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The Regulation of Phospholipid Signaling and Actin Cytoskeletal Remodeling by the Eukaryotic Translation Elongation Factor eEF1A2

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THE REGULATION OF PHOSPHOLIPID SIGNALING AND ACTIN CYTOSKELETAL REMODELING BY THE EUKARYOTIC TRANSLATION ELONGATION FACTOR eEF1A2

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Thesis submitted to the Faculty of Graduate and Postgraduate Studies
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Biochemistry

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

December, 2008

Your file Votre référence
ISBN: 978-0-494-51814-4
Our file Notre référence
ISBN: 978-0-494-51814-4

Abstract

The regulation of the actin cytoskeleton is vital in several cellular and physiological processes from cell movement and phagocytosis to embryonic development and immune response. Thus, it is imperative to not only understand the mechanisms that regulate actin cytoskeletal dynamics but to also identify novel signaling networks and proteins that impinge upon these pathways. Here, we identify the eukaryotic elongation factor, eEF1A2, as a mediator of actin cytoskeleton remodeling through the phosphatidylinositol signaling pathway.

EEF1A2, the gene encoding eukaryotic elongation factor 1 alpha 2 (eEF1A2), is one of two functional transcripts of *EEF1A*. During protein translation, eEF1A2 binds to amino-acylated tRNAs and recruits them to the ribosome. Aside from protein synthesis, eEF1A proteins from several genera and species associate with the actin cytoskeleton by binding and bundling actin. This suggests that eEF1A proteins may regulate actin cytoskeleton dynamics in addition to, or independent of, protein translation. Furthermore, recent work has identified *EEF1A2* as an oncogene of the ovary, lung and breast, however the mechanism by which eEF1A2 contributes to oncogenesis has yet to be elucidated.

The work presented in this thesis provides a mechanism by which eEF1A2 promotes oncogenesis. We first demonstrate that eEF1A2 can regulate phospholipid signaling by interacting with, and activating, phosphatidylinositol-4 kinase III beta (PI4KIIIβ), a lipid kinase that is important in the phosphatidylinositol signaling pathway. Through a series of biochemical, molecular, and imaging assays, we describe and detail this interaction and provide evidence that eEF1A2-mediated PI4KIIIβ activation results in an increased pool of

phosphatidylinositol-4 phosphate (PI4P), the product of PI4KIIIβ lipid kinase activity. We then go on show that the interaction between eEF1A2 and PI4KIIIβ leads to the production of finger-like, actin-based membrane protrusions termed filopodia. Specifically, eEF1A2-mediated filopodia formation occurs in a phosphatidylinositol-4,5 bisphosphate ($PI(4,5)P_2$)- and Cdc42-dependent manner. Furthermore, we show that PI4KIIIβ activation is necessary for eEF1A2-mediated filopodia development. Lastly, we describe some of the ways eEF1A2 regulates breast oncogenesis. We show that ectopic eEF1A2 expression in breast cancer cells increases *in vitro* growth rates, inhibits anoikis, and activates the Akt serine-threonine kinase. Importantly, eEF1A2 is shown to be a valid therapeutic target as ablation of eEF1A2 decreases tumour formation in mice. Taken together, the work presented in this thesis provides new insights into ways eEF1A2 contributes to oncogenesis and provides a mechanism as to how eEF1A2 regulates the actin cytoskeleton.

Acknowledgements

This thesis is one of the greatest and most enjoyable accomplishments of my life, and would have been impossible without the help and support of a number of people who have had a tremendous impact on my life. This is my opportunity to happily express my gratitude and sincerest thanks to all of them for making my graduate experience a wonderful time.

To my supervisor, Jonathan Lee, I am grateful for having you as a mentor. Your enthusiasm, guidance, and views on research and science have strengthened my confidence and belief of pursuing this as a career. I thank you for providing me with an opportunity to experience graduate school in your lab. I also appreciate that you let me explore and test many new ideas and hypotheses, as crazy as they may have been, while reminding me to finish off what I had started. I have learned a lot working with you and hope to use these experiences and lessons in my future endeavours.

I also thank my committee members, Drs. Barbara Vanderhyden and Stephen Lee, for all their help throughout my stay at the University of Ottawa. You both have provided a great deal of insight and assistance to my research and have made my work that much better. I also am appreciative to CIHR / Ontario Women's Health Council, the US Army, and the Canadian Breast Cancer Research Initiative for funding my research.

I have been extremely fortunate for the people I have met throughout my years at McMaster and the University of Ottawa. To my friends and labmates at McMaster – Steve Innocente, Nisha Anand, Howard Cukier, Tahir Feroz, Dan Purcell, Sarah Thornton, and Jun Ly - I thank you for all your help and support. Not only were Steve, Nisha, Howard, and Tahir my mentors when I started my work, they were also instrumental in teaching me the basics of research. I thank them for providing me with a view of science and research that I

have, to this day, never forgotten. I especially miss all those Pocket Tank tournaments we used to have. The friendly atmosphere they generated in and out of the lab was a joy to be around, and I have tried to bring and spread that environment in Ottawa.

My choice to come to Ottawa to continue my graduate studies here was one of the best decisions in my life. My time here has been a blast. Two people who have played a key role in that are Dixie Pinke and Anne Morrow. I will definitely miss the laughter, joy, and craziness that we shared in the lab. Not only did they help me with the science-related issues, but they made the lab seem like a family. I have no doubt that they will succeed in whatever they choose to do. I would also like to take the time to apologize for all of my crazy antics in the lab. I don't know how you managed to stay sane despite my strange antics but both of you never let it show (aside from the Candy Cane Incident of 2008). Thanks for putting up with me. I am also grateful to Geeta Kulkarni for her humour and friendship during the years she was in Ottawa. Geeta was the first student in the Ottawa lab and she, along with Jessica Rousseau, helped me get started by introducing me to the people on the floor. I also cannot forget the Science Goddess, Nadine Wiper-Bergeron, for being such a great role model in how to enjoy science. Her humour and great wit were a great combination to balance my wacky character. I really hope her "Four Protein Theory" gets accepted by the scientific community and that she gets the respect she is overdue. Finally, to Sanaa Noubir, Sandy Szeto, Jessica Rousseau, Elnaz Attabaksh, Tanja Francetic, Claire Kuzmochka, Farahnaz Noei, Anahita Amiri, and Julie Leblanc, thanks for memories you have given me. I will miss all of you.

While the relationships with the members of my lab have been instrumental in making my stay in Ottawa a positive one, I am also appreciative to the rest of the BMI

department staff and students, both past and present. I have always been amazed at the friendliness of the BMI faculty and their willingness to share their experiences and wisdom with me. To Drs. Bennett, Baetz, Skerjanc, Mah, Harper, Brown, Pelchat, Altosaar, Couture, Rudner, Figueys, Filion, Sattar and Yao, I thank you for having an "open-door" policy with me and letting me ask of you questions that I had about your work as well as mine. Your enthusiasm for research and science made me realize that there are people out there who are as crazy, and as excited, as I am when it came to science. It is an amazing feeling to know and learn from such great scientists. Similarly, to all of my friends on the floor, and BMI at large, I am glad to have met you all. To name each and every one of you would fill pages in this thesis. Instead, I hope that you recognize the great impact you have had on my life. I have learned and taken a lot from you (joy, laughter, fun, excitement, hope, (insert any other positive adjective), and yes, even science products) during my stay in Ottawa. I just hope that I was able to give you at least a fraction of it in return. Last, but not least, my sincerest thanks to Carol Ann Kelly and Nicole Trudel, for all of their help during my transition here from McMaster, and during my stay here at Ottawa. You are definitely the hearts of the department.

Finally, at the base of my tree of support, are my friends and family. To Ganeshwaran uncle and Saraswati aunty, and to Uthayan uncle and Sugi aunty, thank you for your support. To my grandparents, Nadarajah and Rajeshwary, and my cousins in Australia and Sri Lanka, thank you. To my sister, Rukshi, thanks for always keeping me grounded and reminding me that there's more to the world than a PhD. Most importantly, this thesis is dedicated to my parents. Not only have you provided me with everything I need to get to this point, but you have always given me the strength, freedom, and

confidence to pursue a career that I love and enjoy. The person I am today is due to your help and guidance. I thank you and love you very much.

I would like to end by listing three quotes that I have tried to follow both in science and in life. The quotes describe and define me, how I choose to live, and how I approach any issue or question that I face.

To see a world in a grain of sand,
And a heaven in a wild flower,
Hold infinity in the palm of your hand,
And eternity in an hour.

- William Blake (from *Auguries of Innocence*)

Choose a job you like, and you will never have to work a day in your life.

- Confucius (551-479 BCE)

Seek the wisdom of the ages, but look at the world through the eyes of a child.

- Ron Wild

Table of Contents

Contributions of Collaborators

This thesis was written my me, Sujeeve Jeganathan, with editing done by Dr. Jonathan Lee. The work presented in Chapter Two was done by myself. Work done in Chapter Three, specifically experiments shown in Figures 3.2A and 3.6, were done by Anahita Amiri and Anne Morrow, respectively. Experiments shown in Figures 4.1 and 4.3 were done by Carolina Perez-Iratxeta (breast tumour data analysis) and by Geeta Kulkarni and Anahita Amiri (Akt data). All other figures were generated by me. I would like to thank Heidi McBride and Astrid Schauss for assistance with the quantitative confocal microscopy, Meenakshi Sundaram for help with the lipid kinase assays, Jessica Rousseau for the eEF1A2 adenovirus, and Geeta Kulkarni for the GST-eEF1A2 plasmid and eEF1A2 antibody. I also thank Tamas Balla and Nadine Wiper-Bergeron for various plasmid constructs, as well as Joseph Nevins for making the breast cancer gene expression data publicly available. I am grateful to Dixie Pinke, Anne Morrow, Nadine Wiper-Bergeron, Heidi McBride, Zemin Yao and David Huntsman for helpful discussions and critical reviews of manuscripts. This work was supported by funding from the National Cancer Institute of Canada, the US Army, the Canadian Breast Cancer Research Alliance and a studentship from the CIHR Institute of Gender and Health and the Ontario Women's Health Council.

List of Abbreviations

ABP	Actin-binding protein
ARF	ADP ribosylation factor
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
C. elegans	*Caenorhabditis elegans*
C-terminal	Carboxy-terminal
CFP	Cyan fluorescent protein
$CHCl_3$	Chloroform
CMV	Cytomegalovirus
DAG	Diacylglycerol
D. discoideum	*Dictyostelium discoideum*
D. melanogaster	*Drosophila melanogaster*
DMEM	Dulbecco's Modified Eagle's Medium
DTT	Dithiothretiol
EDTA	Ethylenediaminetetraacetic acid
eEF	Eukaryotic elongation factor
eIF	Eukaryotic initiation factor
eIF4E-BP	eIF4E Binding Protein
eRF	Eukaryotic release factor
F-actin	Filamentous actin
G-actin	Globular actin
GBD/CRIB	GTPase-binding domain/Cdc42 and Rac interactive binding
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GST	Glutathione-S-Transferase
GTP	Guanosine triphosphate
HCl	Hydrochloric acid
HER2	Human epidermal growth factor receptor 2
HOAc	Acetic acid
HRP	Horseradish peroxidase
ILK	Integrin-linked kinase
IP_3	Inositol trisphosphate
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
K_{cat}	Catalytic rate constant

K_d	Dissociation constant
kDa	Kilo-Dalton
K_M	Michaelis constant
LBA	Luria Bertani - Ampicillin
M	Molar
MeOH	Methanol
Met-tRNA	Methionyl-tRNA
MOI	Multiplicity of infection
mRFP	Mitochondrial red fluorescent protein
mRNA	Messenger RNA
mTOR	Mammalian target of Rapamycin
MW	Molecular weight
N-terminal	Amino-terminal
NCS-1	Neuronal calcium sensor-1
P site	Peptidyl site
PBS	Phosphate buffered saline
PDK1	Phosphoinositide-dependent kinase 1
PH	Pleckstrin homology
PI	Phosphatidylinositol
PIK	Phosphatidylinositol kinase
PI3K	Phosphatidylinositol-3 kinase
$PI(3,4,5)P_3$	Phosphatidylinositol-3,4,5 trisphosphate
PI4P	Phosphatidylinositol-4 phosphate
$PI(4,5)P_2$	Phosphatidylinositol-4,5 bisphosphate
PI4K	Phosphatidylinositol-4 kinase
PI5K	Phosphatidylinositol-5 kinase
PLC	Phospholipase C
PTEN	Phosphatase and tensin homolog deleted on chromosome ten
RIPA buffer	Radioimmunoprecipitation assay buffer
S. cerevisiae	*Saccromyces cerevisiae*
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
Ser	Serine
SHIP	SH2-containing 5'-inositol phosphatase
shRNA	Short-hairpin RNA
siRNA	Short-interfering RNA
TEF	Translation elongation factor
Thr	Threonine
TLC	Thin-layer chromatography
tRNA	Transfer RNA
ts	Temperature-sensitive

V_{max}	Maximum velocity
WASP	Wiskott Aldrich Syndrome protein
WH1/EVH1	WASP homology/Ena VASP homology

List of Figures

Chapter 1

Introduction

1.1 Phosphatidylinositols - Introduction

Phosphatidylinositols (PIs) are negatively charged, membrane-bound phospholipids that serve as regulators of multiple signaling pathways (41, 158, 159, 276, 284, 320). PIs are composed of an inositol ring covalently bound to a lipid phosphatidic acid backbone by a phosphodiester bond at the inositol D1 carbon (Fig. 1.1A). Although PIs make up only a small fraction of cellular phospholipids (2-12%, depending on the type of cell) (24, 59, 282, 309), they are of great importance in regulating cellular processes. These include calcium signaling (82, 455), vesicular trafficking (457, 492), proliferation (38, 39), apoptosis (91), motility (109, 308), and adhesion (247, 453), as well as mediating morphological changes to the actin cytoskeleton (384, 423). Accordingly, the list of diseases involving these phospholipids and PI-metabolizing enzymes continues to grow from diabetes (55, 266) and a variety of cancers (38, 91, 316, 448), to diseases of the eye (24, 92), kidney (489), muscle (231, 433), nervous system (23, 381), and the gastrointestinal tract (435).

Approximately 5-10% of phosphatidylinositols in mammalian cells are phosphorylated at one, two, or three positions of the inositol ring (41). These modifications can occur, either at the D3, D4, or D5 carbons of the inositol ring (Fig. 1.1B), and each phosphorylation event is carried out by a specific lipid kinase enzyme. Moreover, all of the phosphorylated species (also known as phosphoinositides) can rapidly interconvert through specific kinases and phosphatases (described below). Although there are eight different phosphoinositide combinations (including PI itself), the most common species in decreasing cellular abundance are phosphatidylinositol (PI), phosphatidylinositol-4 phosphate (PI4P) and phosphatidylinositol-4,5 bisphosphate ($PI(4,5)P_2$), and phosphatidylinositol-3,4,5 trisphosphate ($PI(3,4,5,)P_3$) (41, 116).

1.1.1 The Phosphatidylinositol Signaling Pathway

In eukaryotic cells, there exists a complex network of pathways that lead to the generation of each of the phosphoinositides. One common pathway (Fig. 1.2) begins with the phosphorylation of PI by phosphatidylinositol-4 kinase (PI4K) to yield PI4P. This is followed by phosphorylation by phosphatidylinositol-5 kinase (PI5K) to give $PI(4,5)P_2$. Lastly, $PI(4,5)P_2$ is phosphorylated by phosphatidylinositol-3 kinase (PI3K) to yield $PI(3,4,5)P_3$. In addition, phosphatases such as Sac1, SHIP (SRC Homology 2 Domain-containing 5'-inositol phosphatase) and PTEN (phosphatase and tensin homolog deleted on chromosome ten), are responsible for converting PI4P to PI, $PI(4,5)P_2$ to PI4P, and $PI(3,4,5)P_3$ to $PI(4,5)P_2$, respectively (241).

1.1.2 Phosphatidylinositol Kinases

As described above, phosphatidylinositol kinases phosphorylate specific carbon residues on the inositol sugar and thus, are named accordingly. While the kinases act on specific carbons, they can also function in sequence. Thus, while PI3Ks, PI4Ks, and PI5Ks can produce PI3P, PI4P, and PI5P, sequential phosphorylation events can lead to biphosphoinositides, $PI(3,4)P_2$, $PI(3,5)P_2$, and $PI(4,5)P_2$, and the triphosphoinositide, $PI(3,4,5)P_3$.

1.1.2.1 Phosphatidylinositol-3 Kinases

Phosphoinositol-3 kinases (PI3Ks) are lipid kinases that regulate several signaling pathways controlling critical cellular processes including cell growth (123, 356), proliferation (225, 263), embryogenesis (64, 253, 465), angiogenesis (187, 188), differentiation (194, 382), motility (233, 280, 403, 456), survival (53, 110, 484) and intracellular trafficking (102, 201, 240). PI3K-catalyzed reactions generate the lipid second

messengers, PI3P, $PI(3,4)P_2$, $PI(3,5)P_2$ and $PI(3,4,5)P_3$ that arbitrate signal transduction. PI3P, for example, is known to play a role in the endocytic pathway (138, 160, 259, 336). It is enriched in early endosomes, membrane-bound compartments inside cells that sort material before transport to lysosomes or the plasma membrane (160). Petiot and colleagues showed that while PI3P signaling does not regulate the core machinery of endosome biogenesis and transport, it does control the sorting of receptors in early endosomes (336). Similarly, $PI(3,4,5)_3$ been shown to be important in multiple signaling pathways that regulate the actin cytoskeleton, cell survival, cell growth, vesicular trafficking and cytokinesis (28, 58, 68, 81, 179). One of $PI(3,4,5)P_3$'s major roles in the cell is in the activation of the Akt serine threonine kinase, a key activator of several cell survival pathways and inhibitor of multiple apoptotic pathways (91, 112, 326, 448).

Very little is known about the two 3-phosphorylated biphosphoinositides, $PI(3,4)P_2$ and $PI(3,5)P_2$. While $PI(3,5)P_2$ was discovered over a decade ago, much remains unknown about this phospholipid. What is known is that $PI(3,5)P_2$ regulates trafficking from distal compartments to organelles that are more proximal in the endocytic/lysosomal system (though the mechanism remains unclear) (90, 386). Another function mediated by $PI(3,5)P_2$ involves the regulation of organelle morphology in response to stress. Wild type yeast cells have multiple, small vacuoles that serve as sites for protein degradation and osmoregulation whereas cells with defects in regulating protein turnover or membrane trafficking and signaling have enlarged vacuoles (461). Rudge and colleagues reported that *Saccharomyces cerevisiae* cells deficient in $PI(3,5)P_2$ synthesis exhibit a grossly enlarged vacuole morphology whereas increased levels of the phospholipid provokes the formation of multiple, small vacuoles (371). Furthermore, Bonangelino and colleagues found that, in

response to hyperosmotic stress, there is a nearly 20-fold increase in $PI(3,5)P_2$ levels with a subsequent increase in small vacuole formation (30). These observations suggest that $PI(3,5)P_2$ regulates proper vacuole function in yeast. Similarly, while the role of $PI(3,4)P_2$ in cells remains uncharacterized, Traynor-Kaplan and colleagues showed a transient increase in $PI(3,4)P_2$ levels upon human neutrophil activation (442). An increase in $PI(3,4)P_2$ levels was also observed in human platelets stimulated by thrombin, but the mechanism involved is currently unknown.

1.1.2.2 Phosphatidylinositol-5 Kinases

Phosphatidylinositol-5 kinases (PI5Ks) are responsible for phosphorylating the D5 carbon of the inositol sugar. Phosphoinositides produced as a result of PI5K activity include PI5P, $PI(3,5)P_2$, $PI(4,5)P_2$, and $PI(3,4,5)P_3$. PI5Ks and their products regulate various cellular processes such as actin cytoskeletal dynamics, vesicle trafficking, ion channel activity, gene expression, and cell survival (116, 126, 319). Recently, Bunce and colleagues identified a novel mechanism to stimulate Cul3-SPOP ubiquitin ligase activity in multiple human cell lines through PI5K-mediated PI5P production (36). There are also reports suggesting that PI5P can regulate the actin cytoskeleton in yeast, rodent and human cell lines (75, 374) but the exact mechanisms remain unidentified. Conversely, the importance of $PI(4,5)P_2$ has been well established and will be discussed below. One of the ways to generate $PI(4,5)P_2$ is through PI5K (also referred to as phosphatidylinositol phosphate kinase type I (PIPKI)) activity on PI4P (248).

PIPKIs synthesize most $PI(4,5)P_2$ *in vivo* (255). There are three PIPKI isoforms: α, β, and γ (248). Each isoform shows distinct subcellular distribution and functions in distinct cellular processes. PIPKIα, for example, is found in the nucleus and in the cytoplasm where

it is found on membrane ruffles and phagocytic structures (31, 62). Conversely, PIPKIβ localizes to the perinuclear region and is contained within vesicular structures (89), suggesting a role in the endosomal pathway. Similarly, PIPKIγ targets to focal adhesions where it mediates focal adhesion assembly by binding to talin, a cytoskeletal protein, and strengthening the link between the cytoskeleton and integrins (247, 303). Taken together, these findings suggest that PI5Ks play a role in cytoskeletal reorganization and adhesion, processes intricately linked with cell motility.

1.1.2.3 Phosphatidylinositol-4 Kinases

Phosphatidylinositol-4 kinases (PI4Ks) catalyze the first committed step in the phosphatidylinositol signaling pathway. They phosphorylate the D4 carbon of the inositol sugar in PI to yield PI4P. Since PI4P abundance controls the levels of all subsequent downstream phosphoinositides, PI4P levels are tightly regulated in the cell. As such, cells have developed multiple mechanisms to control the activities of PI4Ks.

1.1.2.3.1 PI4K Family Members

There are four PI4K isoforms that have been cloned (Fig. 1.3). These are PI4KIIIα (formerly PI4K230), PI4KIIIβ (formerly PI4K92), PI4KIIα (formerly PI4K55α) and PI4KII β (formerly PI4K55β). The four proteins are quite different from each other with respect to sequence homology, tissue expression profiles and intracellular localization. The classification of the various isoforms as type II or type III relate to their biochemical activities. Type II enzymes have their activities inhibited by adenosine (K_i between 10-20μM), are insensitive to wortmannin and phenylarsine oxide (K_i values between 500-800μM for each compound), and are detergent-activated. Type III enzymes are barely

sensitive to adenosine (K_i between 1500-1600μM), are not detergent-activated, and are

sensitive to wortmannin (K_i between 0.03-1μM) and phenylarsine oxide (K_i between 5-20μM) (21, 101, 124, 161).

1.1.2.3.2 Tissue Distribution and Intracellular Localization of PI4K Enzymes

PI4KIIIα mRNA is highly expressed in mammalian heart, brain, skeletal muscle, placenta, kidney, spleen, thymus, prostrate, ovary, small intestine, and colon, whereas it is barely detectable or absent in the lung, liver, pancreas, testis, and leukocytes (22, 300). PI4KIIIβ also shows a wide tissue distribution similar to PI4KIIIα, with the exceptions being the brain and kidney, where no mRNA is detected (22, 281, 299). Conversely, PI4KII enzymes do not show ubiquitous tissue distribution. Their mRNA has been detected in placenta, heart, skeletal muscle, and colon (470).

Intracellular studies of PI4KIIIα have shown that the enzyme is largely membrane-bound. PI4KIIIα has been found on the membranes of the rough endoplasmic reticulum, mitochondria, and multivesicular bodies (18, 463, 496). Additionally, the kinase has also been reported to be localized in the nucleolus of neuronal cells (496). In contrast, the PI4KIIIβ isoform is found on Golgi membranes and in the cytoplasm (463). Moreover, in neuronal cells, the enzyme is also found in membranes of the mitochondria, rough endoplasmic reticulum, and on the cytoplasmic surface of the nuclear envelope (463, 471, 496). PI4KIIα is predominantly located in the Golgi while PI4KIIβ is found in lipid rafts on the plasma membrane, Golgi, and endoplasmic reticulum (458). Additionally, both isoforms have been detected in early endosomes and nuclear endosomes (17).

1.1.2.4 Phosphatidylinositol-4 Kinase III Beta

Mammalian PI4KIIIβ shows a ubiquitous tissue expression profile. PI4KIIIβ mRNA can be detected in the placenta, lung, liver, pancreas, skeletal muscle, spleen, thymus, prostrate, testis, ovary, small intestine, and colon (124). In these tissues, PI4KIIIβ protein is found predominantly associated with the Golgi but cytosolic PI4KIIIβ staining has also been observed (471). Most studies involving PI4KIIIβ have been in the context of the Golgi. The Golgi complex is the central organelle of the secretory pathway in eukaryotic cells (370). Proteins and lipids pass through the Golgi on their way to their final cellular destinations. While in the Golgi, molecules are post-translationally modified (glycosylation, for example) by several Golgi enzymes (267). In mammalian cells, the Golgi is a ribbon-like structure composed of stacks of flattened, adherent cisternae embedded in a matrix (391). Individual cisternae are attached to each other through direct tubular connections (111). Many components of this matrix are highly dynamic, transitioning from membrane-associated to cytoplasmic states (267). PI4KIIIβ has been identified to play an essential role in maintaining the structural integrity of the Golgi complex and vesicular trafficking (75, 161, 490). Studies by Godi and colleagues using brefeldin A (BFA) showed that PI4KIIIβ is a key regulator of Golgi disintegration and reintegration (129). BFA is a lactone antibiotic produced by fungal organisms that inhibits protein transport from the endoplasmic reticulum to the Golgi apparatus by preventing the formation of transport vesicles (210). A consequence of this is that some Golgi proteins and lipids, among them galactosyl transferase and ceramide, remain in the endoplasmic reticulum unable to properly form the

Golgi (86, 210, 425). Godi and colleagues showed that the ADP ribosylation factor (ARF), a GTPase, was able to recruit and activate PI4KIIIβ to the Golgi complex, resulting in the generation of a pool of PI4P and $PI(4,5)P_2$ on the Golgi membrane. Since ARF proteins are known to be critical for membrane budding and fusion events associated with Golgi function (195, 351), the authors proposed that the increase in membrane PI4P and $PI(4,5)P_2$ then recruited cytoskeletal proteins, such as spectrin, to serve as scaffolds to help organize cisternae (129). Furthermore, expression of a kinase-dead PI4KIIIβ in COS-7 cells delayed and distorted the reassembly of tubular structures within the Golgi complex after BFA treatment and removal, again suggesting a role for PI4KIIIβ in regulating Golgi structural integrity (129).

