 2002). Cancer cells remove Rb's inhibitory ability to allow their cells with damaged DNA to continue through the cell cycle (Alberts *et al* 2002).

A key issue associated with cancer is metastasis as tumour cells tend not to stay in their primary locations (Oppenheimer 2006). Metastasis, is the process in which cells spread to distant areas of the body using the bloodstream, lymphatic systems or through body spaces (Oppenheimer 2006). There are 5 stages to metastasis: 1) the tumour cells detach from the primary tumor, 2) tumour

cells migrate from their primary sites by penetrating into the lymph or blood vessels and disseminate into the distant areas, 3) the tumour cells lodge themselves into the blood vessels of distant organs, 4) the tumour cells then invade the tissues of the secondary sites through the vessel walls, and 5) secondary tumour cells begin to grow at the new secondary site (Oppenheimer 2006). Metastasis is being heavily studied as to better understand how these cancer cells are able to survive the migration to the secondary site (Oppenheimer 2006). Only about 1% of cancer cells travelling in the bloodstream make it to the secondary sites (Oppenheimer 2006). A majority of them die in the bloodstream, making researchers believe that the blood serum contains waste products or other substances that are toxic to the cancer cells (Oppenheimer 2006). This has brought about the idea that cancer cells might travel as a cluster then (Oppenheimer 2006). As the cluster migrates, the cells on the outside receive all the toxic effects protecting the interior cells from the hostile environment (Oppenheimer 2006). This then allows the interior cancer cells in the cluster to then exit the bloodstream with no issues and begin growing in its secondary location. The determination of secondary sites has yet to be fully understood. There are 2 means in which a cancer cell determines its secondary site: 1) the relative geometry of the primary and secondary site and 2) selective stickiness (Oppenheimer 2006). The first is based on the simple geometry of the body and the natural channels such as veins and the lymphatic system (Oppenheimer 2006). Here the cells simply look for the first available space and then migrate in (Oppenheimer 2006). As for selective adhesion that has yet to be fully understood but there is some evidence showing that cancer cells are selective for the lungs, brain, adenal and ovary (Oppenheimer 2006). In an experiment, it was found that when collecting cancer cells from the lung in mice, then injecting them back into other mice and then collecting cancer cells from the lungs, by the 10^{th} mice there were more secondary melanoma in

the lung of the 10th mice than in the first (Oppenheimer 2006). This showed that these cancer cells had developed a lung-seeking ability and would repeatedly migrate towards the lungs to metastasize (Oppenheimer 2006).

1.6.1 Cancer and mitochondria

Many tumour cells rely on metabolism reprogramming to support their survival and thereby rely on the reprogramming centers, the mitochondria, to facilitate this action (Vyas *et al* 2016). Most tumour cells push glycolytic interactions into the pentose phosphate pathway, serine biosynthesis, and lipid biosynthesis instead of completing oxidation by mitochondrial respiration (Vyas *et al* 2016). This is usually done by limiting how much pyruvate is used by the mitochondria. Pyruvate kinase (PKM), regulates how much pyruvate is available for mitochondrial oxidation (Vyas *et al* 2016). This is usually the final step in glycolysis as it ends up generating pyruvate necessary for the citric acid cycle (Vyas *et al* 2016). In cancer cells PKM2 isoforms are upregulated and their low activity allows other upstream intermediates to accumulate and be used for anabolic processes (Vyas *et al* 2016). Additionally, the mitochondria pyruvate carriers (MPC1 and MPC2) are either completely gone or downregulated in several kinds of cancers (Vyas *et al* 2016).

Additionally, mitochondria have been linked to malignant transformation, which is the conversion of a normal cell into a neoplastic precursor (Porporato *et al* 2018). These neoplastic precursors, when not monitored by the body for defects can lead to additional alterations that allow unrestricted proliferation, dissemination and formation of distant macrometastases (Porporato *et al* 2018). It is worth noting that only carcinogenic models of oncogenesis can recapitulate malignant transformation (Porporato *et al* 2018). The mitochondria contribute to malignant turnover by: 1) ROS favouring the increase in oncogenic DNA defects and activation

of their signalling pathways, 2) an abnormal accumulation of fumarate, succinate and 2-hydroxyglutarate, and 3) the functional defects in MOMP or mitochondrial permeability transition (MPT) (Porporato *et al* 2018). In ROS, mitophagy is commonly studied. Studies have found that when knocking down or out genes essential for mitophagy it promoted oncogenesis (Porporato *et al* 2018). Additionally, when looking at Fanconi anemia genes there was evidence to suggest their involvement in mitophagy (Porporato *et al* 2018). Fanconi anemia genes are mutated or silenced genes found in a majority of human tumours (Porporato *et al* 2018). Thereby finding this link it suggests that the oncosuppressive activity of these genes are rooted in the removal of damaged mitochondria overproducing ROS (Porporato *et al* 2018). Succinate dehydrogenase complex iron sulfur subunit B(SDHB), and fumarate hydratase (FH) are usually loss of function mutations that result in the accumulation of fumarate and/or succinate (Porporato *et al* 2018). These metabolites then allow their accumulation to trigger malignant turnover and inhibit α-ketoglutarate dependent enzymes which serve to control gene expression (Porporato *et al* 2018). Additionally, this accumulation of fumarate can lead to a process known as succination where fumarate induced a non-enzyme post-translational protein modification (Porporato *et al* 2018). Succination of kelch like ECH-associated protein 1 activates NFE2, an oncogenic transcription factor (Porporato *et al* 2018). The alterations needed for mitochondria to go through MOMP or MPT is best described by the overexpression of BCL2 (Porporato *et al* 2018). BCL2 is an apoptosis regulator that localizes to the mitochondrial membrane (Porporato *et al* 2018). The overexpression of BCL2 allows the malignant precursor cells to remain resistant against regulated cell death (Porporato *et al* 2018). Thereby using the mitochondria in these aspects, the cells undergoing malignant turnover, begin to prepare to become cancer cells (Porporato *et al* 2018).

1.6.2 Cancer and the ER

As mentioned earlier, the ER is the primary organelle responsible for protein folding and the maturation and the maintenance of cellular homeostasis (Yadav *et a* 2014). The ER like many other organelles can be under stress, which results in the activation of the unfolded protein response (UPR) (Yadav *et a* 2014). The ER begins stress responses when there are biochemical environmental changes or DNA damage (Yadav *et a* 2014). The UPR serves to restore homeostasis or start cell death, which seems to be avoided in cancer cells (Yadav *et a* 2014). The UPR starts by the simultaneous dissociation of the Grp78 and binding immunoglobulin protein, Bip, to 3 membrane-bound ER stress sensors, PERK, ATF6, and IRE1α (Yadav *et a* 2014). The disassociation/binding action to PERK serves to block general protein synthesis by phosphorylating eIF2α and inhibiting NF-κB (Yadav *et a* 2014). The binding to ATF6 then leads to the transcription factor to regulate gene expression (Yadav *et a* 2014). Lastly, the binding to the IRE-1α leads to the splicing of XBP1 which translocates to the nucleus to activate transcription factors encoded for chaperones or folding enzymes needed in protein folding and secretion (Yadav *et a* 2014).

In cancer cells, they adapt to the microenvironment through activating UPR and macrophages to create an environment that is favourable for them (Yadav *et a* 2014). Cancer cells in ER stress induce cyclooxygenases-2 expression through the NF-κB pathway, switching the ER stress response into an antiapoptotic role (Yadav *et a* 2014). Additionally, cancer cells continue to manipulate the UPR by acting on Grp78 and PERK (Yadav *et a* 2014). The exact mechanisms that Grp78 and PERK act to promote cancer cell growth and survival is still being studied. However, studies have found Grp78 expression to be elevated in metastatic cancer cell lines, lymph node metastasis and that any reduction or knockdown of Grp78 inhibited tumour cell

invasion, formation and growth (Yadav *et a* 2014). In regard to PERK, a study found that when PERK was absent it affected the ability of mammary carcinoma cells to create solid tumours (Yadav *et a* 2014). PERK continued to be implicated in tumours through the stabilization of HIF1α (Yadav *et a* 2014). Hypoxia is very common in a tumour environments, this causes HIF1α to be stabilized and fully activates UPR through PERK phosphorylating elf2α, ATF4 and GADD34 (Yadav *et a* 2014). Protein synthesis is inhibited by the phosphorylation of elf2α but ATF4 is activated (Yadav *et a* 2014). ATF4 is a transcription factor that has been linked to cancer cell proliferation and survival in a nutrient deficient environment (Yadav *et a* 2014).

1.7 Research hypothesis and objectives

Evidently the ER and the mitochondria play key roles in allowing cancer cells to survive. Not only are their functions often manipulated to promote survival and growth, but their dynamic structures have been linked to cancer cell migration. It is seen that when the MCU is silenced or other mitochondria fission or fusion proteins are removed cell migration is altered. Additionally, mitochondria and the ER have an important role in calcium which is another factor in cell migration. As metastasis continues to be the key issues associated to cancer treatment, we believe the relationship the mitochondria and the ER have on cell migration should be studied to shed light on these organelles ability to lead to metastatic potential. Based on these findings we believe that the ER in combination with mitochondria have a role in regulating cell migration. As previously highlighted, each organelles function is manipulated by cancer cells and it is believed that an organelles shape determines its function. Therefore, we sought to determine the relationship the mitochondria and the ER's morphology have on the cells ability to migrate. We hypothesize that mammalian cell migration is regulated by directed mitochondrial movement and by endoplasmic reticulum (ER) mediated mitochondrial fission. The objectives of this study are

to:

1) characterize and quantitate mitochondrial morphology and movement in migratory cells.

2) determine how changes in ER structure affect mitochondria morphology and cell migration.

3) determine if migration parameters are controlled by ER morphology or mitochondria morphology

Chapter 2: Materials and Methods

Plasmids and Antibodies. All plasmids except CLIMP63-GFP and OPA-GFP were bought from Addgene. CLIMP63-GFP was gifted from the Nunnari lab in Davis University and OPA-GFP was gifted from the Slack lab at the University of Ottawa. RTN4A-GFP(61807), RTN4A-mCherry (86683), Cerulean TOMM20 (55449), Drp1-mCherry (49152), Cortactin-GFP(50728), mEmerald Talin (54266) and mEmerald Tubulin (54292). Plasmids were purified using a Qaigen mini prep kit (27104) in LB broth. Mini prep samples were digested with their respective restriction enzymes and electrophoresed in a 2% gel. The best digestion and concentration from the mini prep will be used for a maxi-prep. Maxi-prep is done using a Qaigen kit (12663). Concentrations are determined using a Nanodrop (Thermo Scientific, Model 2000). We used antibodies for Calnexin (abcam, ab22595) and Cortactin (Invitrogen, PA5-29799)

Tissue Culture. NIH3T3, mouse embryo fibroblasts, were used throughout this study from ATCC (CRL-1658). Cells were grown using Dulbecco's modified Eagle's medium (DMEM) (Thermo Scientific, D5796) that was supplemented with 10% FBS (Gibco, 12483-020), 1mM of sodium pyruvate (Gibco, 11360020) and 1mM of penicillin-streptomycin (Fisher, SV30010). For sub-culturing cells were removed from the plate using 0.25% EDTA-trypsin (Thermo Scientific, 25200-056).

Transfection. NIH3T3 cells are split from 80-100% confluent plates and seeded at 10,000 cells per 35mm dish (ibidi, 81158) or slide (Azer Scientific, ES0117580) and left to adhere to the surfaces over night. The next day cells are transfected to their optimized Lipofectamine:DNA ratio, see chart below for exact amounts and ratios. First a Lipofectamine-OptiMem mix is created which consists of 50µl of OptiMem (Thermo Scientific, 31985-070) per well or plate or slide and respective amounts of Lipofectamine 2000 (Invitrogen, 11668-019) based on amount of

DNA(ng). Lipofectamine-OptiMem mixture incubates for 10minutes at room temperature. During incubation a DNA and Optimum mixture was made. DNA volume is calculated based on the amounts needed for the experiment which is listed above. The volume of OptiMem is the same as above; 50µl per well or plate or slide. Lipofectamine-OptiMeM and OptiMeM-DNA mixtures are combined and incubated for 20 minutes at room temperature. During Lipofectamine-Optimum-DNA incubation, aspirate media from plates or wells and replace with DMEM media supplemented with 10% FBS and 1Mm NaPy (Antibiotic Free DMEM). 1ml of Antibiotic Free DMEM media in 35mm^2 dishes, 500µl in 12 well plates (VWR, 29442-040) and 75µl in 96 well Imagelock plates (Essenbio, 4379). After incubation 100µl of Lipofectamine-Optimum-DNA mixture is added to each plate and 12 well plate or 75µl in 96 well plates. Incubate for 4hrs at 37˚C with 5% CO_2. After incubation, Antibiotic Free Media is aspirated and replaced with fresh supplemented DMEM.

Experiment Type	Lipofectamine:DNA ratio	DNA Amount (ng)
CLIMP63 or RTN4A w/ mitotracker stain experiments or cortactin stain	1:2.5	1000ng
Incucyte Free Range and Wound	1:1.5	150ng
Janelia Images	1:4	25ng
Cortactin w/ mitotracker stain experiments	1:2.5	500ng
Focal adhesions w/ mitotracker stain	1:4	850ng
CLIMP63+Drp+Tomm20 or RTN4A+OPA+Tomm20	1:4	800ng

Fixing Cells. Cell are fixed with 4% PFA (Sigma, 30525-89-4) for 15 minutes at room temperature. PFA is then aspirated and slides washed using PBS (Biobar, 31142500**)** three times. Coverslips are mounted on slides using ibidi mounting media (ibidi, 50001). Coverslips are sealed using clear nail polish.

Incucyte and Tracking. Wound experiments were imaged using the incucyte on 96 well Imagelock plates (Essenbio, 4379). Cells were transfected using the same methods stated above. After transfection cells were left to grow until 80% confluency. At 80% confluency using the scratch maker, all 96 wells were scratched and imaged every 10 minutes for 24hrs or until the wound closed. Each well had 2 images per well taken for every 10 minutes. For free range migration, cell density for 96 well Imagelock plates (Essenbio, 4379) were 750 cells per well on the day of transfection. Incucyte Zoom version 2018A acquisition parameters is 3 images across the well every 15 minutes for 72 hours. Post acquisition individual images are exported as an image sequence through ImageJ to create a movie to be used for tracking. Using Matlab version R2019b and CellTracker version 1.1, cells were manually tracked by clicking on the center of the nucleus creating the track the cell migrates. Speed, angle, total distance and displacement were analyzed using a single tail t-test.

Live Cell Imaging. Experiments with CLIMP63, Cortactin, or RTN4A transfectants used Mitotracker Red CMXRos (Molecular Probes Fisher, M7512). Mitotracker Media was made using Mitotracker Red CMXRos and phenol red-free DMEM (Thermo Scientific, 31053-028) at a 1:10,000 dilution. The day after transfections, cells are incubated in Mitotracker media for 30 mins. After incubation Mitotracker media is replaced with imaging media, which is phenol red-free DMEM, 10% FBS and penicillin-streptomycin. Live cell images are taken on LMS 8800 Airyscan Confocal microscope using Zen black edition. Fission/fusion images were taken every 7 seconds for 18mins in non-transfected cells and every 10 seconds for 8mins in CLIMP63 and RTN4A cells. Triple transfection cells of RTN4, OPA, Tomm20 or CLIMP63, Drp1, Tomm20 were imaged every 45 seconds for 15 minutes. Live cell images taken on the Delta Vision were imaged every 45 seconds for 25 mins using FITC filter at 2% transmitted light and 1.0 exposure

time and mCherry filter with a 2% transmission ad 0.8 exposure time. Fixed cell imaging used EGFP and mCherry signals fully imaging the entire cell at 500µs for each channel. Tubulin and mitochondria experiments were taken on the Lattice Light Sheet (LLSM) at Janelia Research Campus every 4 seconds for 30 minutes (https://www.aicjanelia.org/llsm).

Image Processing/Deconvolution and Analysis. Images were processed using AutoQuant version X3, Zen 3.1 version blue and the Delta Visions Deconvolution processing was done using the Softworx software version 7.0. AutoQuant parameters were 15 iterations at high noise to background levels. Zen Blue parameters were Fast Iterative with a bad pixel correction applied. Delta Vision deconvolution parameters were 10 iterations on aggressive mode. Analysis of mitochondrial length was done using the measure function in ImageJ after individual mitochondria are selected. Fission and fusion events were scored manually by monitoring individual mitochondria fuse and fizz. 3D surfaces were created with Imaris version 9.5.1.

Janelia Imaging Cell Culturing and Image Analysis. Cells were grown on 5mm coverslips (Thomas Scientific, 64-0700) that were placed in 12 well plates and left to adhere overnight. The next day tubulin and mitochondria were transfected following the transfection procedure listed above. DNA-Lipofectamine-OptiMeM complex is added to coverslips and left to incubate for 30 minutes. Then 500µl of Antibiotic Free Media is added to the wells with the coverslips and the coverslips are left to incubate for 3.5hrs with the Antibiotic Free Media and complex. After the incubation the wells were aspirated of all liquids and given fresh media. The next day the slides are imaged on the LLSM following the imaging parameters listed above.

Images were deconvolved using the Richardson-Lucy algorithm supplied by employees of Janelia Research Institute. This deconvolution produces tiff and klb files that were analyzed on ImageJ and Imaris; the tiff versions were used for the analysis as they were faster to open on

ImageJ. Using File->Import -> Image Sequence, each individual timepoint is uploaded into a stack. To create the hyperstack, we used the following commands: Image-> Hyperstacks-> Stack to Hyperstack. A pop up will come on listing channel, slices and frames. Based on each cell's parameters of slices and frames, those are inputted into the pop up and the order sequence is selected from the drop down menu. This creates the hyperstack needed for Imaris. Using the Plugin-> Image to Imaris, the hyperstack is sent to Imaris. On Imaris, surfaces were created using the surfaces tab. Files were exported using the Animation option to create an avi file of the time lapse with the surfaces.

