CONTROL OF MORPHOGENESIS AND NEOPLASIA BY THE ONCOGENIC TRANSLATION FACTOR eEF1A2

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ABSTRACT

The eukaryotic elongation factor 1 alpha 2 (eEF1A2) is a protein normally expressed only in the brain, heart and skeletal muscle. eEF1A2 is likely to be a breast and ovarian cancer oncogene based on its high expression in these malignancies and its *in vitro* transforming capacity . The goal of my thesis is to understand eEF1A2's role in oncogenesis.

In order to determine if eEF1A2 was a prognostic marker for ovarian cancer, we examined eEF1A2 expression in 500 primary human ovarian tumours. We show that eEF1A2 is highly expressed in approximately 30% of ovarian tumours. In serous cancer, high expression of eEF1A2 was associated with an increased 20-year survival probability. Expression of eEF1A2, in a clear cell carcinoma cell line, SK-OV-3, increased the cells ability to form spheroids in hanging drop culture, enhanced *in vitro* proliferative capacity, increased stress fiber formations, and reduced cell-cell junction spacing. Expression of eEF1A2 did not alter sensitivity to anoikis, cisplatin, or taxol.

In order to examine the role of eEF1A2 in breast cancer, we used a three-dimensional culture system. The ability to disrupt the *in vitro* morphogenesis of breast cells cultured on reconstituted basement membranes is a common property of breast oncogenes. I found that phosphatidylinositol 4-kinase (PI4KIIIβ), a lipid kinase that phosphorylates phosphatidylinositol (PI) to PI(4)P, disrupts *in vitro* mammary acinar formation. The PI4KIIIβ protein localizes to the basal surface of acini created by the human MCF10A cells

and ectopic expression of PI4KIIIβ induces multi-acinar formation. Expression of the PI4KIIIβ activator, eEF1A2, also causes a multi-acinar phenotype. Ectopic expression of PI4KIIIβ or eEF1A2 alters PI(4)P and PI(4,5)P_2 localization, indicating a role for these lipids in acinar development.

Therefore, eEF1A2 is highly expressed in ovarian carcinomas and its expression enhances cell growth *in vitro*. eEF1A2 expression is likely to be a useful ovarian cancer prognostic factor in ovarian patients with serous tumours. Furthermore, PI4KIIIβ and eEF1A2 both have an important role in the disruption of three-dimensional morphogenesis of MCF10A cells. Additionally, PI4KIIIβ and eEF1A2 likely have an important role in mammary neoplasia and development and could be anti-cancer targets.

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LIST OF ABBREVIATIONS

AAD	Adult Atopic Dermatitis
ABP	Actin binding protein
Arf	ADP ribosylation factor
AP	Adapter protein
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BLISS	Bacus Laboratories Inc. Slide Scanner
BRMS1	Breast cancer metastasis suppressor 1
DAG	Diacylglycerol
d.c.m.	Disintegrations per minute
DMSO	Dimethylsulfoxide
ECM	Extracellular matrix
EDTA	Ethylene diaminetetraacetic acid
eEF	Eukaryotic elongation factor
EHS	Engelbreth-Holm-Swarm
eIF	Eukaryotic initiation factor
ER	Endoplasmic Reticulum
eRF	Eukaryotic release factor
FAPP	Four adaptor protein
FISH	Fluorescent in situ hybridization
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
HRP	Horseradish peroxidase
MCS	Multi-cellular spheroids
MDCK	Madin-Darby Canine Kidney
mRNA	Messenger RNA
NCS-1	Neuronal Calcium Sensor-1
OIS	Oncogene induced senescence
OSBP	Oxysterol binding protein

PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PH	Pleckstrin homology
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol 3-kinase
PI(3)P	Phosphatidylinositol 3-phosphate
$PI(3,4,5)P_3$	Phosphatidylinositol 3,4,5-trisphosphate
PI4K	Phosphatidylinositol 4-kinase
PI(4)P	Phosphatidylinositol 4-phosphate
$PI(4,5)P_2$	Phosphatidylinositol 4,5-bisphosphate
PI5K	Phosphatidylinositol 5-kinase
PKD	Protein kinase D
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl fluoride
Prdx1	Peroxiredoxin 1
PTEN	Phosphatase and tensin homolog deleted on chromosome ten
PVDF	Polyvinylidene fluoride
Rb	Retinoblastoma
rictor-mTOR	rictor-mammalian target of rapamycin
RIPA buffer	Radioimmunoprecipitation assay buffer
RT	Room temperature
S1P	Sphingosine 1-phosphate
SDS	Sodium dodecyl sulfate
SK	Sphingosine kinase
TMA	Tissue microarray
tRNA	Transfer RNA
wst	Wasted
ZO	Zonula Occluden

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1: GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Breast and Ovarian Cancer

The Canadian Breast Cancer Foundation statistics indicate that breast cancer is the most commonly diagnosed malignancy in Canadian women and rates second for mortality (48). The foundation estimates that approximately 445 Canadian women are diagnosed with breast cancer every week. While widespread screening results in earlier detection of the disease and new treatment options are increasing survival rates, it is estimated that 5 300 Canadian women will die from the disease this year. As such, further research into early detection and more efficacious treatments will lead to increased survival rates.

Breast cancer is a heterogeneous disease with varied morphological appearances, molecular features, behaviour and response to therapy (250). Adjuvant systemic therapy reduces the risk of both recurrence and death from breast cancer, however, it does have associated risks and thus it is beneficial for patients to be optimally selected for these treatments (65). Prognostic factors may select cancer patients most likely to reoccur without adjuvant therapy and benefit from it, while predictive factors identify the appropriate therapy for the individual (65, 162). A prognostic factor is considered to be any measurement available at the time of surgery that correlates with disease-free or overall survival in the absence of systemic adjuvant therapy and a predictive factor is any measurement associated with the response of the therapy (5, 65). When assessing prognosis and determining appropriate treatment, certain factors are assessed in combination to group patients into risk

categories. While the risk categories aid in assessing prognosis in groups of patients, they do not determine the prognosis and risk of the individual patient (261). More recent studies show that gene expression profiling has been increasingly helpful in refining breast cancer classification and assessing the individuals' prognosis and response to therapy (261).

The above statements also apply to ovarian cancer. Ovarian cancer accounts for approximately 4% of female cancers and has the highest fatality-to-case ratio of all gynaecological cancers (147, 200, 286). The high fatality rate is in part due to the failure to detect ovarian cancer at the early stages. Although a number of molecular mechanisms of ovarian cancer have been identified, the heterogeneous nature of ovarian cancer makes it difficult to treat effectively (286). Studies exploring the role of specific genes associated with these malignancies will help to establish improved detection and diagnosis, more personalized treatments, and enhance survival and quality of life of those diagnosed with breast and ovarian cancer.

1.2 Protein Translation Factors and Cancer

In the search for specific genes associated with malignancies, scientists have discovered that many protein translation factors are overexpressed in cancer cell lines and malignant tissues. Intrinsically, cancer cells proliferate at a faster rate and thus require an increase in protein translation. In general, an increase in protein synthesis is required for the cell to double its protein content and size prior to cell division so that an average cell size is maintained (256). However, while normal cells are able to downregulate protein translation and withdraw into quiescence, transformed cells do not markedly decrease protein synthesis

upon growth factor deprivation (256). Translation factors whose overexpression is associated with cancers include: translation initiation factors 2α, 2C2, 4E, 5A2, 4GI and translation elongation factors 1A2 and 1δ (2, 145, 265, 266). Interestingly, the translation elongation factor 1A2 exhibited the most significant alteration in expression, displaying a 10 to 2000 fold overexpression in nine out of ten cancer cell lines analyzed (145).

One of the first and most extensively studied oncogenic translation factors is the eukaryotic initiation factor 4E (eIF-4E). The overexpression of eIF-4E is observed in human tumours of the breast, head and neck, colon, prostate, bladder, cervix and lung (74, 319). While the normal cellular function of eIF-4E is to regulate the recruitment of mRNA to the ribosome, it contributes to malignancy by enabling the translation of proteins involved in cellular growth (c-myc, cyclin D1), angiogenesis (vascular endothelial growth factor), survival (Bcl-2) and invasion (matrix metalloprotease 9) (74, 115). Furthermore, the MAP kinase and PI3 kinase pathways can induce eIF-4E activity (319). In wildtype cells, the translation of most mRNAs is controlled at the rate limiting step of initiation (266).