Consistent with the above findings, the yeast PI4KIIIβ homologue, Pik1, also shows similar effects on the Golgi complex. Studies with a temperature sensitive Pik1 in *Saccharomyces cerevisiae,* show defects in secretion, endocytosis, and Golgi membrane dynamics in cells at the restrictive temperature (12, 148, 307, 383, 450). Interestingly, the morphology observed in the Pik1 mutant cells is similar to cells lacking a functional ARF, confirming the importance of both proteins in proper Golgi function (12).

Another protein known to interact with PI4KIIIβ is the neuronal calcium sensor-1 (NCS-1). NCS-1 proteins from several organisms, from *Drosophila* (168, 273), yeast (4, 162), and mammals (72, 349, 490), have been reported to bind to, and activate respective PI4KIIIβ homologues. For example, mammalian NCS-1 was shown to interact and regulate

PI4KIIIβ activity in COS-7 cells (490). The biochemical interaction data were supported by colocalization assays showing both proteins in the Golgi. Similarly, in *Drosophila*, the binding and activation of PI4KIIIβ by NCS-1 is important in enhancing the efficiency of synaptic-vesicle-mediated neurotransmitter release (342). Finally, the yeast NCS-1 / Pik1 interaction is thought to provide a feedback mechanism, through calcium mobilization, to improve exocytic-vesicle reformation (162). In the latter case, NCS-1 was shown to bind to the Pik1 N-terminal domain and promote a conformational change that facilitated kinase activation (177, 416).

1.1.3 The Phosphoinositide Phosphatases

While the focus of this thesis will be on phosphatidylinositol kinases, this section will highlight the roles of the various phosphoinositide phosphatases in the phosphatidylinositol signaling pathway.

Like their kinase counterparts, phosphoinositide phosphatases are specific in their activities. Phosphatases are classified based on which inositol carbon they dephosphorylate. Thus, 3'-phosphatases dephosphorylate the D3 carbon, 4'-phophatases dephosphorylate the D4 carbon, and 5'-phosphatases dephosphorylate the D5 carbon.

1.1.3.1 The 3'-Phosphatases

The most studied mammalian 3'-phosphatases are PTEN and the myotubularin family phosphatases (53, 359). As described earlier, PTEN (phosphatase and tensin homolog deleted on chromosome ten) is a 3'-phosphatase that specifically dephosphorylates the D3 carbon of phosphoinositides. PTEN substrates include $PI(3,5)P_2$, $PI(3,4)P_2$ and $PI(3,4,5)P_3$ (28, 240, 355). Due to its role in regulating cellular $PI(3,4,5)P_3$ levels, and thus

Akt levels, PTEN has been extensively studied in terms of its role in various diseases, among them cancer (188, 207, 333). For example, deletions in 10q23 (*PTEN* locus in humans) occur in multiple tumour types, most prominently advanced glial tumours (glioblastoma multiforme) but also prostrate, endometrial, renal and small cell lung carcinoma, melanoma and meningioma (243, 410, 434). It should also be noted that, to date, PTEN has not been shown to dephosphorylate PI3P and convert it to PI. This activity is regulated by the myotubularin family phosphatases.

The myotubularin family of phosphatases consists of myotubularin (MTM) and myotubularin-related (MTMR) phosphatases (359). Originally discovered to specifically target PI3P (29, 433), it is now known that MTMs and MTMRs also dephosphorylate $PI(3,5)P_2$ (377). As indicated earlier, PI3P and $PI(3,5)P_2$ play important roles in the endosomal-lysosomal pathway. As such, mutations in MTM/MTMR genes lead to defects in membrane trafficking (333, 359).

1.1.3.2 The 5'-Phosphatases

The 5'-phosphatases dephosphorylate the D5 carbons of phosphoinositides. Bacterial, yeast and mammalian 5'- phosphatases have been shown to use $PI(3,5)P_2$, $PI(4,5)P_2$, and $PI(3,4,5)P_3$ as substrates (103). However, to date, no 5'-phosphatase in any organism has been found to convert PI5P to PI.

The most studied 5'-phosphatases are the SHIP (SRC Homology 2 Domain-containing 5'-inositol phosphatase) phosphatases and the yeast synaptojanin family of phosphatases (9, 368). Both of these phosphatase families predominantly use $PI(4,5)P_2$ and $PI(3,4,5)P_3$ as substrates. There are two proteins in the SHIP family phosphatases: SHIP1 and SHIP2 (103, 368). SHIP1 displays 5'-phosphatase activity specifically with $PI(3,4,5)P_3$

and does not appear to use $PI(4,5)P_2$ as a substrate whereas SHIP2 can use both as substrates (103, 368). Due to the importance of $PI(4,5)P_2$ and $PI(3,4,5)P_3$ in vesicular signaling, actin cytoskeleton reorganization and cancer, SHIP phosphatases have been looked at as potential therapeutic targets.

Synaptojanin1 was originally identified in yeast and was initially found to have 5'-phosphatase activity on $PI(4,5)P_2$ alone (407). Since then, other members have been identified in yeast and mammals that can hydrolyze the phosphates from any of the three positions that may be phosphorylated (D3, D4 or D5). Thus, for example, Sjl2 and Sjl3 can dephosphorylate $PI(3,5)P_2$ (5'-phosphatase activity) in addition to PI3P (3'-phosphatase activity) (144, 328, 394, 411).

In addition to the above two families, a novel 72kDa 5'-phosphatase (72-5ptase) has been identified in adipocytes that targets specifically $PI(3,5)P_2$ and converts it to PI3P (214, 215). However, more research is needed to identify whether this protein is unique to adipocytes and whether it belongs to a new class of 5'-phosphatases.

1.1.3.3 The 4'-Phosphatases

To date, the only 4'-phosphatases that have been identified are those that convert $PI(3,4)P_2$ to PI3P, $PI(4,5)P_2$ to PI5P and PI4P to PI. The phosphatases that use the biphosphoinositides as substrates are called 4-ptase I and 4-ptase II (444). Both these enzymes have been identified in mammals and localize to late endosomal membranes (387, 388). Ungewickell and colleagues showed that 4-ptase I and II affect the lysosomal degradation of internalized plasma membrane receptors (444). Likewise, the phosphatase that hydrolyzes the phosphate on PI4P and converts it to PI, Sac1, is a 67kDa membrane protein found in the endoplasmic reticulum and Golgi. Sac1 mutant yeast strains exhibit

mating and sporulation defects, inositol auxotrophy, and have defects in the endocytic pathway and vacuolar trafficking (56, 175, 212, 217, 269, 305, 421). Rhode and colleagues cloned the human Sac1 protein and showed that it behaved similarly to its yeast homologue in terms of its substrate specificity and subcellular localization (367). However the effects upon hSac1 protein ablation or overexpression were not looked at.

1.1.4 Phosphatidylinositol-4,5 Bisphosphate

Phosphatidylinositol-4,5 bisphosphate ($PI(4,5)P_2$), comprises >99% of the doubly phosphorylated phosphoinositides and is the major polyphosphoinositide in mammalian cells (275). Found predominantly on plasma and organelle membranes (172), $PI(4,5)P_2$ concentrations range from 10 – 40μM, accounting for 1-3% of total membrane phospholipids (275). While low in abundance, $PI(4,5)P_2$ is of critical importance in cell life as seen by its involvement in multiple cellular processes such as proliferation (117), apoptosis (278), cell morphology (353), motility (179), adhesion (459), endocytosis/exocytosis (274) and so on.

In mammalian cells, $PI(4,5)P_2$ levels are predominantly maintained by four major processes - two that generate the lipid and two that deplete its levels. Two processes that increase $PI(4,5)P_2$ levels are (a) phosphatidylinositol-4 phosphate-5 kinase (PI4P5K) action on phosphatidylinositol-4 phosphate to generate $PI(4,5)P_2$, and (b) PTEN activity on phosphatidylinositol-3,4,5 trisphosphate to yield $PI(4,5)P_2$. Two processes that decrease cellular $PI(4,5)P_2$ levels are (a) phosphatidylinositol-3 kinase (PI3K) action on $PI(4,5)P_2$ to generate $PI(3,4,5)P_3$ and (b) hydrolysis of $PI(4,5)P_2$ by phospholipase C (PLC) to produce diacylglycerol and inositol 1,4,5-trisphosphate.

While $PI(4,5)P_2$ is involved in various aspects of cell physiology, one important role

– regulation of the actin cytoskeleton – shall be highlighted below due to data in Chapter Three that show increased $PI(4,5)P_2$ production in eEF1A2-expressing cells$I(4,5)P_2$ Actin cytoskeleton remodeling by $PI(4,5)P_2$ occurs primarily through its ability to induce actin filament assembly and / or prevent its disassembly (275, 353).

1.1.4.1 $PI(4,5)P_2$ and the Cytoskeleton – Rho Family GTPases

The role of $PI(4,5)P_2$ in regulating the actin cytoskeleton has been extensively studied. One of the ways that $PI(4,5)P_2$ accomplishes this function is by interacting with the Rho-family GTPases Rho, Rac, and Cdc42. These low molecular weight GTPases participate in several cellular processes that regulate the actin cytoskeleton (311). Specifically, Rho GTPases are important mediators in events that require rapid actin polymerization such as the formation of stress fibers, focal adhesions, membrane ruffles, lamellipodia and filopodia (146, 311, 312, 430).

The first indication of a biological interaction between Rho GTPases and $PI(4,5)P_2$ was when Chong and colleagues showed that inhibition of Rho function abolished calcium release in response to platelet-derived growth factor (PDGF) stimulation (52). Since hormone-mediated calcium release is regulated by $PI(4,5)P_2$ levels (287, 431), this group showed that inhibiting Rho (by preventing it from binding GTP) resulted in reduced $PI(4,5)P_2$ levels and decreased calcium mobilization in response to PDGF. Conversely, microinjecting an activated Rho mutant into cells stimulated phosphatidylinositol-4 phosphate-5 kinase (PI4P5K) activity and reversed the decrease in calcium mobilization (52). These results suggested Rho influenced the phosphatidylinositol signaling pathway by regulating $PI(4,5)P_2$ levels through PI4P5K.

While the above data provided evidence for the association of Rho GTPases with

$PI(4,5)P_2$, Hartwig and colleagues demonstrated the link between Rho proteins, $PI(4,5)P_2$, and the actin cytoskeleton (155). By stimulating the thrombin receptor in blood platelets, they found that there was a rapid increase in actin polymerization correlating with an increase in $PI(4,5)P_2$ levels. Importantly, the elevated levels of $PI(4,5)P_2$ led to a rapid increase in free (or uncapped) growing ends – or plus ends – on actin filaments. Furthermore, the addition of a constitutively active mutant Rac increased the rate of actin monomer addition onto the plus ends of filaments and rapidly induced $PI(4,5)P_2$ synthesis (155). This provided evidence of a pathway for actin assembly through Rac and $PI(4,5)P_2$ signaling.

Analogous to the relationship between $PI(4,5)P_2$ and Rho or Rac, $PI(4,5)P_2$ also modulates the activity of Cdc42. Zheng and colleagues demonstrated that, *in vitro*, $PI(4,5)P_2$ was able to stimulate GDP release from Cdc42 in a concentration-dependent manner, thereby enabling the GTPase to bind GTP and become activated (493). Zigmond and colleagues provided further evidence for the $PI(4,5)P_2$/Cdc42/actin association (495). This group showed that the addition of GTPgammaS (a nonhydrolyzable analog of GTP that acts through small GTPases) to *Dictyostelium* induced the formation filamentous actin (495). Furthermore, using a cell-free system, they showed that recombinant Cdc42 induced actin polymerization *in vitro* and this was inhibited when a Cdc42-binding competitor peptide was added. Lastly, they observed that the addition of $PI(4,5)P_2$ was able to rescue the actin polymerization phenotype seen in the presence of the competitor (495), providing further evidence of a link between $PI(4,5)P_2$, Rho GTPases, and actin.

In addition to the above data, there have been numerous studies to provide compelling support for the close association between the three components. For example,

Rac stimulates actin reorganization by associating with PI4P5K (or PIPKI) *in vitro* (42, 437-439). Similarly, Rho and Cdc42 have also been shown to interact with and activate PI4P5K in multiple mammalian cell lines and cell types (7, 52, 172, 319, 460). In all of these cases, the interaction with the kinase leads to (a) the recruitment of the kinase to the plasma membrane, (b) the activation of the kinase to generate $PI(4,5)P_2$, and (c) changes to the actin cytoskeleton.

1.1.4.2 $PI(4,5)P_2$ and the Cytoskeleton – Actin Binding Proteins

In addition to remodeling cell morphology through Rho GTPases, $PI(4,5)P_2$ also regulates the actin cytoskeleton by affecting the function of several actin-associated cytoskeletal proteins. $PI(4,5)P_2$ regulates a number of proteins involved in the generation and maintenance of the actin cytoskeleton by controlling the activities of proteins that sequester actin monomers, bind or crosslink actin filaments, and cap and/or sever these filaments. In most cases, it does so by directly binding to these proteins through pleckstrin homology (PH) or other (PDZ, C2, ENTH, ANTH and FERM) $PI(4,5)P_2$-binding motifs (63, 170, 293, 419, 422, 485). For example, $PI(4,5)P_2$ binds to profilin, an actin binding protein that sequesters actin monomers and suppresses actin nucleation (469). Skare and colleagues showed that one of the domains in profilin that mediates profilin/$PI(4,5)P_2$ interaction partially overlaps with the actin-binding site, suggesting a competition for the protein by the phospholipid and actin (396). Similarly, $PI(4,5)P_2$ also associates with several actin filament-severing proteins, among them gelsolin (420), villin (226), severin (98), and cofilin (80). For example, by binding to cofilin through its acyl chain and head group, $PI(4,5)P_2$ is able to abrogate cofilin's ability to sever actin filaments and decrease the rate of actin monomer dissociation from minus ends (or slow-growing ends) (132). As in the case with

profilin, Ojala and colleagues showed that the $PI(4,5)P_2$ binding site in yeast cofilin overlaps with areas important in actin binding, suggesting that $PI(4,5)P_2$ competes with actin for cofilin (314). Similarly, gelsolin, an actin-binding protein that severs and caps actin filaments and nucleates actin assembly, has its activity inhibited by $PI(4,5)P_2$ (183, 420). In contrast, the binding of $PI(4,5)P_2$ to vinculin, a cytoskeletal protein found at focal adhesions and adherens junctions, dissociates vinculin's head-tail interaction, thereby unmasking its actin-binding sites and promoting focal adhesion assembly (77, 125). In addition to binding vinculin, $PI(4,5)P_2$ also binds to talin and strengthens its ability to associate with other cytoskeletal proteins (125). Talin is a cytoskeletal protein that links actin filaments to the plasma membrane by crosslinking actin filaments into both networks and bundles (488). Thus, by interacting with several key actin binding proteins, $PI(4,5)P_2$ plays a crucial role in coordinating and regulating cytoskeletal dynamics by promoting actin polymerization.

1.1.4.3 $PI(4,5)P_2$, WASP Proteins, and Actin Polymerization

The Wiskott Aldrich Syndrome protein (WASP) family of proteins has been identified as important mediators of *de novo* actin nucleation downstream of Cdc42 (366). They are the link between GTPases (like Cdc42) and the actin cytoskeleton. WASP proteins bind to active Cdc42 through their GBD/CRIB (GTPase-binding domain/Cdc42 and Rac interactive binding) domains (285, 286). The cooperative effect of these two proteins in actin cytoskeleton regulation was detected in COS-7 cells where overexpression of both proteins induced longer actin protrusions than when Cdc42 alone was overexpressed (424).

In addition to its GBD/CRIB domain, WASP proteins also contain a WH1/EVH1 (WASP homology/Ena VASP homology) domain, a basic region, a proline-rich region, a verprolin-homology (V) region, a cofilin-like (C) region, and an acidic (A) region (Fig. 1.4).

The last three are usually referred together as the VCA domain (424). In unstimulated conditions, WASP exists in an auto-inhibitory state. NMR studies have shown that the VCA region interacts with the GBD/CRIB domain, thus blocking interaction with, and preventing the activation of the Arp2/3 complex (described later). Upon stimulation, however, active Cdc42 binds to the GBD/CRIB domain, thus exposing the VCA region. Furthermore, studies have also shown that $PI(4,5)P_2$ binds to the basic region and helps anchor WASP to the plasma membrane. Moreover, when active Cdc42 is present, the binding of $PI(4,5)P_2$ to the basic region also helps expose the VCA region and enables subsequent Arp2/3 complex

activation. Thus, the cooperative effect of Cdc42 and $PI(4,5)P_2$ both regulate changes to the actin cytoskeleton through WASP (14, 464).

When WASP exposes its VCA region, members of the Arp2/3 complex can bind to it. The Arp2/3 complex is composed of two actin-related proteins, Arp2 and Arp3, which act as templates for new actin filaments, and other additional subunits that serve various structural and regulatory roles (261, 325). The complex contains several sites for binding to nucleation-promoting factors and actin filaments, which allow the complex to nucleate the formation of actin filament arrays in response to several signals. As actin monomers bind to the newly created actin filaments (capped by the Arp2/3 complex at the minus end), the filaments push the membrane forward. Continued actin polymerization results in the formation of finger-like protrusions called filopodia (described below) (393, 412). The process of filopodia formation described above is summarized in Figure 1.5.

1.2 Cell Movement

Cell movement is a dynamic phenomenon that is essential for a variety of biological processes such as embryonic morphogenesis (143, 200, 237, 350), immune response (3, 242, 272), wound healing (83, 87, 344) and cancer metastasis (361, 479). Cell movement occurs in response to an external signal in the cell's surrounding environment. The process of cell movement involves the constant restructuring of the actin cytoskeleton and can be conceptually divided into three stages. First, there is a forward extension of the cell membrane. This occurs by the cell orienting and reorganizing the actin network at its leading edge. The second stage involves the adhesion of the cell to the substrate at the leading edge and release of the cell body at the rear. Lastly, contractile forces generated by the actin-myosin network pull the cell forward (234, 235, 398, 399).

1.2.1 Actin and Cell Movement

In cells, actin exists as one of two forms: globular actin or filamentous actin. Globular actin (or G-actin) are monomeric actin units while filamentous actin (or F-actin) consists of actin monomers that are assembled into helical filaments (339). The polymerization of actin monomers into filaments occurs in an organized fashion and can be conceptually separated into two stages: a) actin nucleation and b) actin filament elongation (218). The initial formation (nucleation) of an actin filament requires actin monomers to associate in sequential steps to form dimers and then trimers (218). Since the individual actin monomers bind in a head-to-tail orientation, the generation of these intermediates polarizes these new, short filaments into a plus (or fast-growing) end and a minus (or slow-growing) end. It is during the formation of dimers and trimers that actin-binding proteins or actin filament severing proteins, such as profilin, gelsolin, villin and cofilin influence whether the new filaments continue to grow or depolymerize. If polymerization is needed, then filament elongation occurs, and actin monomers bind to the plus ends at a rate roughly ten times the rate of addition at the minus ends (340). For example, at 30μM actin monomer concentrations, the rate of actin polymerization is about 350 subunits per second (or about 1 micron of polymer length per second) (340).

As described earlier, cell movement is dependent on the dynamic nature of the actin cytoskeleton. Actin filaments can be assembled at the plasma membrane into multiple structures, among them sheet-like structures (lamellipodia and ruffles) and filopodia (51). Lamellipodia are surface-attached, sheet-like membrane protrusions observed during cell motility. In these structures, actin filaments form a branching, mesh-like network, with the plus ends pointing out towards the leading edge (10, 468). Lamellipodia are generally the

first membrane protrusions created during cell movement, and start at the leading edge and extend several micrometres back (1). Their thickness can vary from between 100-160nm at the edge to greater than 200nm away from the edge (1). Lamellipodia also have varying adhesive properties. At the leading edge, they are weakly adherent to the substratum to permit rapid, dynamic changes and movements, whereas away from the leading edge, they are strongly adherent to ensure proper traction and stability (15, 141). In contrast to lamellipodia, membrane ruffles are sheet-like membrane protrusions that do not attach at all to the substratum (60, 357). While ruffles also contain a branched, mesh-like actin filament network, ruffles are transient structures that assemble at the leading edge of motile cells and move backwards (51, 468). Ruffles form when lamellipodia at the leading edge lose attachment with the substratum and move rearward due to myosin II-based contraction (49). In contrast, filopodia have diameters less than 200nm and contain between 10-30 actin filaments whose plus ends point towards the membrane (51). In motile cells, filopodia protrude outward from the leading edge, sometimes emanating from lamellipodia, and attach to the substratum (106).

1.2.2 Filopodia

Filopodia are long, thin cell extensions that are supported by tight, dynamic, parallel bundles of actin filaments (140). They are found in various organisms and cell types and play roles in cell-cell communication (369) and cell movement (140, 415, 472). For example, thin extensions, termed myopodia, emanate from muscle cells during innervation by the neuron (358). The contact-mediated interaction between the axon and muscle cell myopodia allows for the possibility of mutual exchange of information between the two cells, eventually leading to the maturation and stabilization of a neuromuscular junction

(358). During chemotaxis, the directed movement of cells in chemical gradients, filopodia form in the direction of movement (203). Chemotaxis allows *Dictyostelium* to find their bacterial prey and, when starved, to aggregate into multicellular masses using cyclic AMP signals (119, 147, 216, 467).

Filopodia were first observed in the blastula stage of sea urchin embryo development by Gustafson and Wolpert (142, 143). They were seen extending from primary mesenchymal cells (PMC) that were migrating up the interior wall of the blastocoelic cavity. The filopodia were 5-35 nm in length, very dynamic, and appeared to be exploring the substrate over which the PMCs were migrating. Since these initial observations, similar actin protrusions have been detected in many other cell types and have been shown to play important biological roles from embryonic development to cell motility and cancer metastasis (415, 472).

1.2.2.1 Mechanism of Filopodia Formation

One of the proteins involved in regulating filopodia formation is the Rho family GTPase, Cdc42. Cdc42 is an ubiquitously expressed, essential protein found in multiple organisms. Cdc42's role in filopodia formation is to promote new actin polymerization (through $PI(4,5)P_2$-mediated recruitment to the plasma membrane and subsequent Arp2/3 complex activation) and to stimulate the uncapping or severing of filaments (424). Expression of constitutively active Cdc42 can lead to filopodia formation with or without concomitant lamellipodia formation depending on the cell type. For example, Kozma and colleagues found that microinjection of GTP-activated Cdc42 into mouse fibroblasts induced filopodia formation at the cell periphery (134, 220, 222, 376). Conversely, filopodia formation in response to agonists was suppressed when a dominant-negative

Cdc42 was expressed (127, 146, 302, 312, 430). Furthermore, Chen and colleagues showed that Cdc42-null mouse embryonic stem cells lack filopodia that are found in wild type cells (46). Thus, Cdc42 is an important mediator of filopodia development.

1.2.2.2 Alternate Mechanisms of Filopodia Development

While the model for filopodia development presented above has been well investigated, there are a few controversies. The issues are related to the importance and necessity of certain proteins in filopodia formation as well as the existence of alternate pathways.

1.2.2.2.1 Cdc42- / WASP-independent Mechanisms

While there are numerous studies demonstrating the need for Cdc42 in filopodia formation, there are a few reports suggesting that the protein is not essential. For example, Czuchra and colleagues showed that Cdc42 is not essential for filopodia formation in fibroblastoid cells (69). This group generated Cdc42-null cells and demonstrated that while they had an altered morphology, the cells were capable of generating filopodia and lamellipodia. Furthermore, Ellis and colleagues have shown that another Rho-family GTPase, Rif (Rho in filopodia), can regulate actin-based membrane rearrangements in a Cdc42-independent manner (100, 330). Filopodia observed in Rif-overexpressing HeLa cells were not affected by coexpression of a dominant-negative mutant of Cdc42 (100). Additionally, unlike Cdc42, Rif did not directly interact with WASP (100) and the sequestration of Arp2/3 complex proteins did not affect filopodia formation (330). Thus, Rif-mediated filopodia formation does not proceed via the aforementioned Cdc42/WASP/Arp2/3 pathway.

While Rif regulates filopodia completely independent of Cdc42, WASP, and

Arp2/3, there are other proteins that activate WASP-Arp2/3 independently of Cdc42. One such group of proteins is the SH3-containing proteins (Nck, Grb2, Src-like kinases and WASP-interacting SH3 protein (WISH)) (40). Using purified proteins and cell models, Fukuoka and colleagues showed that WISH interacted with WASP through its SH3 domain (118). Furthermore, they demonstrated that this interaction led to WASP-induced Arp2/3 complex activation *in vitro*. While the addition of GST-WISH to WASP increased Arp2/3 complex-induced actin polymerization, addition of Cdc42 did not increase this activity any further, suggesting that WISH can expose the VCA region of WASP independently of Cdc42. Finally, co-expression of WISH and a mutant WASP, which is unable to bind Cdc42, in COS-7 cells led to filopodia formation, confirming previous observations that Cdc42 is not essential for WISH-mediated filopodia development (118). Taken together, the above data suggest that there are alternative, Cdc42- and Cdc42/WASP/Arp2/3 complex-independent mechanisms to regulate filopodia development.

1.2.2.2.2 Arp2/3 and Formins

The role of Arp2/3 complex proteins in filopodia formation has also been a point of controversy. For example, as described earlier, Arp2/3 complex proteins bind to the minus (or slow-growing) ends and to the sides of actin filaments. However, Arp2/3 prefers to nucleate new filaments at 70^0 angles (325, 418). This promotes actin branching and creates mesh-like actin networks – structures found in lamellipodia but not in filopodia. Furthermore, there are reports that Arp2/3 proteins may not be involved with, or found in, filopodia. Steffen and colleagues showed that while siRNA-mediated knockdown of the Arp2/3 complex proteins, Arp3 and p21-Arc, in mouse melanoma cells abrogated lamellipodia formation, neither filopodia development nor the frequency of filopodia

formation were affected (412). Thus, it is unclear whether Arp2/3 proteins are needed for filopodia growth and, if they are, how they would be involved in the polymerization of straight actin filaments.