Chapter 3: Results

3.1 Mitochondria migrate towards the biochemically dense leading edge of the cell.

Naturally a cell creates a defined leading edge and a trailing edge which requires mitochondria to provide energy for the movement of the cell. We were curious to know if mitochondria move into one protrusion specifically during migration. To establish mitochondria movement to these protrusions, MitoTracker stained mitochondria in a NIH3T3 cell were monitored for over 25mins. As the cell migrates towards the bottom left of the screen (blue arrows), the mitochondria migrate in the same direction into the leading edge (Figure 3.1A). The blue to green in the track indicates the time point as the mitochondria migrate in this tracked path. At the beginning of the movie, indicated by the dark blue colour in the track, the mitochondria start their migration in the perinuclear area of the cell. As the movie continues, the track changes colour to show the progression of time with the light green indicating where the mitochondria stopped migrating after 25 mins. As the leading edge is known to be biochemically different from other parts of the cell, we wanted to establish is the mitochondria migration seen in Figure 3.1A a response to cell polarization. Using cortactin, a polarization marker, it was seen that regardless of the starting location (trailing edge, perinuclear area, or leading edge) all mitochondria have a directional based migration towards the biochemically dense leading edge (Figure 3.1B). 3 mitochondria were tracked for the duration of their lifespan, this created the blue track for the mitochondria in the trailing edge, a white track for the mitochondria in the perinuclear area and the green track for the mitochondria in the leading edge. As seen by the tracks, each mitochondrion makes turns as they migrate but continue to migrate towards the biochemically dense leading edge indicated by the red arrowhead. Additionally, in a cell with oscillating movement, mitochondria change their direction of migration as the cell oscillates

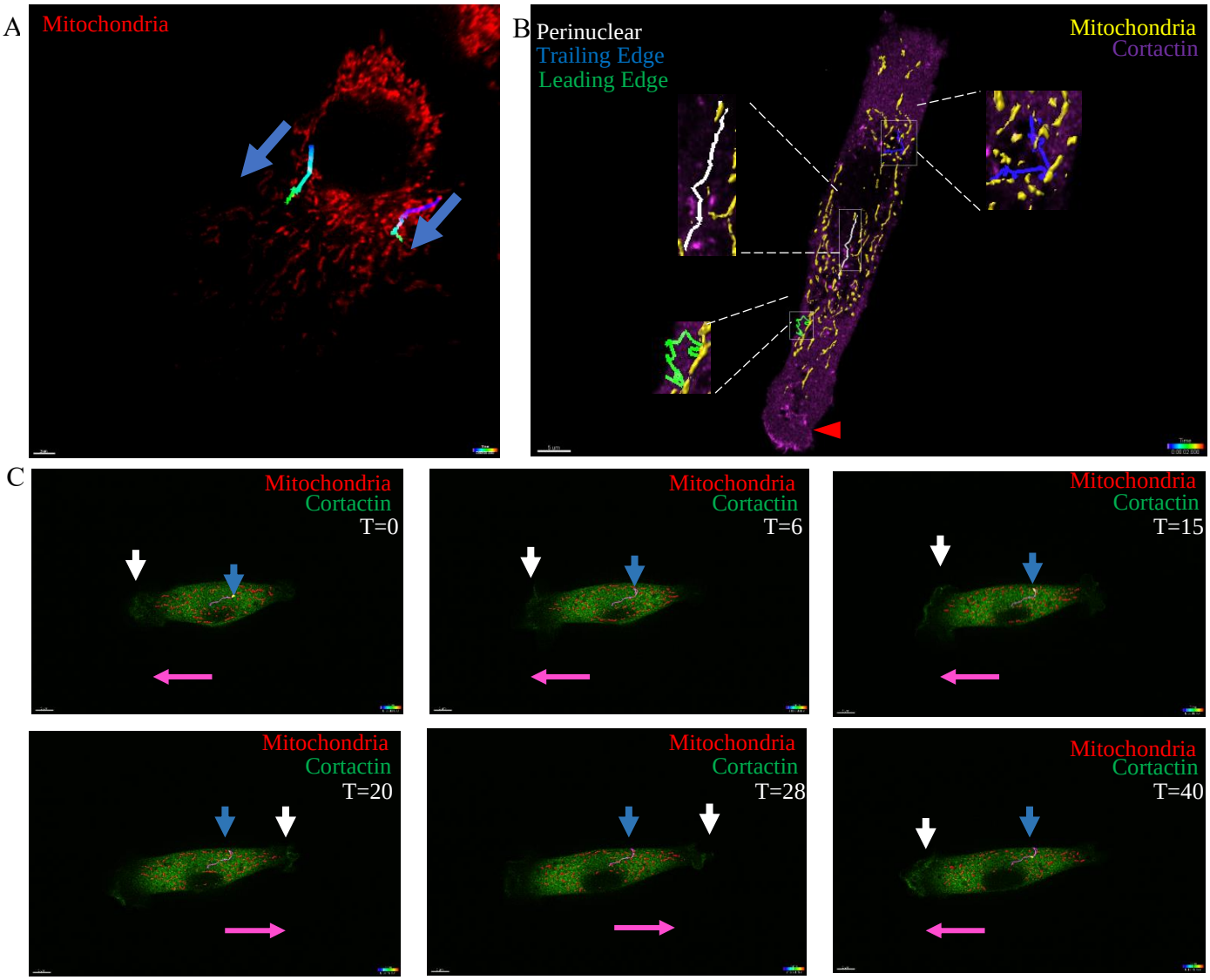
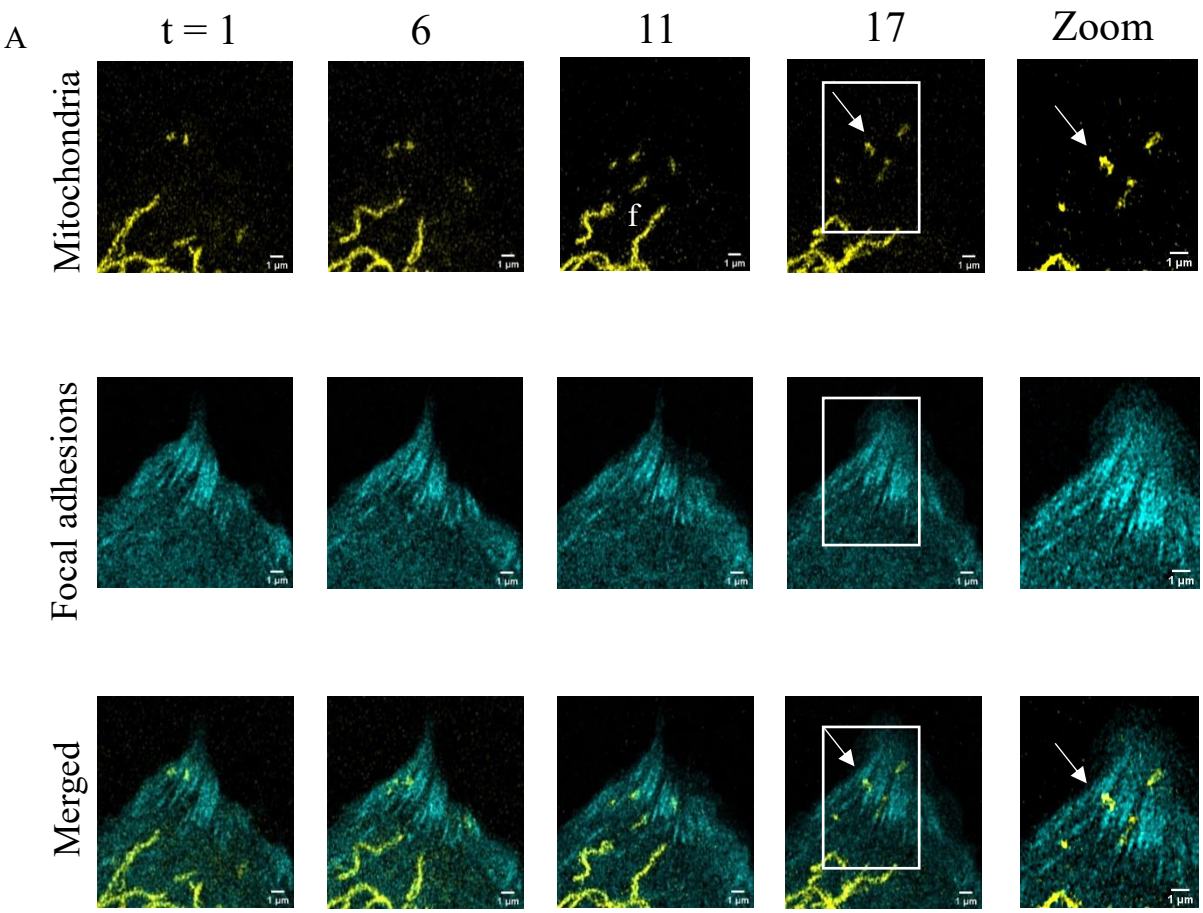

A
Mitochondria
B
Perinuclear
Trailing Edge
Leading Edge
Mitochondria
Cortactin
C
Mitochondria
Cortactin
T=0
Mitochondria
Cortactin
T=6
Mitochondria
Cortactin
T=15
Mitochondria
Cortactin
T=20
Mitochondria
Cortactin
T=28
Mitochondria
Cortactin
T=40

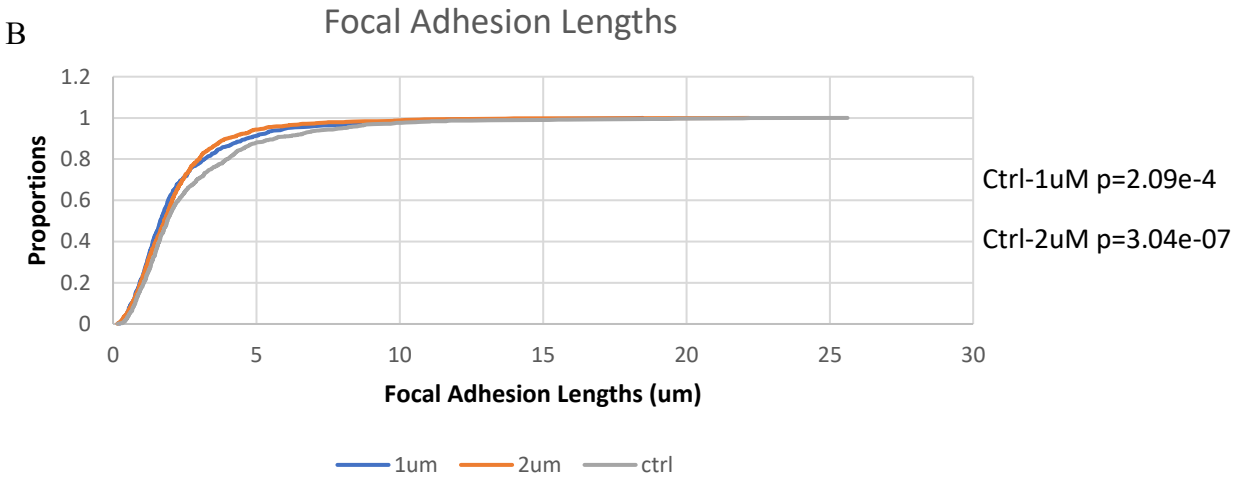
Figure 3.1: Mitochondria move to the polarized leading edge of the cell. A) Mitochondria migrate to the leading edge of the cell indicated by the blue arrows. The path of the mitochondria are indicated by the lines with each colour of the line representing the start of different timepoints. Dark blue indicates the first 5 minutes and green indicate the last 5 minutes in a 30 minute timespan movie. B) Mitochondria starting at the trailing edge (blue), perinuclear area (white), or the leading edge (green) all migrate towards the polarized leading edge of the cell (red arrow head). C) Mitochondria oscillate direction during migration**.** Live cell image of NIH3T3 cells transfected with cortactin (green) and stained mitochondria (red), showing that mitochondria alter migration paths when polarity signal changes from the leading edge to the trailing edge. Purple line indicates the path the mitochondria (yellow) took, white arrow shows polarized end, blue arrow shows mitochondria, t is in minutes, pink arrow is the direction the cell is migrating.

(Figure 3.1C). Using cortactin to mark the polarized edge, this NIH3T3 had its leading edge change throughout the movie. In the beginning of the movie cortactin is densely expressed on the left side of the cell (white arrow), by 20 minutes it changes to the right side and by the end of 40 minutes it is on the left side again. This change in the leading edge also changes the direction the cell is migrating (pink arrow), first the cell is migrating to the left and at 20 minutes it begins to migrate to the right and changes direction to the left at 40 minutes. This change in direction and density leads to a change in the mitochondria's path indicated by the purple track. At the beginning of the movie, the mitochondria (blue arrow) is migrating to the leading edge seen on the left side of the cell. Again, at the 20 minute mark the mitochondria (blue arrow) makes a turn and starts migrating to the right towards the new leading edge and then at 40 minutes makes another turn towards the new leading edge at the left side of the cell.

3.2 Mitochondria and focal adhesions interact at the leading edge of the cell.

One of the many structures involved in cell migration are focal adhesions. Focal adhesions tend to be on the peripheries of the cell which includes the leading edge as they are the points of interaction between the cell and the substrate during migration (Burridge *et al* 1997). As we believe that mitochondria are involved in cell migration and have numerous organelle interactions, we wanted to determine whether the mitochondria interacted with focal adhesions and if this interaction would further identify what aspects of cell migration mitochondria are involved in. Using live cell microscopy, we visualized focal adhesions using talin, a protein involved in the linking of integrin β subunit to actin filaments and stained mitochondria. We found that as the cell migrates, mitochondria infiltrating the leading edge localize with focal adhesions (Figure 3.2A). The dense teal colour indicates focal adhesions specifically at the


A
t = 1
6
11
17
Zoom
Mitochondria
Focal adhesions
Merged
f


B
Focal Adhesion Lengths
Proportions
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
30
Focal Adhesion Lengths (um)
1um
2um
ctrl
Ctrl-1uM p=2.09e-4
Ctrl-2uM p=3.04e-07

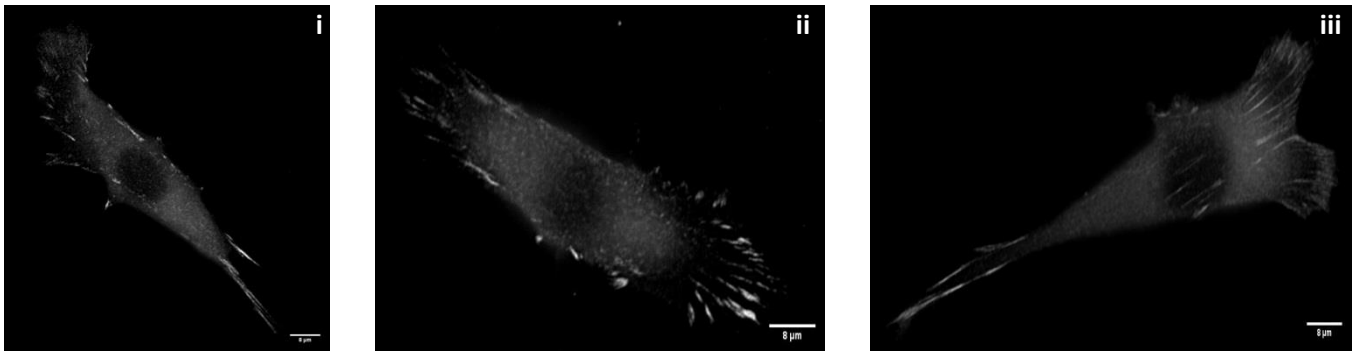

i
ii
iii

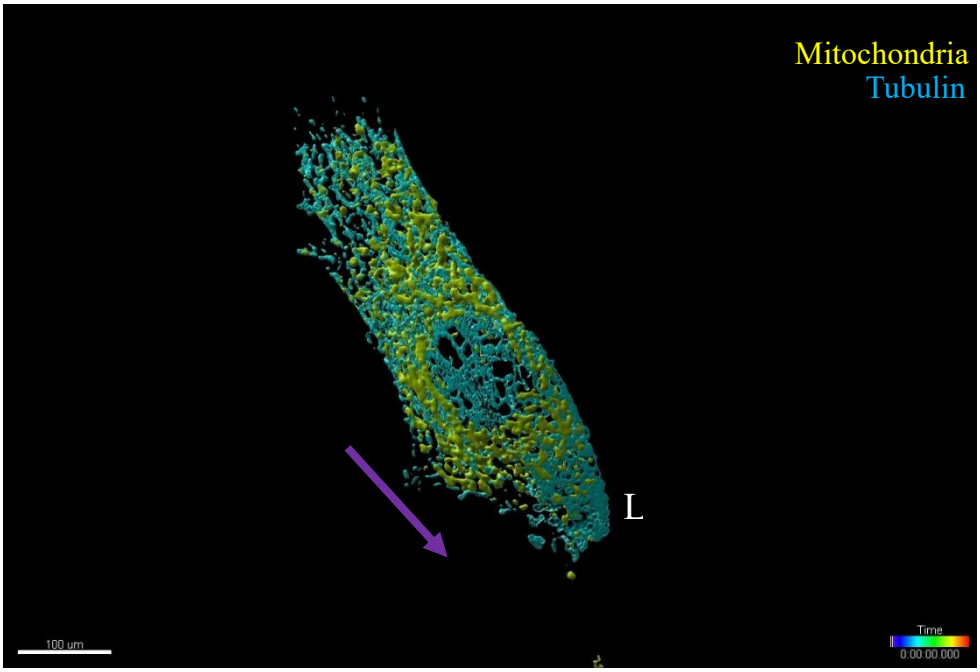
Figure 3.2: Mitochondria and focal adhesions interact at the leading edge of the cell. A) Still images of a live cell movie of NIH3T3 cell transfected with mApple-Tomm20(mitochondria in yellow) and mEmerald-Talin (focal adhesions in teal). As mitochondria migrate they come in contact with focal adhesions in the leading edge. B) Cumulative distribution function and representative images of focal adhesion lengths when exposed to 1μm or 2μm of mitochondrial inhibitor, oligomycin. Oligomycin creates short, fragmented focal adhesions. i is the 1μm representative image, ii is the 2μm representative image and iii is the control representative image.

leading edge of the cell. By 6 seconds the mitochondria undergo fission (white f) and the new daughter mitochondria migrates further into the leading edge. As the daughter mitochondria continues to migrate, by 11 seconds it localizes with the focal adhesion (white arrow). This process of mitochondria infiltration and focal adhesion localization continues as seen by the 17 second time frame. The white box indicates the area of the zoomed in images, showing closely the overlap of the mitochondria (yellow) and the focal adhesions (teal) indicating their interaction. To further explore this relationship, we were interested in seeing what functional role mitochondria have on focal adhesions. We used oligomycin, an ATP synthase inhibitor, at 1μM or 2μM. Using fixed cell microscopy, NIH3T3 cells were transfected with talin and later treated with oligomycin. When treating cells with 1μm or 2μm oligomycin, focal adhesion length decreased. (Figure 3.2B). On average control cells had a length of 2.78μm, cells treated with 1μM of oligomycin had a length of 2.33μm and cells treated with 2μM of oligomycin had a length of 2.22μm. These lengths are represented in Figure 3.2B i-iii, where NIH3T3 cells transfected with talin (dense white lines) have shorter focal adhesions once treated with oligomycin. Altogether, this data presents focal adhesions as a new organelle mitochondrion interact with and that mitochondria regulate migration through this contact.

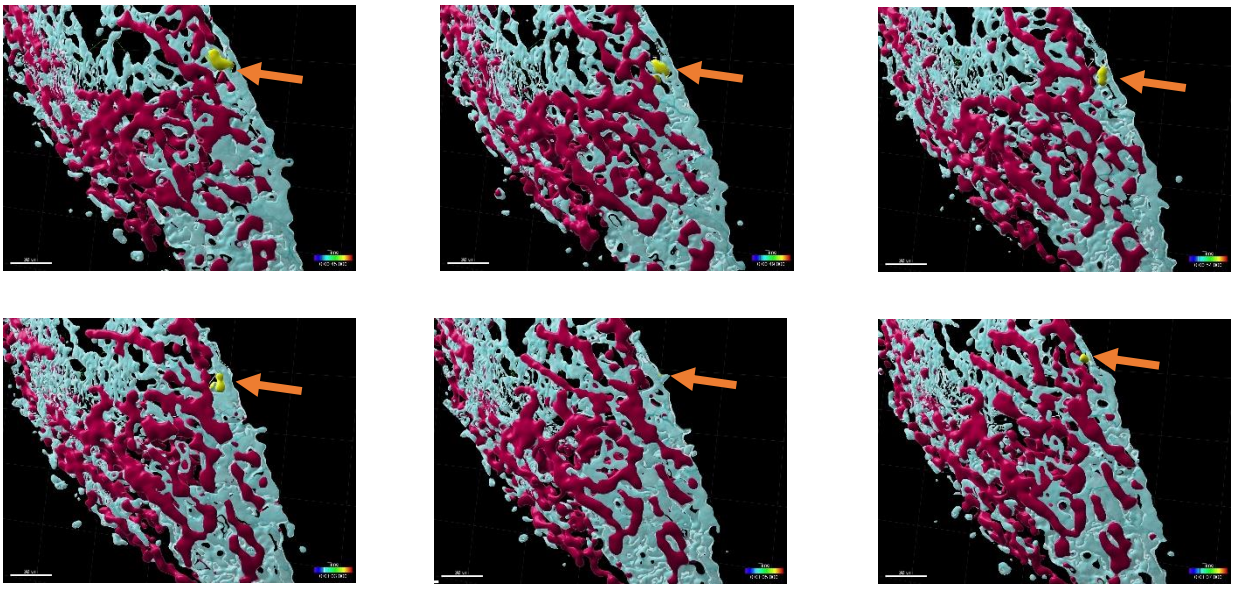
3.3 Mitochondria migrate along microtubules.