1.3 Protein Translation

Protein translation is the process by which the mRNA of a gene is converted to its corresponding protein. Protein synthesis is conceptually divided into three stages: initiation, elongation and termination. The initiation stage involves recognition of the start codon and assembly of the large and small ribosomal subunits into a complete and fully functional ribosome (4, 162). The binding of the mRNA to the ribosome requires several initiation factors. Initially, the 43S ribosome pre-initiation complex must form, which consists of the

40S small ribosomal subunit, the initiating methionyl tRNA and a group of eukaryotic initiation factors (eIFs), including eIF2 and eIF3 (234, 266). The 43S pre-initiation complex then binds to the mRNA which, in the case of cap-dependent translation, occurs by a mechanism that involves the recognition of the m^7G cap at the 5'-terminus of the mRNA by the subunit of eIF4F, eIF4E, which is a cap binding subunit of the initiation factor (193, 234). The eIF4F cap-initiation complex is comprised of eIF4E, the cap binding subunit, as well as eIF4G, a scaffolding protein for translational machinery assembly and eIF4A, a helicase which unwinds the mRNA (2, 74, 266). Furthermore, the eIF4F complex interacts with the polyA tail binding protein at the 3' polyadenylated tail of the mRNA (266). The mRNA-bound ribosomal complex then moves along the 5' untranslated region from where it initially bound until it recognizes an initiation codon which base pairs to the initiator tRNA to form the 48S initiation complex (234). The displacement factors on the 48S complex and the amalgamation of the 60S subunit forms the fully functional 80S ribosome with the initiator tRNA in the P site of the ribosome (234). Ribosomes have three tRNA binding sites, the aminoacyl site (A site), the peptidyl site (P site) and the exit site (E site) (146).

Translation elongation is the process where additional amino acids are added to the growing polypeptide chain. This stage involves the expenditure of metabolic energy due to the formation of multiple bonds, in particular the amino acyl-tRNA to mRNA bond and the translocation of the amino acid to the growing chain (42). When the eukaryotic elongation factor (eEF) 1A is in a high energy GTP-bound state, it is responsible for the recruitment of amino-acylated tRNAs to the A site of the ribosome and eEF1B promotes the generation of eEF1A-GTP complexes (2). The translocation of the peptidyl-tRNA from the A site to the P site of the ribosome and the movement along the mRNA is mediated through eEF2 which

also requires GTP (2). Following the formation of a peptide bond, the ribosome has a deacylated tRNA in the P site and an aminoacylated tRNA in the A site (146). Vacating the A site, so the binding of the subsequent aminoacylated tRNA may occur, is proposed to occur in two steps (146). The first step occurs following the peptidyl transferase reaction when the acceptor ends of the two tRNAs (one deacylated and the other aminoacylated) move relative to the large subunit from the A and P site to the E and P sites respectively, while the anticodons remain bound to the small subunit (146). The second step involves the anticodon arms of the two tRNAs to move relative to the small subunit (146). Once the tRNAs have translocated, the deacylated tRNA moves to the E site where it dissociates from the ribosome (146).