Recent studies suggest that another actin nucleator may be involved in directing the formation of straight actin filaments. Formins are a family of cytoskeleton-organizing, multidomain proteins that direct the rapid assembly of actin structures for a variety of cellular functions, including cell division, migration, cell morphogenesis and adhesion (105, 131, 218). Unlike Arp2/3 complex proteins, formins nucleate actin filaments from the plus ends. This is especially ideal for efficient generation of long actin filaments in the presence of actin capping proteins (which bind the plus ends of actin filaments) (154, 219).

Due to the necessity of formins in the formation of a variety of cellular actin structures, studies have found formins and formin homologues in multiple organisms, among them *Saccharomyces cerevisiae* (two formins), *Saccharomyces pombe* (three formins), *Drosophila melangaster* (six formins), *Caenorhabditis elegans* (six formins), *Dictyostelium discoideum* (10 formins), and mammals (15 formins) (166, 451). All of these formins are defined by a unique, ~400-amino acid C-terminal formin homology 2 (FH2) domain preceded by an N-terminal, proline-rich FH1 domain (218). Analyses of the FH2 domain have led to the classification of the 15 mammalian formins into seven groups: the Diaphanous formins (Dia1, Dia2 and Dia3), the forming-related proteins identified in leucocytes (FRL1, FRL2 and FRL3), the disheveled-associated activators of morphogenesis (DAAM1 and DAAM2), Delphilin, the "inverted" formins (INF1 and INF2), the formin homology domain-containing proteins (FHOD1 and FHOD2), and the original formins (FMN1 and FMN2) (131).

Evidence linking formins and filopodia development was first found by Peng and colleagues. They found that microinjection of anti-mDia2, a mouse formin, or the expression of a dominant-negative mDia2, blocked Cdc42-induced actin reorganization (334). While the necessity of Cdc42 in filopodia development is not clear, Higashida and colleagues found that overexpression of EGFP-tagged mDia1 in *Xenopus* fibroblasts led to a massive formation of filopodia-like structures with EGFP staining at their tips (164). In *Dictyostelium* cells, disruption or elimination of the dDia2 gene led to defects in filopodia production while overexpression of GFP-tagged dDia2 promoted filopod development (378). Furthermore, like mDia1, both endogenous and GFP-tagged dDia2 were enriched at the tips of filopodia. Lastly, Rif, described earlier, is a modulator of filopodia formation independent of the Cdc42/WASP/Arp2/3 pathway. Pellegrin and colleagues showed that mDia2 is required for Rif induction of filopodia (330). Upon co-expression of active Rif and mDia2 in NIH 3T3 cells (which lack endogenous mDia2), mDia2 translocated to the plasma membrane and the previously observed Rif filopodial phenotype was produced. Conversely, expression of a dominant-negative mDia2 in HeLa cells blocked induction of filopodia by active Rif. These and other observations provide growing evidence of formin involvement in controlling filopodial dynamics.

While evidence for the involvement and necessity of formins in filopodia development continues to grow, the mechanism of how these proteins modulate actin assembly is not fully understood. It is known that the FH2 domain mediates actin polymerization (218). FH2 domains alter actin polymerization dynamics in three ways: a) accelerating *de novo* filament nucleation, b) altering filament elongation/depolymerization rate, and c) preventing filament plus-end capping by capping proteins (165). The

importance of FH2 domains in actin assembly was first shown in yeast (104, 345, 373). Data from these studies showed that the FH2 domain of the yeast formin, Bni1, was sufficient to nucleate unbranched actin filaments *in vitro*. Bni1's FH1 and FH2 domains were observed to be associated with the growing plus ends of new filaments (345). Furthermore, while the new actin filaments were assembled independently of Arp2/3 complex proteins, the actin-monomer-binding protein, profilin, was required for actin polymerization (104, 373). In fact, using mutant profilins, Sagot and colleagues demonstrated that intact FH1/profilin interactions were required for Bni1 FH1-FH2 to efficiently assemble filaments from profilin-bound actin monomers (373). Furthermore, when filament plus ends were saturated with Bni1, filament elongation still occurred at 50% of the normal rate of free plus end growth, suggesting that the FH2 domain associates with the plus end not just during nucleation but during filament elongation as well (345). Taken together, these studies revealed a novel mechanism for actin nucleation independent of Arp2/3.

Structural and kinetic modeling studies have shown that formins nucleate actin filaments by stabilizing actin dimmers and trimers (318, 478). Through crystal structure studies, Xu and colleagues reported that the Bni1 FH2 domain forms a "tethered dimer" in which two elongated actin binding heads are tied together at either side by an unusual "lasso and linker" structure that provides flexibility to each half of the dimer (478). It is believed that each half of the FH2 dimer interacts with two actins in an orientation similar to that in an actin filament, leading to an "actin nucleus" (318).

After filament nucleation, formins maintain their association with plus ends, processively moving with the growing filament end and enabling rapid addition of actin

subunits ("processive capping") (296). This continued association with the filament ensures that actin-capping proteins can not bind and inhibit actin polymerization and makes sure that the plus end of the filament continues to grow. Live imaging studies using GFP-labeled mDia1 in rodent cells provide further support for the "processive capping" model (164). Higashida and colleagues showed that GFP-mDia1 dots traveled on linear paths over long distances (>10μm), moving at the same rate as actin elongation / polymerization (164), suggesting that actin filaments grown by plus end addition while remaining attached to formins.

1.3 eEF1A – Eukaryotic Elongation Factor 1 Alpha

Our lab became interested in cell motility and actin cytoskeletal dynamics when we found that the protein we were studying, eueEF1A2, regulated filopodia formation (5). More specifically, ectopic expression of ***e***ukaryotic ***e***longation ***f***actor **1** ***a***lpha **2** (eEF1A2) in Rat2 rat fibroblasts and in the BT549 ductal carcinoma cell line stimulated Akt-dependent filopodia formation, cell invasion and cell migration (5). eEF1A2 is one of two isoforms of eEF1A, a protein translation elongation factor that plays an important role in protein synthesis. eEF1A is a GTP-binding protein that, when GTP-bound, binds amino-acylated tRNAs and recruits them to the ribosome. The hydrolysis of GTP results in the binding of the tRNA to its respective mRNA codon and the released GDP-eEF1A is then converted back to GTP-eEF1A through interactions with other elongation factors.

eEF1A proteins are among the most abundant proteins in the cytoplasm, comprising 1-2% of total cellular protein (61, 99). eEF1A proteins are highly conserved, from lower to higher eukaryotes (99, 304, 348). For example, human eEF1A proteins share 81% amino acid homology with those of yeast (50), 91% with those of zebrafish (121), and 99% with

those of rats (390). The eEF1A protein carries an overall positive charge, which facilitates its interaction with the negatively charged ribosome and/or high molecular weight mRNAs (304).

The human *EEF1A* gene family consists of two functional transcripts, *EEF1A1* and *EEF1A2*, and more than 18 homologous sequences that are thought to encode non-functional pseudo-genes (258). Human *EEF1A1* and *EEF1A2* map to chromosomes 6q14 and 20q13 respectively, and share greater than 95% DNA and amino acid identity, with the majority of differences occurring in the C-terminal end (6, 27, 211, 258, 443). Like in humans, both transcripts have been found in the mouse (204), rat (390), rabbit (196), salmon (315), *Drosophila melangaster* (171) and *Saccharomyces cerevisiae* (198). Consistent with their close sequence homology, eEF1A1 and eEF1A2 are functionally identical in terms of their roles in protein translation elongation (43). However, eEF1A1 and eEF1A2 proteins display differential expression profiles in mammals. While eEF1A1 is expressed ubiquitously in all tissues, eEF1A2 mRNA is detectably expressed only in the tissues of the heart, brain, and skeletal muscle (6, 27, 44, 205, 236, 324). In these three tissues, eEF1A1 expression is markedly lower, yet still detectable (27).

1.3.1 eEF1A and Protein Synthesis

Protein translation is the process by which messenger RNA (mRNA) is converted to its protein product. It is a dynamic process that is conceptually divided into three stages: translation initiation, elongation, and termination. Each of these steps involve ribosomal subunits, transfer RNAs (tRNA), amino acids and a diverse array of step-specific protein factors that catalyze the whole process (163).

Translation initiation begins with the binding of a 40S preinitiation complex to the

mRNA transcript (190, 426). The preinitiation complex consists of the 40S ribosomal subunit associated with a GTP-bound initiation factor (eIF2A)-methionyl-tRNA (Met-tRNA) complex (426). The binding of the preinitiation complex to mRNA is mediated by eIF4F, a heteromeric complex composed of eIF4E, eIF4G, eIF4A, and eIF4B (265). eIF4E recognizes the m^7GpppN-cap structure located at the 5' end of eukaryotic mRNAs. This cap is a 7-methyl guanosine (m^7) linked to any nucleotide by a triphosphate bond. The binding of the eIF4F complex to the 5' cap of an mRNA commits the protein synthesis machinery to translation of that particular mRNA. Binding of the 5' cap by eIF4E is recognized as the rate-limiting step in protein synthesis since eIF4E provides the link between the mRNA and eIF4F (145, 401, 449). Once bound to mRNA, the ribosome is thought to locate the AUG start codon by an ATP-dependent mechanism known as mRNA scanning (262, 329, 335). Although the precise mechanism of how the AUG start codon stops the scanning process is not known, it is believed that the RNA-RNA interaction between the AUG and CAU anticodon (on the Met-tRNA) stops the ribosome at the initiation site (262, 335). The ribosome structure and other initiation factors themselves may also contribute to proper recognition of the start codon, although the precise mechanisms are not fully understood (365). Upon Met-tRNA / AUG codon interaction, dissociation of eIF2A from the Met-RNA allows for the 60S ribosomal subunit to bind to the 40S subunit, generating the functional 80S ribosome (71).

The assembly of the 80S ribosome signals the start of the elongation phase of protein synthesis. During elongation, amino acids are sequentially added to the C-terminus of the growing polypeptide chain. The amino acids that are added are determined by the codon sequence of the mRNA. As in translation initiation, elongation also requires a number of

protein factors (termed elongation factors), as well as amino-acylated tRNAs (acylated tRNA molecules complexed with their respective amino acids via aminoacyl-tRNA synthetase enzymes).

Upon binding to an amino-acylated tRNA, GTP-eEF1A recruits it to the ribosome. If the appropriate mRNA codon is available and recognized, then the GTP is hydrolyzed, freeing the GDP-eEF1A from the ribosome. The guanine nucleotide exchange factor, eEF1B, stimulates the regeneration of GTP-eEF1A (33, 337).

The formation of the peptide bond between the new amino acid and the existing peptide chain is governed by the peptidyltransferase activity of the ribosome and by eEF2 (25, 360). Following peptide bond formation, GTP-bound eEF2 enters the ribosome and, upon GTP hydrolysis, translocates the peptidyl-tRNA and moves the ribosome down one codon on the mRNA towards the 3' terminus. Therefore, eEF2 is responsible for the translocation process and ribosomal movement, while the peptidyltransferase in the 60S ribosomal subunit catalyzes the peptide bond formation between the amino acids (192, 404, 432). The deacylated-tRNA is then released into the cytoplasm for reacetylation and the GDP-eEF2 leaves the A-site for the recruitment of subsequent aminoacylated-tRNAs (by first being recycled to GTP-eEF2) (192).

The elongation process continues until the ribosome encounters one of three stop codons: UAA, UAG, or UGA (209). At this point, protein synthesis enters the termination stage. Upon recognition of a stop codon, the *e*ukaryotic *r*elease *f*actor 1 (eRF1) binds to the codon (26). eRF1 has a similar tertiary structure and dimensions of tRNA, and thus mimics tRNA (26). This binding triggers the hydrolysis of the ester bond between the tRNA and the polypeptide located in the P-site, resulting in the release of the newly synthesized

polypeptide (354). Following the hydrolysis of peptidyl-tRNA, GTP-eRF3 facilitates the dissociation of eRF1 from the ribosome (via GTP hydrolysis) (178, 291). The 80S ribosome is then separated into its 40S and 60S subunits.

1.3.2 eEF1A - Domain Structure

The various protein domains of mammalian eEF1A proteins have not been precisely defined. However, based on conserved nucleotide and amino acid sequence homology with eEF1A proteins from prokaryotes to lower and higher eukaryotes (99, 304, 348), our lab has generated a putative protein domain map of human eEF1A (Fig. 1.6). For example, eEF1A contains the consensus sequences characteristic of all G-proteins: the G-1 motif, which consists of a GxxxxGKS sequence, which mediates GTP phosphate binding, the G-3 motif, which facilitates GTP hydrolysis, and the G-4 motif, consisting of NKxD, that interacts with the guanine ring of GTP (405). Furthermore, studies of the prokaryotic eEF1A protein, EF-Tu, identified the amino acid residues responsible for tRNA binding (191, 406). Finally, studies on *Dictyostelium* and yeast eEF1A proteins have identified two actin binding domains (78, 95, 136, 480). Based on the above information, we have identified the corresponding amino acid residues responsible for mammalian eEF1A's GTP-binding, GTP-hydrolysis, tRNA-binding, and actin-binding domains (Fig. 1.6).

1.3.3 eEF1A2

As described earlier, *EEF1A2*, the gene encoding eEF1A2, is the second functional transcript of eEF1A (196). *EEF1A2* maps to 20q13.3 and the protein is detectably expressed in mammalian heart, brain, and skeletal muscle (44).

1.3.3.1 eEF1A2 and the Wasted Mouse

Wasted (*wst*) is an autosomal recessive mutation that consists of a 15.8 kilobase

deletion that removes the promoter and first non-coding exon region of the mouse *Eef1a2* gene (44, 306). This abolishes transcription of the gene, and thus eliminates eEF1A2 activity. Homozygous *wst/wst* mice appear normal until 21 days, though they develop progressive atrophy of the spleen and thymus and show a concomitant decrease in circulating lymphocytes levels (44, 306). After three weeks, the mice develop substantial neurological defects, muscle wasting, and immune system abnormalities (including a defective response to DNA damage in lymphoid cells). Histological examinations reveal extensive neuronal vacuolar degeneration of anterior horn cells of the spinal cord with less severe abnormalities of motor nuclei in the brain stem. The mice lose weight, develop progressive paralysis, and eventually die by 30 days of age (44, 306). These results suggest that eEF1A2 is involved in maintaining neuromuscular integrity, regulating immunological functions, and controlling lymphoid tissue apoptosis (44, 306).

1.3.3.2 eEF1A2 and Cancer

The identification of eEF1A2 as an oncogene was first shown by Anand and colleagues in 2002 (6). Ectopic expression of eEF1A2 in rodent fibroblasts resulted in greater proliferative capability, enabled anchorage-independent growth, and induced tumour formation when xenografted in nude mice. Moreover, *EEF1A2* gene amplification is observed in 25-33% of ovarian tumours and cell lines (6). Since this initial study, others have found overexpression of eEF1A2 protein and mRNA in up to 75% of ovarian clear cell carcinomas (440), and overexpression of protein in greater than 85% of serous and endometroid cancers (338). In serous cancers, eEF1A2 has been shown to be an independent prognostic marker for survival; patients with serous tumours showing high eEF1A2 protein expression have increased probability of 20-year survival (338).

More recently, eEF1A2 has been implicated in lung cancer (245, 494). Comparative genomic hybridization studies, as well as genomic, transcript, and tissue microarray analyses, have identified *EEF1A2* as a lung oncogene. Lung adenocarcinomas show *EEF1A2* gene amplifications, increased mRNA transcript, and increased protein levels (245). Furthermore, elevated protein levels correlated with higher tumour grade, more advanced disease stage, and a shorter survival of patients, suggesting that eEF1A2 is a marker for poor prognosis in lung cancer (245).

In the above two cases, eEF1A2 appears to have differential effects. While being an oncogene in both the ovary and lung, its effects in cells are different. For example, eEF1A2 ablation in lung cancer cell lines using short-interfering RNA (siRNA) suppresses cell proliferation and induces apoptosis . However, while overexpression of eEF1A2 in ovarian cancer cell lines increases cell proliferation, it does not confer resistance to drug-induced cell death or anoikis (338). Similarly, as noted above, while eEF1A2 is a marker for poor prognosis in lung cancer, it is a marker for good prognosis for patients with serous tumours (338). Therefore, the microenvironment within which eEF1A2 is expressed appears to play an important role.

1.3.3.2.1 eEF1A2 and Breast Cancer

Amplification of the 20q13 locus is a marker for breast cancer and is correlated with poor clinical prognosis and increased tumour aggressiveness (65, 427-429). Thus, it is not surprising that eEF1A2 mRNA and protein have been found to be overexpressed in breast cancer (130, 193, 224, 441). Tomlinson and colleagues showed that while eEF1A2 mRNA is found in extremely low levels in normal breast and benign breast tumours, there is a 30-fold increase in malignant tumour samples (441). Moreover, there was a positive correlation

between eEF1A2 expression and estrogen-receptor positive tumours while there was no correlation between eEF1A2 expression level and tumour grade or lymph node positivity (224). Independently, our lab has shown that eEF1A2 mRNA is highly expressed in ductal carcinomas, metastases, and lobular carcinomas (224). However, we have found no correlation between eEF1A2 expression and tumour size or grade, nodal status, and,contrary to the Tomlinson study, estrogen receptor status (224). More importantly, we have found that high eEF1A2 expression is a significant predictor of outcome. Women with tumours showing high eEF1A2 levels have an increased probability of 20-year survival than women with low eEF1A2 expression in their tumours (224).

1.3.4 eEF1A - Non-Canonical Roles

Aside from its role in translation elongation, eEF1A and eEF1A-like proteins have several other roles. These include regulating the actin (96, 136, 297) and tubulin (94, 294, 313) cytoskeleton, promoting the degradation of damaged proteins (45, 230, 372, 436), mediating apoptosis (385), inducing the heat shock response (352, 481, 482) and promoting phosphatidylinositol signaling (313).

1.3.4.1 eEF1A and the Actin Cytoskeleton

eEF1A proteins from several genera are known to associate with actin and tubulin, and mediate cytoskeletal remodeling. This was first observed in sea urchin eggs, in which a GTP-binding protein that was structurally and functionally similar to yeast eEF1A was found associated with the mitotic apparatus (specifically in centrosomes and bound to astral and spindle microtubules) (84). Soon thereafter, an actin-binding protein, ABP-50, similar in nucleotide sequence to mammalian eEF1A, was found in *Dictyostelium discoideum* (95, 480). ABP-50 was described as an abundant actin filament bundling protein that, in addition

to binding cytosolic monomeric actin, was found to be present in filopodia and other cortical regions that contained actin filament bundles (84). More specifically, Dharmawardhane and colleagues showed that upon the addition of cAMP, a chemoattractant, ABP-50 localized to filopodia that were extended as a response to stimulation, whereas in unstimulated cells, ABP-50 exhibited a diffuse distribution in the cytosol (84). Furthermore, ABP-50 binding to, and colocalization with, actin filaments in filopodia occurred with concomitant cell movement (13, 228). These studies were the first to show an interaction between eEF1A proteins and the cytoskeleton, as well as an association between eEF1A2 and cell movement (via filopodia production). Since these original findings, eEF1A proteins from protozoans (136, 297), yeast (57), plants (5, 136, 249), and mammals (84) have been shown to associate *in vitro* and *in vivo* with the actin cytoskeleton through co-immunoprecipitation assays and colocalization studies (immunofluorescence and electron microscopy).

The nature of the eEF1A-actin interaction has also been well characterized. Immunoprecipitation of cytosolic *Dictyostelium* eEF1A using specific antibodies show co-precipitation of actin in whole cell lysates (84). Dharmawardhane and colleagues showed that purified eEF1A bound to monomeric actin (G-actin) with a K_d of approximately 0.09μM and at a cytosolic molar ratio of 1:2 (eEF1A:actin) (84). Furthermore, purified eEF1A inhibits actin polymerization both in *Dictyostelium* and as a recombinant GST-fusion protein *in vitro* at cytosolic molar ratios, while nucleating actin polymerization at higher molar ratios to actin (298). Murray and colleagues proposed that eEF1A's ability to inhibit actin polymerization was related to its actin bundling activity (298). There are several reports of eEF1A inducing rapid actin bundle formation *in vitro* (78, 84, 97, 298, 480). Electron micrographs of *in vitro* actin polymerization show significant actin bundling within

20 seconds in the presence of eEF1A (298). Furthermore, EF1A proteins have also been shown to crosslink with actin filaments in such a way to prevent other actin-binding proteins from binding. Using purified actin and *Dictyostelium* eEF1A, Owen and colleagues found that the resulting actin bundles contained crosslinked actin filaments that were rotated 90° relative to one another (250, 252). These findings provided further rationale for the observations of Murray and colleagues that actin bundles became progressively more ordered up to 60 seconds after the introduction of eEF1A (298). Taken together, these results suggested that eEF1A sequestered in actin bundles played a role in the organization of actin networks. Furthermore, Owen and colleagues proposed that this specific type of actin crosslinking by eEF1A presents an environment that is highly conducive and specific for mRNA translation. However, this model implies that eEF1A's role in actin binding is related to its role in protein synthesis. Described below is another model that suggests that eEF1A's role in protein translation is distinct and independent of its role in regulating the actin cytoskeleton.

Sequence analyses of various prokaryotic and eukaryotic eEF1A proteins have led to the identification of two actin-binding domains (95, 249, 252, 480). Using sedimentation assays and immunofluorescence microscopy, Edmonds and colleagues found that eEF1A's affinity for actin filaments decreases (from a K_d of around 0.2μM to greater than 2.2μM) *in vitro* and in cells, when the pH increased from 6.2 to 7.8 (97). Furthermore, Liu and colleagues showed that the pH dependence of eEF1A/actin binding was sufficient to shift the binding of eEF1A from actin filaments to aminoacylated-tRNA (252). Coupled with *in vitro* competition binding experiments, eEF1A's binding to actin filaments and aminoacylated tRNA was found to be mutually exclusive (252). Crystal structure studies of

the bacterial eEF1A, EF-Tu, depicts that, in the EF-Tu-aminoacylated-tRNA complex, the tRNA binding interface comprises parts of actin binding domain (310). Similarly, Edmonds and colleagues demonstrated that actin filament-bound eEF1A had a nearly seven-fold decrease in the affinity for GTP or GDP (K_d values of 0.17μM (no GDP or GTP) to between 1.2μM and 1.3μM (when bound to GTP or GDP, respectively)), suggesting that actin-binding and GTP/GDP binding are also mutually exclusive (96). Taken together, these results suggest that when bound to actin filaments, eEF1A proteins are unavailable for protein synthesis. However, upon stimuli to start protein synthesis, eEF1A proteins dissociate from actin filaments and participate in peptide elongation (61). Additionally, having eEF1A proteins bound to actin filaments permits for a concentrated supply of eEF1A for protein synthesis should the need arise. Thus, this model describes a scenario by which the protein synthetic machinery and the actin cytoskeleton interact and crosstalk but do not function simultaneously.

1.3.4.2 eEF1A and Phosphatidylinositol Signaling

In 1993, Yang and colleagues purified a phosphatidylinositol-4 kinase (PI4K) activator, PIK-A49, from carrot cells grown in suspension culture (481, 482). PIK-A49 had a mass of 49kDa and was able to induce a two-four-fold activation of carrot plasma membrane PI4K as measured by an *in vitro* kinase assay. Interestingly, the authors also found that PIK-A49 was an abundant cytosolic protein, constituting nearly 1% of total soluble protein. Furthermore, PIK-A49 had 69% amino acid homology to the *Dictyostelium* actin-binding protein ABP-50, and greater than 90% identical to the elongation factor 1A from carrot, tomato, and *Arabidopsis* (481, 482).

Consistent with the sequence similarity to other actin-binding proteins and

elongation factor 1A proteins, PIK-A49 was able to function in an *in vitro* translation elongation assay, cooperate with other elongation factors, and was able to bind actin and facilitate actin polymerization (27, 258, 436). These results suggested that an actin-binding protein with translational activity was able to regulate phospholipid metabolism through PI4K activation.

While the above data brought to light an intriguing link between a participant in protein synthesis, the actin cytoskeleton, and phosphatidylinositol signaling, not much was followed. For example, the full length cDNA of PIK-A49 was never fully cloned to confirm eEF1A homology. Moreover, the ability to activate PI4K was never checked with other, non-carrot plant or mammalian eEF1A proteins. Lastly, since there are multiple isoforms of PI4K (discussed later), it is not clear whether PIK-A49 is an activator of all PI4Ks or specific isoforms. Thus, much is still unclear about the relationship between eEF1A proteins and PI4K.

1.4 Research Hypotheses and Objectives

At the outset of this thesis, the following was known about the protein translation factor, eEF1A and, in particular, eEF1A2. First, eEF1A proteins regulated the actin cytoskeleton by binding and bundling actin. Second, in carrots, an eEF1A-like protein activated PI4K. Third, *EEF1A2* was an ovarian oncogene. Lastly, eEF1A2 promoted filopodia development and increased the rates of cell migration and invasion. The overarching hypothesis of my research was that eEF1A2's primary role in oncogenesis was to promote cell migration and invasion through phosphatidylinositol-dependent actin cytoskeletal reorganization. More specifically, I hypothesized that by activating PI4K in mammalian cells, eEF1A2 enhanced phosphatidylinositol signaling. A consequence of this

increased signaling is the generation of several important downstream lipid products that regulate the cell's cytoskeletal architecture, cell movement, cell growth, and oncogenesis. Furthermore, I hypothesized that eEF1A2's role in these various processes was independent of its role in protein translation. Finally, I hypothesized that in addition to being an ovarian oncogene, *EEF1A2* was also an oncogene of the breast due to amplifications of its locus in breast cancers. Therefore, the goals of my PhD research were three-fold. First, I wanted to investigate whether eEF1A2 activated PI4KIIIβ in mammalian cells. Second, I wanted to determine whether eEF1A2-mediated filopodia formation occurred through a phosphoinositide-dependent mechanism. Specifically, I wanted to determine whether filopodia generated in eEF1A2-expressing cells was due to eEF1A2 / PI4KIIIβ interaction. Lastly, I wanted to describe some *in vitro* and *in vivo* effects of eEF1A2 expression in breast cancer cell lines.