One of the biggest questions in mitochondrial migration was how do they migrate? Extensive work has been done showing that microtubules serve as the highways for this movement and rely on mitochondrial adaptor proteins and kinesin-1 to mediate this action. To ensure this same mechanism was working in our NIH3T3 cells, we did a double transfection to visualize mitochondria and tubulin. After we obtained our image, we did a 3D surface rendering of the cell using Imaris (Figure 3.3). As the cell migrated, the mitochondria were seen to migrate and only

A


Mitochondria
Tubulin
L
100 um
Time
0 00 00 000

B



C

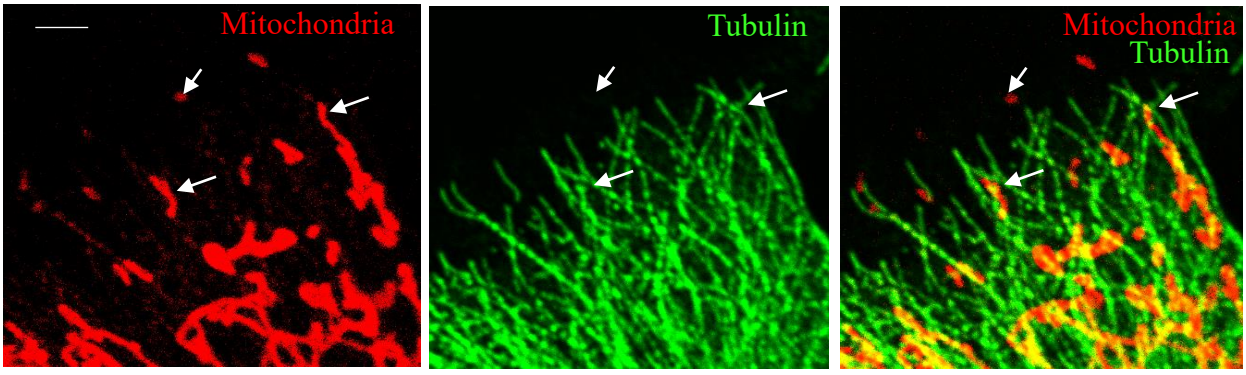
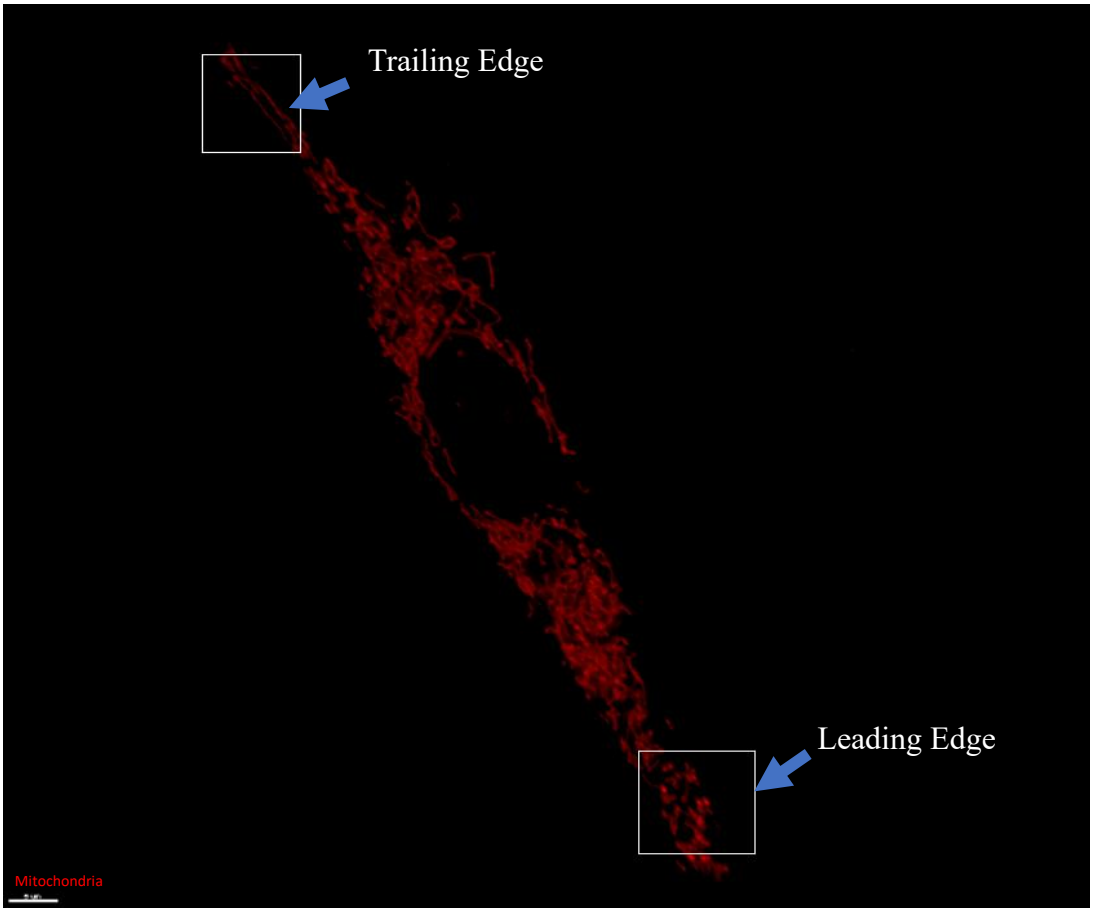

Mitochondria
Tubulin
Mitochondria
Tubulin

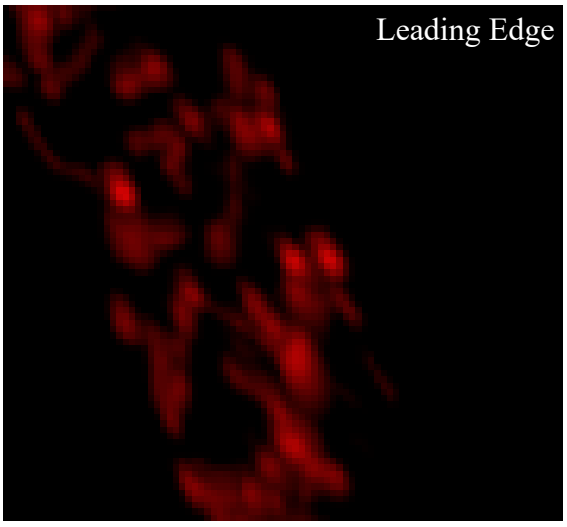
Figure 3.3: Mitochondria migrate along microtubules. A) Still images of a live cell movie of NIH3T3 cell transfected with mApple-Tomm20(mitochondria in yellow) and mEmerald-Tubulin (tubulin in teal). As mitochondria migrate they make contact with tubulin to reach the leading edge (white L). Migratory direction is the purple arrow. B) Shows the highlighted mitochondria (yellow) (orange arrow) associated with microtubules(teal) migrating over a period of time. C) Still image of mitochondria (red) in the leading edge are not tethered to microtubules (teal).

contacted the microtubules. Figure 3.3B show tracked mitochondria (yellow) (orange arrow) seen directly localizing on the microtubules (teal). With each time frame showing the mitochondria moving, it was clear that the mitochondria continued to localize to the microtubules showing that our cells rely on the microtubules to be a highway for the mitochondria. As the mitochondria migrate along the microtubules, once they reach the leading edge, we found that the mitochondria no longer associate with the microtubules (Figure 3.3C). In the merged image it is clear that the mitochondria (red) are in the leading edge but not touching the microtubules (teal) as they are in free space.

3.4 Mitochondria in the leading edge are smaller and undergo more fission events.

Mitochondria have a wide range of morphologies, so we were curious to determine if mitochondria shape changes in a motile cell. Using fixed cell microscopy, mitochondria were stained in NIH3T3 cells. We found that in a motile cell where a distinct leading edge and trailing edge is created the mitochondria have different morphologies. The mitochondria in the trailing edge are long tubular structures while the mitochondria in the leading edge are punctate or short tubules (Figure 3.4). As mitochondria are dynamic organelles, we believed the morphological changes seen in Figure 4 may be associated to mitochondrial dynamics, fission and fusion. To further identify the role fission and fusion has in the leading and trailing edge, we monitored stained mitochondria in non-transfected NIH3T3 cells. While monitoring mitochondrial fission and fusion, we found that both events occur in the leading edge and the trailing edge. During fission the mitochondria can be seen thinning and pulling away from each other (Figure 3.5A). Focusing on the mitochondria in the green box, at the beginning of the clip the mitochondria appears to be a short tubular structure. By 7 seconds we see that the mitochondria begin to thin, and fission is completed by 21 seconds creating 2 new daughter mitochondria. During fusion,


Trailing Edge
Leading Edge
Mitochondria


Leading Edge

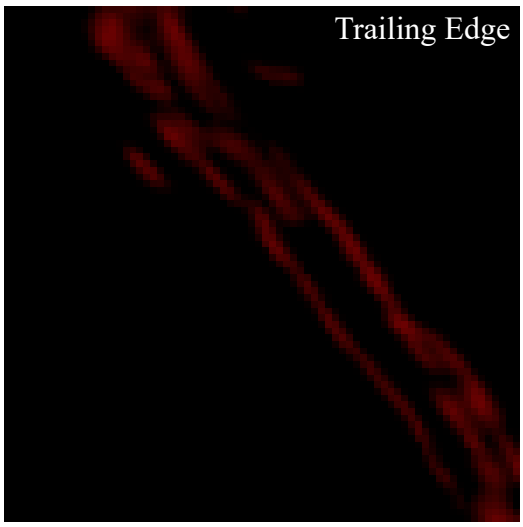
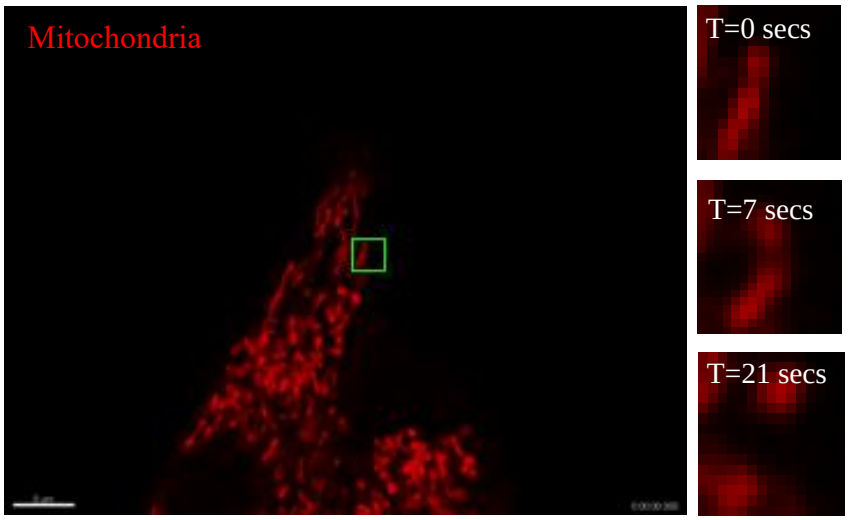
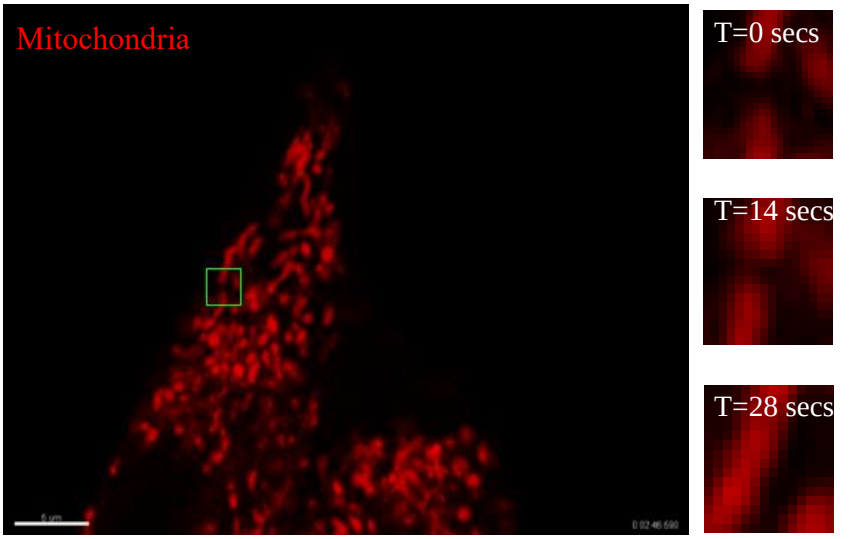

Trailing Edge

Figure 3.4: Mitochondria have different morphologies depending on the region of the cell. A) Mitochondria (red) moving to the leading edge are punctate and mitochondria in the trailing edge and perinuclear area are more tubular.

A


Mitochondria
T=0 secs
T=7 secs
T=21 secs

B


Mitochondria
T=0 secs
T=14 secs
T=28 secs

C

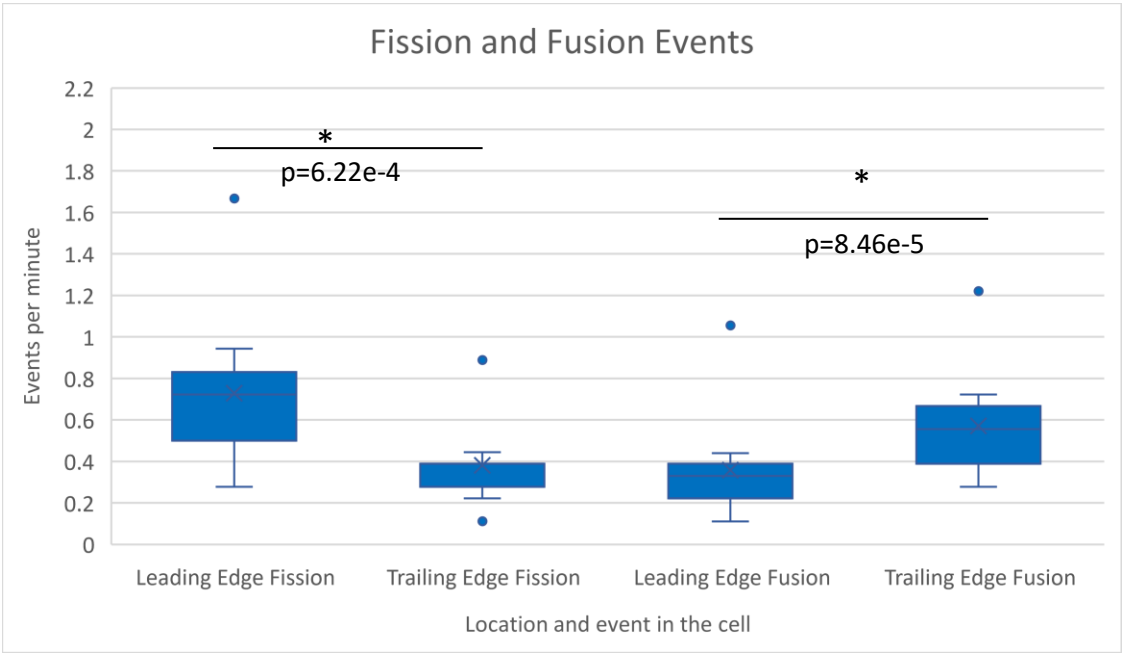

Fission and Fusion Events
Events per minute
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
p=6.22e-4
*
p=8.46e-5
Leading Edge Fission
Trailing Edge Fission
Leading Edge Fusion
Trailing Edge Fusion
Location and event in the cell

Figure 3.5: Mitochondria undergo more fission events in the leading edge. A) Going down the panels, mitochondria (red) undergo fission and become two separate mitochondria in the leading edge. B) Going down the panels, mitochondria (red) fusing with neighboring mitochondria in the leading edge. C) Quantitative analysis of fission and fusion events in the leading and trailing edge of the cell. Greater fission events per minute in the leading edge and greater fusion events per minute in the trailing edge. Stars indicate $p<0.0001$, one-tailed paired t test.

neighboring mitochondria get close to each other and create branches to pull neighboring mitochondria together to fuse (Figure 3.5B). Again, looking at the mitochondria in the green box we see two separate mitochondria. As time progresses the neighboring mitochondria migrate closer together. By the end of the fusion process at 28 seconds, these two individual mitochondria have completely fused and become one. When analyzing these figures, we scored the number of times a fission or fusion event occurred in the leading edge or trailing edge occurred per minute. The leading edge had an average of 0.73 fission events per minute and the trailing edge had an average of 0.38 fission events per minute (Figure 3.5C). Therefore, the greater number of fission events in the leading edge result in the short tubules or punctate mitochondria seen in the Figure 3.3. The leading edge had an average of 0.36 fusion events per minute and the trailing edge had an average of 0.57 fusion events per minute (Figure 3.5C). Therefore, the greater number of fusion events in the trailing edge result in the mitochondria being long tubules as seen in Figure 3.3.