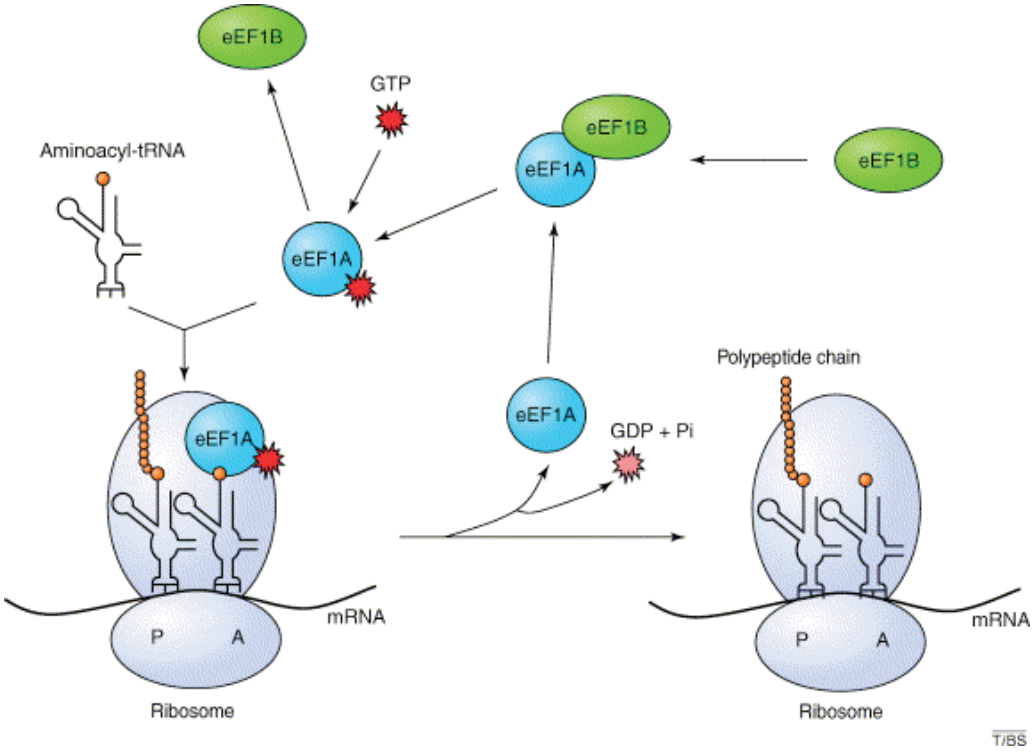
The termination step occurs when a stop codon enters the A site of the ribosome. At this point, termination factors cause a dissociation of the translation machinery and the newly synthesized protein is released (60). The termination step is mediated through a eukaryotic release factor (eRF) 1, which is also dependent on GTP, and the binding of eRF1 stimulates the cleavage of the bond between the tRNA and the peptide chain, releasing the newly synthesized peptide (2).

1.4 Eukaryotic elongation factor 1A in protein translation

Eukaryotic elongation factor 1 alpha (eEF1A) is the second most abundant eukaryotic protein after actin, and comprises 1-2% of total cellular protein (69, 96). As described above, during elongation, eEF1A binds to an amino-acylated tRNA and recruits it to the A site of the 80S ribosome (Figure 1.1) (206, 269). It has been shown that eEF1A can

Figure 1.1: The role of eEF1A in protein translation elongation.

Eukaryotic Elongation Factor 1A (eEF1A), in a high energy GTP-bound state, binds its cognate amino-acylated tRNA and recruits it to the A site of the 80S ribosome. When appropriate codon-anticodon recognition occurs, GTP is hydrolyzed and eEF1A-GDP is released from the ribosome. eEF1B then interacts with eEF1A to promote the exchange of GDP for GTP to regenerate the active eEF1A-GTP. Adapted from (2).


eEF1B
GTP
eEF1B
eEF1A
eEF1B
Aminoacyl-tRNA
eEF1A
Polypeptide chain
eEF1A
GDP + Pi
eEF1A
mRNA
P
A
Ribosome
mRNA
P
A
Ribosome
TiBS

form complexes with free tRNA and it is hypothesized that eEF1A and tRNA initially form a complex that is subsequently amino-acylated (96). The addition of the amino acid to the growing polypeptide chain via eEF1A is a high energy process requiring GTP to bind the eEF1A protein (231). Upon recognition of the cognate codon-anticodon, GTP is hydrolyzed and the eEF1A-GDP complex is released (28). When bound to GDP, eEF1A has been shown to interact with deacylated tRNA and can transport it to aminoacyl tRNA synthetase for recycling (231, 235). The GTP dependent activity of eEF1A is assisted by the eukaryotic elongation factor 1B, which promotes the regeneration of the high energy eEF1A-GTP complex (2).