1.5 Summary of Thesis

The understanding of cell motility and the signals that regulate it are critical in understanding diseases such as cancer. The first step in cell movement is the creation of plasma membrane protrusions in response to migratory and/or chemotactic stimuli. Filopodia, long, thin cell extensions that are supported by tight, dynamic, parallel bundles of actin filaments, are one type of these protrusions. As such, the identification of proteins that regulate filopodia formation, and the mechanisms through which they do so, are important. In this thesis, I provide evidence that the protein elongation factor eEF1A2 regulates filopodia formation. Furthermore, I show that eEF1A2's ability to regulate filopodial dynamics is dependent on the phosphatidylinositol signaling pathway, specifically, the activation of the lipid kinase, PI4KIIIβ. Finally, I present some data on eEF1A2's role in

breast oncogenesis.

In Chapter Two, I characterize a physical and functional interaction between eEF1A2 and the lipid kinase, PI4KIIIβ. I show that eEF1A2 binds to, and activates this kinase *in vitro* and in cells. In Chapter Three, I describe how the activation of PI4KIIIβ leads to filopodia formation seen in eEF1A2-expressing cells. I show that eEF1A2-mediated activation of PI4KIIIβ is necessary for eEF1A2-induced filopodia development. In Chapter Four, I highlight some of the effects of abnormal eEF1A2 expression in breast cancer cell lines and provide evidence of how eEF1A2 could be used as a therapeutic target in breast oncogenesis. Finally, Chapter Five summarizes and discusses the implication of the provided data. I propose and describe a new model for eEF1A2-mediated regulation of the actin cytoskeleton. This chapter also focuses on some unpublished data and links them with future research areas.

Chapter 2

Binding of elongation factor eEF1A2 to phosphatidylinositol-4 kinase III beta stimulates lipid kinase activity and phosphatidylinositol-4 phosphate generation*

*A modified version of this Chapter has been published.

Jeganathan, S. and J.M. Lee. 2007. Binding of elongation factor eEF1A2 to phosphatidylinositol-4 kinase beta stimulates lipid kinase activity and phosphatidylinositol-4 phosphate generation. J Biol Chem 282:372-80.

2.1 Introduction

eEF1A2 is one of two members of the eEF1A family of proteins (eEF1A1 and eEF1A2). During protein translation elongation, eEF1A proteins bind amino-acylated tRNA and facilitate their recruitment to the ribosome (163). Aside from their canonical role in protein translation, eEF1A proteins have other functions, including binding actin and inducing rearrangements of the actin and tubulin cytoskeleton (61, 389). The inactivation of the mouse eEF1A2, *Eef1a2*, leads to immunodeficiency and death by 30 days of age (44, 392).

Mammalian eEF1A2 mRNA can be detected only in normal heart, brain and skeletal muscle tissues (196, 211, 239). However, high levels of eEF1A2 protein and mRNA are observed in 30-60% of ovarian, breast and lung tumours (6, 223, 244, 441). We have previously reported that eEF1A2 has transforming properties: ectopic expression of wild type human eEF1A2 in rodenet firoblasts enables anchorage-independent growth and enhances tumourigenicity in nude mice (6). Thus, eEF1A2 has an important role in promoting tumour development. However, the mechanism by which eEF1A2 promotes oncogenicity remains unclear.

It has previously been reported that an eEF1A-like protein purified from carrots, PIK-A49, binds and activates carrot phosphatidylinositol-4 kinase (PI4K) (481, 482). This suggests an important relationship between translation elongation and phosphatidylinositol (PI) generation. PIs are negatively charged, membrane-bound phospholipids that serve as regulators of multiple signaling pathways (41, 116, 276, 320). PIs are composed of an inositol ring linked to a lipid phosphatidic acid backbone by a phosphodiester bond at the inositol D1 carbon. Inositol phosphorylation occurs at the D3, D4, or D5 carbons. Specific

kinase families are responsible for phosphorylation at each of these sites. Phosphatidylinositol-3 kinases (PI3K), phosphatidylinositol-4 kinases (PI4K), and phosphatidylinositol-5 kinases (PI5K) phosphorylate the D3, D4, and D5 inositol carbons respectively (41, 116, 276).

PIK-A49 showed *in vitro* translation elongation factor activity and an ability to activate *in vitro* PI4K lipid kinase activity (481, 482). However, PIK-A49 does not have complete amino acid sequence identity with wild-type carrot eEF1A and has yet to be cloned as a full-length cDNA. It is therefore unclear whether PIK-A49 is a bona fide eEF1A protein. Neither is it known whether wild-type carrot eEF1A, or eEF1A proteins from non-plant species, participate in PI4K activation. In addition, there are three identified sub-families of PI4K proteins, PI4KIIIα, PI4KIIIβ and PI4KII (16, 161), and it is unclear which PI4K isoform(s) are activated by PIK-A49 or other eEF1A proteins. Moreover, *in vitro* PI4K activation by PIK-A49/eEF1A has unknown physiological significance.

Here we report that human eEF1A2 can directly bind and activate PI4KIIIβ. Ectopic expression of eEF1A2 in rodent and human cells increases overall PI4K activity and cellular PI4P generation. Furthermore, eEF1A2 ablation reduces endogenous PI4K activity. This suggests eEF1A2 is a physiological regulator of PI4KIIIβ.

2.2 Materials and Methods

2.2.1 Cell lines

MCF7, BT549, and Rat2 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and grown according to their instructions.

2.2.2 Adenoviral vectors

eEF1A2 was subcloned into the pShuttle-IRES plasmid (*EcoRV/XhoI*) with a Flag epitope tag. eEF1A2 and GFP virus were manufactured by the Adenoviral Core Facility of the University of Ottawa. For viral transduction, BT549 and Rat2 cells were infected with Ad-eEF1A2 or Ad-GFP at a multiplicity of infection (MOI) of 200 (BT549) or 500 (Rat2) in complete media. Cells were incubated with virus for a minimum of 24 hours.

2.2.3 Antibodies

Antibodies used for experiments are as follows: human PI4KIIIβ (Upstate Cell Signaling Solutions, Charlottesville, VA, USA), β-actin (Sigma, Oakville, ON, Canada), HRP-conjugated goat anti-mouse IgG (Upstate Cell Signaling Solutions), HRP-conjugated anti-rabbit IgG, (Cell Signaling Technology, Danvers, MA, USA). The generation of the rabbit polyclonal eEF1A2 antibody and its validation in western blotting, immunoprecipitation, and immunohistochemistry is described elsewhere (223).

2.2.4 GST fusion proteins.

eEF1A2 cDNA was cloned into the *EcoRI/NotI* site of pGEX-4T2 (Pharmacia, Kirkland, QC, Canada). GST-eEF1A2 was transformed into *E coli* BL21DE3 and grown in LBA to A_{600}. ~0.7. 0.5mM IPTG was added for 2 hours at 25°C. Bacteria were lysed in 25mM HEPES; pH7.9, 100mM KCl, 2mM EDTA, 20% glycerol, 2mM DTT, and 1X protease inhibitor cocktail (Roche, Laval, QC, Canada). Glutathione Sepharose 4B beads

(Amersham Biosciences, Piscataway, NJ, USA) were equilibrated in lysis buffer and mixed with sonicated suspensions. PI4KIIIβ in pGEX-6P-3 was a gift of T. Balla (19, 491). The GST-PI4KIIIβ fusion protein was purified as described (491). GST-C/EBPβ was a kind gift from Dr. N. Wiper-Bergeron (University of Ottawa). To remove the GST moiety, 100μg of GST-eEF1A2 was incubated overnight with 1U of Thrombin (Amersham) in 1x PBS at room temperature. PI4KIIIβ was generated by cleaving 100μg of GST-PI4KIIIβ with 1U of PreScission Protease (Amersham) in 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.0 at 4°C.

2.2.5 *In vitro* lipid kinase assay

10μl of recombinant protein in PBS, at concentrations indicated in the figure legends, was added to 35μl of kinase buffer (1mM EDTA, 30mM HEPES; pH 7.4, 100mM NaCl, 2mM $MgCl_2$, and 0.2% Triton X-100), 3mM PI (or as indicated in the figure legend) and 5μl of 10mM ATP containing 10μCi of ^{32}P-ATP, and incubated for 60 minutes. The reaction was stopped by the addition of 60μl of 1N HCl. Phospholipids were extracted by adding 160μl of $CHCl_3$:MeOH (1:1, v/v). After brief vortexing, samples were centrifuged for 10 minutes at 10,000 x *g*. Aliquots of the organic phase (10-20μl) were spotted onto TLC plates (Sigma) and placed in a pre-equilibrated tank containing $CHCl_3$:acetone:MeOH:HOAc:water (46:17:15:14:8, v/v). Prior to use, TLC plates were precoated with 1% potassium oxalate, 3mM EDTA in methanol:water (2:3, v/v) for one hour and allowed to air dry overnight. Plates were activated by baking for 1 hour at 110°C. Phosphatidylinositol standards were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). PI4P spots were scraped and dissolved in 1-2 ml of water. Aliquots were then diluted in BetaMax scintillation fluid (ICN Biomedicals, Irvine, CA, USA) and scintillation

counts were taken using the Wallac 1414 Liquid Scintillation Counter (Fisher Scientific Limited, Ottawa, ON, Canada). Values of K_m, V_{max}, and K_{cat} were determined using the GraphPad Prism software (San Diego, CA, USA). For assays involving cell lysates, recombinant eEF1A2 and the cell lysate was added to a total volume of 10μl.

2.2.6 Crosslinking studies

Purified eEF1A2 and/or PI4KIIIβ (without GST) were incubated in PBS and crosslinked with 30mM dimethyl pimelimidate (DMP; Pierce, Rockford, IL, USA) in 0.2M ethanolamine, pH 8.0 at room temperature. Reactions were stopped by addition of glacial acetic acid at a 1:4 (v/v) to the sample. Proteins were concentrated to a final volume of 50μl with Microcon Centrifugal Filter Devices (Millipore, Billerica, MA, USA) and solubilized using 5% (w/v) sucrose in water and electrophoresed in a continuous, non-denaturing 5% (40% (29:1) acrylamide/bis-acrylamide) polyacrylamide gel using 100mM sodium phosphate (pH 7.0) running buffer as previously described (120)..

2.2.7 Cell lysis and co-immunoprecipitation

For co-immunoprecipitation, cells were grown to 80-95% confluence in 100mm cell culture plates. Cells were lysed by sonication on ice in detergent-free buffer (137mM NaCl, 8mM KH_2PO_4 (pH 7.5), 2.7mM KCl, 2.5mM EDTA, and 1% Aprotonin, 1mg/ml leupeptin, 50mM NaF, 1mM Na_3VO_4, 10μg/ml pepstatin, 1mM PMSF). Lysates were centrifuged at 10,000x*g* for 20 minutes to remove membranes. Supernatants were collected and protein levels were quantified using a Bradford assay (BioRad, Mississauga, ON, Canada) according to the manufacturer's instructions. 100 μg of total protein was pre-cleared with protein G sepharose (Amersham) for one hour at 4°C. Following this, 2-4μg of PI4KIIIβ, Flag, or eEF1A2 antibody coupled to beads was added and incubated overnight at 4°C.

Beads were washed 3X in PBS, centrifuged, boiled for 5 minutes in sample buffer, and the supernatant subjected to SDS-PAGE. The covalent coupling of the eEF1A2 antibody to protein A agarose beads was performed using a previously described protocol (153). Western blot detection was according to manufacturers' instructions or as described for the eEF1A2 antibody (223).

For Western blotting, cells were lysed in RIPA buffer (50mM Tris–Cl; pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1mM EDTA; pH 7.0, 150mM NaCl and 1% Aprotonin, 1mg/ml leupeptin, 50mM NaF, 1mM Na_3VO_4, 10μg/ml pepstatin in ethanol, and 1mM PMSF in DMSO).

2.2.8 siRNA transfections

Sequences of the eEF1A2 siRNAs are 5'-UGGUCC-UUUUGUCAAUACCtc-3' (siRNA #1) and 5'–UCGAACUUCUCAAUGGUCCtt-3' (siRNA #2). The negative control (NC) siRNA was purchased from Ambion (Cat. # 4611, Austin, TX, USA). siRNA transfections were performed using siPORT Lipid (Ambion) according to manufacturer's instructions.

2.2.9 Immunofluorescence

To detect PI4P levels, Rat2 and BT549 cells were transfected with either eEF1A2-pcDNA3.1 (6) or GFP-pcDNA3.1 using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions Five hours post-transfection, transfection media was removed and complete media added. The next day, cells were fixed with 3.7% formaldehyde/PBS and permeabilized with 0.1% Triton-X/PBS. Cells were then blocked with 10% goat serum / PBS for 30 minutes at 37°C and anti-PI4P IgM antibody added (1:100 in PBS, overnight; Echelon Biosciences Inc., Salt Lake City, UT, USA). Goat anti-

mouse IgM, R-phycoerythrin (Caltag Laboratories, Carlsbad, CA, USA) was then added at 1:100 in PBS for 30 minutes. eEF1A2 was detected with a monoclonal anti-V5 antibody (1:500 in PBS, 1 hour; Sigma) followed by an AlexaFluor 488 goat anti-mouse IgG (1:450 in PBS, 1 hour; Invitrogen). Cell nuclei were stained with Hoescht 33258 (20μg/ml; Sigma) for 10 minutes at room temperature. Slides were viewed with a Leica DM-1L Fluorescence microscope and deconvolved using Improvision 3.1 software (Richmond Hill, ON, Canada) or an Olympus FluoView FV1000 Laser Scanning Confocal microscope. Fluorescence intensity was quantified with the confocal microscope using Olympus software (FV1000 Ver.01.04a, Center Valley, PA, USA).

2.3 Results

2.3.1 eEF1A2 increases PI4KIIIβ lipid kinase activity

To determine whether eEF1A2 could activate PI4KIIIβ, we purified recombinant GST-eEF1A2 and GST- PI4KIIIβ. The proteins were isolated both with and without their GST moiety. A Coomassie Blue-stained gel of the purified proteins is shown in Fig. 2.1A. The predicted molecular weight of full-length, wild-type eEF1A2 is ~54 kDa and that of PI4KIIIβ is ~100 kDa.

We first determined whether eEF1A2 could increase PI4K activity in cell lysates. As shown in Fig. 2.1B, addition of GST-eEF1A2 to a lysate of BT549 breast carcinoma cells reproducibly doubled *in vitro* phosphatidylinositol-4 phosphate (PI4P) generation compared to the addition of GST alone or GST-coupled to the C/EBPβ transcription factor. A representative TLC plate is also shown in the right panel of Fig. 2.1B.

Bacterially expressed PI4KIIIβ has previously been reported to be active in *in vitro* lipid kinase assays (19, 491). As shown in Fig. 2.1C, our GST-PI4KIIIβ is active *in vitro* and its kinase activity is inhibitable by 0.2μM Wortmannin. Wortmannin inhibition indicates that we are measuring the lipid kinase activity of a type III PI4 kinase, the family of PI4 kinases to which PI4KIIIβ belongs. To investigate whether purified eEF1A2 could directly activate PI4KIIIβ, we next added recombinant eEF1A2 (without GST) to purified PI4KIIIβ (without GST). As shown in Fig. 2.1D, eEF1A2 increased PI4KIIIβ lipid kinase activity in a concentration-dependent manner. PI4KIIIβ activity was increased approximately two-fold. 100-200nM eEF1A2 is required to maximally activate a 100nM solution of PI4KIIIβ. No enhancement of PI4KIIIβ activity was observed in the presence of bovine serum albumin (BSA).

In order to further determine how eEF1A2 affected PI4KIIIβ activity, we experimentally determined the PI4KIIIβ K_m, V_{max}, and K_{cat} for phosphatidylinositol with and without eEF1A2. Bovine serum albumin served as the control. As shown in Fig. 2.1E, eEF1A2 (without GST) reproducibly increased the V_{max} of PI4KIIIβ (without GST) from 0.62 to 1.11 μmole/min/mg. This doubling of V_{max} was also mirrored in the increase of K_{cat} from 0.82 to 1.48 sec^{-1}. The PI4KIIIβ K_m was unchanged.

2.3.2 eEF1A2 binds PI4KIIIβ

We next tested whether eEF1A2 could directly interact with PI4KIIIβ in a cell-free system. As shown in Fig. 2.2A, purified PI4KIIIβ (without GST) was precipitated by GST-eEF1A2 and eEF1A2 (without GST) was precipitated by GST-PI4KIIIβ. Neither PI4KIIIβ nor eEF1A2 were precipitated by GST alone. We next investigated the stoichiometry of eEF1A2 / PI4KIIIβ interaction. We incubated recombinant eEF1A2 and PI4KIIIβ (both without GST) and chemically crosslinked the resulting complex. As seen in Fig. 2.2B, after an hour of incubation, most of the added PI4KIIIβ and eEF1A2 are found in a complex with an apparent molecular weight (MW) of 165kDa. The formation of the complex is time-dependent and a greater amount of complex is detected at 60 minutes of incubation relative to the 30 minute time point. eEF1A2 has an apparent MW of ~60kDa and that of PI4KIIIβ is ~100kDa. The ~165kDa MW of the complex indicates that eEF1A2 and PI4KIIIβ associate with a 1:1 stoichiometry. Neither eEF1A2 nor PI4KIIIβ formed detectable multimers on their own. We were unable to detect protein complexes with a MW greater than 165 kDa, indicating that multimers containing more than one molecule each of eEF1A2 or PI4KIIIβ do not form.

2.3.3 eEF1A2 interacts with PI4KIIIβ in mammalian cell lines

To determine whether eEF1A2 and PI4KIIIβ associate in cells, we first identified mammalian cells that express eEF1A2. In rodents and humans, eEF1A2 shows detectable mRNA and protein expression only in the tissues of the heart, brain and skeletal muscle (196, 211, 239). We have also observed eEF1A2 expression in many breast cancer cell lines (223). As shown in Fig. 2.3A, we detect eEF1A2 protein expression in the MCF7 human breast carcinoma cell line but not in BT549 breast carcinoma cells nor in the non-transformed Rat2 rat fibroblast cell line.

We then used co-immunoprecipitation to determine whether wild-type eEF1A2 and PI4KIIIβ associate in MCF7 cells. We used detergent-free cell lysis for these experiments. As shown in Fig. 2.3B, eEF1A2 detectably immunoprecipitates with PI4KIIIβ and PI4KIIIβ reciprocally immunoprecipitates with eEF1A2. Neither protein binds to the Flag antibody control. While immunoprecipitation is only semi-quantitative, we estimate that ~10% of endogenous PI4KIIIβ associates with eEF1A2 under detergent-free lysis conditions. A similar ratio of eEF1A2 is associated with PI4KIIIβ.

Both PI4KIIIβ and eEF1A2 have been previously reported to be diffusively cytoplamsic, although PI4KIIIβ has some concentration in the Golgi apparatus (6, 16, 161). We attempted to visualize eEF1A2 and PI4KIIIβ co-localization via immunofluorescence confocal microscopy but were unable to detect significant co-localization between PI4KIIIβ and eEF1A2 (Appendix A; Figure A.1). Co-localization detection is complicated by the diffuse cytoplasmic staining pattern of the two proteins and the likelihood that only a small fraction of each exists in an *in vivo* PI4KIIIβ /eEF1A2 complex. Nonetheless, the detectable

amount of eEF1A2 that co-precipitates with *in vivo* PI4KIIIβ indicates that

PI4KIIIβ/eEF1A2 complexes do exist in MCF7 cells.

2.3.4 eEF1A2 is sufficient to increase cellular PI4K activity

To determine whether eEF1A2 expression was sufficient to increase PI4K activity in mammalian cells, we used an adenovirus to express eEF1A2 in BT549 and Rat2 cells. Neither cell line detectably expresses eEF1A2 without transduction (Fig. 2.3A). At a low multiplicity of infection (MOI) of 20 virus particles per cell, infection had little effect on PI4K activity; lipid phosphorylation was similar between eEF1A2-infected cells and those infected with GFP (Fig. 2.4A). However, increasing the MOI to 200 reproducibly doubled cellular PI4K activity in eEF1A2-infected cells relative to those infected with GFP (Fig. 2.4A). Similarly, infection of Rat2 cells with the eEF1A2 adenovirus doubled PI4K activity relative to GFP controls (Fig. 2.4B). Adenoviral infection by eEF1A2 does not detectably alter total PI4KIIIβ protein levels (Fig. 2.4C). Thus, eEF1A2 expression can increase PI4K activity in these mammalian cell lines.

2.3.5 eEF1A2 is a physiological PI4K regulator

To determine whether eEF1A2 had a physiological role in regulating PI4K activity, we reduced eEF1A2 protein levels in MCF7 cells. MCF7 cells express abundant eEF1A2 (Fig. 2.3A). For this purpose, we designed two eEF1A2-specific siRNAs. As shown in Fig. 2.5, both siRNAs substantially reduce eEF1A2 protein levels compared to those cells treated with a control siRNA or untreated cells. Some residual eEF1A2 remains, however. The siRNA-mediated decrease in eEF1A2 protein levels leads to a concomitant two-fold decrease in PI4K activity relative to controls.

2.3.6 eEF1A2 increases cellular PI4P generation

We next determined whether eEF1A2 could increase intracellular PI4P abundance. To this end, we transiently transfected Rat2 and BT549 cells with an eEF1A2 plasmid and stained these cells with an antibody specific for PI4P. As shown in Fig. 2.6A, BT549 and Rat2 cells transiently expressing eEF1A2 show PI4P staining that is visibly brighter than in untransfected cells, indicating greater abundance of PI4P upon eEF1A2 expression. The increase in PI4P occurs throughout the cytosol (Fig. 2.6A). Transfection with GFP had no visible effect on PI4P levels (Fig. 2.6B). To quantify the eEF1A2-dependent increase in PI4P generation, we divided each transfectant pool into groups based on expression of the transfected protein and quantified PI4P fluorescence in individual cells from each group. As shown in Fig. 2.6C, cells expressing moderate or high levels of eEF1A2 each have a significantly higher level of PI4P than those that express no/low eEF1A2 ($p< 0.001$, Mann-Whitney). This increase of PI4P generation occurred in both Rat2 and BT549 cells transfected with eEF1A2. GFP had no effect on PI4P generation. Thus, eEF1A2 expression increases intracellular PI4P abundance.

2.4 Discussion

Phosphatidylinositol lipids are well-documented participants in pathways that regulate organelle biogenesis, cell morphology, proliferation and oncogenesis (333). In this report, we show that eEF1A2 binds phosphatidylinositol-4 kinase III beta (PI4KIIIβ), increases its *in vitro* lipid kinase activity and can increase intracellular PI4P generation in rat and human cells.

We find that purified eEF1A2 can increase recombinant PI4KIIIβ activity *in vitro*. Two other direct PI4KIIIβ activators have been previously identified: ADP Ribosylation Factor (ARF) and Neuronal Calcium Sensor-1 (NCS-1) (129, 490). ARF, a small GTPase, regulates Golgi function through PI4KIIIβ activation (129). NCS-1 controls IgE-mediated exocytosis in mast cells through PI4KIIIβ (199). Since we find that eEF1A2-directed siRNA reduces PI4K activity and that ectopic eEF1A2 expression increases this activity, it is likely that eEF1A2 is a novel and physiological PI4KIIIβ activator.

In human cells, the majority of PI4K activity is associated with the plasma membrane and the membranes of the nucleus, lysosome, Golgi, and endoplasmic reticulum (139, 161, 232, 251, 347). PI4Ks are also known to localize and be active in several specialized organelles and secretory vesicles (139). The yeast homolog of mammalian PI4KIIIβ, *PIK1*, has been studied in great detail (12, 122, 148, 307, 383, 450). Pik1p, the protein encoded by *PIK1*, is essential for normal secretion, Golgi and vacuole membrane dynamics, and endocytosis (12, 122, 148, 307, 383, 450). Pik1p localizes to the nucleus and the *trans*-Golgi (122, 450). $Pik1^{ts}$ cells exhibit a defect in secretion of Golgi-modified secretory pathway cargos (307). Mammalian PI4KIIIβ is concentrated in the Golgi apparatus (34, 462), although detectable amount of protein is found in the non-Golgi

cytoplasm. However, we have not seen appreciable eEF1A2 protein in the Golgi and we have observed no consistent Golgi defects in eEF1A2-expressing cells. This suggests that eEF1A2's capacity to increase PI4P formation is regulating some non-Golgi aspect of cell physiology. Active PI4K is observed in the non-Golgi cytosol (93) and we find that eEF1A2 expression increases PI4P generation throughout the cell.

The molecular mechanism by which eEF1A2 activates PI4KIIIβ is currently unknown. eEF1A proteins bind and hydrolyze GTP, and bind tRNA. They have not been shown to have phospho-transferase activity or other capacity to covalently modify proteins. Thus it is unlikely that eEF1A2 affects PI4KIIIβ activity by post-translationally modifying it. Because eEF1A2 expression does not increase steady-state PI4KIIIβ protein levels (Fig. 2.5C), it is unlikely that eEF1A2 expression increase cellular PI4P generation by increasing the amount of PI4KIIIβ message being translated. We propose that direct interaction between eEF1A2 and PI4KIIIβ leads to a conformational change in PI4KIIIβ that increases its catalytic activity. This hypothesis is consistent with our observations that eEF1A2 increases the overall PI4KIIIβ catalytic rate (K_{cat}) and makes PI4KIIIβ a more efficient kinase (V_{max}/K_m). While the possibility of this conformational change remains an open one, we do not believe that eEF1A2 is a general phosphatidylinositol kinase activator. We have been unable to detect physical interaction between eEF1A2 and PI3K nor have we observed activation of this kinase by eEF1A2 (data not shown).