3.5 Mitochondria interact with the ER and microtubules.

As stated earlier, mitochondria interact with several organelles. We identified focal adhesions as a new organelle however, a very noted organelle that the mitochondrion interacts with is the ER. These interaction sites have been called ER-mitochondria contact sites (ERMCS) and are the locations of mitochondrial dynamics, fission and fusion. Additionally, the mitochondria has been linked to using microtubules as a means to travel through the cell. We wanted to observe whether the mitochondria simultaneously interacts with the microtubules and the ER. In a fixed NIH3T3 cell, we stained the ER, mitochondria and microtubules and observed several contact sites between all three organelles (Figure 3.6). In this motile cell, we observe two contact sites (white arrowhead) between all three organelles before the mitochondria has undergone fission. On the

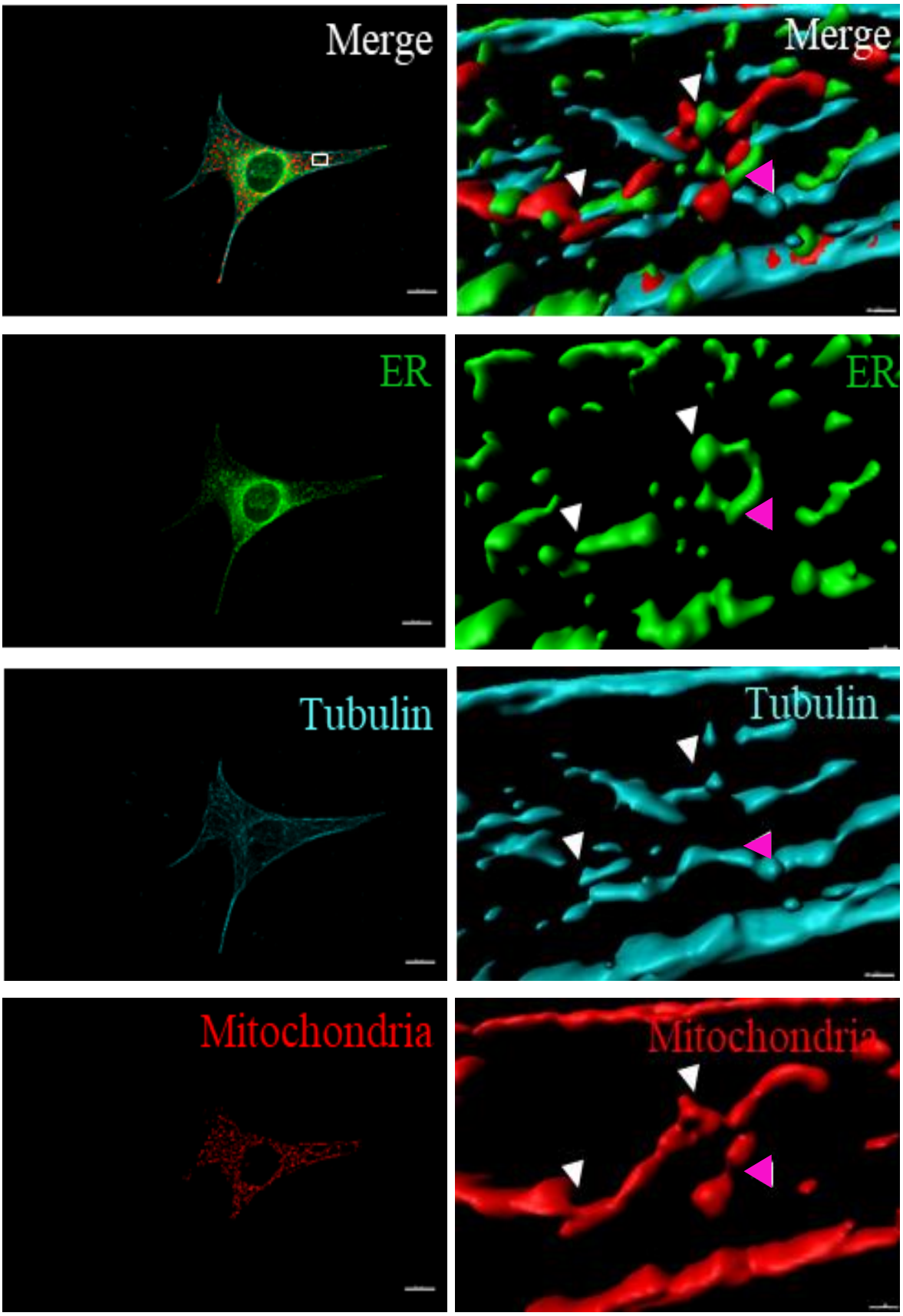
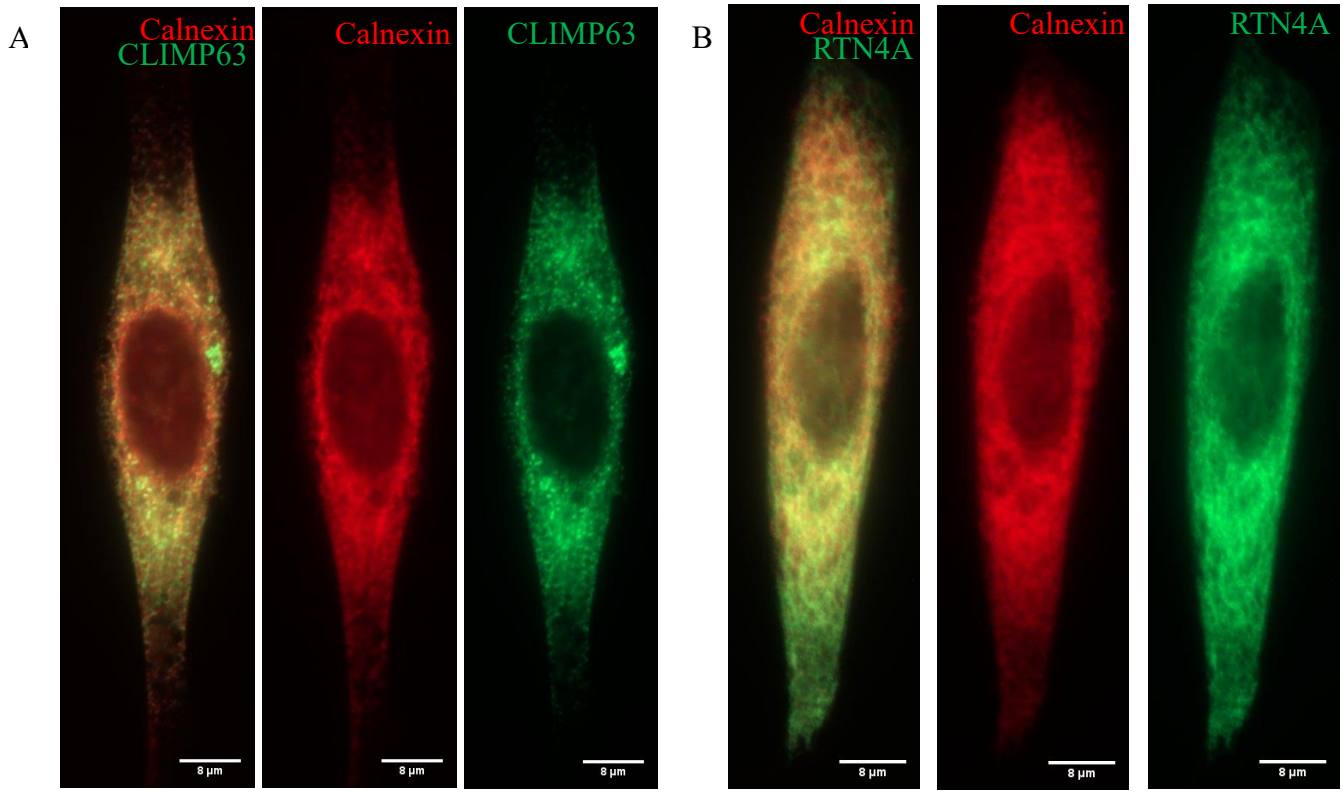

Merge
Merge
ER
ER
Tubulin
Tubulin
Mitochondria
Mitochondria

Figure 3.6: Mitochondria interact with the ER and the microtubules in protrusions. Fixed cell image of ER (green) and microtubules (teal) making contact with mitochondria (red) in various spots (white arrow heads) throughout the protrusion.

other hand we observe a contact site between all three organelles during mitochondrial fission (pink arrowhead). This has us believing that as the cell migrates, the mitochondria use microtubules as a path for their migration into the leading edge. During this migration, when the mitochondria interacts with the ER, mitochondrial fission or fusion can occur and the new mitochondria will continue to migrate along the microtubules to the leading edge.

3.6 Mitochondria dynamics and morphology are dependent on ER structure.

As mentioned above, the ER and mitochondria have several contact sites which result in fission and fusion events to occur. To further explore this relationship, we wanted to see whether altered ER structure changes mitochondria structure. Before observing the mitochondria, we needed to determine if the ER structure could be successfully altered in NIH3T3 cells. The ER has 2 structures: sheet-like (rough) or tubular (smooth). To observe these structures, we transfected our cells with CLIMP63, a transmembrane protein, or RTN4A, a reticulon protein involved in determining ER tubule to sheet ratio. Calnexin, a transmembrane protein that is involved in ER quality control, is used as a control for this experiment as it does not stabilize only one ER structure. NIH3T3 cells transfected with CLIMP63 stabilize the sheet-like ER (Figure 3.7A). The merge image shows that CLIMP63 is present throughout the entire ER by overlapping with calnexin however, separating the merge image shows that CLIMP63 successfully stabilized one ER structure and the calnexin image has varied ER structures. On the other hand, RTN4A stabilized the tubular structure of the ER (Figure 3.7B). Similar to CLIMP63, the merged in image of RTN4A and calnexin shows that RTN4A is present throughout the entire ER and has only stabilized one ER structure that is different from the ER structure seen by CLIMP63. After stabilizing, the sheet-like ER or the tubular ER we examined stained mitochondria through live cell imaging. We observed that cells transfected with CLIMP63 had long tubular mitochondria


A
Calnexin
CLIMP63
Calnexin
CLIMP63
B
Calnexin
RTN4A
Calnexin
RTN4A
8 µm

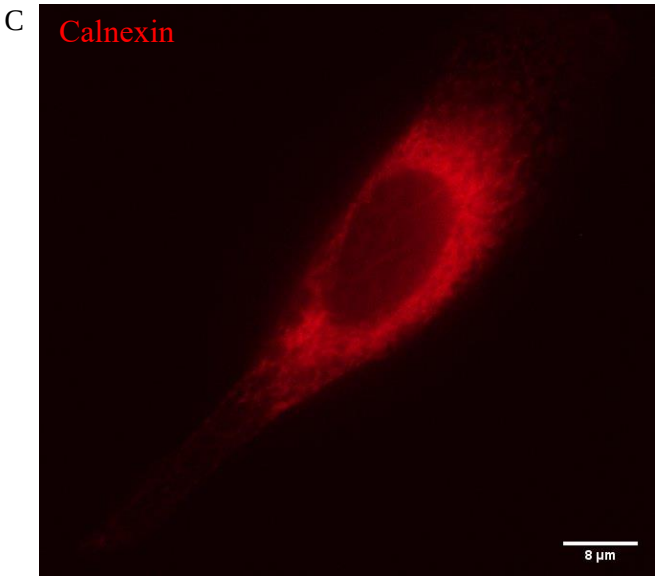

C
Calnexin
8 µm

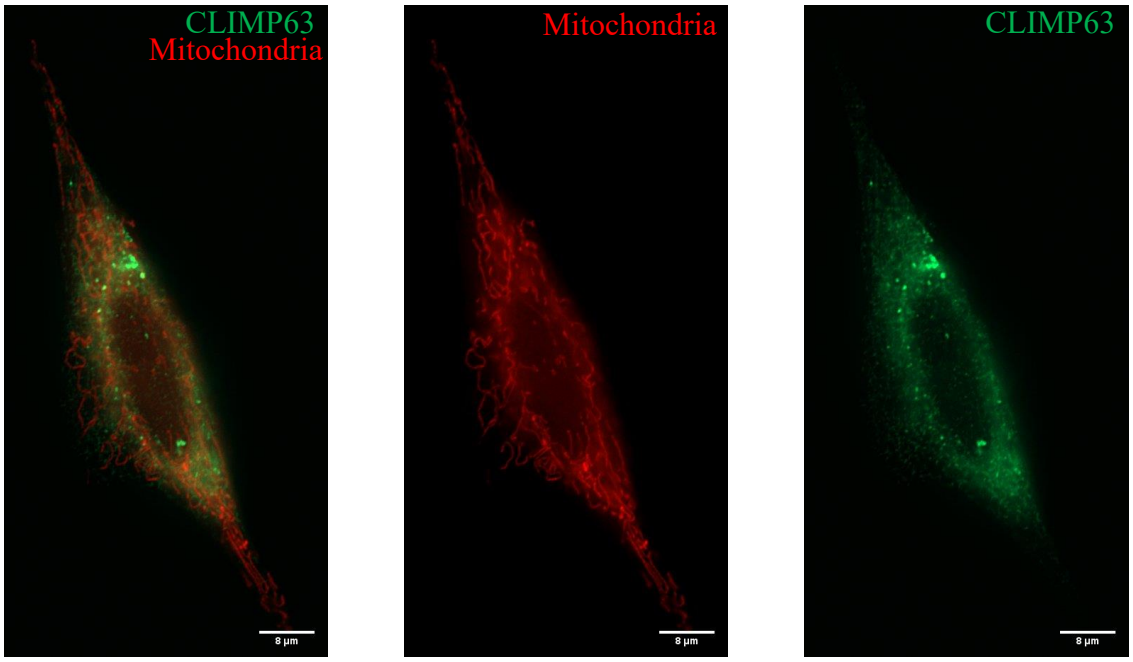
Figure 3.7: CLIMP63 and RTN4A alter ER structure. A) NIH3T3 cells were transfected with CLIMP63 (green) and calnexin (red) were stained. CLIMP63 stabilizes sheet-like structure of the ER. B) NIH3T3 cells were transfected with RTN4A (green) and calnexin (red) were stained. RTN4A stabilizes tubular-like structure of the ER. C) Calnexin stain in control NIH3T3 cells.

throughout the entire cell while cells transfected with RTN4A had short tubular mitochondria (Figure 3.8A-B). Using ImageJ mitochondria length was measured, showing that CLIMP63 have the longest mitochondria at an average of 2.34μm and RTN4A has the shortest mitochondria at 1.26μm when compared to control(non-transfected) cells at 1.85μm (Figure 3.8C). Therefore, ER structure alters mitochondria morphology. As we mentioned earlier, mitochondria morphology is a result of fission or fusion through ER contact so we wanted to determine whether fission or fusion rates were altered in our cells with altered ER structure causing the change in mitochondria lengths. Fission and fusion analysis was processed on these images similar to the analysis done on Figure 3.5. The number of fission and fusion events per minute were scored for CLIMP63, RTN4A and control non-transfected cells. Fission events per minute were similar in CLIMP63, RTN4A and control(non-transfected) cells (Figure 3.9A). On average CLIMP63 cells had an average of 4.15 fission events per minute, RTN4A cells 4.39 fission events per minute and control cells 4.4 fission events per minute. However, fusion events per minute were much larger in CLIMP63 cells than RTN4A and control (Figure 3.9B). On average CLIMP63 had 5.63 fusion events per minute, RTN4A had 2.89 fusion events per minute and control cells had 3.58 fusion events per minute.

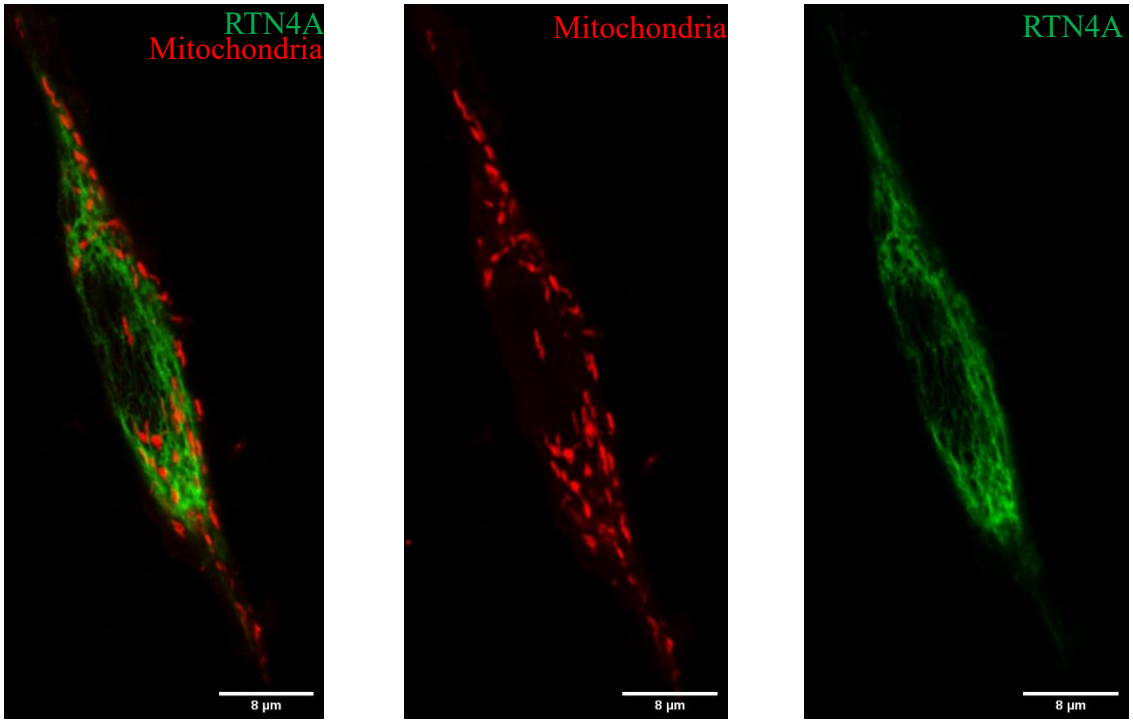
3.7 Altering ER structure and mitochondria morphology changes migration parameters.

As previously determined, altering ER structure altered mitochondria morphology. As mitochondria are necessary for cell migration, their altered structure could change cell migration parameters, such as total distance traveled, displacement, speed and angles turned. To explore whether these altered structures changed cell migration, we transfected CLIMP63 or RTN4A

A


CLIMP63
Mitochondria
Mitochondria
CLIMP63
8 µm
8 µm
8 µm

B


RTN4A
Mitochondria
Mitochondria
RTN4A
8 µm
8 µm
8 µm

C

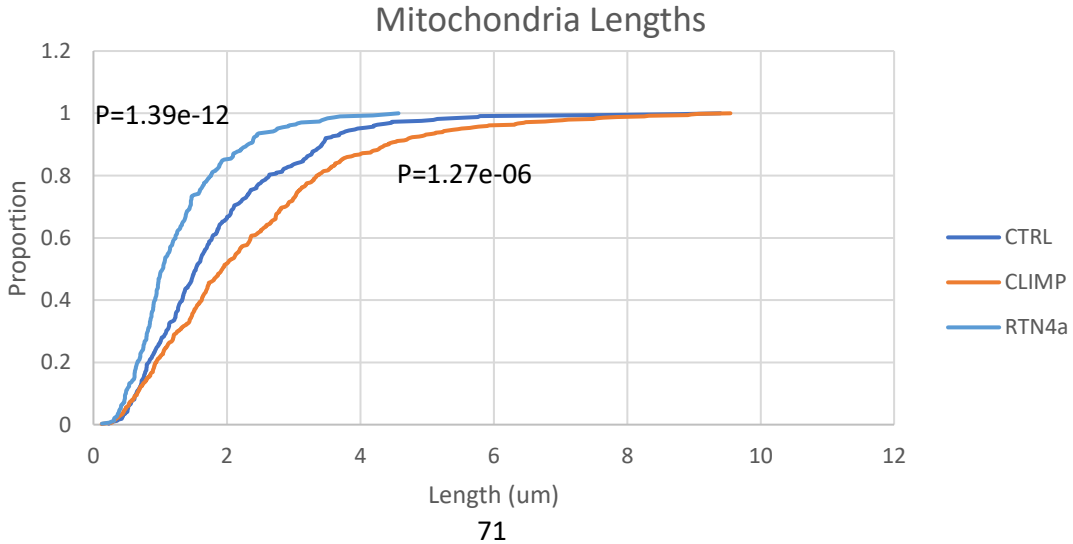
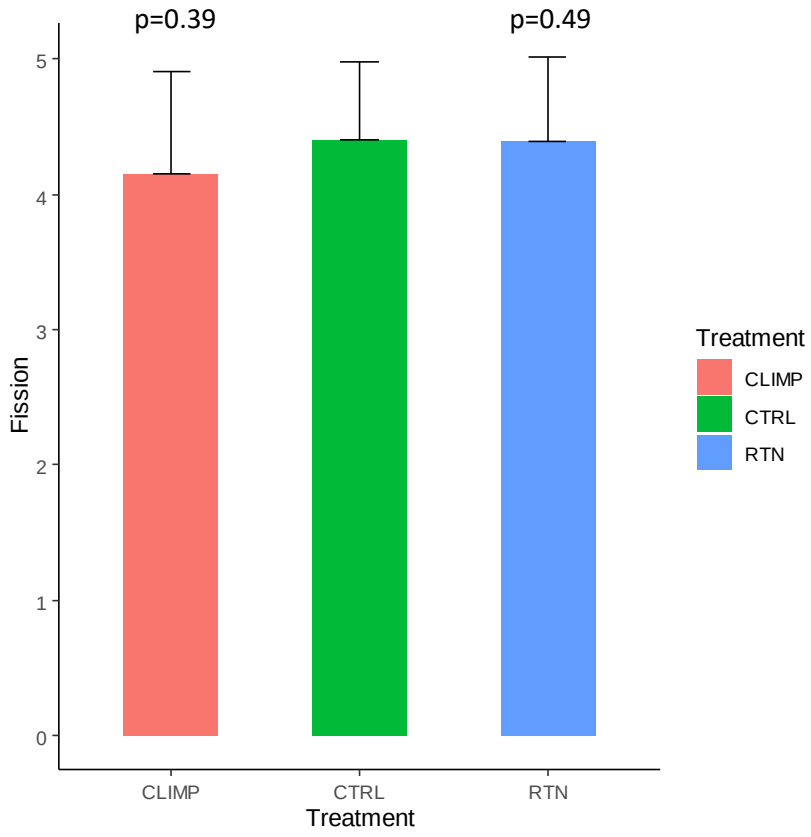

Mitochondria Lengths
1.2
1
0.8
0.6
0.4
0.2
0
Proportion
P=1.39e-12
P=1.27e-06
0
2
4
6
8
10
12
Length (um)
CTRL
CLIMP
RTN4a

Figure 3.8: Altered ER structure changes mitochondria morphology. A) Mitochondria (red) are short tubules when expressing RTN4A(green). B) Mitochondria (red) are long tubules when expressing CLIMP63(green). C) Quantitative analysis of mitochondrial lengths when ER structure was altered. Mitochondrial mean length is 17.6412um in CLIMP63 cells, 9.82905um in RTN4A cells and 13.4457um in control cells. Statistical significance found when comparing control cells with CLIMP63 and RTN4A, $p<0.0001$, one-tailed paired t test.