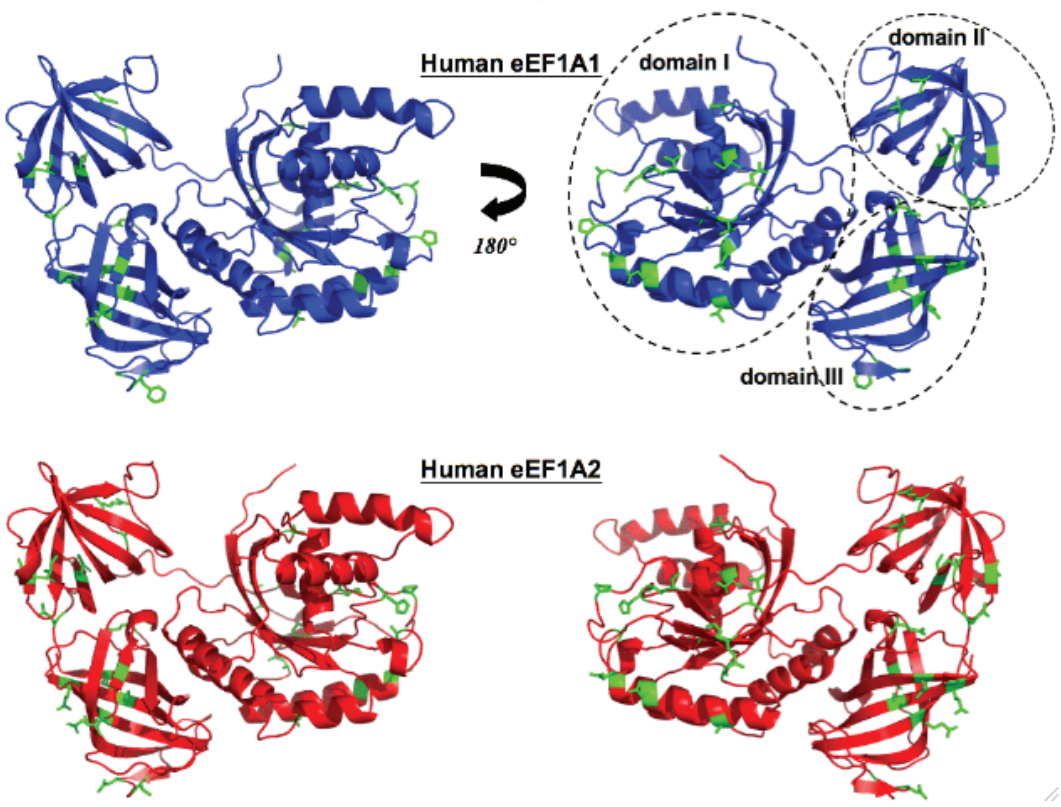
1.5 Two mammalian forms of eEF1A

eEF1A is encoded by a small multigene family. It has been reported that there are at least two isoforms in humans, four in rice, five in fish and more than ten in maize (96, 138). All isoforms from the various species are highly similar and conserved between organisms. While genes within a multigene family may share a related function, the individual genes within the family may also have distinct functions and/or expression patterns (247).

As previously mentioned, there are two mammalian isoforms of eEF1A, eEF1A1 and eEF1A2. The two isoforms share more than 90% identity at the amino acid level and are believed to have essentially the same function in protein translation (Figure 1.2) (6, 158). However, the two mammalian isoforms vary primarily in their mutually exclusive expression pattern. eEF1A1 is expressed ubiquitously except for brain, heart and skeletal muscle where eEF1A2 is normally expressed (144, 240, 282). The two isoforms map to

Figure 1.2: The two isoforms of eEF1A.

The three-dimensional models for eEF1A1 (blue, top panel) and eEF1A2 (red, lower panel). For each protein, two views, rotated by 180° around the y-axis is displayed. The differences between the two isoforms side-chains are shown in stick representation in green. Adapted from (269).


Human eEF1A1
domain I
domain II
domain III
180°
Human eEF1A2

different chromosomes, eEF1A1 maps to 6q14 and eEF1A2 maps to 20q13.3 (195, 282). Interestingly, the only other tissue specific translation factor found in humans is eEF1Bα which has a specific isoform for brain and muscle as well (241).