Our *in vitro* results suggest that eEF1A2 and PI4KIIIβ bind in a 1:1 stoichiometry and the majority of recombinant eEF1A2 and PI4KIIIβ are competent to physically interact with each other. However, up to a two-fold molar excess of recombinant eEF1A2 is required to maximally increase *in vitro* PI4KIIIβ activity. Thus, it is possible that some

post-translational modification of eEF1A2 may enhance its ability to activate PI4KIIIβ. Yang and colleagues showed that dephosphorylation of PIK-A49 prevented it from activating PI4KIIIβ, and that activation could be restored by *in vitro* phosphorylation of PIK-A49 by a calcium-dependent protein kinase (481). The possibility of post-translational modification of eEF1A2 enhancing its ability to enhance PI4KIIIβ activity is intriguing. However, no post-translational modifications of eEF1A2 have yet been identified and our results indicate that post-translational modification of eEF1A2 is not obligatory for PI4KIIIβ activation. Post-translational modification could also regulate PI4KIIIβ /eEF1A2 interaction. We find that only a small fraction (<10%) of total eEF1A2 is in a complex with PI4KIIIβ in MCF7 cells (and vice versa). We speculate that post-translational modification of either protein could enhance cellular complex formation and therefore PI4P generation.

eEF1A2 is highly expressed in 30-60% of breast, ovary and lung tumours (6, 223, 244, 441). We have previously reported that eEF1A2 has transforming properties (6) and that its inactivation in mice increases lymphoid apoptosis (343). This identifies eEF1A2 as an important human oncogene. We hypothesize that eEF1A2 activates oncogenesis through PI4K activation and PI4P generation. Our observation that eEF1A2 expression is sufficient to increase both PI4K activity and PI4P levels indicates that high eEF1A2 expression in human tumours likely leads to an overall increase in cellular PI4P production. We propose that increased production of PI4P could increase the abundance of $PI(4,5)P_2$ and $PI(3,4,5)P_3$. Both these phospholipids are second messengers that regulate actin cytoskeletal organization, intracellular vesicular trafficking and proliferation (21, 41, 124, 161, 176, 179, 268). Of particular importance to oncogenesis would be $PI(3,4,5)P_3$, the abundance of which controls cell growth, apoptosis and cell invasiveness. While PI3K activity has long

been thought to be the rate-limiting step in the production of $PI(3,4,5)P_3$, this may not be the case in tumours that have high PI3K activity through oncogenic mutation of the kinase or have inactivating mutations in the PTEN lipid phosphatase (477). In tumours with constitutively active PI3K or inactive PTEN, increased PI4K activity would be predicted to increase overall $PI(3,4,5)P_3$ abundance and activate $PI(3,4,5)P_3$-dependent signaling. This idea is consistent with a recent report by Pendaires and colleagues who showed that the phosphatidylinositol-5 phosphate produced by the *Shigella* pathogen is sufficient to activate Akt (332), a serine threonine kinase whose activity is dependent on $PI(3,4,5)P_3$ abundance (91). No PI4K has been reported to activate $PI(3,4,5)P_3$ production, nor have PI4K genes been reported to be transforming or to be highly expressed during oncogenesis. However, this idea has not been fully explored.

eEF1A proteins have a well characterized ability to bind and bundle F-actin (61, 389). It has been proposed that eEF1A2 couples the protein translation machinery to actin cytoskeleton assembly. Because we find that eEF1A2 directly binds PI4KIIIβ, it is probable that eEF1A2 links protein translation to phosphatidylinositol generation and the actin cytoskeleton.

In summary, we have identified eEF1A2 as a direct activator of PI4KIIIβ. Ectopic expression of eEF1A2 increases cellular PI4K activity and increases cellular PI4P abundance. Furthermore siRNA- mediated eEF1A2 inactivation decreases PI4K activity. This is consistent with the idea that eEF1A2 functionally and physiologically regulates PI4K function. We propose that PI4KIIIβ activation has an important role in eEF1A2-mediated tumourigenesis and transformation.

Chapter 3

Elongation factor eEF1A2 cooperates with phosphatidylinositol-4 kinase III beta to stimulate filopodia production through increased $PI(4,5)P_2$ generation

*A modified version of this Chapter has been published.

Jeganathan, S., A. Morrow, A. Amiri and J.M. Lee. 2008. Elongation factor eEF1A2 cooperates with phosphatidylinositol-4 kinase III beta to stimulate filopodia production through PI(4,5)P2 generation. Mol Cell Biol 28(14):4549-4561.

3.1 Introduction

Filopodia are finger-like projections from the plasma membrane that are the first cellular structures to reach new space during cell migration. Filopodia are composed of bundled actin filaments and actin associated proteins (106, 140). Transmembrane receptors within filopodia respond to extracellular cues and guide directional movement towards chemoattractants (246). In addition, filopodia contain abundant adhesion molecules that regulate cellular attachment to growth substrates and cell-cell interaction (413). As such, filopodia regulate several key physiological processes, including cell migration, wound healing and development. For example, filopodia are essential for neurogenesis in mice (289) and for cell-cell adhesion during *Drosophila melangaster* embryogenesis (350).

We have previously described a role for eEF1A2 (eukaryotic elongation factor alpha 2) in filopodia initiation and maintenance (5). In several mammalian cells, eEF1A2 expression is sufficient to stimulate filopodia formation (5). eEF1A2 is one of two members of the eEF1A family of proteins , eEF1A1 and eEF1A2. During the elongation phase of protein synthesis, GTP-bound eEF1A proteins interact with amino-acylated tRNA and recruit them to the ribosome (44, 163, 258). While eEF1A1 and eEF1A2 are believed to have equivalent roles in protein translation, their tissue-specific expression pattern is markedly different. eEF1A1 is expressed ubiquitously whereas eEF1A2 is detectably expressed only in normal tissues of the mammalian heart, brain and skeletal muscle (6, 44). Homozygous deletion of eEF1A2 occurs in the wasted mouse. These mice develop normally but suffer from neuromuscular abnormalities, immunodeficiency and die at approximately one month of age (44, 343, 392). Importantly, eEF1A2 is likely to be a human oncogene and it is highly expressed and its gene amplified in 30-60% of human

tumours of the breast, ovary and lung (6, 224, 245, 441). eEF1A2 is transforming and its expression in mammalian cells increases their *in vitro* growth rate, allows them to grow in soft agar and enhances their tumourigenicity in xenograft models (6).

The mechanism by which eEF1A2 stimulates filopodia production is currently unclear. Filopodia production is regulated, in major part, by the plasma membrane abundance of phosphatidylinositol-4,5 bisphosphate ($PI(4,5)P_2$) (106). $PI(4,5)P_2$ co-operates with WASP family proteins and the Cdc42 GTPase to stimulate the ability of the Arp2/3 complex to assemble actin (106). As such, proteins that control $PI(4,5)P_2$ abundance are likely to have critical roles in controlling filopodia initiation. Consistent with a role for eEF1A2 in phospholipid signaling, we have previously reported that eEF1A2 binds to the lipid kinase PI4KIIIβ and increases its kinase activity (184). PI4KIIIβ is a member of the PI4K family of lipid kinases that phosphorylates the D4 carbon of the inositol ring in phosphatidylinositol to yield phosphatidylinositol-4 phosphate (PI4P) (21, 161). PI4Ks are emerging as important mediators of cell physiology because PI4P is itself a regulatory phospholipid and additionally an obligate precursor for $PI(4,5)P_2$ and $PI(3,4,5)P_3$ (20).

Here, we investigate whether eEF1A2 might activate filopodia production through PI4KIIIβ. We find that eEF1A2 expression is sufficient to increase the cytoplasmic and plasma membrane abundance of $PI(4,5)P_2$. This increase in plasma membrane $PI(4,5)P_2$ is necessary for eEF1A2-induced filopodia formation. eEF1A2-mediated filopodia formation and $PI(4,5)P_2$ accumulation are both dependent on PI4KIIIβ. Cdc42 activation is also required for eEF1A2-induced filopodia formation and eEF1A2 expression is sufficient to activate Cdc42. Furthermore, we find that PI4KIIIβ expression is sufficient to induce filopodia formation and plasma membrane $PI(4,5)P_2$ localization. Our work is consistent

with a model for filopod production where eEF1A2 stimulates filopod production through Cdc42 activation and a PI4KIIIβ–mediated increase in $PI(4,5)P_2$ abundance. Our work also suggests an important regulatory role for PI4KIIIβ in controlling actin remodeling.

3.2 Materials and Methods

3.2.1 Cell lines and vectors

Rat2, BT549, and MCF7 cells were obtained from the American Type Culture Collection (Manassas, VA) and grown according to their instructions. MCF7 cell lines with stable eEF1A2 ablation were generated using the pSilencer Neo siRNA expression vector (Ambion) with eEF1A2-directed siRNA using sequences previously described (184). Bradykinin was purchased from purchased from Calbiochem (San Diego, CA). The generation of the eEF1A2 plasmid (eEF1A2-pcDNA3.1) is described in Anand *et al.* (6). The generation of the adenoviral vectors (Ad-eEF1A2 and Ad-GFP) is described elsewhere (5, 224). The PI4KIIIβ-expressing stable cell lines and the vector controls were generated using the pLXSN retroviral system described in Grignani *et al.* (135).

3.2.2 Antibodies

The generation of the rabbit polyclonal eEF1A2 antibody and its validation in western blotting and immunohistochemistry are described elsewhere (224). eEF1A2 expression is also detected using a V5-HRP antibody (Invitrogen or Sigma) . The PI4KIIIβ and goat anti-mouse, horseradish peroxidase-conjugated IgG antibodies were from Upstate Cell Signaling Solutions (Charlottesville, VA). Actin antibody was from Sigma (Oakville, Ontario) and horseradish peroxidase-conjugated anti-rabbit IgG from Cell Signaling Technology (Danvers, MA). $PI(4,5)P_2$ was visualized using an anti-$PI(4,5)P_2$ IgM antibody (Echelon Biosciences Inc., Salt Lake City, UT) and a goat anti-mouse IgM, phycoerythrin secondary antibody (Caltag Laboratories, Carlsbad, CA).

3.2.3 siRNA and transfections

The sequence of the PI4KIIIβ siRNA is 5' – GGAGGUGUUGGAGAAAGUCtt - 3'.

This siRNA and the negative control siRNA (Cat. # 4611) were purchased from Ambion (Austin, TX). siRNA transfections were performed using siPORT Lipid (Ambion) according to manufacturer's instructions.

During the immunofluorescence experiments, parental Rat2 cells were triply-transfected with the PLCδ-PH-GFP plasmid (kind gift from T. Balla), eEF1A2-pcDNA3.1, and either the negative control siRNA or PI4KIIIβ siRNA. The eEF1A2 or vector plasmids were always in a 10-fold molar excess over the PLCδ-PH-GFP plasmid. When the Rat2 cells stably expressing eEF1A2 were used, the transfections were done using only the PLCδ-PH-GFP plasmid and the siRNA. For transfections, total plasmid amount was 4μg. When using the stable cell lines for the siRNA experiments, cells were transfected with 3.7μg of the PLCδ-PH-GFP plasmid and 20nM of either the negative control siRNA or the PI4KIIIβ siRNA. To study $PI(4,5)P_2$ levels upon transient eEF1A2 or empty vector expression, Rat2 cells were transfected with the PLCδ-PH-GFP plasmid and either an eEF1A2-pcDNA3.1 or pcDNA3.1 plasmid. In all transient eEF1A2 transfections, the eEF1A2 or vector plasmids were in a 10-fold molar excess over the other plasmids. All transfections were performed using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol.

3.2.4 Immunofluorescence

Cells were plated in 6 well plates containing coverslips. The next day, cells were fixed in 3.7% paraformaldehyde (15 minutes), permeabilized with 0.1% Triton-X (20 minutes) and blocked with 5% FBS/PBS (1 hour, 37^0C). Following staining, cells were mounted on slides using fluorescent mounting medium (Dako Cytomation, Golstrup, Denmark). Actin was stained with a Phalloidin 546 or Phalloidin 594. In experiments

where eEF1A2 was analyzed, eEF1A2 was stained in one of two methods. For the first, we used an eEF1A2-specific rabbit polyclonal antibody (MCF7 cells - 1:100, overnight) followed by an Alexa Fluor 488 goat anti-rabbit IgG secondary antibody (1:300, 2 hours, room temperature). In the second case we used a monoclonal anti-V5 antibody (Rat2 cells – 1:1000, 1 hour, room temperature), followed by an Alexa Fluor 680 goat anti-mouse IgG secondary antibody (1:300, 1 hour, room temperature). The $PI(4,5)P_2$-specific antibody was used at 1:100 (overnight) followed by the goat anti-mouse IgM, phycoerythrin secondary antibody (1:500, 1 hour, room temperature). All images were acquired using an Olympus FluoView FV1000 laser-scanning confocal microscope. $PI(4,5)P_2$ Fluorescence was quantified with the Olympus software (FV1000, version 01.04a, Center Valley, PA) and analyzed using GraphPad Prism 4 (San Diego, CA).

3.2.5 Phosphoinositide labeling

Near confluent Rat2 cells had their media removed and replaced with phosphate-free DMEM (Invitrogen) and labeled inorganic phosphate (40μCi/ml, GE Healthcare, Piscataway, NJ) for four hours. Following the incubation, cells were pelleted, and phospholipids were extracted with 40μl of 1:1 methanol:water, 10 μl of saturated NaCl, 2ul of glacial acetic acid, and 40μl of chloroform. The sample was vortexed vigorously and frozen at -20°C. Samples were thawed at room temperature, and 40μl aliquots of the organic phase were spotted onto pre-coated TLC plates and the plates were placed in the appropriate solvent system. The pre-coating protocol, as well as details of the solvent system are described elsewhere (184). The phosphatidylinositols were visualized with Storage Phosphor Screens (GE Healthcare), which were placed on top of the plates for a 24 hour period. For the adenoviral experiments, Rat2 cells were incubated with the Ad-eEF1A2

or Ad-GFP virus (MOI of 500) when they were 50-60% confluent for an overnight period. The next day, media was replaced with phosphate-free media and the above procedure was followed.

3.2.6 *In vitro* PI4KIIIβ lipid kinase assay

Total cellular protein (100μg in 20μl volume) from PI4KIIIβ or vector stable cells were added to 35μl of kinase buffer (1mM EDTA, 30mM HEPES, pH7.4, 100mM NaCl, 2mM $MgCl_2$, and 0.2% Triton X-100) and 5μl of 10mM ATP containing 10μCi of [^{32}P]ATP. Following a 20 minute incubation, the reactions were stopped by the addition of 60μl of 1N HCl. Phospholipids were then extracted by adding 160μl of chloroform/methanol (1:2, v/v). After a brief vortex, samples were centrifuged for 10 minutes at 10,000 x *g*. Aliquots of the organic phase were spotted onto pre-coated TLC plates and the plates were placed in the same solvent system as mentioned previously. Once complete, the plates were placed in a cassette and covered with a Phosphor Screen (GE Healthcare) for a 24 hour period. The screen was then analyzed with a Storm 860 Phosphorimager (GE Healthcare).

3.2.7 Generation of eEF1A2 mutants

eEF1A2 mutant proteins were generated using a PCR-mediated deletion protocol (152). Briefly, we used the eEF1A2pLXSN retroviral plasmid (5) as the template (50ng) and *Pfu* Turbo DNA polymerase (Stratagene) with the appropriate primers (0.2μM each), 1X *Pfu* buffer and 0.2mM dNTP. The 5'-phosphorylated primers used were: 5'-TCGGTGAAGGACATCGGTAAGCCTATCCCTAACCCTCTC-3'(Δ321-464, forward), 5'-AGGGATAGGCTTACCGATGTCCTTCACCGACACGTTCTT-3' (Δ321-464, reverse), 5'-ACAGAGCCGGCCTACGGTAAGCCTATCCCTAACCCTCTC-3' (Δ163-464,

forward), 5'-AGGGATAGGCTTACCGTAGGCCGGCTCTGTGGAGTCCAT-3' (Δ163-464, reverse), 5'-GCGCCGGAATTCATGCGGCGGGGCAACGTGTGTGGGG AC-3' (Δ1-320, forward), and 5'-CACGTTGCCCCGCCGCATGAATTCCGGCGCCT AGAGAAG-3' (Δ1-320, reverse). The PCR reaction was carried out as follows: denaturation at 95^{0}C for 45 seconds, annealing at 43^{0}C (Δ163-464 and Δ321-464) or 55^{0}C (Δ1-320) for 45 seconds, and elongation at 72^{0}C for 14 minutes (2 minutes/kb). Following the PCR, the 50μl reaction was digested with *Dpn*I for 1-2 hours at 37^{0}C and heat-inactivated for 20 minutes at 80^{0}C. 1-2μl of the treated reaction were then transformed into competent cells. Rat2 cells stably expressing the mutants were generated as described elsewhere (5).

3.2.8 Co-immunoprecipitation

For co-immunoprecipitation, cells were grown to 80-95% confluence in 100mm cell culture plates. Cells were lysed by sonication on ice in detergent-free buffer (137mM NaCl, 8mM KH_2PO_4 (pH 7.5), 2.7mM KCl, 2.5mM EDTA, 1% aprotinin, 1mg/ml leupeptin, 50mM NaF, 1mM Na_3VO_4, 10μg/ml pepstatin, 1mM phenylmethylsulfonyl fluoride) and centrifuged at 10,000 x *g* for 20 minutes to remove membranes. Supernatants were collected, and protein levels were quantified using a Bradford assay (Bio-Rad) according to the manufacturer's instructions. 250μg of total protein was precleared with protein A-Agarose (Amersham Biosciences) for one hour at 4°C. Following this, 2-4μg of PI4KIIIβ antibody (rabbit polyclonal, Upstate), anti-V5 agarose affinity gel (Sigma) or anti-FLAG affinity gel (Sigma) were added and incubated overnight at 4°C. Beads were washed three times in PBS, centrifuged, and boiled for 5 minutes in sample buffer, and the supernatant was subjected to SDS-PAGE. Antibodies used to detect the proteins were mouse anti- PI4KIIIβ antibody

(BD Biosciences), anti-V5-HRP (Invitrogen), and anti-FLAG-HRP (Sigma).

3.3 Results

3.3.1 eEF1A2 regulates filopodia formation

We have previously reported that eEF1A2 expression induces filopodia formation in rodent and mammalian cell lines (5). For example, stable expression of eEF1A2 in the Rat2 rat fibroblast cell line and BT549 breast carcinoma cell line visibly increases the number and size of filopodia (Fig. 3.1A). This also occurs in Rat2 cells transiently transfected with eEF1A2 (Fig. 3.1A). In general, eEF1A2-expression causes a significant, eight to nine-fold increase in the number of cells with filopodia greater than three microns in length relative to empty vector controls ($p < 0.05$, Student's *t*-test). To further investigate a role for eEF1A2 in filopodia production, we studied the MCF7 human breast cancer cell line. These cells express abundant eEF1A2 message and protein (224). We generated MCF7 variants where endogenous eEF1A2 expression had been stably reduced using RNA interference (Fig. 3.1B). While abundant filopodia are observed in control cells, their size and number is visibly reduced upon eEF1A2 reduction (Fig. 3.1C). To quantify the reduction in filopodia, eEF1A2-deficient and control cell lines were incubated with bradykinin, a soluble stimulator of filopod formation (221). After incubation with 100nM bradykinin, there was a significant, nine-fold decrease in the number of eEF1A2-ablated MCF7 cells with filopodia and pseudopodia relative to control MCF7 cells. (Fig. 3.1D). Bradykinin had no observable effect on eEF1A2 protein levels (Fig. 3.1E). Thus, eEF1A2 likely has a physiological role in filopodia formation. For the most part, the eEF1A2 protein is diffusely cytoplasmic but can be found within filopodia and also in the bases and edges of pseudopods, co-localizing with actin there (Fig. 3.1F).

3.3.2 eEF1A2 regulates filopodia development through Cdc42 activation

One of the key proteins that regulates filopodia formation is the Rho-family GTPase Cdc42 (106). Bradykinin, for example, stimulates filopodia through Cdc42 activation (221). eEF1A2 expression is sufficient to increase Cdc42 activity (Fig. 3.2A). In four separate experiments, eEF1A2 expression increased eEF1A2 expression 2-8 fold relative to GFP controls. To investigate whether Cdc42 played a role in eEF1A2-dependent filopod production, we inhibited Cdc42 activity in eEF1A2-expressing Rat2 cells using a dominant-negative, GFP-tagged Cdc42 (DN-GFP-Cdc42) (417). Untransfected eEF1A2-expressing cells or those transfected with a GFP control plasmid show prominent filopodia, but those expressing DN-GFP-Cdc42 show visibly reduced filopodia number (Fig. 3.2B). Moreover, the filopodia that remain are reduced in length relative to controls. Overall, there is a significant, eight-fold decrease ($p < 0.05$, Student's t-test) in the number of eEF1A2/DN-GFP-Cdc42-expressing cells with filopodia greater than three microns in length relative to eEF1A2/GFP-expressing control cells (Fig. 3.2B). DN-GFP-Cdc42 expression had no visible effect on overall shape or cytoplasmic actin architecture in the vector control cell lines. To confirm the specificity of these effects for dominant-negative Cdc42, we repeated them with dominant-negative Rac (DN-GFP-Rac) or a dominant-negative Rho (DN-GFP-Rho). Cells expressing DN-GFP-Rac show no significant decrease in filopodia size or length relative to GFP-expressing controls (Fig 3.2C). However, there is a nearly two-fold decrease in the number of DN-GFP-Rho-expressing cells with filopodia greater than three microns in length compared to GFP controls (Fig 3.2C). This is still four-fold more filopodia than in cells expressing DN-GFP-Cdc42 (Fig. 3.2B), suggesting that either eEF1A2 may regulate filopodia development through a mechanism that is partially Rho-

dependent or that DN-Rho is interfering with Cdc42 function. Taken together however, our findings indicate that eEF1A2 regulates filopodia primarily through Cdc42.

3.3.3 eEF1A2 stimulates PI(4,5)P_2 accumulation and membrane localization

A major pathway of filopodia production is controlled through Cdc42 and the abundance of phosphatidylinositol-4,5 bisphosphate (PI(4,5)P_2) (106). Active Cdc42 and PI(4,5)P_2 cooperate to activate actin nucleation by the Arp2/3 complex (167). We have previously found that eEF1A2 can activate PI4KIIIβ, leading to an increase in the intracellular abundance of phosphatidylinositol-4 phosphate (PI4P) (184), a precursor for PI(4,5)P_2. We reasoned that eEF1A2 might be inducing filopodia through an increase in cellular PI(4,5)P_2 abundance. To test this idea, we first used thin-layer chromatography to measure total PI(4,5)P_2 levels in Rat2 cells transduced with an eEF1A2 adenovirus and in Rat2 cells stably expressing eEF1A2. We observed an increase in PI(4,5)P_2 levels upon eEF1A2 expression relative to controls (Fig. 3.3A; 2.1 ± 0.4 fold in adenovirally transduced cells and 1.5 ± 0.2 fold in the eEF1A2 stable cell line). Because Cdc42 co-operates with membrane-bound PI(4,5)P_2 during filopod generation, we next investigated PI(4,5)P_2 localization in eEF1A2-expressing cells. To this end, cells were transfected with a fluorescent PI(4,5)P_2 reporter constructed from the fusion of the PI(4,5)P_2-binding pleckstrin homology (PH) domain of phospholipase C delta (PLCδ) and GFP as described (446). PLCδ-PH-GFP binds to membrane-bound PI(4,5)P_2. When Rat2 cells were co-transfected with eEF1A2 and PLCδ-PH-GFP, we detected prominent PI(4,5)P_2 at the cell membrane (Fig. 3.3B). The staining in eEF1A2-expressing cells was visibly thicker and more intense than that found in control cells transfected with an empty vector and the reporter. Furthermore, the ratio of PI(4,5)P_2 staining in the membrane relative to that in the

cytosol was greater in eEF1A2-expressing cells than in vector controls. A similar increase in membrane PI(4,5)P_2 staining was observed in cell lines stably expressing eEF1A2 (Fig. 3.3C). PI(4,5)P_2 can also be visualized within filopodia-like structures and at their bases (Fig. 3.3D). To further extend these studies, we used a PI(4,5)P_2 antibody to quantify cytosolic PI(4,5)P_2. This antibody does not detect PI(4,5)P_2 at the cell membrane. We transiently transfected Rat2 cells with either GFP or eEF1A2 and then stained fixed cells with this antibody (Fig. 3.3E). Cells transfected with eEF1A2 show visibly more PI(4,5)P_2 staining compared to untransfected cells or those transfected with GFP. We quantified PI(4,5)P_2 fluorescence intensity in individual eEF1A2-transfected and untransfected cells as well as in GFP-transfected and untransfected cells (Fig. 3.3F). PI(4,5)P_2 fluorescence intensity is significantly higher ($p < 0.0001$, Mann Whitney) in eEF1A2-expressing cells (2160 ± 712 fluorescence units) than in either untransfected (1522 ± 268 fluorescence units) or GFP-transfected cells (1441 ± 261 fluorescence units). Thus, eEF1A2 expression increases both plasma membrane-bound and cytosolic PI(4,5)P_2 abundance.

3.3.4 eEF1A2-mediated filopodia formation is dependent on PI(4,5)P_2

To determine whether the eEF1A2-mediated increase in PI(4,5)P_2 levels was necessary for filopodia production, we used a rapamycin-inducible system to decrease membrane-bound PI(4,5)P_2. This system uses two plasmids: PM-FRB-CFP and mRFP-FKBP-5-ptase (447). The mRFP-FKBP-5-ptase contains the phosphoinositide-5 phosphatase domain of the inositol polyphosphate 5-phosphatase enzyme fused to the rapamycin binding FKBP12 protein and the red fluorescent protein (mRFP). The PM-FRB-CFP is a fusion of the plasma membrane-bound FRB domain of mTOR (containing the palmitoylation sequence of the human GAP43 protein) and cyan fluorescent protein (CFP).