A


p=0.39
p=0.49
Fission
0
1
2
3
4
5
CLIMP
CTRL
RTN
Treatment
Treatment
CLIMP
CTRL
RTN

B

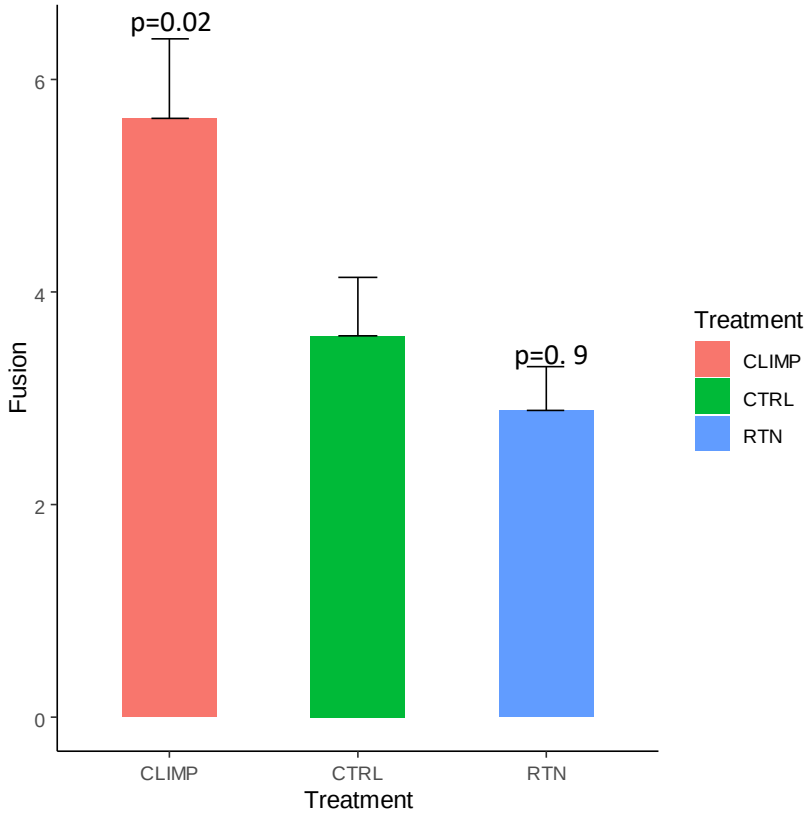

p=0.02
p=0. 9
Fusion
0
2
4
6
CLIMP
CTRL
RTN
Treatment
Treatment
CLIMP
CTRL
RTN

Figure 3.9: CLIMP63 has more fusion events than fission events. A) CLIMP63 has slightly less fission events compared to RTN4A and control cells. B) CLIMP63 has more fusion events compared to RTN4A and control cells.

into our cells and observed their free range migration over 72 hours. In CLIMP63 cells where the mitochondria are long tubules, cells had a smaller displacement of 5.12μm, travelled a shorter total distance of 15.99μm and had more turns that are less then 90˚ and between 180˚ and 270˚ (Figure 3.10). RTN4A cells where the mitochondria are short tubules, had a larger displacement of 6.04μm and travelled greater total distances of 16.95μm (Figure 3.10A and B). RTN4A cells had relatively similar proportions of angles turned when compared to control cell (Figure 10C). Additionally, cell speed did not change significantly between groups (Figure 3.10D). Altogether this highlights that ER structure and mitochondria structure have a role in cell migration. Although the changes in distance are not high the changes in displacement are statistically different implying that depending on the ER structure a cells path can be altered.

3.8 CLIMP63 cells make sporadic turns in a wound.

We observed our above changes in migration when the cells were in free range. It is known that cells migrate differently when in a wound so we were curious to determine if the observed changes in angles turned would also be seen in a wound migrating cell. Using a scratch maker, a wound was made, and cells transfected with CLIMP63 or RTN4A were monitored for 24hrs. The changes we observed in Figure 3.8C were similar to the results seen in the wound images. CLIMP63 cells were seen to sporadically change the angles turned while migrating into the wound (Figure 3.11A). The cell (white arrow) starts on the outside of the wound and orients itself to go forward. During the time course of this cell's lifespan, it changes direction and begins migrating towards the left and then changes direction to begin migrating to the right. On the other hand, RTN4A cells were observed to migrate in a straight line and make a 90 degree turn (Figure 3.11B). The RTN4A cell begins at the wound already oriented to move vertically and continues along this path in a relatively straight line. Eventually the cell makes a 90 degree turn

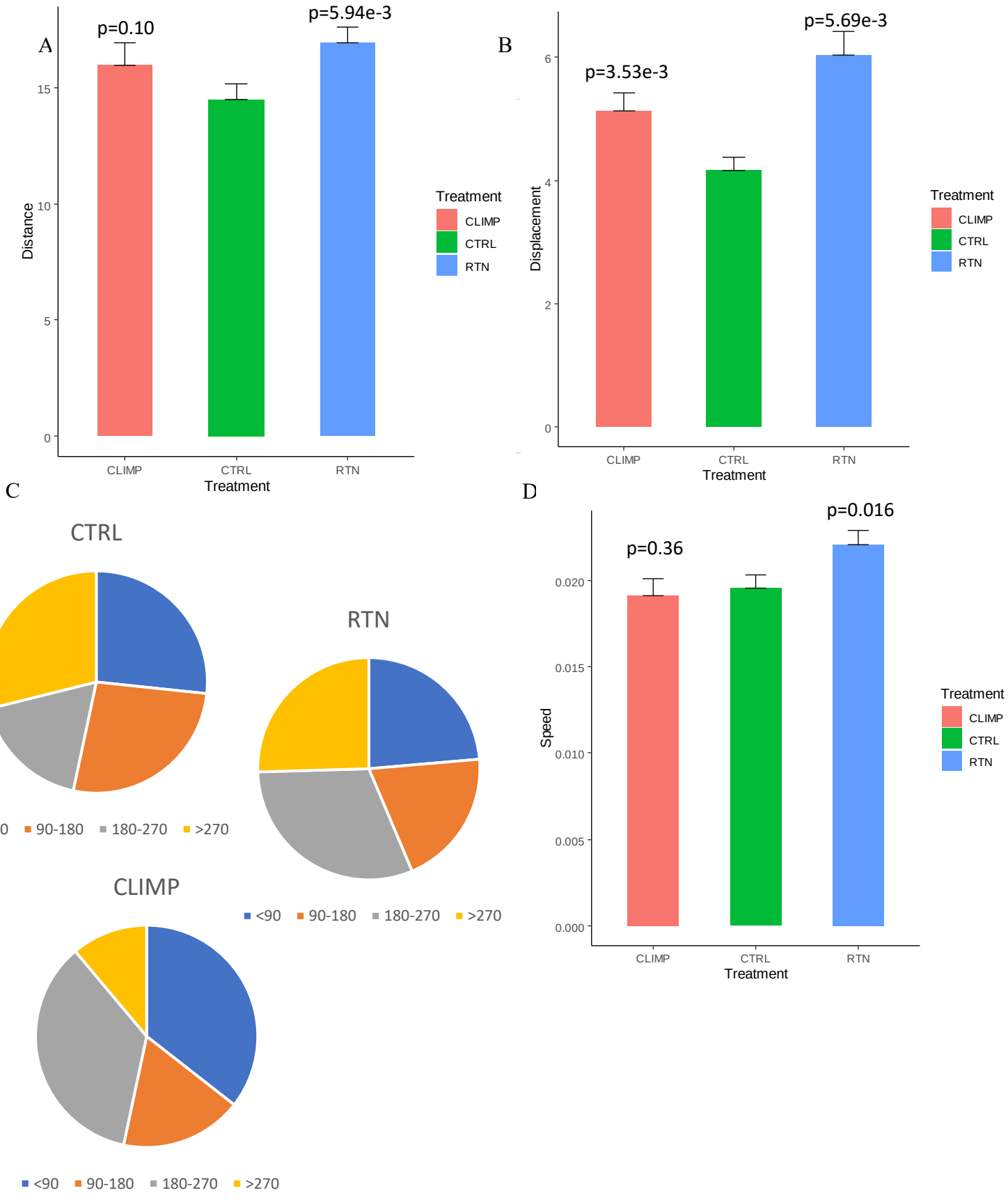
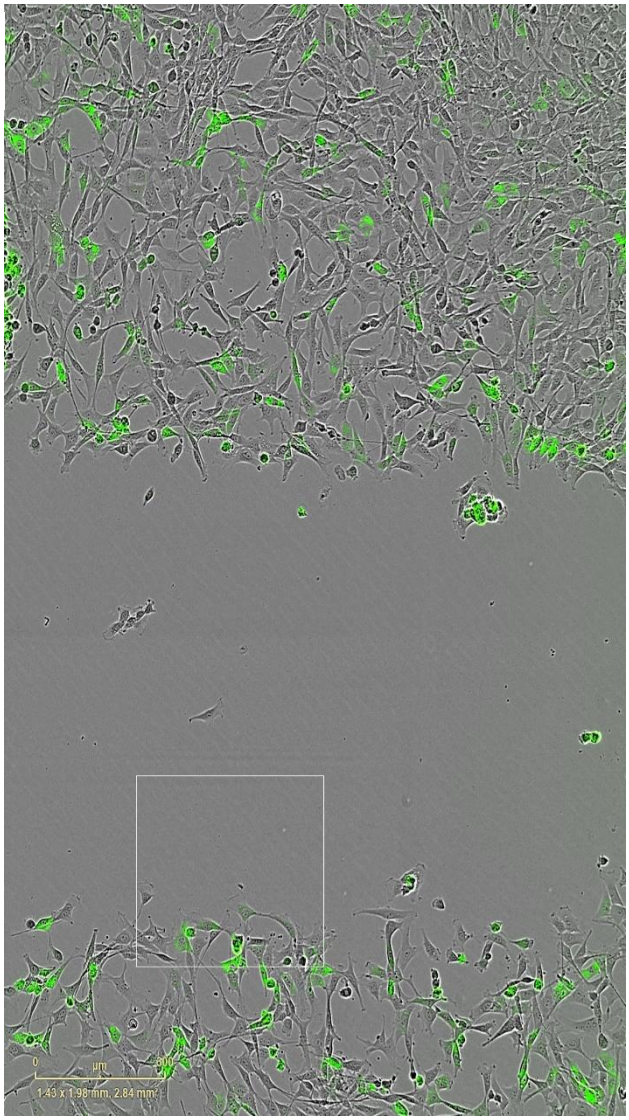
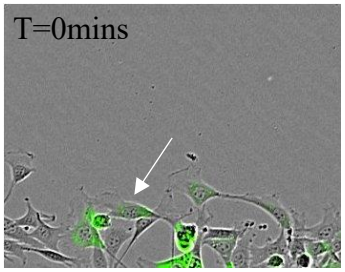
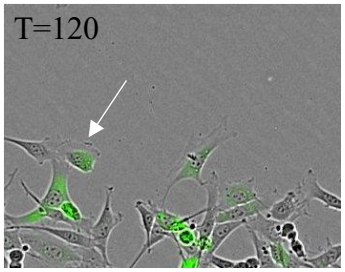

A
p=0.10
p=5.94e-3
Distance
Treatment
CLIMP
CTRL
RTN
B
p=3.53e-3
p=5.69e-3
Displacement
C
CTRL
RTN
CLIMP
<90
90-180
180-270
>270
D
p=0.36
p=0.016
Speed

Figure 3.10: Stabilizing the ER as either tubular or sheet-like alters displacement, distance and angles turned in migratory cells. Analysis of cells transfected with CLIMP63, RTN4A or non-transfected cells showing A) CLIMP63 travel shorter distances that control and RTN4A cells B) CLIMP63 cells have lower displacements than the control and RTN4A cells. C) CLIMP63 cells have more turns that are less than 90 degrees and between 180 and 270 degrees compared to control and RTN4A cells. D) Speed remained relatively similar between CLIMP63, RTN4A and control cells. Statistical significance found when comparing control cells with CLIMP63 and RTN4A, $p<0.01$, one-tailed paired t test.

A




T=0mins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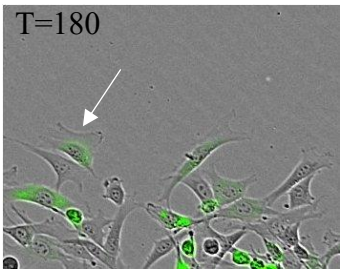
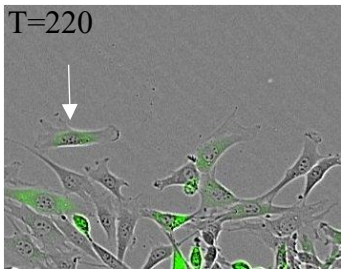
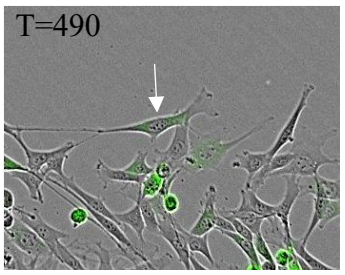
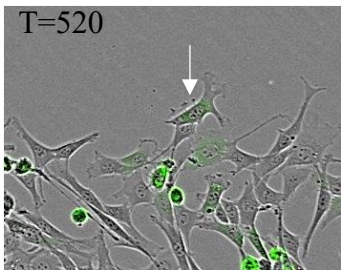
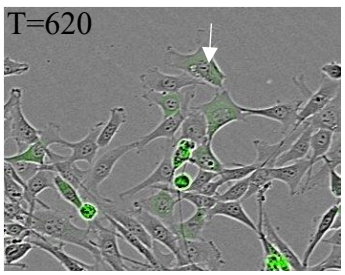
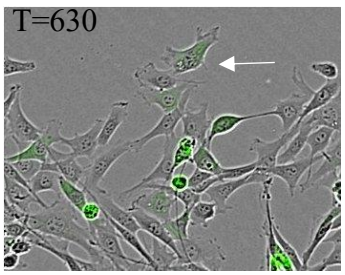

T=180


T=220

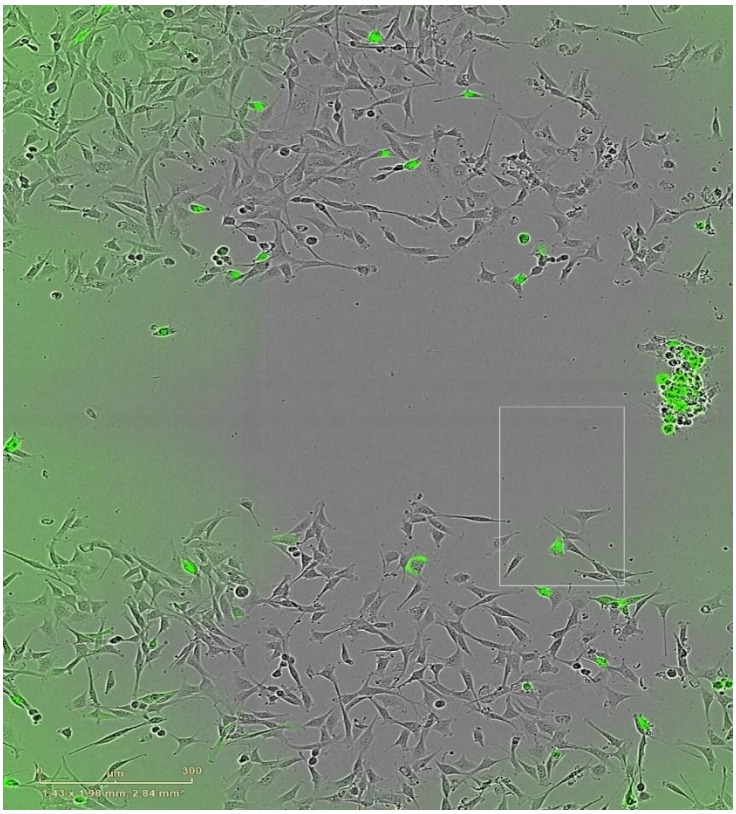

T=490

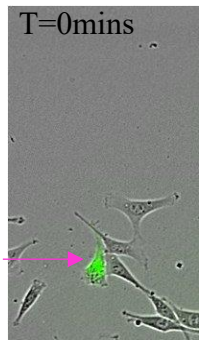

T=520


T=620


T=630

B

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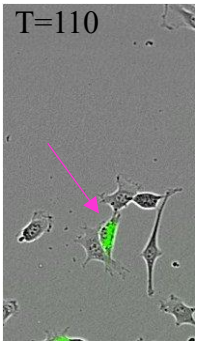
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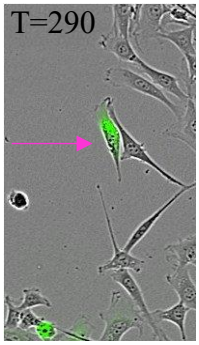
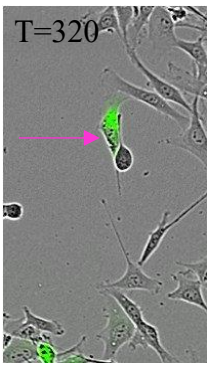

T=290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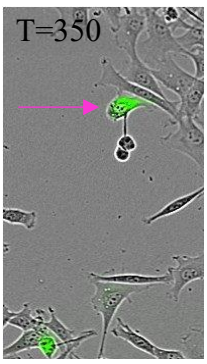
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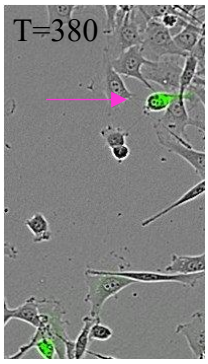

T=380

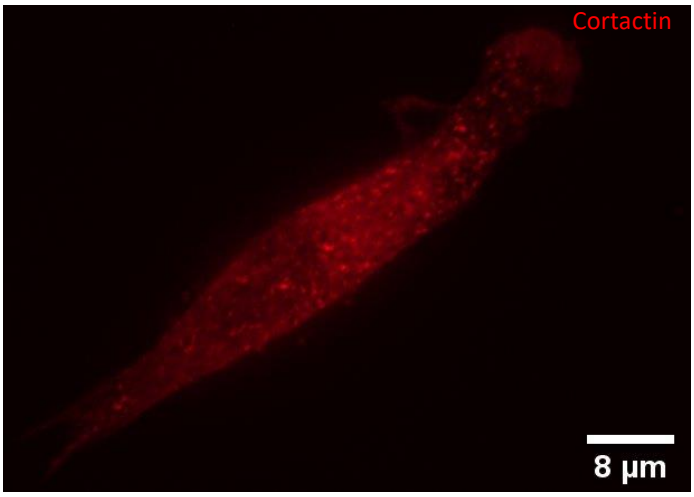
Figure 3.11: In wound healing experiment, CLIMP63 cells had sporadic turns while RTN4A had guided 90 degree turns. A) Time series of CLIMP63 cell (white arrow) starting at the edge of a wound and alternating directions twice. Starts moving forward then changes direction to go left then changes direction again to turn right. B) Time series of RTN4A cell (pink arrow) starting in a wound, migrating straight and making a 90 degree turn.