The two isoforms may exhibit similar translational activities but differ in their affinity of GTP and GDP. eEF1A1 binds to GTP more strongly than GDP, while the inverse is true for eEF1A2, the GDP dissociate rate constant is approximately seven times higher for eEF1A1 than for eEF1A2 (155, 269). As there have been many reported modifications of eEF1A, including methylation, acetylation, phosphorylation and myristoylation there are likely many novel functions of eEF1A yet to be discovered. While the best characterized function of eEF1A is protein translation, it has been associated with other functions as well (206).

There has been evidence that indicates that eEF1A interacts with the cytoskeleton. eEF1A was first isolated as an actin binding protein in *Dictostelium discodieum* and was given the moniker actin binding protein 50 (ABP-50) in part due to its molecular weight (85). Co-precipitation assays in whole cell extracts indicate that the two precipitate in a 1:2 molar ratio of eEF1A:actin (219). This interaction is not dependent on GTP and the ability for eEF1A to interact with actin efficiently requires the absence of amino-acylated tRNAs, indicating that the binding of these two elements to eEF1A is mutually exclusive (94, 190). It is hypothesized that through the interaction with the cytoskeleton, eEF1A binds and bundles actin and stabilizes microtubules (219, 269).

Additionally, eEF1A has been shown to play a role in apoptosis. Apoptosis is a term for a distinct type of programmed cell death to remove damaged or unnecessary cells (97). There have been contradictory reports on the effect of eEF1A on apoptosis. Initially, it was

reported that high levels of eEF1A was associated with increased cell death (93), while later studies then demonstrated that eEF1A was associated with survival (279). Originally, the discrepancy in how eEF1A was contributing to apoptosis was hypothesized to be due to the presence of the two different isoforms. However, more recent studies have shown that eEF1A2 has different effects on apoptosis depending on the tissue type it is expressed in as well (246, 257).

Several other interesting non-canonical roles have been elucidated for eEF1A. For example, eEF1A, a primarily cytoplasmic protein, has been associated with the nuclear export of amino-acylated tRNA (121). Since this discovery, eEF1A was also shown to participate in the nuclear export of transcription-dependent proteins and recently detected in the nucleus of yeast (152, 206). Furthermore, eEF1A has been found to be anchored to the cytoplasmic side of the endoplasmic reticulum by what appears to be an ethanolamine bridge between an aspartate residue and phosphoinositol (128). Additionally, eEF1A has been implicated in many human diseases including: Adult Atopic Dermatitis (AAD), diabetes, and cancer (96). Patients suffering from AAD showed elevated levels of an anti-eEF1A autoantibody (227). Furthermore, type 1 and 2 diabetes patients showed a decrease in protein synthesis and unbalanced expression of eEF1A mRNA, which was two to six fold overexpressed, has been observed in the skeletal muscle of both types of diabetes (95). While these new roles for eEF1A present novel links between protein translation and other cellular processes, supplementary research is required to fully understand the mechanism by which this is occurring.

1.6 eEF1A2

The focus of my project is on the eEF1A2 isoform. As previously mentioned, eEF1A2 is normally expressed in the brain, heart and skeletal muscle (1, 8). In these tissues, eEF1A2 expression is upregulated during terminal differentiation, and thus, this is the isoform observed in post-mitotic myotubules, cardiomyotubules and neurons (28, 177, 178). Originally, these tissues express the eEF1A1 isoform, downregulation of eEF1A1 occurs as eEF1A2 is upregulated to participate in protein translation (28). The variable tissue expression of eEF1A2 raised the possibility that it has an important role in regulating the differentiation and function of the tissue.