Upon addition of rapamycin, mRFP-FKBP-5-ptase translocates to the membrane where it heterodimerizes with the PM-FRB-CFP protein via the FRB domain. Once localized to the plasma membrane, the phosphatase in mRFP-FKBP-5-ptase depletes plasma membrane PI(4,5)P_2 by converting it to PI4P. We transfected Rat2 cells stably expressing eEF1A2 with these plasmids (Fig. 3.4A). In the presence of rapamycin, eEF1A2-expressing cells transfected with PM-FRB-CFP and mRFP-FKBP-5-ptase show visibly decreased plasma membrane-bound PI(4,5)P_2 and a concomitant reduction in filopodia number and length relative to cells without rapamycin. Rapamycin treatment of eEF1A2-expressing cells without PM-FRB-CFP and mRFP-FKBP-5-ptase was unable to elicit changes in filopodia appearance or PI(4,5)P_2 membrane localization (Fig. 3.4B). Therefore, the induction of filopodia by eEF1A2 is dependent on plasma membrane PI(4,5)P_2.

3.3.5 eEF1A2-mediated filopodia formation is dependent on eEF1A2 interaction with PI4KIIIβ

Thus far, we have determined that eEF1A2 can increase PI(4,5)P_2 production and that membrane PI(4,5)P_2 is required for eEF1A2-mediated filopodia formation . Because we have previously shown that eEF1A2 can increase the cellular pool of PI4P by binding to and activating PI4KIIIβ (184), we next investigated the role of PI4KIIIβ in eEF1A2-dependent PI(4,5)P_2 generation and filopodia production. We first designed eEF1A2 variants that did not interact with PI4KIIIβ. Based on homology with yeast and *Dictyostelium* eEF1A, the eEF1A2 protein contains domains for GTP binding, GTP hydrolysis, tRNA binding and two putative actin binding domains (Fig. 3.5A). We generated mutants lacking amino acids 163-464, 321-464 and 1-320 (Δ163-464, Δ321-464, and Δ1-320) and tagged with a C-terminal V5 epitope. We generated polyclonal cell lines expressing each of these proteins; protein

expression levels in the polyclonal cell lines are shown in Fig. 3.5B. To determine which of these mutants interact with PI4KIIIβ, we performed co-immunoprecipitation assays in each of these cell lines. As shown in Fig. 3.5B, full length eEF1A2 and the Δ321-464 mutant bind to PI4KIIIβ but the Δ163-464 and Δ1-320 mutants do not. Thus, a putative PI4KIIIβ interaction domain is likely contained within eEF1A2 residues 163-320.

We next investigated whether these eEF1A2 mutants were competent for increasing $PI(4,5)P_2$ or filopodia production. As shown in Fig. 3.5C, the full length eEF1A2 and the Δ321-464 mutant both activate filopodia production and membrane PI(4,5)P2 accumulation but the Δ163-464 and Δ1-320 do not. Overall, wildtype eEF1A2-expressing cells and Δ321-464-expressing cells had a significant, ~four-fold increase ($p < 0.05$, Student's *t*-test) in the number of cells showing filopodia greater than three microns in length (Fig. 3.5D). The Δ163-464 and Δ1-320 expressing cells were no different from vector controls with respect to filopodia appearance (Fig. 3.5D). Thus, eEF1A2 proteins that do not interact with PI4KIIIβ activate neither $PI(4,5)P_2$ production nor filopodia generation.

To further study the requirement for PI4KIIIβ interaction in eEF1A2-medated filopodia formation, we used short-interfering RNA (siRNA) to decrease PI4KIIIβ protein levels in Rat2 cells (Fig. 3.5E). The siRNA decreased PI4KIIIβ protein levels by ~60%. We transfected the eEF1A2-expressing Rat2 stable cell lines with PLCδ-PH-GFP, to visualize $PI(4,5)P_2$ production, and either PI4KIIIβ or negative control siRNA (Fig. 3.5F). Cells with the PI4KIIIβ siRNA show a marked decrease in the intensity of $PI(4,5)P_2$ fluorescence relative to control siRNA treated cells. Importantly, the number of cells with filopodia was similarly decreased. This attenuation was not absolute, however, likely

because some PI4KIIIβ was still present in the cells. To confirm these results, we repeated this experiment with Rat2 cells co-transfected with wildtype eEF1A2 and the siRNAs (Fig. 3.5G). As with the eEF1A2 stable cell lines, eEF1A2-expressing cells transfected with PLCδ-PH-GFP and the PI4KIIIβ siRNA had decreased filopodia compared to those with the control siRNA. In general, eEF1A2-expressing cells with ablated PI4KIIIβ protein levels had a significant, two to three-fold decrease ($p < 0.05$, Student's *t*-test) in the number of cells with filopods greater than three microns in length (Fig. 3.5H).

3.3.6 PI4KIIIβ is sufficient for filopodia formation

The data thus far suggests that PI4KIIIβ is necessary for eEF1A2-induced filopodia development. We thus decided to investigate whether PI4KIIIβ alone was sufficient to activate both $PI(4,5)P_2$ generation and filopodia production. We generated Rat2 cell lines that stably overexpressed PI4KIIIβ (Fig. 3.6A). These cells have an ~ 1.5-fold increase in PI4KIIIβ lipid kinase activity (Fig. 3.6B). Like eEF1A2-expressing cells, there is a significant, ~ four-fold increase ($p < 0.05$, Student's *t*-test) in the number of PI4KIIIβ-expressing cells with filopodia greater than three microns in length (Fig. 3.6C) as well as an increase in membrane-bound $PI(4,5)P_2$ staining when compared to vector controls (Fig. 3.6D). The $PI(4,5)P_2$ also shows some degree of colocalization with the actin in the filopodia (Fig. 3.6D). PI4KIIIβ has previously been reported to be enriched in the trans-Golgi network and to have roles in vesicular trafficking (157, 463). However, we have observed no visible changes in Golgi appearance in cells ectopically expressing eEF1A2 or PI4KIIIβ. Taken together, these results indicate that PI4KIIIβ is sufficient to increase $PI(4,5)P_2$ levels and activate filopodia development.

3.4 Discussion

eEF1A2 is a transforming gene, highly expressed in ~50% of breast, ovarian and lung tumours (6, 224, 245, 441). eEF1A2 expression also stimulates actin remodeling, cell invasion and migration (5). Here, we propose a pathway of actin remodeling where eEF1A2 stimulates filopodia extrusion through PI4KIIIβ and PI4KIIIβ–mediated accumulation of $PI(4,5)P_2$. This leads to filopodia creation through a Cdc42-dependent pathway. We also identify a region within the eEF1A2 protein necessary for its interaction with PI4KIIIβ.

We have previously found that eEF1A2 increases cellular PI4P abundance in a PI4KIIIβ–dependent manner (184). Here we report that eEF1A2 expression increases $PI(4,5)P_2$ abundance as well. eEF1A2 mutants that are unable to interact with PI4KIIIβ activate neither $PI(4,5)P_2$ generation nor filopodia production. Furthermore, PI4KIIIβ ablation using RNA interference attenuates the eEF1A2-mediated increase in $PI(4,5)P_2$ abundance and PI4KIIIβ expression is sufficient to increase $PI(4,5)P_2$ levels at both the cytosol and cell membrane. The simplest interpretation of these findings is that the increased $PI(4,5)P_2$ abundance upon eEF1A2 expression is a direct consequence of the larger PI4P precursor pool created by PI4KIIIβ activation. The K_M of mammalian PI4P5-kinases, the enzymes responsible for converting PI4P to $PI(4,5)P_2$, are in the 10-45μM range (180). Thus, an increase in PI4P concentration will stimulate $PI(4,5)P_2$ accumulation wherever PI4P concentrations are in the magnitude of 10μM or less. In eukaryotic cells, phosphatidylinositol concentrations range from 0.5–2.5 μmoles/gram (283), with an overall concentration of ~10-500pM in cells with a volume range of 500-3000μm^3 (108). PI4P compromises ~10-20% of total phosphatidylinositols, suggesting that overall cellular PI4P abundance is in the 1-100pM range. However, plasma membrane concentrations of PI4P

may be much higher. Stephens and colleagues, for example, estimated that PI4P concentrations in the inner plasma membranes of unstimulated neutrophils approach 3mM (414). Here, we find that eEF1A2 expression doubles both total cellular PI4P and $PI(4,5)P_2$ abundance in cells. Furthermore, increased levels of $PI(4,5)P_2$ are distributed along the plasma membrane as well as in the cytoplasm. *In vitro*, purified eEF1A2 doubles the V_{max} of recombinant PI4KIIIβ (184). Thus, PI4P concentration is likely to be limiting in the generation of $PI(4,5)P_2$ in our cell lines. Broadly consistent with this idea, previous reports had suggested the existence of an intracellular pool $PI(4,5)P_2$ in mammalian cells that was under the control of a wortmannin-sensitive PI4 kinase (22, 281) later identified as PI4KIIIβ (19).

The mechanism of PI4KIIIβ activation by eEF1A2 is unknown. Because purified eEF1A2 increases the lipid kinase activity of PI4KIIIβ *in vitro*, it is likely that eEF1A2 induces a conformational change in PI4KIIIβ that increases its activity. For example, the small GTPase Ras activates PI3K via a change in conformation, so this method of activation is not unprecedented (323). We have mapped residues necessary for eEF1A2/ PI4KIIIβ interaction to amino acid residues 163-320. It is likely that amino acid residues therein are likely to directly bind PI4KIIIβ and induce an as yet uncharacterized structural change in the protein to stimulate kinase activity.

The eEF1A2 protein is diffusely cytoplasmic (6, 224) whereas PI4KIIIβ is largely localized in the Golgi (471). Thus, it was somewhat of a surprise to us that PI4KIIIβ can stimulate plasma membrane $PI(4,5)P_2$ accumulation and activate filopodia generation there. Inactivation of PI4KIIIβ in both yeast and mammalian cells impairs Golgi structure and function (450). Furthermore, the increased cytosolic $PI(4,5)P_2$ accumulation we observed

with the $PI(4,5)P_2$ antibody did not appear to be like the distribution observed for the Golgi apparatus. Since eEF1A2 or PI4KIIIβ expression stimulates production of filopodia, we favor the idea that the $PI(4,5)P_2$ generated upon eEF1A2 or PI4KIIIβ expression has primary importance at the cell membrane. eEF1A2/ PI4KIIIβ interaction, mediated through amino acid residues 163-320 in eEF1A2, and activation could occur on organelle membranes in the cytosol. The resulting PI4P would then be rapidly shuttled to the plasma membrane to generate filopodia. It may even be possible that the PI4P is converted to $PI(4,5)P_2$ coincident with its transport to the plasma membrane. This scenario may explain why we observe a general increase in overall membrane-bound $PI(4,5)P_2$ levels and not distinct pools of the lipid on the membrane. PI4KIIIβ's ability to affect the plasma membrane may not require substantial localization of the protein there. For example, PI4KIIIα is primarily localized to the ER in mammalian cells (19, 471) but controls plasma membrane PI4P levels (301) and is part of the P2X7 ion channel (208). Thus, the interaction between eEF1A2 and PI4KIIIβ could occur away from the plasma membrane even though the $PI(4,5)P_2$ generated would be functional there. Alternatively, while the eEF1A2 and PI4KIIIβ proteins are found in the cytoplasm, the interaction and activation of the kinase may occur transiently, near the plasma membrane.

While we propose that eEF1A2-mediated activation of PI4KIIIβ leads to $PI(4,5)P_2$ accumulation due to a larger PI4P substrate pool, other possibilities do exist. For example, PI4P could also be activating PTEN, the 5'-phosphatase responsible for converting $PI(3,4,5)P_3$ to $PI(4,5)P_2$, thereby increasing $PI(4,5)P_2$ abundance. Alternatively, PI4P could be directly activating a PI4P5K. Monophosphoinositides can influence the activity of other proteins involved in bi- and tri-PI generation. For example, Pendaries and colleagues have

recently reported that an increase in cellular PI5P levels upon *Shigella flexnari* infection leads to an activation of a class 1A PI3-kinase, resulting in an increased level of active Akt, a downstream target of PI3-kinase (331). Thus, it is possible that the generated pool of PI4P could be indirectly regulating $PI(4,5)P_2$ levels.

We have previously reported that eEF1A2 expression can cause activation of the Akt serine threonine kinase (5). Akt/PKB is a regulator of cell proliferation, insulin responsiveness and apoptosis (156, 274, 326). Akt activation is dependent, in major part, on plasma membrane levels of $PI(3,4,5)P_3$. The mechanism by which eEF1A2 activates Akt is currently unknown, but we speculate that eEF1A2 activates Akt indirectly since we have been unable to co-immunoprecipitate or co-localize eEF1A2 with Akt (not shown). We hypothesize that eEF1A2 can increase membrane abundance of $PI(3,4,5)P_3$ in the same manner that it does with $PI(4,5)P_2$.

The ability of PI4KIIIβ to increase filopodia production also suggests that transcriptional or post-transcriptional control of PI4KIIIβ abundance could be an important facet of both actin remodeling and PI signaling control. To the best of our knowledge, however, no extracellular stimulus is known to increase the abundance of PI4KIIIβ message or protein. It is also unclear whether the ability of eEF1A2 to activate PI4KIIIβ and PI signaling contributes to its ability to transform cells *in vitro* or enhance their tumourigenicity. Moreover, eEF1A2 might have effects on other aspects of cell physiology that depends on $PI(4,5)P_2$. For example, hydrolysis of $PI(4,5)P_2$ by phospholipase C, yields diacylglycerol (DAG) and inositol trisphosphate (IP_3). IP_3 is known to release calcium from its storage pools in the endoplasmic reticulum (107, 287). Similarly $PI(4,5)P_2$ is known to mediate endocytosis/exocytosis and vesicular transport (274). Thus, eEF1A2 could have

multiple effects on plasma membrane physiology.

There may be other pathways of eEF1A2-dependent actin remodeling. For example, purified eEF1A proteins bundle actin *in vitro* and in *Dictyostelium* sp., they become localized in filopodia extended in response to cAMP stimulation (84). This suggests that direct actin bundling by eEF1A2 could also contribute to filopodia production. The localization of eEF1A2 to filopodia bases and within filopodia suggests that both actin bundling and phospholipid regulation may contribute to actin remodeling.

In summary, we have shown that eEF1A2 regulates filopodia formation through binding and activating PI4KIIIβ. This activation is required to generate a pool of cytosolic and membrane-bound $PI(4,5)P_2$. The membrane $PI(4,5)P_2$ then induces filopodia formation through a Cdc42-dependent mechanisms. Moreover, we also show that PI4KIIIβ itself can regulate filopodia formation, a novel function for this enzyme. This work provides additional evidence of the link between protein translation, phosphatidylinositol signaling, actin remodeling, and oncogenesis.

Chapter 4

eEF1A2 is an Akt/PKB activator that inhibits anoikis and is a potential therapeutic target for breast cancer

4.1 Introduction

Breast cancer is the most common malignancy among North American women (37, 186). The Canadian Cancer Society estimates that, in 2007, breast cancer will kill more than 5300 Canadian women and a further 22000 will be diagnosed with the disease (37). It is expected that one in nine women will be diagnosed with, and 1 in 27 will die, of breast cancer in their lifetime (37). The identification of causative factors and elucidating their roles in the disease are key to understanding breast oncogenesis. Furthermore, it also provides novel therapy targets for individualized disease management.

We have previously reported that *EEF1A2*, the gene encoding protein elongation factor eEF1A2, is amplified and overexpressed in ~30% of ovarian tumours and ~60% of primary breast tumours (6, 224). eEF1A2 is one of two members of the eEF1A family of proteins (eEF1A1 and eEF1A2) that bind amino-acylated tRNA and facilitate their recruitment to the ribosome during translation elongation (163). The two isoforms share greater than 90% sequence identity but have different expression patterns: eEF1A1 is ubiquitously expressed while eEF1A2 is detectably expressed only in the heart, brain and muscle (211). eEF1A2 proteins have other functions not related to translation as well. Inactivation of the mouse eEF1A2 homolog, *Eef1a2*, leads to immunodeficiency and neural/muscular defects and death by 30 days of age (44, 392). eEF1A2 can also induce changes to the actin cytoskeleton (5). We have previously found that eEF1A2 expression in mammalian and rodent cells can induce filopodia formation *in vitro* through the activation of phosphatidylinositol-4 kinase III beta (PI4KIIIβ) (5, 184, 185). Importantly, eEF1A2 has transforming properties: ectopic expression of wild-type eEF1A2 in mammalian cells enabled growth in an anchorage-independent manner and enhanced tumourigenicity in nude

mice (6). These results suggest that eEF1A2 is a valid therapy target.

In this report, we find eEF1A2 mRNA is highly expressed in ~30% of primary human breast tumours and metastases and cell lines but not in normal breast epithelium or immortalized cell lines. We also find that eEF1A2 is a novel inhibitor of anoikis, the apoptosis induced by loss of a solid growth substrate (115, 137). In addition, we find that eEF1A2 is an activator of the Akt/PKB serine threonine kinase, a well documented regulator of cell growth and oncogenesis (91, 150). Importantly, we find that xenografts of cell lines in which eEF1A2 has been inactivated using RNA interference leads to smaller or no tumour formation compared to control cell lines. These results suggest that eEF1A2 has an important role in regulating breast oncogenesis and can be used as a therapeutic target in breast cancer.

4.2 Materials and Methods

4.2.1 Cell lines

Cell lines were obtained from the American Tissue Culture Collection (ATCC). The MCF10AT cells were a gift from Dr. Janusz Rak (McMaster University) and grown in DMEM/F12 (1:1) media supplemented with 5% horse serum, 29uM HEPES, 10ug/ml insulin, 20ng/ml EGF, 0.5ug/ml hydrocortisone, and 100ng/ml cholera toxin. Cells were kept in a 37°C incubator at 5% CO_2. BT549 and MCF10AT cells were transfected with a V5-tagged *EEF1A2*- or pCDNA3. plasmid using Lipofectamine 2000 (Invitrogen). For adenoviral infection, eEF1A2 was cloned into pShuttle-IRES (*EcoRV/XhoI*) and the virus was manufactured by the University of Ottawa. eEF1A2 and GFP control adenoviruses were used at a multiplicity of infection of 200.

4.2.2 RNA purification

60mm plates of cells were lysed with 1 ml of TRIzol (Invitrogen). 10ug of cell RNA or human skeletal muscle mRNA (Stratagene) was loaded per lane and transferred to a GeneScreen membrane. Membranes were prehybridized at 63°C in Church's buffer for three hours and hybridized at 63°C overnight with a 598 base pair *Bam*HI/*Pst*I fragment of *EEF1A2*.

4.2.3 Western blotting

Proteins were extracted using RIPA buffer. An anti-V5-HRP antibody (Invitrogen; 1:5000 in TBST (100mM Tris-Cl, 0.9% NaCl, 0.1% Tween-20, pH 7.5)) and ECL (Amersham) were used to detect eEF1A2 expression. Antibodies for beta actin (Sigma), PARP (USBiological), Akt, phospho-Akt, PI3K and Akt2 (Cell Signaling Technology), goat anti-mouse IgG, HRP-conjugate (Upstate Cell Signaling Solutions) and anti-rabbit IgG,

HRP-linked antibody (Cell Signaling Technology) were used according to manufacturers' directions.

4.2.4 Growth and apoptosis assays

Cells were grown in 96-well plates. At each time interval, wells were washed with PBS and kept at -80°C. After thawing, 100ul of double distilled water was added to each well for 1 hour. Hoescht 33258 (Sigma) staining solution (20ug/ml; 100ul) was then added for 10 minutes and plates were analyzed with the FluorSTAR Galaxy fluorometer. For apoptosis, cell lines were plated in triplicate in 96-well plates for six hours. Drug was added to the wells and 24 h later, plates were analyzed as above. For anoikis, cells were placed in standard tissue culture dishes on an orbital shaker at 37°C. Cell viability was analyzed every four hours by trypan blue exclusion. Cell counts were performed blind in triplicate. An Apoptosis Detection Kit (Sigma-Aldrich) was used to measure Annexin staining according to the manufacturer's instructions.

4.2.5 siRNA

The antisense sequences of the two *EEF1A2*-directed siRNAs are 5'-UGGU-CCUUUUGUCAAUACCTC-3' (siRNA#1), and 5'-UCGAACUUCUCAAUGGUCCTT-3' (siRNA#2). The negative control siRNA was purchased from Ambion. siPORT Lipid (Ambion) was used for transfection. MCF7 cell lines with stable eEF1A2 ablation (MCF-1A and MCF-2A) were generated using the pSilencer Neo siRNA expression vector (Ambion) the two eEF1A2-directed shRNAs described above. The control MCF7 cell line (MCF-NC) was generated with the negative control shRNA.

4.2.6 Tumour studies

Six to eight week old, female SCID-BEIGE (C.B-*Igh*-1b/GbmsTac-*Prkdc*^{scid}-*Lyst*^{bg}

N7) mice were purchased from Taconic Farms (Hudson, New York) and left to acclimatize in the University of Ottawa Animal Care Facility for one week. 17 β-Estradiol pellets (90-day release; 0.72mg/pellet total dose; Innovative Research of America) were implanted into the backs of the mice and, after three days, cells were xenografted into the right hind legs (1.5×10^6 cells in 250μl PBS). Each cell line (MCF-NC, MCF-1A and MCF-2A) was xenografted into three mice. Tumour volumes were calculated using the formula $V = (0.5)(4\pi/3)(L/2)(W/2)(H)$ where L, W, and H represent the length, width, and height (respectively) of the tumour. Tumour tissue processing and Hematoxylin and Eosin staining were done by the Department of Pathology at the University of Ottawa.

4.2.7 Gene expression analysis

Gene expression data in Fig 1 was extracted from the GeneExpress database from GeneLogic (Gaithersburg, MD) for probe set 204540_at on the HG-U133A GeneChip from Affymetrix (Santa Clara, CA). Microarray data values were generated by the MAS5 signal algorithm. Data were extracted for samples available as of February 2003. HER-2 status was assessed using the expression of probe set 216836_s_at and a threshold of 8000. eEF1A2 expression data from Fig. 2 were obtained from Huang and colleagues (173); we analyzed those tumours with at least 45 months of follow-up. EEF1A2 expression was determined using hybridization to the U95Av2 Affymetrix GeneChip array and the 35174_i_at probeset. Of the 65 tumours analyzed, 57 are infiltrating ductal carcinomas, one each are Papillary, Apocrine and Cribriform carcinomas, and the remaining 5 are classified as Other.

4.3 Results

4.3.1 eEF1A2 expression is elevated in human breast tumour tissue independent of HER2

To determine whether eEF1A2 was involved in breast cancer, we analyzed eEF1A2 mRNA expression in human breast tissue derived from normal and tumour samples (n=345). We hypothesized that a role for eEF1A2 in breast oncogenesis could be inferred from high tumour-specific gene expression, an approach used to identify other candidate oncogenes (476). eEF1A2 expression was measured from Affymetrix U133 array hybridization (Fig. 4.1A). eEF1A2 expression was very low in normal breast epithelium (n=22) and in fibroadenomas (n=13). However, approximately 30% of ductal carcinomas (73/251), lobular carcinomas (10/30) and breast tumour metastases (10/29) had eEF1A2 expression greater than 2σ above the normal tissue expression mean. This high eEF1A2 expression implicates eEF1A2 as a breast cancer oncogene.

Approximately 30% of breast tumours have high expression of the HER-2 receptor tyrosine kinase due to amplification of the *HER-2* gene (6). Overexpression of HER-2 is an important marker in breast cancer aggressiveness (397). We next examined whether eEF1A2 expression correlated with HER-2 status (Fig. 4.1B). HER-2 positive (n=41) and HER-2 negative (n=210) ductal carcinomas had a similar fraction of tumours with eEF1A2 expression above normal and the distribution of eEF1A2 expression between populations was not significantly different by Kolmogorov-Smirnov ($p<0.39$) analysis. This suggests that any role of eEF1A2 in breast oncogenesis is HER-2 independent.

4.3.2 eEF1A2 expression enhances growth in human breast cell lines

To investigate a causal role for eEF1A2 in breast oncogenesis, we investigated the

effect of eEF1A2 expression or ablation in breast cancer cell lines. We first sought to identify breast tumour cell lines that do and do not express eEF1A2. By northern analysis, three of five breast lines we tested showed detectable eEF1A2 mRNA expression (MCF7, BT474, and MBMDA231) while two (BT549 and MCF10AT) showed no expression (Fig. 4.2A). This observation agrees with previously reported eEF1A2 mRNA and protein expression profiles (5, 184, 224, 409). We generated eEF1A2-expressing lines from two of the eEF1A2 negative lines (BT549 and MCF10AT) with eEF1A2 under the control of the cytomegalovirus (CMV) promoter. eEF1A2 protein expression in three independent lines of BT549 and MCF10AT cells are shown (Fig 4.2B). In both the BT549 and MCF10AT cell lines, eEF1A2-expressing clones showed a greater proliferation rate than parental cells (Fig 4.2C). The BT549 and MCF10AT cell lines with the highest eEF1A2 expression (BT549-5 and MCF10AT-P5) had doubling times of 26$\pm$3 hours and 17$\pm$1 hours respectively, whereas the vector controls had doubling times of 47$\pm$4 hours and 26$\pm$2 hours. We then used two short-interfering RNAs (siRNAs) against eEF1A2 to determine what effect eEF1A2 inactivation might have on cell growth. These siRNAs decreased eEF1A2 protein levels in BT549-5 cells and reduced eEF1A2 mRNA levels in MCF7 cells (Fig. 4.2D). eEF1A2 inactivation in MCF7 cells reduced their growth rate (Fig. 4.2E). Thus, eEF1A2 is a positive activator of breast cell proliferation.

4.3.3 eEF1A2 expression inhibits anoikis in human breast cell lines

We next investigated the role of eEF1A2 on anoikis. Anoikis, apoptotic death induced by the lack of a solid growth substrate, has an important role in controlling tumour development and metastasis (115). We induced anoikis by shaking cultures of BT549 cells to prevent adhesion. eEF1A2-expressing BT549 cell lines consistently showed a greater

fraction of viable cells than controls in adhesion-limited growth (Fig. 4.3A). After 15 hours in adhesion-limited culture, parental and vector cell survival was ~20% while all eEF1A2-expressing cells had ~60% survival. To confirm that this death was apoptotic, we stained cells for Annexin-V. Ten hours after culture, fewer eEF1A2-expressing BT549 cells were apoptotic compared to controls (Fig. 4.3B). To confirm that this resistance to anoikis was specific for eEF1A2, we used siRNA to reduce eEF1A2 levels in BT549 cells. siRNA treatment increased the observed anoikis in the eEF1A2-expressing BT549 cells to almost wildtype cell levels (Fig. 4.3C). Moreover, both siRNAs increased cell death in MCF7 cells in response to adhesion-limited culture (Fig. 4.3D), indicating that eEF1A2 inactivation increased sensitivity to anoikis. Ablation of eEF1A2 in mice leads to an increase in lymphoid apoptosis (343) and we investigated whether eEF1A2 could regulate the apoptosis induced by anticancer agents. We used Cisplatin and Taxol to induce apoptosis in BT549 cells. We observed that eEF1A2 expression had no effect on sensitivity to apoptotic death induced by either drug (Fig. 4.3E). Moreover, eEF1A2 downregulation in MCF7 cells had no measurable effect on sensitivity to Cisplatin or Taxol (not shown). These observations indicate that the ability of eEF1A2 to inhibit anoikis is unlikely to be due to a generalized inhibition of all forms of apoptosis.