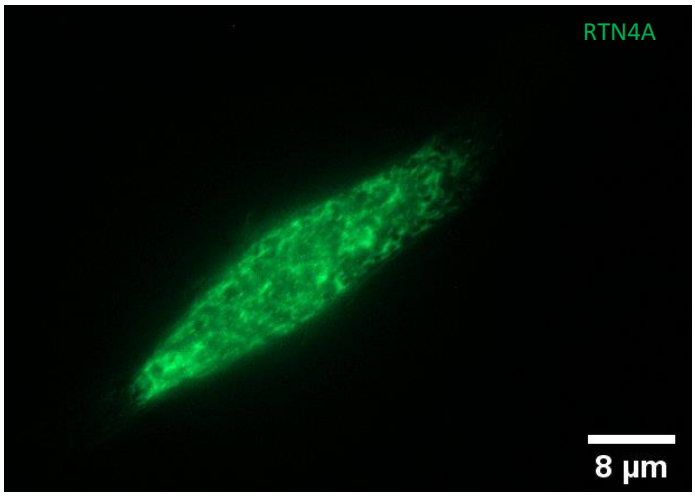
and begins travelling horizontally in a relatively straight path. Therefore, regardless of the environment CLIMP63 cells continue to have altered and disoriented turns compared to RTN4A and controlled cells.

3.9 Altering ER structure alters cell polarity.

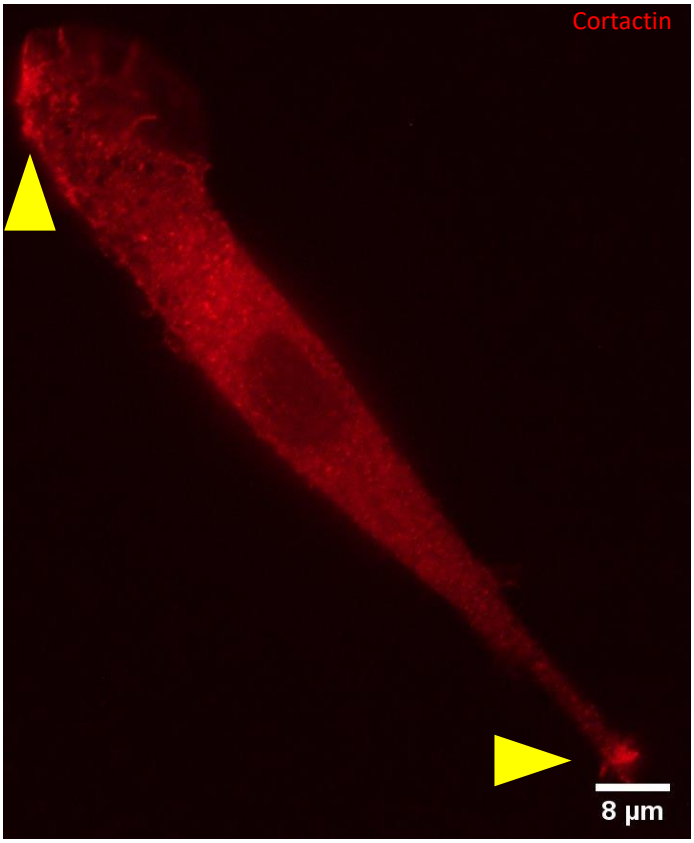
As mitochondrial and cell migration is regulated by polarity, and we previously saw that altering ER structure altered cell migration, we were curious to identify what other aspects of migration, such as polarity might be altered. Previously we had show polarization by transfecting our cells with cortactin, however rather than looking at overexpression we wanted to identify polarity in these experiments based on endogenous levels. We continued to transfect our cells with CLIMP63 or RTN4A and then following IF protocols stained for endogenous levels of cortactin, the biochemical maker used earlier. We observed non-polar cells as cells that did not exhibit a dense pigmentation of cortactin at the leading edge as seen in Figure 3.12A panel labelled Cortactin. On the same slides we observed polarized cells that exhibited the dense pigmentation (yellow arrowhead) at the leading edge (Figure 3.12B). After establishing our criteria of polarized and non-polarized, we scored the number of CLIMP63, RTN4A, GFP and control(non-transfected) cells that were either polarized or non-polarized. Proportionally, CLIMP63 cells were hyperpolarized compared to the other cells, explaining why these cells had obscure angles turned as they lack the ability to determine directionality (Figure 3.12C). On average CLIMP63 had 50.33% more polarized cells versus non-polarized, RTN4A had 34%, GFP had 24.61% and control(non-transfected) cells had 21.83%.

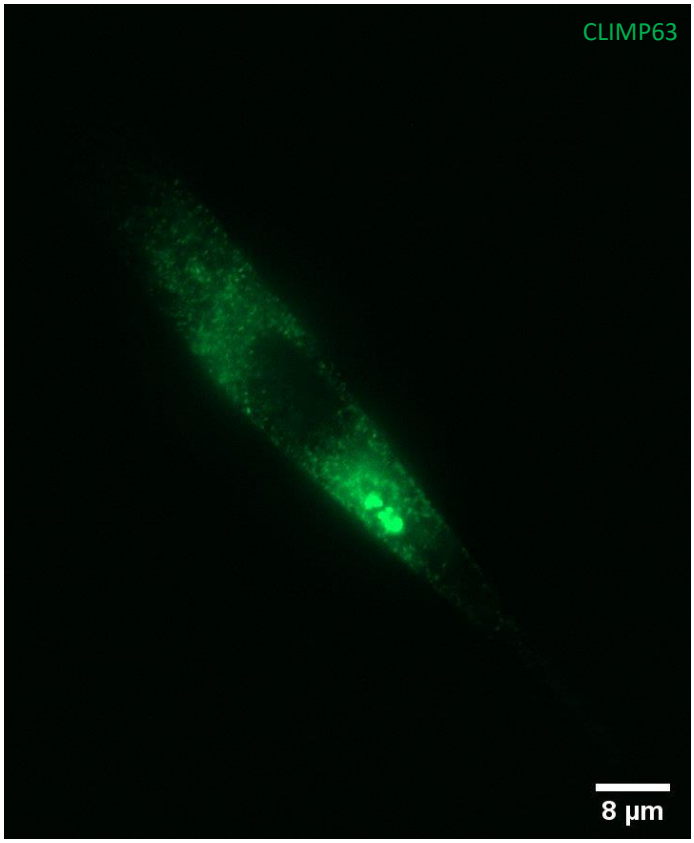
A


Cortactin
8 µm


RTN4A
8 µm

B


Cortactin
8 µm


CLIMP63
8 µm

C

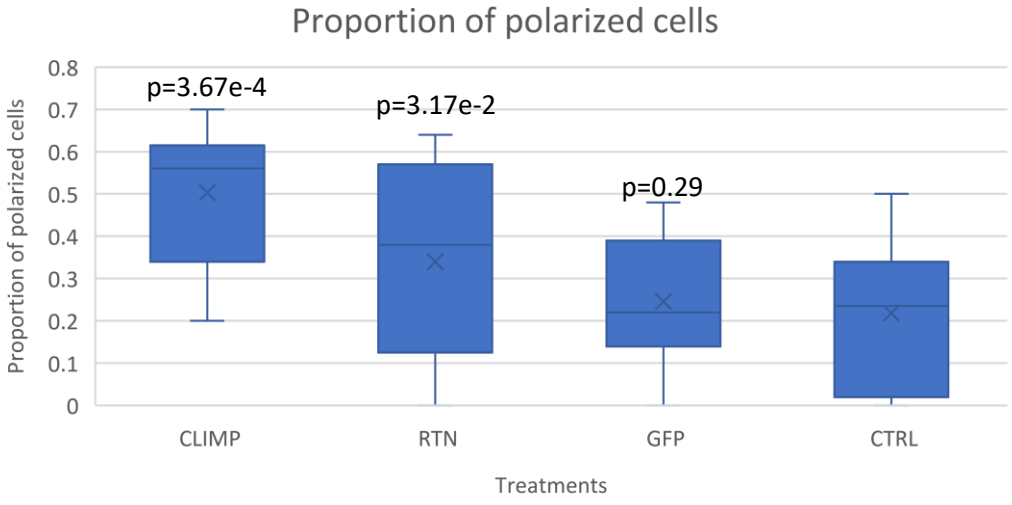
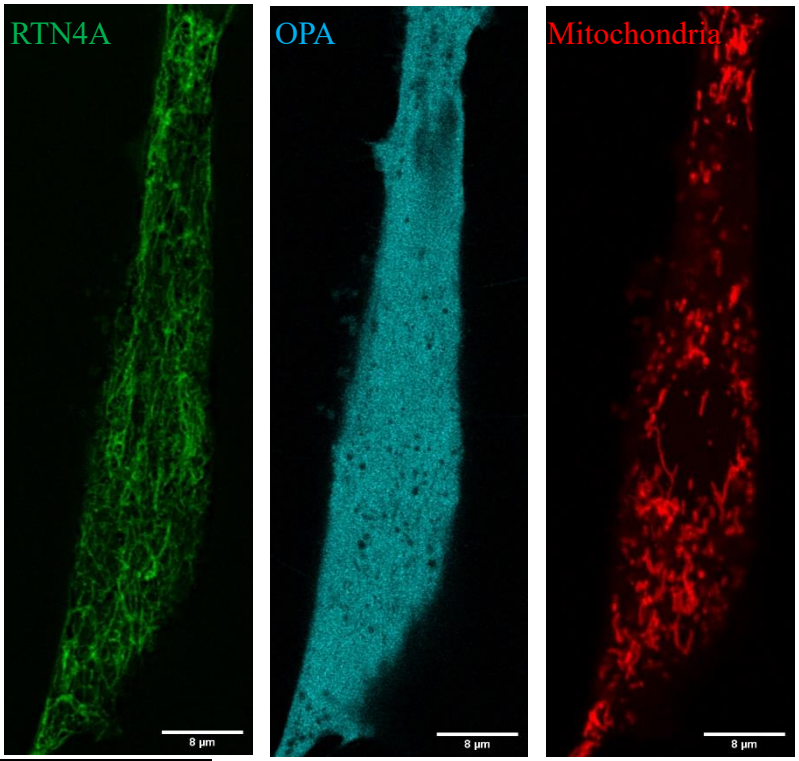

Proportion of polarized cells
Proportion of polarized cells
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
p=3.67e-4
p=3.17e-2
p=0.29
CLIMP
RTN
GFP
CTRL
Treatments

Figure 3.12: CLIMP63 cells are hyperpolarized. Representative images of A) non polarized and B) polarized cells in NIH3T3 cells transfected with CLIMP63. Polarized end indicated with yellow arrow head. C) Quantitative analysis of polarized cells in CLIMP63, RTN4A, GFP or control cells. Stars indicate $p<0.01$, one-tailed paired t test.

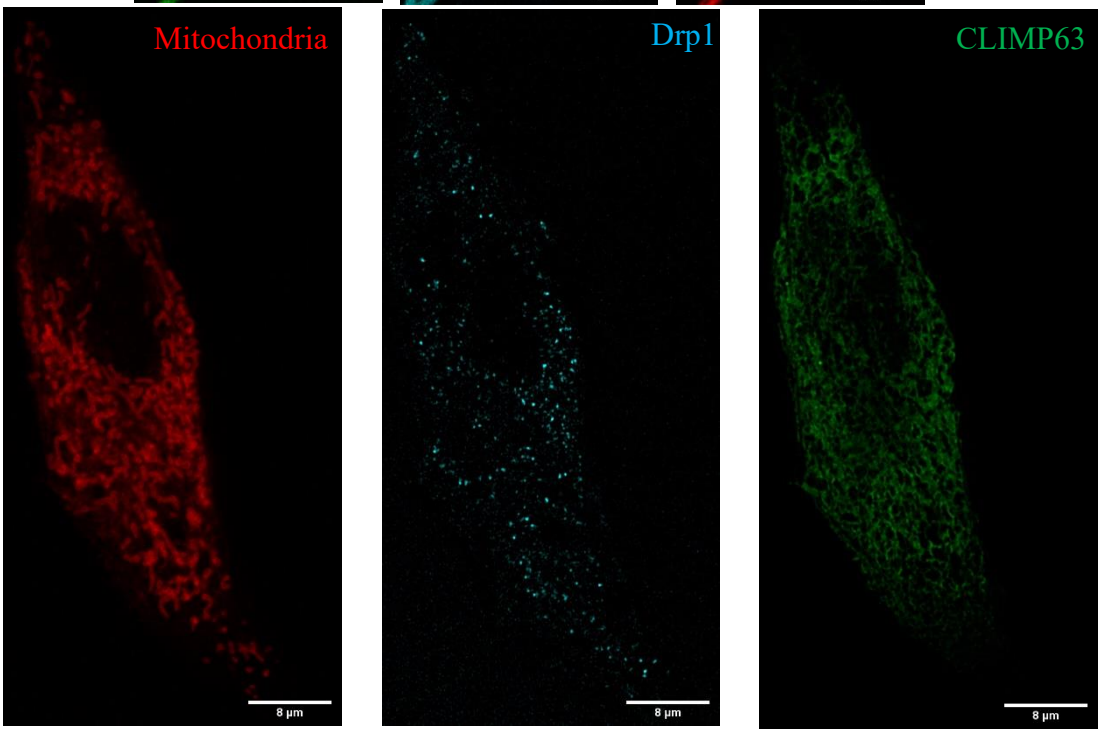
3.10 ER structure determines cell displacement and total distance travelled, while mitochondria structure determines angles turned.

Initially, we believed these changes in migration parameters were based on the relationship of the ER and mitochondria structure. However, we did not understand whether these changes were due entirely to the altered ER structure, only due to the mitochondria structure or if it was both of their structures working simultaneously to cause these changes. Therefore, mitochondria fusion and fission proteins were used to determine whether cell migration parameters changed because of the mitochondria shape. NIH3T3 cells were transfected with either OPA, a mitochondrial fusion protein, or drp1, a mitochondrial fission protein. First, we wanted to observe a change in mitochondria morphology using these fusion and fission proteins in our CLIMP63 or RTN4A cells. NIH3T3 cells were triple transfected with OPA, RTN4A and TOMM20, to see if OPA will overpower the RTN4A mitochondria phenotype or Drp1, CLIMP63 and TOMM20, to see if Drp1 would overpower the CLIMP63 mitochondria phenotype. The triple transfection with Drp1 had much smaller mitochondria than the original CLIMP63 cells and the OPA triple transfection had some mitochondria that were phenotypically longer than the original RTN4A cells (Figure 3.13A and B). We found mitochondria in RTN+OPA(RO) cells had an average length of 1.36μm and mitochondria in CLIMP63+Drp1(CD) cells had an average length of 1.18μm. Mitochondria in control cells had an average length of 1.85μm (Figure 3.13C). The mitochondria in the CD cells are much shorter than those in the original CLIMP63 and the mitochondria in the RO cells are longer than the original RTN4A mitochondria. This shows that the presence of these mitochondrial dynamic proteins overpowers the ER's ability to change mitochondrial morphology. This then allowed us to repeat our free range migration experiment to determine whether it is solely mitochondrial morphology that alters cell migration. When comparing cell

A


RTN4A
OPA
Mitochondria
8 µm
8 µm
8 µm

B


Mitochondria
Drp1
CLIMP63
8 µm
8 µm
8 µm

C

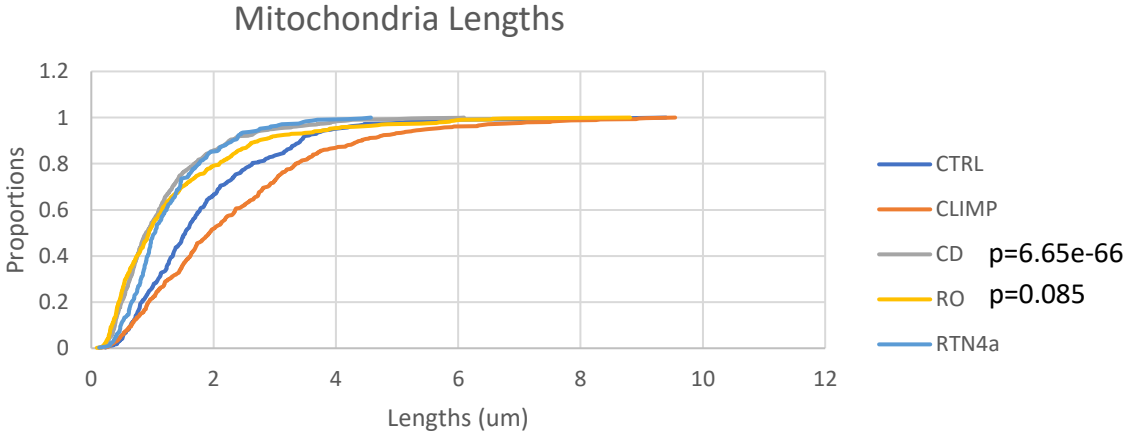

Mitochondria Lengths
Proportions
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
Lengths (um)
CTRL
CLIMP
CD
p=6.65e-66
RO
p=0.085
RTN4a

Figure 3.13: Mitochondria in CLIMP63+Drp1 and RTN4A+OPA cells have similar lengths to control cells. A) Live cell image of a NIH3T3 cell transfected with OPA (cyan), CLIMP63(green) and TOMM20(red). B) Live cell image of a NIH3T3 cell transfected with Drp1(cyan), CLIMP63(green) and TOMM20(red). C) RTN4a+OPA and CLIMP63+Drp1 cells have mitochondria lengths similar to each other and control cells.

migration parameters, it became clear that it was the ER structure that determined distance and displacement rather than mitochondrial morphology. Cells transfected with CLIMP63 and Drp1 favoured CLIMP63 wildtype parameters while cells transfected with RTN4A and OPA favoured RTN4A wildtype parameters rather than Drp1 and OPA wildtype respectively. CLIMP63+Drp1 cells had an average distance of 14.28μm and an average displacement of 4.99μm, Drp wildtype had an average distance of 14.90μm and an average displacement of 5.98μm, RTN4A+OPA had an average distance of 15.68μm and an average displacement of 5.79μm, and OPA wildtype had an average of 12.88μm and an average displacement of 4.83μm (Figure 3.14A and B). The displacement of CLIMP+Drp1(4.99μm) cells were closer to the displacement of CLIMP63 (5.12μm) cells and the displacement of RTN+OPA (5.79μm) cells was closer to the displacement of RTN4A (6.04μm) cells, therefore it is the CLIMP63 or RTN4A structure that determines this cell migration attribute rather than mitochondrial length. When comparing angles turned, the CLIMP63 phenotype was saved with the addition of Drp1(Figure 3.14D). We also observed that adding OPA to our RTN4A cells obscured the cells ability to have proportional turns. Based on this evidence, aspects associated to long mitochondria result in unproportionable angles turned therefor leading to the cells lack directionality. Altogether, this means that the ER structure is responsible for the cell's migration path in terms of distance and displacement, but the mitochondria structure is responsible for its directionality along this path. When comparing speed there were no differences between control and the 6 treatment groups (Figure 3.14C).