The spontaneous deletion of eEF1A2 in mice resulted in a phenotype termed wasted (*wst*) mice (55, 197). The homozygous *wst/wst* mouse appear completely normal until the weaning age, at approximately 21 days, when at this point the mice develop tremors and ataxia (55, 197). These mice have neurological defects, including weak synaptic transmission, show muscle wasting, have immune system abnormalities, which includes a defective response to DNA damage in lymphoid cells, become paralyzed and this mutation is lethal by day 28 (1, 55, 153). In the *wst* mutant tissues, the decrease in eEF1A1 expression occurs although the eEF1A2 isoform is not expressed. Normally, in the wildtype mice eEF1A2 is the only isoform present in the tissues by day 14 (153). These findings suggest that eEF1A2 in the brain, heart and skeletal muscle is the adult form of the elongation factor, while eEF1A1 is the embryonic isoform (153). Interestingly, when muscles have been subjected to denervation or toxic injury they express eEF1A1, reverting back to eEF1A2 expression only upon recovery (53, 154). The onset of the wasted phenotype correlates with the decline of eEF1A1 expression, indicating that the deletion of eEF1A2 results in the loss

of the ability to synthesize protein and that the lack of protein synthesis is responsible for the numerous defects seen in these mice.

1.7 eEF1A2 and Ovarian Cancer

The human eEF1A2 gene, *EEF1A2*, maps to 20q13 and an increase in copy number at that locus occurs in several tumour types, implicating genes at that locus in cancer (70, 89, 171, 270). As 20-30% of ovarian tumours showed this amplification, Anand et al. attempted to characterize the genes found at this locus which are relevant to ovarian cancer (8). Using fluorescent in situ hybridization (FISH) they found that 25% of primary ovarian tumours had eEF1A2 amplifications (8). It was then discovered that eEF1A2 also had transforming properties. Rodent fibroblast cells ectopically expressing eEF1A2 were able to form colonies in soft agar and proliferated at an accelerated rate (8). Additionally, eEF1A2 expressing cells were able to form foci, a marker of cell transformation, and when injected subcutaneously into nude mice was sufficient to induce *in vivo* tumourigenicity (8). This indicates that while eEF1A2 is traditionally viewed as an translation factor, it displays the transforming properties associated with oncogenes.

Further work by Tomlinson and colleagues tried to establish the mechanism by which eEF1A2 is being abnormally expressed in ovarian tissues (286, 287). They determined that the increased copy number at the eEF1A2 locus does not correlate with expression levels, as several instances where there was a normal copy number had high expression, and that no functional mutations were found (286). Additionally, they found that the gene is unmethylated in normal and tumour DNA, indicating that overexpression is

likely not due to genetic or epigenetic modifications (286). The overexpression of eEF1A2 in ovarian cancer and its transformational activity indicates that eEF1A2 has a causal role in ovarian tumour development.

While there is a great deal unknown as to how eEF1A2 is being expressed in the ovarian tissue and how the elongation factor is leading to oncogenesis, there have been positive findings as well. Recently resveratrol, a phytoalexin present in grapes and recently reported to possess chemopreventative and chemotherapeutic activities, has been shown to be a potential inhibitor of the oncogenic properties of eEF1A2 (175). Lee and colleagues found that pre-treatment of cells with resveratrol inhibited the cells ability to form colonies in soft agar. Moreover, administration of resveratrol via intraperitoneal injection retarded the growth of ovarian cancer cells in nude mice (175). These findings indicate that while the mechanism by which eEF1A2 is leading to oncogenesis remains mostly unknown, it may be a potential target for the anti-proliferative effects of resveratrol.

1.8 eEF1A2 and Breast Cancer

The amplification of chromosome 20q13.3 is not only observed in ovarian tumours but breast tumours as well (176). When the genes localizing to this locus were analyzed in breast tumours, eEF1A2 displayed the highest alteration of expression in mammary cancer cell lines (145, 176). Initial studies by Abbott et al. on a small subset of breast tumours demonstrated that two-thirds of breast tumours expressed eEF1A2 at moderate to strong levels (287). They also found that over-expression was associated with estrogen receptor positivity (287). When a larger scale study was performed a similar proportion of breast

tumours was found to express eEF1A2 (162). However, tumours with high eEF1A2 expression were found to be independent of HER-2 protein expression, tumour size, lymph node status and estrogen receptor expression (162). Moreover, it was found that eEF1A2 expression in breast tumours correlated with an increased 20-year survival probability (162).