4.3.4 eEF1A2 expression leads to AKT activation

To identify a mechanism by which eEF1A2 could enhance growth and inhibit anoikis, we investigated a role for eEF1A2 in Akt/PKB activation. Akt is a serine threonine kinase regulating multiple pathways of cell growth and apoptosis (91, 151). Infection of BT549 cells with a GFP-tagged eEF1A2 adenovirus markedly increased phosphorylation of Akt residues serine 473 (S473) and threonine 308 (T308) relative to control GFP-infected

cells while overall protein levels of Akt did not change (Fig. 4.4A). Phosphorylation of these residues are markers for Akt activation (150). Increases in S473 and T308 phosphorylation were also observed in the BT549 cells stably expressing eEF1A2 (Fig. 4.4B) and no changes in overall Akt, Akt2 or PI3 kinase protein levels were observed. These observations are consistent with the idea that eEF1A2 is a novel activator of Akt and provides an explanation for how eEF1A2 regulates cell growth and anoikis.

4.3.5 eEF1A2 inactivation decreases *in vivo* tumourigenecity

To investigate whether eEF1A2 is a valid target for therapy, we determined the effect of eEF1A2 ablation in tumour formation. MCF7 breast cancer cells express abundant eEF1A2 protein (184). We generated MCF7 variants where endogenous eEF1A2 expression had been stably reduced using RNA interference (Fig. 4.5A). Xenografts of eEF1A2-ablated cells (MCF-1A and MCF-2A) into immunocompromised mice showed delayed tumour formation or no tumour formation compared to control MCF7 cells (MCF-NC) (Fig. 4.5B). On average, by day 108, tumours formed by eEF1A2-ablated cells were ~5-fold smaller than those formed by cells with wildtype eEF1A2 levels (Fig. 4.5B and C; 154.2 ± 56.1 mm^3 versus 812.4 ± 56.1 mm^3, excluding mice with no tumours). These results indicate that inactivation of eEF1A2 reduces *in vivo* tumour formation. Furthermore, Hematoxylin and Eosin staining of the tumours showed decreased invasion into skeletal muscle and adipose tissues in eEF1A2-ablated tumours as opposed to controls (Fig. 4.5D), suggesting that inactivation of eEF1A2 reduces tumour metastasis.

4.4 Discussion

The protein elongation factor eEF1A2, is an important ovarian, lung and breast oncogene (6, 224, 245, 441). *EEF1A2* maps to chromosome 20q13.3 (258). Amplifications of 20q are frequently observed in primary human breast tumours and are associated with poor clinical prognosis (169). Several candidate transforming genes within 20q might have a putative role in breast oncogenesis (236). In this report we find that eEF1A2 is highly expressed in ~30% of primary human breast tumours and metastases but not in normal breast epithelium. Furthermore, we also find eEF1A2 expression in several breast cancer cell lines. Ectopic expression of eEF1A2 increases *in vitro* cell growth rates, inhibits anoikis but not drug-induced cell death, and leads to Akt activation. Importantly, we find that inactivation of eEF1A2 decreases tumour formation and growth in mice. Taken together, our data indicate that eEF1A2 has an important role in regulating breast oncogenesis, and is a potential therapeutic target.

eEF1A2 inhibits anoikis, the form of apoptosis induced by inadequate mechanism contact with a solid growth substrate (113). Anoikis can be suppressed by the activation of multiple transforming genes (113, 115) and resistance to anoikis confers a growth advantage to tumour cells by allowing them to survive during tumour invasion and metastasis. The ability of eEF1A2 to inhibit anoikis suggests a plausible mechanism through which eEF1A2 increases tumour aggressiveness. The actin cytoskeleton exerts great control over anoikis. Integrins and other adhesion molecules modulate anoikis as do Cdc42 and Rac1, GTPases that modulate actin polymerization and bundling (48, 114). eEF1A2 binds to both actin and tubulin (61) and has been shown to induce filopodia formation in a PI3K/Akt-dependent manner (5). Thus, eEF1A2 could regulate anoikis through control of the cytoskeleton.

Another pathway of anoikis inhibition involves the activation of the Akt serine/threonine kinase (115). Akt regulates apoptosis and cell proliferation by regulating the activity of several transcriptional and translational regulators (91). The principle pathway for Akt activation occurs through the phosphatidylinositol signaling pathway, more specifically, through phosphatidylinositol-3 kinase (PI3K) (91, 448). We find that eEF1A2 expression is sufficient to induce phosphorylation of Akt on serine 473 (S473) and threonine 308 (T308); phosphorylation of these residues is a marker for Akt activation. T308 phosphorylation is controlled by phosphoinositide-dependent kinase 1 (PDK1) while S473 is phosphorylated by the integrin-linked kinase (ILK) (151, 448). The activity of both PDK1 and ILK is governed, at least in part, by PI3K-dependent signaling (151, 448). While the mechanism by which eEF1A2 activates Akt is unknown, we speculate that it occurs through phosphatidylinositol signaling. We have previously reported that eEF1A2 activates phosphatidylinositol-4 kinase III beta (PI4KIIIβ) (184). We also know that eEF1A2-mediated activation of PI4KIIIβ leads to downstream effects; the increased PI4KIIIβ activation leads to increased plasma membrane-bound phosphatidylinositol-4,5 bisphosphate ($PI(4,5)P_2$) and filopodia development (185). $PI(4,5)P_2$ serves as the principal substrate for PI3K, which converts it to phosphatidylinositol-3,4,5 trisphosphate ($PI(3,4,5)P_3$), a necessary lipid for Akt activation (91). Thus, eEF1A2 could indirectly activate Akt through PI4KIIIβ activation.

The high expression of eEF1A2 in breast cancer suggests that it may be a target for therapy over and above any potential as a prognostic marker. Ablation of eEF1A2 using RNA interference inhibits cellular proliferation and sensitizes breast cells to anoikis. More importantly, eEF1A2-ablated cells lead to slower tumour formation and growth when

xenografted into mice. These results suggest that eEF1A2 is a valid therapy target in breast cancer. However, siRNA are problematic therapy agents because they induce a vigorous immune response and have difficult pharmacokinetics (32, 327). The Didemnin family of natural marine products may be one possible eEF1A2-inactivating agent. Didemnin B is a 7-amino acid cyclic depsipeptide that inhibits cell cycle progression and is also an immune suppressant. Didemnin B inhibits protein translation elongation, and thus protein synthesis, by binding to eEF1A proteins (67, 395). Didemnin B was withdrawn from clinical trials because of toxicity but a Didemnin B derivative, Aplidin, has lower toxicity and is currently in phase II trials for cancer (189). It is possible that Aplidin may be particularly efficacious in those breast tumours with high eEF1A2 expression.

In summary, we present data suggesting that eEF1A2 is a breast cancer oncogene and a potential therapeutic target. Moreover, since eEF1A2 expression was observed in breast cancers independent of HER-2, its usefulness as a marker can be applied to a broad spectrum of breast tumours. Furthermore, we show that eEF1A2 is an Akt activator, providing further evidence of crosstalk between eEF1A2 and the phosphatidylinositol signaling pathway.

Chapter 5

Discussion

5.1 Summary of Thesis

The actin cytoskeleton plays a crucial role in cell physiology. Alterations of the cytoskeleton are necessary for multiple cellular processes, from phagocytosis and cytokinesis to cell movement and cancer metastasis (277, 384, 399, 400). Therefore, the identification of signaling pathways and proteins that regulate actin cytoskeletal dynamics is critical to our understanding of various aspects of cell physiology. Filopodia, long, thin cell extensions that are supported by tight, dynamic, parallel bundles of actin filaments, are a type of actin protrusion seen during cell movement (106, 140, 472). In this thesis, we identify that eEF1A2 is a novel regulator of filopodia formation.

The data presented in this thesis suggests that eEF1A2 is a regulator of the actin cytoskeleton and phosphatidylinositol signaling. Specifically, I show that eEF1A2 mediates filopodia development by activating phosphatidylinositol-4 kinase III beta (PI4KIIIβ). In Chapter Two, I characterized a physical and functional relationship between eEF1A2 and the phosphatidylinositol-4 kinase III beta (PI4KIIIβ). I found that eEF1A2 binds to, and activates, PI4KIIIβ, generating a cellular pool of PI4P. I also detail the kinetics of the eEF1A2/ PI4KIIIβ interaction.

In Chapter Three, I extended these findings to show that the activation of PI4KIIIβ by eEF1A2 leads to, and is necessary for, the formation of filopodia seen in eEF1A2-expressing cells. I describe how the increased PI4P generated upon PI4KIIIβ activation stimulates the production and accumulation of plasma membrane-bound phosphatidylinositol-4,5 bisphosphate ($PI(4,5)P_2$. The generated $PI(4,5)P_2$ then leads to filopodia extrusion through Cdc42 activation.

Finally, in Chapter Four, we confirm that eEF1A2 is a breast oncogene and describe

some *in vitro* and *in vivo* effects upon its expression and ablation. We first find that, although eEF1A2 mRNA expression is very low in normal breast epithelium, it is highly expressed in ~30% of ductal and lobular carcinomas and breast tumour metastases. Furthermore, eEF1A2 mRNA is detected in ~60% of breast cancer cell lines. These results confirm that there is a subpopulation of breast tumours and cancer cell lines that show eEF1A2 overexpression, implicating eEF1A2 as a breast oncogene. Upon expression in breast cancer cell lines, eEF1A2 expression increases *in vitro* growth rates, delays the onset of anoikis, but does not affect their sensitivity to drug-induced apoptosis. Furthermore, transient and/or stable eEF1A2 expression in breast cancer cells activates the Akt serine threonine kinase, further suggesting an interaction between eEF1A2 and the phosphatidylinositol signaling pathway. Finally, I provide evidence of eEF1A2 as a valid therapeutic target. Ablation of eEF1A2 in the MCF7 breast cancer cell line decreases tumour formation in mice. Taken together, this thesis presents data describing the various roles of eEF1A2 in cell physiology and breast oncogenesis.

5.2 eEF1A2 and Filopodia Formation – An Alternate Mechanism

Prior to the work done in this thesis, the accepted role for eEF1A proteins in filopodia formation was one of direct association of eEF1A and actin. Studies in *Dictyostelium* showed that eEF1A bound to, and increased its colocalization with, actin filaments upon chemotactic stimulation (84). Moreover, this interaction and colocalization occurred with concomitant filopodia formation and subsequent movement. This and other observations led Condeelis and colleagues to suggest that direct binding and bundling of actin by eEF1A proteins led to eEF1A-mediated cytoskeletal changes (61). Data presented in this thesis provides an alternative method of eEF1A2-mediated filopodia production (Fig.

5.1).

The new model suggests that eEF1A2 mediates filopodia development indirectly through the activation of phosphatidylinositol signaling. We propose that activation of PI4KIIIβ by eEF1A2 leads to the generation of a pool of PI4P, which then serves as a substrate for $PI(4,5)P_2$ production at the plasma membrane. This pool of $PI(4,5)P_2$ then leads to filopodia formation through a Cdc42-dependent mechanism.

While Chapter Three provides some evidence for this new model, it is not complete. For example, while data is shown that there is an increase in active Cdc42 upon ectopic eEF1A2 expression, it is not clear whether Cdc42 translocates to the plasma membrane. Furthermore, we have not shown whether WASP or Arp2/3 complex proteins are involved in eEF1A2-mediated filopodia development. Additionally, it remains unclear whether Arp2/3 proteins are even involved in this process instead of formins. Thus, to further characterize this model, these details need to be clarified. Immunofluorescence assays using fluorophore-tagged antibodies against active Cdc42, WASP, Arp2/3 proteins and formins in cells transiently or stably expressing eEF1A2 will shed light on some of these issues.

Lastly, there is the question of PI4KIIIβ and its involvement in this process. Results in Chapter Three suggests the activation of this kinase by eEF1A2 leads to an increase in cellular PI4P levels. However, the PI4P-specific antibody used in Chapter Four does not allow for detailed visualization of where the newly formed PI4P is found. Balla and colleagues have developed a reporter construct to visualize PI4P in a cell that is similar to that used to observe $PI(4,5)P_2$ localization. This construct consists of the pleckstrin homology domain of FAPP1 fused to the enhanced green fluorescent protein. FAPP1 is an adapter protein that specifically binds to PI4P (128). Thus, by transfecting eEF1A2-

expressing cells with this construct, we can investigate whether eEF1A2-dependent PI4KIIIβ activation leads to the generation of localized, membrane-bound pools of PI4P or whether the newly generated PI4P occurs elsewhere and then translocates to the plasma membrane via vesicles. Conversely, an even more informative experiment would be live-cell imaging of a cell expressing eEF1A2 and the PI4P reporter construct. In this assay, new PI4P (as a result of eEF1A2-mediated PI4KIIIβ activation) would be detected as it is generated, and we would be able to identify regions in the cell where this is occurring. Previous studies with PI4KIIIβ showed that distinct pools of PI4P are generated on the Golgi (75, 463). Furthermore, studies of the PI4KIIIβ activators ARF and NCS-1 have shown that, while generating a Golgi-localized pool of PI4P, the activation of PI4KIIIβ by these proteins also generates a pool of $PI(4,5)P_2$ on the Golgi (129, 490). None of the studies found any pool of PI4P or $PI(4,5)P_2$ on the plasma membrane. Thus, while the interaction between eEF1A2 and PI4KIIIβ is necessary for eEF1A2-mediated filopodia development, critical details are still missing. A preliminary approach to investigate this issue is to first answer whether PI4KIIIβ colocalizes with eEF1A2 and actin in the filopodia. I have already shown in Chapter Three that eEF1A2 and actin show some colocalization within filopodia. Moreover, overexpression studies done in our lab with PI4KIIIβ alone also show colocalization between PI4KIIIβ and actin in filopodia. However, we have not shown that eEF1A2, PI4KIIIβ and actin all colocalize in filopodia. If we see colocalization of all three proteins, then we can deduce that eEF1A2-mediated filopodia formation occurs when eEF1A2 binds to and activates the PI4KIIIβ found near the plasma membrane. If PI4KIIIβ does not show colocalization with eEF1A2 and filopodia actin, then we can deduce that cytosolic eEF1A2 interacts with cytosolic PI4KIIIβ to generate PI4P, of which some then

translocates to the plasma membrane (in vesicles) so that it can be converted into $PI(4,5)P_2$. This series of experiments will help elucidate where in the cell these two proteins interact with respect to filopodia development.

5.3 eEF1A2 and PI4K

Chapter Two describes the physical and functional relationship between eEF1A2 and PI4KIIIβ. While the data provides insight into another non-canonical function for the elongation factor, more questions remain. Further investigations of these questions will help our understanding of eEF1A2-mediated cellular processes and may provide a means to identify novel protein interacting partners.

5.3.1 Domain Interaction Studies

Currently, there is no known consensus eEF1A2-binding domain or a PI4KIIIβ-binding domain. DNA and protein sequence alignment of other PI4KIIIβ activators (ARF and NCS-1) with eEF1A2 show no substantial sequence homology. Similarly, there does not appear to be any common sequence or domain homology between other eEF1A2-binding proteins and PI4KIIIβ. In Chapter Three, I identified a putative ~160 amino acid region in eEF1A2 that that appears to be necessary for PI4KIIIβ binding. This region contains one putative actin-binding domain and one tRNA-binding domain. It also contains regions that are, as of yet, undefined. Thus, further studies should be undertaken to elucidate the exact amino acid residues that are responsible for PI4KIIIβ binding. Additionally, it may also be possible that the residues responsible for binding may differ from those responsible for activation. Thus, these issues must be addressed in order to understand the nature of eEF1A2/ PI4KIIIβ binding.

Another important issue is whether eEF1A2's its protein translation activity is

necessary for its various functions (such as filopodia production). Specifically, are the tRNA-binding, GTP-binding, GTP-hydrolysis, and actin binding domains needed for eEF1A2 to bind to and activate PI4KIIIβ? Chapter Three addresses this question. I believe that eEF1A2-mediated regulation of filopodia formation is independent of its role in protein translation. The putative PI4KIIIβ binding region contains only one of the three tRNA binding sites. Moreover, an eEF1A2 mutant that contained most of the translational domains, but not the putative interacting domain, was not able to bind PI4KIIIβ, generate plasma membrane $PI(4,5)P_2$ nor form filopodia. As well, unpublished data from our lab show that cells expressing eEF1A2 proteins that have point mutations in the tRNA-binding and GTP-binding sites still form filopodia. Taken together, the results suggest that filopodia regulation by eEF1A2 is independent of protein translation. To extend these findings even further, we need to investigate whether eEF1A2's activity in oncogenesis (cell proliferation, anchorage-independent growth and *in vivo* tumour formation) are also outside of its role in translation. These questions can be investigated using the eEF1A2 mutants described in Chapter Three.

In addition to the above studies, the identification of where eEF1A2 binds to PI4KIIIβ could also be pursued. For example, the major domains in PI4KIIIβ have been well characterized (161). One of these is the C-terminal catalytic domain. It would be interesting to know if eEF1A2 binds to this catalytic domain to activate PI4KIIIβ, or whether eEF1A2 binds elsewhere on the protein to induce a conformation change that better exposes the catalytic domain. This knowledge could also help identify and explain how ARF and NCS-1 serve as PI4KIIIβ-activating proteins. Thus, the identification of amino acid residues where eEF1A2 binds to PI4KIIIβ could be of value for our understanding of

these two proteins as well as other proteins that may bind to and activate them.

5.3.2 eEF1A2 and PI4K Isoforms

eEF1A2 is a diffusely cytoplasmic protein (6). The choice of studying PI4KIIIβ (over other PI4K isoforms) was made due to its predominantly cytosolic localization in the cell (463). The other isoforms (PI4KIIIα, PI4KIIα and PI4KIIβ) are predominantly membrane-bound (471). Thus, we believed that two cytosolic proteins had a greater probability of interacting than a cytosolic protein and a membrane-bound protein. However, results obtained in Chapter Three indicate that eEF1A2 is found in filopodia and pseudopodia. This leads to the intriguing possibility that pools of eEF1A2 found on filopodia may interact with other, membrane-bound PI4Ks, perhaps specifically during cell motility. We have antibodies for all PI4K isoforms. Co-immunoprecipitation assays with each isoform and eEF1A2 could yield information as to whether the eEF1A2/PI4KIIIβ interaction is specific or general to all PI4Ks.

5.4 eEF1A2 and PI4KIIIβ - Additional Effects

The results from Chapter Three indicate that one downstream effect of the eEF1A2/PI4KIIIβ interaction is the generation of a plasma membrane-bound pool of $PI(4,5)P_2$. This raises the question as to what happens to this pool. Does all of the $PI(4,5)P_2$ get funneled into regulating cell morphology or is some diverted to other signaling pathways? For example, $PI(4,5)P_2$ has an established role in calcium signaling (254, 475). Upon hydrolysis by phospholipase C, $PI(4,5)P_2$ yields diacylglycerol (DAG) and inositol trisphosphate (IP_3) (254). IP_3 is known to release calcium from its storage pools in the endoplasmic reticulum (2, 107, 379). This calcium then serves as a secondary messenger in a variety of calcium/calmodulin-dependent signaling pathways – one of which includes Akt

activation (483). Does ectopic expression of eEF1A2 lead to an increase in intracellular calcium levels? Plant and protozoan eEF1A proteins have been shown to bind calmodulin (94, 202) and calcium/calmodulin-dependent protein kinases (454). Furthermore, Ransom-Hodgkins and colleagues showed that tomato eEF1A protein was proteolytically degraded when incubated with 1mM calcium in an *in vitro* assay (352), suggesting that a calcium-dependent protease controls plant eEF1A protein levels. Thus, it would be interesting to see whether mammalian eEF1A2 can affect intracellular calcium pools and whether calcium levels can regulate eEF1A2 (perhaps in a negative-feedback loop).

5.5 eEF1A2 and Akt Activation

Results presented in Chapter Four provides another downstream effect of eEF1A2 expression – Akt activation. Akt directly and indirectly controls proliferation, growth, apoptosis and actin filament remodeling, by regulating several transcriptional and translational regulators (91, 150, 346, 448). We have previously reported that eEF1A2-dependent filopodia formation is partly dependent on Akt (5). However, it is not known whether eEF1A2-dependent Akt activation leads to other effects. For example, does the increase in active Akt protein levels lead to the increase in cell growth rates observed in Chapter Four? Furthermore, Akt activation also plays a role in inhibiting various forms of apoptosis (91, 112). While we see in Chapter Four that eEF1A2 expression does not protect against drug-induced cell death, it does protect against anoikis. There are several studies that investigated the regulation of anoikis by Akt or activators of Akt (11, 115, 453, 487). For example, the integrin-linked kinase (ILK) activates Akt by phosphorylating it on serine 473 (151). Attwell and colleagues showed that overexpression of ILK in SCp2 cells (normal mammary epithelial cells) inhibited anoikis (11). Furthermore, they found that this effect

was reversible by the transfection of a dominant-negative, kinase dead ILK or a dominant-negative Akt (11). These results suggest that anoikis can be regulated by Akt. Thus, it would be interesting to determine whether eEF1A2's ability to regulate anoikis in breast cancer cells occurs through Akt activation and, moreover, whether eEF1A2-mediated Akt activation affects other signaling pathways.

5.6 eEF1A2 and Development

eEF1A2 mRNA and protein is detectably expressed exclusively in terminally differentiated cells of mammalian heart (cardiomyocytes), brain (neurons) and skeletal muscle (myocytes) (211, 238, 239). However, during mammalian embryonic development, eEF1A2 expression is undetectable and instead, eEF1A1 mRNA is detected (238, 239). For example, eEF1A1 mRNA levels are higher in embryonic day 20 (E20) embryonic rat brains as compared to newborn (day 1 – 30) or adult brains (six – nine months) (238, 239). Interestingly, eEF1A2 mRNA is detected late during brain development and steadily increases to attain a plateau early during postnatal life (~ day 30) (238, 239). A similar pattern is observed in muscle and heart development where eEF1A1 downregulation coincides with the termination of the myogenic process and an increase in eEF1A2 levels (239). These results suggest that there is a preference for eEF1A2 over eEF1A1 during *in vivo* neurogenesis and myogenesis and that eEF1A2 functions as part of a yet undefined postdifferentiation program. Furthermore, eEF1A2 appears to be upregulated at a point when neurons, cardiomyocytes and myocytes permanently leave the cell cycle (8), perhaps suggesting that eEF1A2 plays a role in regulating permanent growth arrest. Many theories for this switch have been proposed but have not been fully investigated. For example, while eEF1A1 and eEF1A2 share >95% amino acid sequence similarity, they do not show any

similarity in their 3'-untranslated region (UTR) (27, 211). The 3'-UTR contains several regulatory sequences, among them protein and microRNA binding sites, that may affect mRNA stability (317, 452). Thus, it may be the case that neurons, cardiomyocytes and myocytes need eEF1A2 expression to regulate the translation of a specific subset of mRNAs. While the above model provides one explanation, it assumes that the eEF1A1-to-eEF1A2 switch occurs in relation to protein synthesis. Below, we look at other possible reasons for the switch.

5.6.1 Neuritogenesis

The sprouting of neurites, or neuritogenesis, is an important event in early neuronal differentiation (70, 181, 288). Neurites, cylindrical actin-based projections consisting of a growth cone at the distal tip, extend from the cell body of a neuron and can become axons or dendrites (70). Regulation of neuritogenesis is important because neurites form the basis of proper neuronal connectivity and brain function. Improper neurite sprouting or elongation (wrong timing or wrong direction) can severely impact the function of the nervous system. Immediately after neuronal commitment, neuritogenesis occurs with the activation of membrane receptors by extracellular cues. These stimuli activate intracellular signaling cascades that trigger changes to the actin cytoskeleton. As neurites grow and develop, further signaling cascades are activated and the neurites (now axons or dendrites) stabilize (70).

In vivo neuritogenesis can be recapitulated *in vitro* using neurosphere cultures (70). Neurospheres are non-adherent, spherical clusters of CNS stem cells (133). They form when CNS cells (early postnatal) are stimulated to proliferate in tissue culture. *In vitro* neuritogenesis occurs in three stages: 1) the neurosphere is broken down to form a bud, 2)

the bud transforms into a neurite (neurite sprouting), and 3) the neurite transforms into an axon or dendrite (neurite elongation and stabilization) (70). Thus, using neurosphere models, one can recapitulate the stages of neurite formation and eventually, neurogenesis.

As described earlier, neurites are actin-based structures. Studies have shown that when grown in culture, hippocampal or cortical neurons will initially attach to the substrate and form lamellipodia tipped by filopodia (66, 74, 76). The lamellipodia then coalesce to form neurites (88). Kwiatkowski and colleagues showed that filopodia were required for cortical neurite initiation (229). Through a series of studies, they showed that the loss of all three murine Ena/VASP proteins (a family of actin regulatory proteins) led to cortical fiber tract defects arising from neurite initiation failure (229). Furthermore, neurite initiation defects in Ena/VASP-deficient neurons were preceded by a failure to form bundled actin filaments and filopodia (229). Dent and colleagues provided further evidence by rescuing showing neuritogenesis in Ena/VASP-null neurons by ectopically expressing mDia2 (formin) (79). Additionally, the addition of laminin, a promoter of filopodia-like structures, also rescued neuritogenesis in these neurons (79). Thus, in some neurons, filopodia formation is required for neuritogenesis.