3.11 Cells with longer mitochondria take longer to turn.

As the cell requires time to make these turns, we wanted to determine if there were changes in the average time to make turns between 45 and 90 degrees, 91 and 180, 181 and 270 and greater

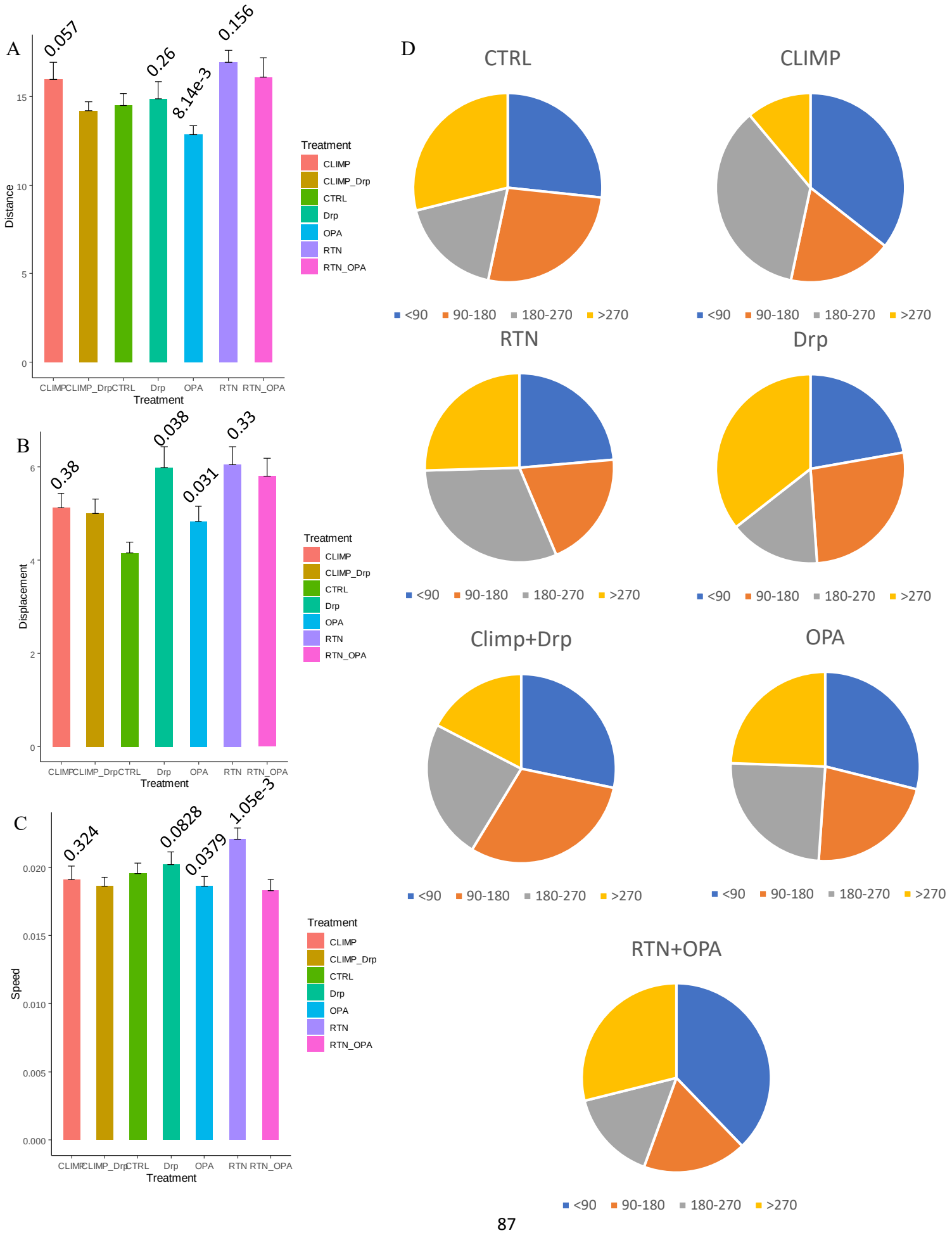
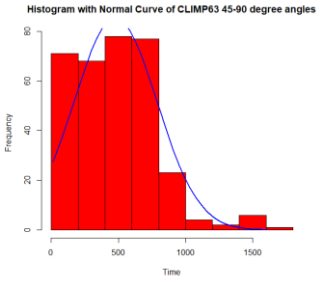

A
0.057
0.26
8.14e-3
0.156
Distance
Treatment
CLIMP
CLIMP_Drp
CTRL
Drp
OPA
RTN
RTN_OPA
B
0.38
0.038
0.031
0.33
Displacement
C
0.324
0.0828
0.0379
1.05e-3
Speed
D
CTRL
CLIMP
RTN
Drp
Climp+Drp
OPA
RTN+OPA
<90
90-180
180-270
>270

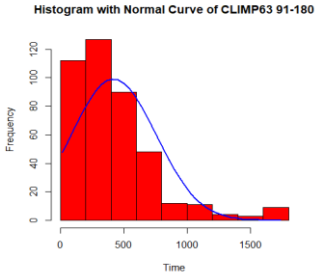
Figure 3.14: RTN4A+OPA and CLIMP63+Drp1 have similar distances and displacements to RTN4A and CLIMP63 respectively. A) RTN4A+OPA and CLIMP63+Drp1 have similar distances to RTN4A and CLIMP63 respectively. B) RTN4A+OPA and CLIMP63+Drp1 have similar displacements to RTN4A and CLIMP63 respectively. C) Speeds are similar in all cell groups and control cells. D) Angles turned for each cell group shows no significant changes.

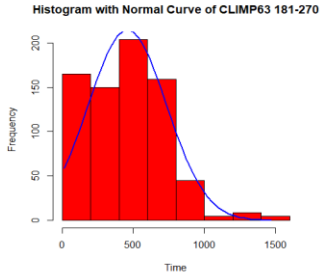
than 270. We found that cells with longer mitochondria, CLIMP63 and RTN+OPA, had longer average times in minutes to make these turns compared to control, RTN, OPA, Drp1, and CLIMP+Drp1. CLIMP63 cells take an average of 478.5minutes for 45 to 90 degrees turned, 419.13minutes for 91 to 180 degrees turned, 458.69 minutes for 181 to 270 degrees turned and 416.41minutes for greater than 270 degrees turned (Table 3.1). RTN+OPA took an average of 453.887 minutes for 45 to 90 degrees turned, 550.05 minutes for 91 to 180 degrees turned, 416.07 minutes for 181 to 270 degrees turned and 441.70 minutes for greater than 270 degrees turned (Table 3.1). We originally noted the cells with longer mitochondria had obscure angles turned causing the cells to have a lack of directionality. This trend is continued with these results to show that these cells take longer to determine in which direction to travel adding to their lack of directionality. This is also observed by the shape of the histograms. The histograms of the CLIMP63 and RO cells show more clustered bars times rather than a relatively even distribution of bars and had a longer x axis for time (Figure 3.15).

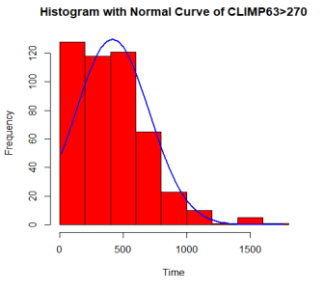
Table 3.1: Average times for each turn division in all cell groups. Climp63 and RTN+OPA cells had the longest times for each turn division.

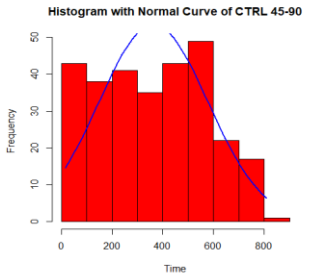
Treatment	45-90	91-180	181-270	>270
CLIMP	478.5	419.13	458.69	416.41
CTRL	363.218	325.158	466.146	409.359
RTN	433.849	139.167	235.909	230.422
RO	453.887	550.046	416.077	441.707
OPA	353.789	429.566	347.391	317.16
CD	384.491	415.675	411.966	356.763
Drp1	452.246	413.802	366.723	479.069

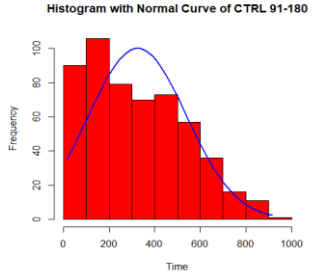

Histogram with Normal Curve of CLIMP63 45-90 degree angles
Frequency
Time

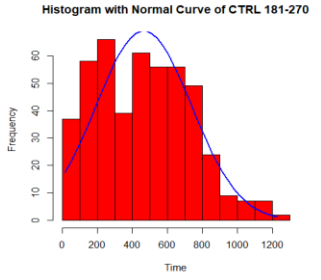

Histogram with Normal Curve of CLIMP63 91-180
Frequency
Time

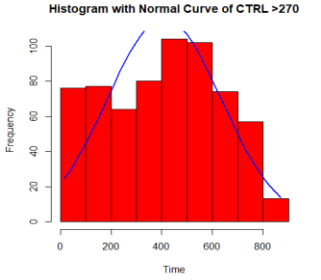

Histogram with Normal Curve of CLIMP63 181-270
Frequency
Time

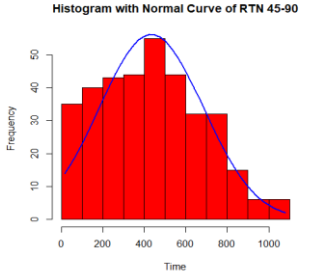

Histogram with Normal Curve of CLIMP63>270
Frequency
Time

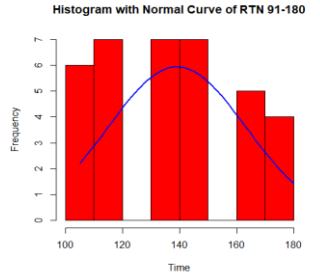

Histogram with Normal Curve of CTRL 45-90
Frequency
Time

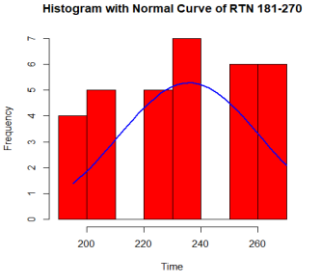

Histogram with Normal Curve of CTRL 91-180
Frequency
Time

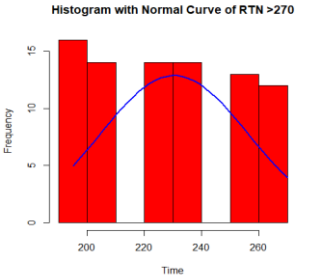

Histogram with Normal Curve of CTRL 181-270
Frequency
Time

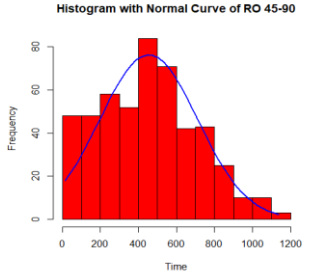

Histogram with Normal Curve of CTRL >270
Frequency
Time

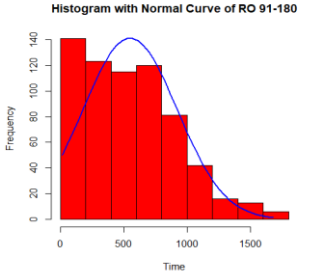

Histogram with Normal Curve of RTN 45-90
Frequency
Time

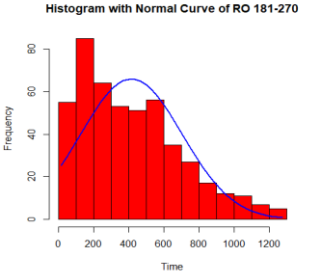

Histogram with Normal Curve of RTN 91-180
Frequency
Time

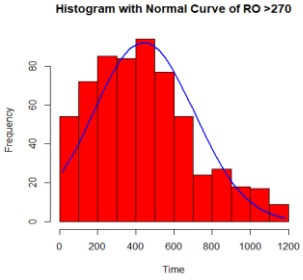

Histogram with Normal Curve of RTN 181-270
Frequency
Time

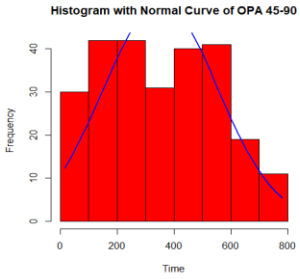

Histogram with Normal Curve of RTN >270
Frequency
Time

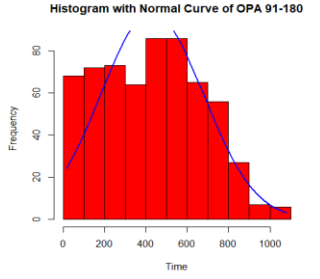

Histogram with Normal Curve of RO 45-90
Frequency
Time

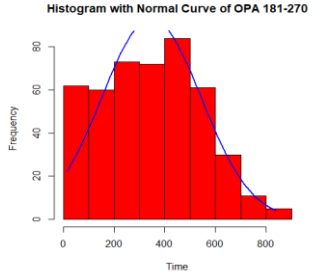

Histogram with Normal Curve of RO 91-180
Frequency
Time

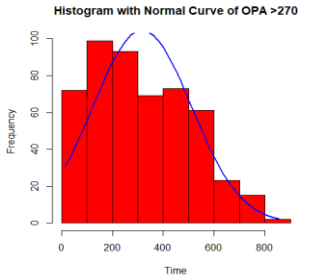

Histogram with Normal Curve of RO 181-270
Frequency
Time

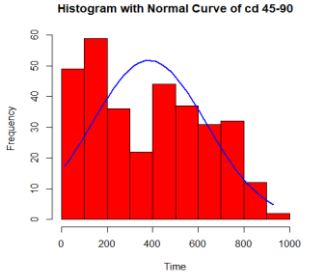

Histogram with Normal Curve of RO >270
Frequency
Time

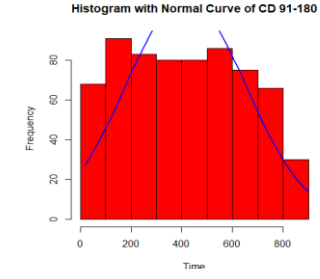

Histogram with Normal Curve of OPA 45-90
Frequency
Time

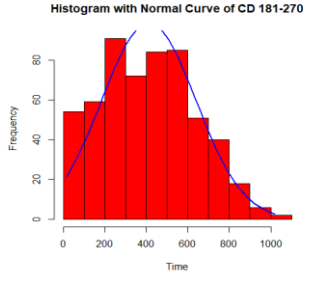

Histogram with Normal Curve of OPA 91-180
Frequency
Time

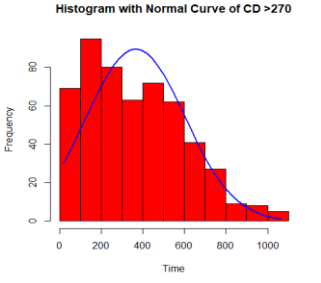

Histogram with Normal Curve of OPA 181-270
Frequency
Time

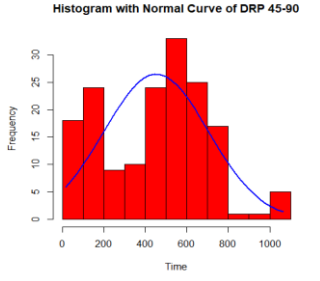

Histogram with Normal Curve of OPA >270
Frequency
Time

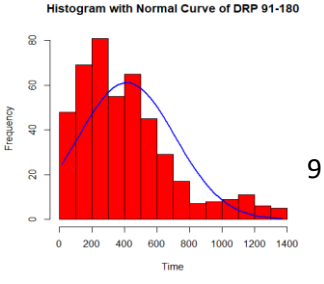

Histogram with Normal Curve of cd 45-90
Frequency
Time

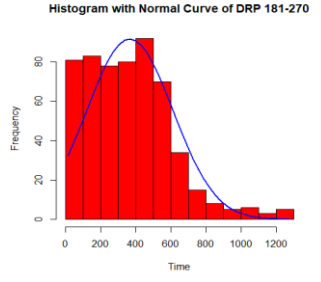

Histogram with Normal Curve of CD 91-180
Frequency
Time


Histogram with Normal Curve of CD 181-270
Frequency
Time


Histogram with Normal Curve of CD >270
Frequency
Time


Histogram with Normal Curve of DRP 45-90
Frequency
Time


Histogram with Normal Curve of DRP 91-180
Frequency
Time


Histogram with Normal Curve of DRP 181-270
Frequency
Time

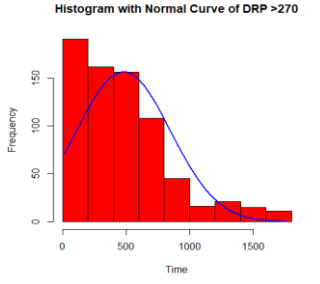

Histogram with Normal Curve of DRP >270
Frequency
Time

Figure 3.15: Long mitochondria mechanisms (RTN+OPA and CLIMP63) cells take on average longer to make turns than shorter mitochondria cells. Histogram with normal distribution function of CLIMP63, RTN4A, control, Drp1, OPA, CLIMP+Drp1 and RTN+OPA for angles turned between 45 and 90, 91 and 180, 181 and 270, and greater than 270. Showing CLIMP63 and RTN+OPA have the longest times and longest x-axis movement.

Chapter 4: Discussion

In this study, we establish that the mitochondria and the ER are simultaneously working together to regulate migration. We found that the mitochondria move into the polarized leading edge and tend to be fragmented. This is consistent with the finds of Cunniff et al 2016. It is interesting to note that the mitochondria changed directions when the cell changed directions. We believe that mitochondria are the drivers of cell migration however, based on Figure 1C both the cell and mitochondria respond to stimuli to direct their migration. Once the mitochondria have fluxed into the leading edge, they continue to drive the cell forward and interact with other organelles along the way. The work done by our summer student Abey Mengeta, highlights that the mitochondria interacting with focal adhesions may be an additional route of mitochondria regulating cell migration. As focal adhesion binding and unbinding are necessary for the movement of a cell, seeing the mitochondria interacting with these organelles brings the prospect of mitochondria regulating the unbinding/binding. The effects of oligomycin, an ATPase inhibitor, on focal adhesion size strengthens the idea that mitochondria are involved in FA unbinding/binding. We found that with various concentrations of oligomycin, FA size decreased and FA number remains unaltered. By decreasing the size of FA it is believed that FA strength is decreased, therefore mitochondria's metabolic properties are controlling the unbinding and binding of FA necessary for cell migration (Stricker *et al* 2011). FA size is also related to cell migration speed. It has been found that when FA size increases cell speed increase until it reaches an optimum speed (Kim and Wirtz 2013). By mitochondria affecting FA size it is indirectly controlling the speed in which the cell travels.