5.6.2 Cell Survival

In addition to the above hypothesis about eEF1A2 being necessary for filopodial-type extensions, another reason for the eEF1A1-to-eEF1A2 switch during the late embryonic to postnatal stages could be for cell survival. The lack of significant regenerative potential in post-mitotic cells demands that they must persist long-term, often for the full lifespan of an organism (473). As such, these cells must employ several mechanisms to refine and regulate apoptosis. eEF1A2 could play a role in delaying cell death. Ruest and

colleagues demonstrated that expression of eEF1A2 in differentiated myotubes protected them against serum deprivation-induced apoptosis, suggesting a pro-survival function for eEF1A2 in skeletal muscle (372). While a mechanism was not given for this pro-survival effect, data presented in Chapter Four and from our lab (5) provides a plausible pathway: Akt activation. Akt is a well known regulator of several proliferative and anti-apoptotic pathways (91, 112). Thus, eEF1A2 expression in terminally differentiated neurons, cardiomyocytes and myocytes may be necessary to prevent the loss of these non-proliferating cells. This theory can be tested by examining these cells during differentiation for changes in Akt activation. If Akt activation increases concurrently with eEF1A2 expression, then eEF1A2 could play a protective role in these post mitotic cells.

5.7 Phosphatidylinositol-4 Kinase III Beta

Although the focus of this thesis has been on eEF1A2, this last section will briefly focus on PI4KIIIβ. While shown to interact with eEF1A2 and provide a potential mechanism for eEF1A2-regulated filopodia formation, PI4KIIIβ itself was shown to recapitulate some of the same effects with respect to $PI(4,5)P_2$ production and filopodia formation (Chapter Three). This suggests that PI4KIIIβ may also play a pivotal role in actin cytoskeleton remodeling and perhaps oncogenesis. Our lab is currently investigating PI4KIIIβ and its potential importance in the regulation of the phosphatidylinositol signaling pathway and oncogenesis. More specifically, we are looking at PI4KIIIβ and its role in regulating the actin cytoskeleton and Akt activation in cancer cell lines.

In addition to looking at PI4KIIIβ, it would also be worth investigating whether other GTPases also interact with PI4KIIIβ. eEF1A2 is considered a Ras-like protein for its structural and functional similarities in GTP-binding and GTP-hydrolysis activity (149).

Downward and colleagues showed that Ras was capable of binding to and activating PI3K (206, 363). Furthermore, this same group showed that the interaction occurred in the PI3K catalytic domain (362, 364), a domain sharing a great degree of homology with PI4 kinases (124). Thus, it might be the case that Ras and other Ras-like proteins may bind and activate PI4K proteins – specifically PI4KIIIβ. This avenue is currently being pursued in our lab.

5.8 eEF1A2, PI4K, and Cell Movement – General Comments

The signaling pathways underlying how cells move and what stimulates cell movement have been extensively studied. For example, studies in *Dictyostelium* cells have shown that the addition of a chemoattractant to a plate results in the directed movement of cells towards the stimulus due to a chemoattractant gradient. The first signal that initiates cell movement is the binding of the ligand to transmembrane receptors and subsequent G protein mediated signaling (182, 445). In eukaryotic cells, and more specifically cancer cells, the chemoattractants are growth factors that bind to tyrosine kinase receptors and lead to autophosphorylation events and subsequent signaling cascades (81, 380). Thus, surface receptors are most often the first point of activation for motility signals due to their roles in sensing gradients.

Interestingly, in most eukaryotic cells, activated receptors are internalized in a matter of minutes in intracellular vesicles. For example, Guglielmo and colleagues demonstrated that upon insulin binding to its receptor, the insulin receptor kinase (IRK), the complex is rapidly (within two to five minutes) internalized into the endosomal apparatus where the receptor can then be recycled back to the membrane or degraded in lysosomes (85). Similarly, the binding of epidermal growth factor (EGF) to its receptor is followed by receptor dephosphorylation and internalization in about ten minutes (174, 375, 408). Thus,

upon binding to a chemoattractant, the receptor is, at least temporarily, unavailable for further ligand binding/sensing. To compensate for this, cells need more receptors at the sensing or leading edge, in order to continue the signaling cues that lead to cell movement. This can occur through accumulation of receptors towards the leading edge through lateral movement along the membrane and / or direct, rapid receptor recycling to the leading edge membrane from intracellular vesicles.

There are several reports describing stimulus-mediated accumulation of proteins on the leading edge of cells. Upon insulin-like growth factor-1 stimulation of MCF7 (breast cancer) cells, Manes and colleagues observed asymmetrical redistribution of the CCR5 chemokine receptor, raft-associated ganglioside GM1, and ephrinB1 proteins to the leading edge (264). Similar effects were observed with EMR2 (epidermal growth factor-like module-containing mucin-like hormone receptor). EMR2 regulates neutrophil responses by potentiating the effects of a number of proinflammatory mediators. Yona and colleagues found that upon neutrophil activation, EMR2 is rapidly translocated to membrane ruffles and the leading edge of cells (486). Thus, the redistribution of proteins towards the leading edge of motile cells is an important event in cell movement. Not only does it lead to an accumulation of receptors for ligand binding, but it also serves as a means to amplify the signaling cascades that will ultimately lead to cell movement.

The work presented in this thesis provides a glimpse at the roles of eEF1A2 and PI4KIIIβ in cell motility. The role of PI4Ks in vesicular trafficking has been well established. The yeast PI4KIIIβ, Pik1, localizes to the trans-Golgi and generates a pool of PI4P on Golgi membranes that is necessary for protein secretion. Hama and colleagues showed that the *Sec14-3* mutant *Saccharomyces cerevisiae* strain, which is deficient for

protein secretion, had markedly reduced PI4P levels compared to wild type strains (148). Furthermore, overexpression of Pik1, or conversely, deletion of Sac1 (the 4'-phosphatase) rescued the growth defects and protein secretion observed in *Sec14-3* mutant cells (148). These results suggest that not only is PI4P limitation the major contributing factor to the secretory defect in *Sec14-3* cells, but that PI4P has an important role in Golgi-to-plasma membrane secretion. The necessity of PI4K activity for secretion is not limited to yeast. Wiedemann and colleagues detected PI4KIIIβ activity on nerve terminal-derived small synaptic vesicles (SSVs), and that inhibition of PI4KIIIβ activity in intact synaptosomes led to an attenuation of the evoked release of glutamate (a consequence of calcium-triggered fusion of SSVs with the presynaptic plasma membrane) (466). Therefore, the role of PI4Ks in protein secretion and vesicular trafficking has been well established. Interestingly, PI4KIIIβ has been shown to play an important role in the endocytosis of muscarinic cholinergic receptors (mAChRs) in neuroblastoma cells (402). Sorenson and colleagues demonstrated that pretreatment of SH-SY5Y neuroblastoma cells with wortmannin or phenylarsine oxide (agents that inhibit type III PI4Ks) resulted in both an inhibition of agonist-induced endocytosis of mAChRs and a decrease in PI4P levels (402). Surprisingly, disruption of the actin cytoskeleton with colchicine or cytochalasin D did not affect receptor internalization, suggesting that PI4K and phosphoinositides (PI4P) modulate endocytosis independently of their roles in maintaining the cytoskeleton (402). Taken together, one can propose a possible model for the involvement of PI4KIIIβ in cell movement. In this scenario, the cell first "senses" a chemoattractant gradient (the ligand binds to its respective receptor that is found on the membrane). Because this gradient will reach the cell in one direction, receptors on a particular area of the cell will be activated. As the ligand/receptor

complex activates downstream cascades that control cell motility (described in earlier chapters), the complex itself can not rest at the membrane. If it does so, then no further signal will be processed by the cell, thus preventing cell motility. To prevent this, the receptor/ligand complex is internalized into intracellular vesicles where the receptor is degraded or recycled back to the membrane to continue receiving signals. If the receptor is degraded, then other receptors, found elsewhere on the plasma membrane, move laterally to replace lost receptors. The continuous recognition of the ligand by receptors creates a leading edge in the cell (filopodia and/or lamellipodia), and eventually, cell movement. The role of PI4KIIIβ in this process is in the formation of these vesicles. Since these vesicles are part of the secretory/vesicular trafficking system, they originate in the Golgi where studies have shown that PI4KIIIβ, and its lipid product, PI4P, play critical roles in maintaining the structural architecture of the Golgi itself, and in Golgi-to-plasma membrane transport of newly synthesized cargoes (129). Taken together, our observations that PI4KIIIβ itself, can regulate filopodia formation (Chapter Four), can be explained by the hypothesis that this regulation occurs indirectly due to constant vesicular trafficking (Golgi to membrane, and vice versa) of plasma membrane bound receptors. Now, one may ask about the role of $PI(4,5)P_2$ in this model. Chapter Four not only provides evidence of PI4KIIIβ regulating filopodia development, but it also suggests that there is an accumulation of $PI(4,5)P_2$ on the filopodia generated due to PI4KIIIβ overexpression. This too, can be explained by the above model. Vesicles traffick proteins in or out of the cell by fusing with, or budding from, the plasma membrane (176). Thus, lipids found on vesicle membranes will end up being part of the plasma membrane. Upon fusion to the plasma membrane, vesicle membrane PI4P will become plasma membrane PI4P. Once localized in the membrane, PI5Ks, which

use PI4P as a substrate and are found in the nucleus, cytosol, and near the plasma membrane (197, 256, 319), can convert PI4P to $PI(4,5)P_2$, leading to an accumulation of the biphosphoinositide at the leading edge of the cell.

A final piece of evidence to support the role of PI4KIIIβ in cell movement described above, come from studies investigating Golgi orientation in motile cells. In moving cells, the Golgi reorients itself to the anterior side of the nucleus so that it faces the direction of forward movement (279). This occurs so as to direct secretory traffic and recycling endosomes to the leading edge. By reorganizing the Golgi to face the direction of forward movement, the cell enables the enrichment of cell-surface receptors at the leading edge and allows for delivery of proteins necessary for the development of the leading edge (227, 279). Thus, by orienting the Golgi to the anterior side of the nucleus, motile cells set up a supply line to the front of the cell. Taken together, one can envision a role for PI4KIIIβ in cell motility that relates vesicular trafficking to the formation of the leading edge.

eEF1A2's role can be viewed in two ways. The first involves the ability of eEF1A2 to activate PI4KIIIβ while the second focuses on eEF1A2's actin-binding activity. The first case provides a simplified view of eEF1A2 in terms of cell motility. Here, eEF1A2 simply activates phosphatidylinositol signaling through PI4KIIIβ. This activation will generate a pool of PI4P which then will regulate creation of the leading edge. However, our results (Chapter Four) suggest that the situation is not as straightforward as described in this model. For example, if eEF1A2's only contribution was to activate PI4KIIIβ, then why does this occur in the cytoplasm but not at the Golgi? Colocalization studies from our lab show no physical interaction between both proteins in the Golgi (Appendix A; Figure A.2). Furthermore, eEF1A2 is found on filopodial edges, tips and bases, suggesting that eEF1A2

is physically present in filopodia. The lack of eEF1A2 on the Golgi apparatus suggests that either eEF1A2 does not affect PI4KIIIβ's role in maintaining Golgi structural integrity, or that eEF1A2 only activates the cytosolic pool of PI4KIIIβ. However, this still does not explain why eEF1A2 is found in filopodia. I believe that the answer to this puzzle is that eEF1A2 can activate multiple PI4Ks. As described earlier in section 5.3.2, we do not know if eEF1A2 activates PI4KIIIβ solely, or whether other PI4Ks can also be similarly activated. PI4KIIIα localizes to plasma membranes (19). Thus, it may be possible that eEF1A2, in addition to activating cytoplasmic PI4KIIIβ (perhaps those found associated with or near vesicles moving between the Golgi and then plasma membrane), can activate plasma membrane PI4KIIIα. The combinatorial activation of multiple PI4K isoforms located in different regions of the cell can generate enough PI4P to not only enhance vesicular trafficking from and to the plasma membrane, but to also generate enough downstream $PI(4,5)P_2$ and $PI(3,4,5)_3$ (through PI5K and PI3K activities, respectively) to initiate the signaling pathways that regulate actin cytoskeletal dynamics. Thus, eEF1A2's role in filopodia development may not be just the activation of general phosphatidylinositol signaling, but perhaps the amplification of regional (area between the Golgi and the leading edge) phosphatidylinositol signaling to ensure that a) protein secretion / receptor recycling occurs at an efficient rate to ensure the continual presence of free receptors at the plasma membrane and b) enough downstream phosphoinositides are generated at the leading edge to maintain and regulate actin cytoskeletal changes. The regulation of these two aspects provides a model as to how eEF1A2 plays an important role in cell motility.

Some evidence for the above model is found in studies by McClatchy and colleagues (270, 271). Both mammalian eEF1A proteins were identified as binding partners to the third

intracellular (i3) loop of the M4 subtype of mAChRs (271). The i3 loops of these receptors contain several small conserved domains that regulate cell surface localization and receptor activity (213, 474). Using the PC12 primary neuronal cell line, McClatchy observed a role for eEF1A proteins in receptor recycling (endosome to plasma membrane) (270). In unstimulated PC12 cells, the M4 subtype of mAChR localizes to the plasma membrane. However, when stimulated with carbachol, M4 internalizes and localizes to endosomes. Furthermore, upon removal of the agonist, M4 recycles back to the plasma membrane (270). McClatchy and colleagues found that while overexpression of eEF1A1 or eEF1A2 in PC12 cells did not affect M4 internalization rates, their expression did inhibit / delay M4 recycling to the plasma membrane (270). These studies demonstrate that eEF1A proteins are involved in the trafficking of internalized M4 receptors from endosomes to the plasma membrane. Furthermore, these studies also revealed that eEF1A inhibits M4 recycling through a direct interaction as eEF1A did not affect the recycling of the M1 mAChR subtype (eEF1A does not bind M1 receptors) (270, 271). Taken together, these observations provide further evidence that eEF1A2 can regulate vesicular trafficking of proteins. Enhancement of receptor recycling could lead to rapid, continuous signal transduction from ligand/receptor complexes found on the leading edge membrane to mediate cell movement. Conversely, inhibition of receptor recycling could lead to pauses during which proteins can effectively alter the actin cytoskeleton to generate filopodia so that recycled receptors can localize within them.

The second role for eEF1A2 in cell movement may relate to its ability to bind and bundle actin. eEF1A proteins from several genera and species bind and bundle actin (35, 96, 136, 198, 257, 295). Actin filaments are key elements in the generation of the leading

edge, and thus, cell movement (290). Actin filament-based structures like filopodia and lamellipodia are often found at the leading edge (73, 292, 341). Results from Chapter Four show that eEF1A2 localizes with actin in filopodia and lamellipodia. Based on the domain structures of eEF1A2 and other eEF1A-like proteins, it appears reasonable that eEF1A2, upon close proximity to actin filaments, would also bind and bundle them through its two actin-binding domain. Interestingly, Owen and colleagues reported that *Dictyostelium* eEF1A proteins crosslink actin in bundles where crosslinked actin filaments are rotated by 90° relative to one another (321). This may explain the localization of eEF1A2 in lamellipodia where actin filaments are organized in a mesh-like network (10). This specific type of actin bundling would create large networks instead of cylindrical, finger-like protrusions (filopodia). So why and how would eEF1A2 be associated with actin in filopodia? A possible answer to both questions can be found in the domain structure of eEF1A2. Chapter Four identifies a putative region within eEF1A2 that binds PI4KIIIβ (Fig. 5.2). This region comprises the smaller of the two actin-binding domains and the linker region separating the two actin-binding domains. Using protein modeling software and the amino acid sequence of human eEF1A2, I generated a putative 3D model of human eEF1A2 (Fig. 5.3). As seen in the model, the two actin-binding domains are perpendicular to each other, in accordance with biochemical data by Owen and colleagues (321). Since eEF1A2 binds to PI4KIIIβ in the region identified in Fig. 5.1, it is possible that, in filopodia, eEF1A2 binds to actin filaments using only one of its actin-binding domain. The other could potentially mediate interaction with, and activation of PI4KIIIβ. Thus, eEF1A2's role in cell motility could be to a) affect vesicular trafficking through PI4KIIIβ activation at or near leading edge protrusions, b) stabilize and bring together actin filaments found in the leading

edge to plasma membrane proteins and lipids that regulate their elongation, and finally c) to synchronize both events so as to ensure proper cell movement in the right direction (towards the original stimulus).

5.9 Concluding Remarks

The data presented in this thesis provides evidence of eEF1A2 being a regulator of phosphatidylinositol signaling and filopodia development. Furthermore, we show that *EEF1A2* is a breast oncogene and is a potential therapeutic target. However, there are more questions about this important oncogene and further research is needed to fully understand the scope of abnormal eEF1A2 expression in cells. Additionally, this thesis also identifies PI4KIIIβ as a regulator of the actin cytoskeleton. Continued study of both proteins will shed light on cancer development and cell movement, and perhaps yield clues on how to effectively target key steps in the oncogenic pathway. The work presented in this thesis increases our understanding of eEF1A2 and its various roles in the cell.

6.0 References

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Appendix A

Supplementary Figures

Appendix B

Published Papers

Appendix C

Curriculum Vitae

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EDUCATION

2005 – Present	**Doctoral Candidate** – University of Ottawa, Ottawa, Ont., Canada Department of Biochemistry, Microbiology and Immunology Principal Investigator – Dr. Jonathan Lee, Ph.D.
2004 – 2005	**Master of Science Candidate** – University of Ottawa, Ont., Canada Department of Biochemistry, Microbiology and Immunology Transferred to Ph.D. program Principal Investigator – Dr. Jonathan Lee, Ph.D.
2003 – 2004	**Master of Science Candidate** – McMaster University, Hamilton, Ont., Canada Department of Molecular Biology, Genetics and Cancer Transferred to M.Sc. program at the University of Ottawa Principal Investigator – Dr. Jonathan Lee, Ph.D.
1998 – 2002	**Honours Bachelor of Science** - University of Toronto, Mississauga, Ont., Canada Specialized in Molecular Biology, Minors in Chemistry and Mathematics

DISSERTATION

Doctoral Research – University of Ottawa, Ottawa, Canada
Research Advisor / Supervisor – Dr. Jonathan Lee

Investigating the regulation of phospholipid signaling and actin cytoskeletal remodeling by the eukaryotic translation elongation factor eEF1A2

This work identified eEF1A2, a protein translation factor, as a regulator of filopodia development through the activation of the phosphatidylinositol signaling cascade. More specifically, I found that eEF1A2 expression mediated filopodia formation by interacting with, and activating, the lipid kinase, PI4KIIIβ. I also investigated the role of eEF1A2 in breast cancer and identified it as a potential therapeutic target. Most of the dissertation has been published in **two first-authored** and **two co-authored** journal articles (see below).

SUBMITTED / PUBLISHED PAPERS

Jeganathan, S., Morrow, A., Amiri, A. and Lee, JM. Eukaryotic elongation factor 1A2 cooperates with phosphatidylinositol-4 kinase III beta to stimulate production of filopodia through increased phosphatidylinositol-4,5 bisphosphate generation. *Mol Cell Biol* 2008, **28**:4549-4561.

Jeganathan, S. and Lee, JM. Binding of elongation factor eEF1A2 to phosphatidylinositol-4 kinase beta stimulates lipid kinase activity and phosphatidylinositol-4 phosphate generation. *J Biol Chem* 2007, **282**:372-380.

Amiri, A., Noei, F., **Jeganathan, S.**, Kulkarni, G, Pinke, DE. and Lee, JM. eEF1A2 activates Akt and stimulates Akt-dependent actin remodeling, invasion and migration. *Oncogene* 2007, **26**:3027-3040.

Kulkarni, G., Turbin, DA, Amiri, A, **Jeganathan, S.**, Andrade-Navarro, MA, Wu, TD, Huntsman, DG, and Lee, JM. Expression of protein elongation factor eEF1A2 predicts favorable outcome in breast cancer. *Breast Cancer Res Treat* 2007, **102**:31-41.

PUBLISHED ABSTRACTS

Jeganathan, S. and Lee, JM. (2006) "Binding of elongation factor eEF1A2 to phosphatidylinositol-4 kinase β stimulates lipid kinase activity and phosphatidylinositol-4 phosphate generation." *4th Euro Fed Lipid Congress.*

Jeganathan, S., Amiri, A., Noei, F., and Lee, JM. (2006) "The eEF1A2 ovarian cancer oncogene activates the AKT kinase, controls cell migration, and stimulates phosphatidylinositol signaling." *2nd International Meeting on Epithelial to Mesenchymal Transition.*

Amiri, A., **Jeganathan, S.**, Noei, F., and Lee, JM. (2006) "eEF1A2 induces actin filament remodeling through PI3K/Akt signaling." *PTEN Pathways Cold Spring Harbor Laboratory.*

Jeganathan, S., Amiri, A., Wu, TM, and Lee, JM. (2005) "Protein elongation factor eEF1A2 is a putative breast cancer oncogene that increases cell growth and cell size." *AACR Proceedings.*

Amiri, A., **Jeganathan, S.**, Noei, F., and Lee, JM. (2005) "eEF1A2 is a novel Akt/PKB activator that regulates cell adhesion and migration and is a HER2-independent marker for breast tumor aggressiveness." *The 3rd National Conference on Ovarian Cancer Research.*

ATTENDED CONFERENCES

2006	**4th Euro Fed Lipid Congress (Madrid, Spain)** Poster Title - *Binding of protein elongation factor eEF1A2 to phosphatidylinositol-4 kinase β stimulates lipid kinase activity*
2005	**American Association for Cancer Research (Anaheim, California, USA)** Poster Title - *Protein elongation factor eEF1A2 is a putative breast cancer oncogene that increases cell growth and enhances cell adhesion.*

TEACHING / WORK EXPERIENCE

2003 – Present	**Graduate Student** – University of Ottawa
Sept. – Dec. 2007	**Teaching Assistant** – University of Ottawa, Ottawa, Canada • **BCH3356 – Molecular Biology Laboratory II**

Jan. – May 2007 **Teaching Assistant** – University of Ottawa, Ottawa, Canada
- **BCH4125 – Cell Regulation and Control**
- **BCH3120 – Cell Metabolism**

Sept. – Dec. 2006 **Teaching Assistant** – University of Ottawa, Ottawa, Canada
- **BCH3356 – Molecular Biology Laboratory II**

Jan. – May 2006 **Teaching Assistant** – University of Ottawa, Ottawa, Canada
- **BCH3346 – Biochemistry Laboratory II**

Sept. – Dec. 2005 **Teaching Assistant** – University of Ottawa, Ottawa, Canada
- **BCH3356 – Molecular Biology Laboratory**

Sept. – Dec. 2003 **Teaching Assistant** – McMaster University, Hamilton, Canada
- **BIOLOGY 1AA3 – Biodiversity, Evolution and Ecology**

1997 – 1998 **Lab Volunteer** – University of Toronto, Toronto, Canada
- Assist graduate and post-doctoral students with their experiments
- Worked with pathogenic fungus, *Ophiostoma novo-ulmi*

OTHER

Jan.2008 - Present **BMI Graduate Student Association (BMIGSA) Member** – University of Ottawa, Ottawa, Canada
- Member at large
- Participate in council meetings, elections, BBQs, Welcome week events, Christmas events, etc.

Jan. – Dec. 2007 **BMIGSA Member / Webmaster** – University of Ottawa, Ottawa, Canada
- Set up, update, and run BMI Graduate Student Association website (http://intermed.med.uottawa.ca/Associations/BMIGSA/Homepage.html)
- Participate in council meetings, elections, BBQs, Welcome week events, Christmas events, etc.

2005 **Let's Talk Science** – University of Ottawa, Ottawa, Canada
- Set up and present simple experiments for Grade 5 students
- Teach Grade 3 students about Animals and their Habitats (Powerpoint presentations with audio/video clips of various animals and their habitats)

AWARDS / HONOURS / ACADEMIC DISTINCTIONS

2006 Ontario Women's Health Council / Canadian Institutes for Health Research Doctoral Research Award (3 years)

University of Ottawa National Excellence Scholarship

Faculty of Graduate and Postgraduate Studies Travel Award (University of Ottawa, Ottawa, Ontario, Canada)

	Department of Biochemistry, Microbiology, and Immunology Travel Award (University of Ottawa, Ottawa, Ontario, Canada)
2005	Faculty of Graduate and Postgraduate Studies Travel Award (University of Ottawa, Ottawa, Ontario, Canada)
	Department of Biochemistry, Microbiology, and Immunology Travel Award (University of Ottawa, Ottawa, Ontario, Canada)
	BMI Poster Day Competition – Best Poster Winner (University of Ottawa, Ottawa, Ontario, Canada)
	Let's Talk Science Achievement Award (University of Ottawa, Ottawa, Ontario, Canada)
2002	Graduated with Distinction (University of Toronto, Mississauga, Canada)
	Let's Talk Science Achievement Award (University of Toronto, Mississauga, Canada)
1999	Doris Geddes Scholarship in Mathematics (University of Toronto, Mississauga, Canada)
1999	Golden Key National Honour Society (University of Toronto, Mississauga, Canada)
1998	Bilingual Diploma (English / French)

TECHNICAL SKILLS

- Southern, Northern, and Western Blotting
- PCR, RT-PCR, real-time PCR
- Immunoprecipitation and co-immunoprecipitation
- Mutagenesis studies (Site-directed and domain mutagenesis)
- Protein purification (GST fusion proteins)
- Protein-protein interaction studies (binding and stoichiometry studies)
- Enzyme kinetics assays
- Cell culture protocols (immortalized and cancer cells)
- Cytotoxicity, cell proliferation, adhesion, apoptosis, anoikis and drug assays
- Proficient with adenoviral, retroviral and lentiviral vector systems for gene delivery *in vitro* and *in vivo*
- Transient and stable gene knockdown assays using antisense oligonucleotides, short-interfering RNAs and short-hairpin RNAs
- Immunofluorescence microscopy (deconvolution and confocal)
- Flow cytometry
- Lipid kinase assays
- Thin layer chromatography
- Proper usage of radioactivity (^{3}H, ^{14}C, and ^{32}P)
- Nude mice (xenografts, pellet implantation, tumour formation studies)

REFERENCES

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