As mitochondria are dynamic organelles, we wanted to observe whether the changes in mitochondria shape in the leading edge (LE) and trailing edge (TE) were based on dynamics. We

found that the LE had more fission events per minute and the TE had more fusion events per minute consistent with our findings in Figure 3.3. Additionally, mitochondria dynamics are related to the association of the ER so we were curious to determine whether the altered mitochondria morphologies were related to mitochondrial dynamic changes. Interestingly, we found that CLIMP63 had the highest fusion events per minute and still experienced fission events but at a lower rate. This is the opposite of what we were expecting as we did not observe many mitochondria in the CLIMP63 cells that had undergone fission. Studies have shown that fission and fusion proteins are both found at ER and mitochondria contact sites (Moletdo *et al* 2019). This may explain why there are still mitochondrial fission products present in CLIMP63 cells as the machinery is still present at the contact sites. Therefore, CLIMP63 may not lead to an increase in Mfn1/2, OPA or other fusion proteins but there might be a pathway involved that leads to the use of fusion proteins at these contact sites more often than the fission ones leading to the long tubular mitochondria seen. Studies are still investigating why fission or fusion is favoured at a given time.

Fragmented mitochondria are most often seen when the cell is stressed or damaged (Zorov *et al* 2019). Throughout these experiments we maintained a healthy cell culture to ensure that the cells would not be stressed, however the RTN4A cells had mitochondria morphologies similar to stressed mitochondria. Although we looked at fission and fusion levels in RTN4A cells we weren't able to associate fission and fusion levels to the observed mitochondria morphology and explain why RTN4A caused the cells to be fragmented. As mentioned earlier fragmented mitochondria are signs of stress and in these situations the mitochondria would undergo mitophagy to remove any damage (Zorov *et al* 2019). However, these cells did not experience higher than normal cell death and they were able to function without any implications of damage.

It is believed that fragmented mitochondria ensure the survival of mitochondrial DNA through the distribution of DNA copies over isolated fragments (Zorov *et al* 2019). This could be a potential explanation as to why the fragmented mitochondria in RTN4A cells were not marked for mitophagy and were able to function normally. Zorov *et al* believe that the most important aspect of mitochondria function may be the maintenance of the stability of mtDNA, which supports the notion that the RTN4A mitochondria may have been a marker of a stressed cell but since it had maintained the stability of mtDNA, mitochondria functionality and indirectly cell migration remained unaltered.

The premise of this project was to better define the relationship of the ER and the mitochondria during cell migration. Based on the cell migration changes seen in displacement and distance we believed that by altering ER structure to alter mitochondria morphology, cell migration was controlled. Initially we found that CLIMP63 had long tubular mitochondria which resulted in shorter displacement and shorter total distances travelled by the cell. CLIMP63 cells also had more turns in the 180 to 270 degree bracket, displaying more of a drastic change in direction. This led us to believe that when the ER is rough and mitochondria are long tubules, the cells have an impaired ability to migrate. As polarization is necessary for cell migration, we looked at endogenous levels of cortactin and found that CLIMP63 were hyperpolarized compared to the other cell groups. This was consistent with the idea that CLIMP63 cells were overstimulated and could not determine a direction to migrate. This led to the many 180 to 270 degrees turned and the shorter displacement and distances. As free range cells do not have a specific stimulus, a wound experiment was used to determine whether the directionality issue was still present. The same observation was seen that CLIMP63 cells in a wound continue to migrate back and forth, lacking the ability to pick a direction and continue to migrate in that lane. We concluded these

experiments believing that the ER was driving the changes in cell migration as in its altered state it changed mitochondria structure and cell migration abilities.

Although we believed that it was the ER driving the changes in cell migration, we needed to test whether it was ER or mitochondria morphology altering cell migration. We monitored cell migration parameters in the presence of mitochondria dynamics proteins, Drp1 and OPA. Here we found that the ER and mitochondria regulate different aspects of cell migration. Initially we believed that by adding Drp1 to CLIMP63 cells we would observe cell migration parameters similar to RTN4A wildtype and by adding OPA to RTN4A cells we would observe similar results to CLIMP63. However, we found that adding Drp1 or OPA didn't alter the cell migration parameters as we expected. We observed that the wildtype Drp1 and OPA had phenotypes that were rescued through the addition of CLIMP63 or RTN4A for displacement and total distance travelled. Adding Drp1 to CLIMP63 saved the cells from making the drastic 180 to 270 degrees turned as observed before. The addition of Drp1 allowed the cells to have a nearly even distribution of angles turned and it decreased the amount of time it took the cell to make any turn. Adding OPA resulted in RTN4A cells to have similar turn patterns observed in CLIMP63 wildtype cells and a longer average of time to make turns. This meant that the longer the mitochondria were the more it lacked directionality. Therefore, we conclude that the ER morphology determines how far the cell will travel but the directionality is determined by the mitochondria length.

Another idea as to why we see changes in directionality could be based off of surface area. Mitochondria are considered to have an ellipse structure so the formula for surface area is $a*b*\pi$. Variables a and b are considered to be units of length, which are the major and minor radius. For this purpose of this idea we will define a as the length observed in our study, therefore the value

of *a* changed whether CLIMP63 or RTN4A was expressed. Qualitatively, the mitochondria did not seem to have a change in the other possible length, *b*. Based on this observation, it is fair to believe that CLIMP63 cells have larger surface areas than RTN4A and control cells. This larger surface area could be the reason why the cells lack directionality as the signalling pathways would be affected and cross talk in polarization from the front and back of the mitochondria could also be affected. I believe that the signalling pathways in the mitochondria are potentially slower moving because the pathway must elicit a signal to the front of the mitochondria that is physically further away. This distance may require the signal to take more time to travel causing the mitochondria to release a signal to the cell at a slower rate as well. By taking longer for the signal to travel to the front of the mitochondria, the cell is left in a confused state unable to make a turn resulting in the longer times it takes for CLIMP63 cells to make any turn. This can also be used to explain why the RTN+OPA cells also had on average longer times to make turns as well. Therefore, it is possible that the longer the mitochondria the slower the signalling pathways work to issue a response to turn causing the cells to take longer to make any turn. Mitochondria naturally are polarized organelles. It is possible that the hyper polarization seen in CLIMP63 cells could be attributed back to surface area as well. As mentioned before, I believe that CLIMP63 cells have a larger surface area. This increase in surface area could translate to an increase in polarization signals being released. As now the cell has more polarized cellular masses its possible this can act to stimulate more polarization responses in the cells.

A key aspect in polarization and signalling is also the cross-talk between the front and the back of the mitochondria. I believe that the cross-talk between the front and the back of the cell necessary for migration is also present in mitochondria because they both share a polarized state. The cross-talk between the front and the back of the mitochondria, like the overall cell, relies on

the signalling pathways and polarization. If the surface area affects these aspects, it is possible that surface area affects the cross-talk as well. This cross-talk can result in the mitochondria responding to changes slower because the cross-talk must also travel a larger distance in CLIMP63 cells. This can explain why the CLIMP63 cells had shorter displacements and distances as the mitochondria did not experience instantaneous communication from the front and the back to cause the cell to respond and continue its migration properly. Rather the cell might have travelled shorter distances between each step because there was a lag in the communication from the mitochondria to the cell to direct it where to migrate. The opposite is seen when the mitochondria is shorter. In RTN4A cells, the mitochondria were much shorter than CLIMP63 and control cells. This decrease in surface area can allow the cross-talk to occur at a faster rate so the mitochondria was able to direct the cell to make larger steps at a faster rate, resulting in the increased displacement and distance. This is an interesting finding/possibility but also a problematic one as well. Numerous studies have highlighted the importance of the mitochondria in cancer cell survival. In my opinion this cross-talk based on surface area/length can highlight a new aspect of the mitochondria being involved in metastasis particularly in RTN4A cells. Cancer cells have the ability to exploit any mechanism available to them in their host. If the cancer cells are able to exploit the ER structure to make the mitochondria shorter, they are creating an opportunity for them to increase their migration abilities. I believe that cancer cells that have manipulated the cell to be the RTN4A ER structure to have the shorter mitochondria lengths, thereby having a decreased surface area will cause there to be a quicker cross-talk experience in the mitochondria. This quicker cross-talk in the mitochondria will then cause the cancer cell to have a quicker response as to where to travel allowing them to migrate from their primary sites faster. As mentioned before a large issue in metastasis is the toxic

environment of the bloodstream. I believe these RTN4A cells have the ability to withstand the toxicity of the bloodstream based on the mitochondria morphology as well. At first glance, RTN4A cells can have punctate mitochondria which is characteristically found in cells that are stressed or when mitochondria have been damaged, thereby resulting in mitophagy and later on potentially apoptosis. However, while conducting these experiments there was no indication that the RTN4A cells were unhealthy or triggering mitophagy. This had me wondering if the cell started to recognize mitochondria that were exhibiting stress phenotypes and then did the cell begin to start stress response mechanisms that ensured the survival of the cell and indirectly the survival of the mitochondria and preservation of the mitochondria shape. If this is occurring than it is possible that this response would be occurring constantly throughout the cell as the mitochondria structure does not change throughout the lifespan of the cell; RTN4A cells consistently had punctate mitochondria. This has me to believe that this stress response machinery if operating continuously could serve to help potential RTN4A cancer cells withstand the toxicity of the bloodstream. This then continues to ensure the metastatic potential of these cancer cells. Based on these ideas, cancer cells that have stabilized RTN4A or overexpression of RTN4A could secure a state in which the mitochondria can direct the cells migration in a quicker manner allowing the cancer cells to migrate faster in time. Additionally, by having this ER and mitochondria structure these cancer cells could migrate further distances to their secondary sites and withstand the toxic affects of the migration. As the ER is linked to cancer there are studies that look at RTN4A involvement. It was found that when RTN4A was knockdown in breast cancer cells, MCF7 and MCF10AT, there was a decrease in cell proliferation and a decrease in migration (Hatakeyama *et al* 2016). This shows that there is a basis on RTN4A's involvement in

cancer cells and that the altered mitochondria morphology and surface area in combination with the RTN4A's presence can lead to a cancer forming cells with great metastatic potential.

Another aspect that connects our findings with cancer is mass. As mentioned before mitochondria have an ellipse structure and the mass again relies on the lengths mentioned above, *a* and *b*. Following the same principles stated above we would assume that CLIMP63 cells had a larger mitochondrial mass than RTN4A cells. This change in mass can link the CLIMP63 structure to tumorigenesis, as studies have found that gain or loss of Myc results in an increase or decrease of mitochondrial mass, respectively (Vyas *et al* 2016). In an oncogenic environment, oncogenic c-Myc can increase cellular biosynthetic and respiratory capacity through upregulating mitochondrial metabolism (Vyas *et al* 2016). This then causes rapid proliferation through c-Myc's effects to stimulate the progression of the cell cycle and glycolytic metabolism to support rapid cell growth (Vyas *et al* 2016). These are characteristics often exploited and seen in cancer cells. Based on this background and our observations in our study, it is possible that CLIMP63 cells could help instigate these processes in an oncogenic environment. If cancer cells are able to stabilize or cause the overexpression of CLIMP63 they would produce mitochondria that are significantly longer thereby increasing mitochondrial mass. Continuing with the idea that the cell is oncogenic, there would be elevated levels of oncogenic c-Myc as well. These cells would then have the stimulation needed to progress in the cell cycle because of c-Myc and would also have the glycolytic support needed to coordinate this rapid cell growth as CLIMP63 increased the mitochondrial mass. Based off of this idea the CLIMP63 ER structure serves as a good starting point for cancer cells to begin proliferation and lead to tumour formation. However, the migration parameters of CLIMP63 do not make cancer cells overexpressing with CLIMP63 favourable for metastasis. These migration parameters could be specific only to

NIH3T3 cells and might be altered in cancer cells. CLIMP63 over expression has been found in cholangio-cellular carcinoma and is related to distant and lymph node metastasis (Sandoz *et al* 2015). This then means that it is possible that if cancer cells overexpress CLIMP63 they increase the mitochondrial mass needed to support proliferation and in cancer cells they might also result in metastasis.

Although RTN4A and CLIMP63 over expression has been linked to metastasis and cancer progression, it does not seem that this over expression increased or decreased patient survival. CLIMP63 expression levels and survival time on a Kaplan-Meier plot indicated that patients with low expression levels had nearly the same months of survival compared to patients with high CLIMP63 expression levels. The same is seen in a Kaplan-Meier plot of RTN4 levels. This suggests that even though CLIMP63 or RTN4A expression can be beneficial in aspects of cancer it is not a defining factor in the severity of the cancer or the survival of the patients. Additionally, when looking at alteration frequencies in cancer patients databases, CLIMP63 and RTN4A frequencies are considerably low ranging from 1%-8% in patient groups. As well, RTN4 alteration frequencies resulted from mutation or amplifications while CLIMP63 alterations were a result of mutation, amplifications, and deep deletions. From all cancer types CLIMP63 alterations were higher in cutaneous squamous cell carcinoma (cscc) while RTN4 alterations were highest in uterine. According to the Canadian Cancer Society, both uterine cancer and cscc have high 5-year net survival at 83% and 95%. This could explain why the Kaplan Meier plots had similar survival rates in both low and high expression as both CLIMP63 and RTN4A seem to be related to cancers with high death rates. These cancers are considered to be effectively treatable in later stages again implying that CLIMP63 and RTN4A expression does not indicate a decrease in survival although they have beneficial properties for cancer cells.

Originally it can be believed that one ER structure is better than the other in terms as one leading to cancer and the other not. Based off of these ideas proposed, it seems that overexpression of either RTN4A or CLIMP63 can be beneficial to cancer cells for either their progression or metastasis. In summary, RTN4A cells create a good basis for metastatic potential in cancer cells and CLIMP63 serves as a good starting point for a cell to become cancerous and potentially metastatic. To fully determine whether one ER structure proves to be easily manipulated it would be interesting to do an individual and co-culturing experiment of CLIMP63 and RTN4A cells in a tumorigenic environment. Based off of these results we can determine whether CLIMP63 and RTN4A cells continue to have the same migration parameters to the original study. Additionally, co-culturing the two different cells can have us determine whether one ER structure is more metastatic favouring than the other. This can shed more light as to which ER structure should be targeted for therapeutic purposes. It would be interesting if neither show differences in these the experiments. If CLIMP63 and RTN4A cells respond similarly when in a tumorigenic environment, this suggests that any overexpression to stabilize one ER structure is favouring a cancer response. This could tie together the findings of CLIMP63 and RTN4A overexpression in other studies and our ideas of CLIMP63 or RTN4A manipulation on mitochondria length, surface area and mass to cancer.

As mitochondria has been linked to many cancer cell migration studies and the ER is beginning to be identified in the process, I believe the work regarding CLIMP63 or RTN4A expression should be replicated in a cancerous cell model, specifically breast cancer as we are a breast cancer lab. As mentioned earlier, more still needs to be know in the context of metastasis and how these cells determine where to migrate. We've created preliminary data to suggest that the ER determines how far the cell will travel in a noncancerous model, therefore we need to

determine whether this remains consistent in a cancer cell. After this identification, determining whether invasiveness is higher in CLIMP63 or RTN4A cells may bring forward which ER structure is more prone to cancer positive situations. CLIMP63 has already be identified to inhibit growth and metastasis of hepatocellular carcinoma, therefore it is likely that CLIMP63 based on this study and my results will continue to decrease metastasis in breast cancer cells (Sandoz and van der Goot, 2015).

Another interesting aspect to study would be determining if there is a mathematic equation that could be used to determine cell speed using FA, mitochondria and the ER. As previously mentioned, Kim and Wirtz, 2013, had determined that FA size and cell speed had a biphasic relationship. As we have identified mitochondria affect FA size which in turns affects cell speed. It would be interesting to see what this relationship would look like mathematically and how the ER plays a part in cell speed as we saw some differences in cell speed in our experiments. As mentioned earlier, mitochondria length variable *b* would likely be a key aspect to this equation for metastatic determination. As based off of our ideas, *b* plays the most prominent role in correlating mitochondrial mass and surface area to metastasis. *b* is regulated by the ER so it's possible that correlating a variable to the ER structure or ER size as sheets or tubules can expand this equation to include the ER into metastatic potential. By establishing this equation, it can be highly utilized to determine whether a cancer patient will experience metastasis and to what extent the cell might travel. If the cell is to travel at a certain speed calculated through this equation, we can determine how long it could take the cell to travel a certain distance to a secondary site. This can determine when to start treatment and when to start to detect for metastasis in patients recovering from treatment. Through the advances in microscopy and the development of software that allow the measuring of various organelles this equation could be

put together to benefit many patients and their doctors during diagnosis and treatment. This equation does not take the bloodstreams toxicity into effect but if the RNT4A cells prove to have beneficial mechanisms in place to survive the bloodstream unlike CLIMP63 cells than it is possible that this could be accounted for by the ER variable.

The last aspect I believe we should identify, would be what relationship CLIMP63 and RTN4A have on mitochondrial dynamic proteins, such as Drp1 and/or Mfn1/2. As mentioned in 1.2, both Drp1 and Mfn1/2 are responsible in regulating cell migration. We observed mitochondria that were long in CLIMP63 and short in RTN4A but did not identify why these changes were observed. It would be interesting to find out whether Drp1 levels were increased in RTN4A leading to the short mitochondria and Mfn1/2 were increased in CLIMP63 leading to long mitochondria. This would help better understand the role the ER plays in cell migration in cancer cells as these studies observed migration in cancerous cells and found that silencing of Drp1 decreased cell migration and silencing of Mfn1/2 lead to an increase (Zhao *et al* 2013, Leal *et al* 2016).

Conclusion

Our conclusions are as follows:

1) mitochondria migrate to the leading edge of the cell,

2) mitochondria in the leading edge undergo more fission events and mitochondria in the trailing edge undergo more fusion events,

3) when the ER is sheet-like the mitochondria are longer tubules and when the ER is tubular the mitochondria a short tubules,

4) cells withs sheet-like ER and longer mitochondria migrate shorter distances and displacements and make more sporadic turns,

5) the ER structure is responsible for distance and displacements while mitochondria are responsible for turns and

6) cells with longer mitochondria (CLIMP63 or RTN4A+OPA) take on average longer to make any turns.

Our study brings into light a new marker that can be used for targeting metastasis. We have identified a means to determine which cell will travel greater distances therefore indirectly determining metastatic abilities in cancer cells.. Metastasis has been linked to the increasing issues of cancer as it responsible for 90% of cancer deaths (Seyfried and Huysentruyt, 2014). By identifying whether cells are over-expressing CLIMP63 or RTN4A, specific cells can be targeted and monitored for their ability to move throughout the host. Although these observations are seen in a noncancerous cell, determining a link to what causes cells to migrate is an essential first step in identifying new means to understand how cancerous cells may migrate as well.

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