Further studies into eEF1A2's role in breast cancer revealed that eEF1A2 is an activator of Akt, which plays a pivotal role in cell survival and proliferation (6, 22). Expression of eEF1A2 induced cytoskeletal rearrangements, as seen through increased filopodia production, and enhanced cell migration and invasion in an Akt and phosphatidylinositol 3-kinase (PI3K) dependent fashion indicating an important role for eEF1A2 in actin remodelling and cell motility (6).

Most research has focused on the role of eEF1A2 in breast and ovarian cancer. However, eEF1A2 is now being reported to have roles in pancreatic, hepatocellular, colon and lung carcinomas as well as multiple myeloma (51, 117, 145, 185, 186). Optimistically, with eEF1A2 now being associated with several other types of cancers, more on the mechanism of oncogenesis can be elucidated.

1.9 eEF1A2 and binding partners

As research on eEF1A2 non-canonical functions within the cells continues, further interesting roles and binding partners are being elucidated for the elongation factor that may provide insight into the mechanism by which it is leading to tumourigenesis. As tyrosine kinases are prominent players in cancer development, a comparison of eEF1A1 and eEF1A2 to be involved in the phoshpho-tyrosine specific signalling process was performed (231). It

was shown that eEF1A2, and not eEF1A1, had the ability to interact with the SH2 domains of Grb2, RasGAP, Shc and Shp2 as well as the SH3 domains of Crk, Fgr, Fyn and PLC-γ1, indicating that eEF1A2 has a greater potential to participate in phospho-tyrosine mediated signalling pathways (231).

In mice, eEF1A2 was shown to co-immunoprecipitate with peroxiredoxin 1 (Prdx1) (59). When eEF1A2 and Prdx1 were double transfected into cell lines, it conferred a higher resistance to peroxide-induced cell death and this protection correlated with a reduced activation of apoptotic factors caspase 3 and 8 as well as an increase in expression of the pro-survival factor Akt (59).

Additionally, it was shown that eEF1A2 directly associates with and increases the catalytic activity of sphingosine kinase 1 (SK1) (168, 169). SK1 catalyzes the generation of sphingosine 1-phosphate (S1P), an active phospholipid involved in calcium mobilization, cell proliferation, apoptosis and cytoskeletal rearrangement (123, 168). Traditionally, SK1 has been involved in determining the balance between pro-apoptotic, pro-proliferative and pro-survival of different signalling molecules, there is now evidence that SK1 plays a role in tumourigenesis and elevated expression is seen in solid tumours (168).

With particular relevance to my project, it has been shown that eEF1A2 binds to and activates the lipid kinase phosphatidylinositol 4-kinase IIIβ (PI4KIIIβ) (142). The two proteins interact with a 1:1 stoichiometry and eEF1A2 doubles the enzymes maximum velocity (142). When cells are transfected with eEF1A2, it increases the activity of PI4KIIIβ and leads to an increase in overall phosphatidylinositol 4-phosphate abundance (142). Furthermore, eEF1A2-dependent $PI(4,5)P_2$ generation of filopodia requires PI4KIIIβ (144).

As such, the interaction between eEF1A2 and PI4KIIIβ is likely to be an important facet of eEF1A2 function.

1.10 Phosphatidylinositol-4 Kinase

Polyphosphoinositides are involved in many signal transduction pathways and the first committed step is catalyzed by phosphatidylinositol 4-kinase (PI4K) (108). The PI4K enzyme catalyzes the phosphorylation of phosphoinositol (PI) at the D-4 position of the inositol ring to produce phosphatidylinositol 4-phosphate (PI(4)P) (Figure 1.3) (313). There are four known mammalian PI4K isoforms, PI4KIIα, PI4KIIβ, PI4KIIIα, and PI4KIIIβ that have been isolated (Figure 1.4). The isoforms have similar C-terminal catalytic domains yet have diverse N-terminal regulatory domains (129). The four isoforms of PI4K are categorized into two types: II and III, which are distinguished from each other based on their sensitivity to inhibitors (75, 129). While type II PI4Ks are inhibited by adenosine, type III is inhibited by wortmannin, and both are activated by non-ionic detergents (75). Each PI4K type also has two independent isoforms, alpha and beta. The majority of PI4K activity in human cells is membrane bound and activity is detected in most membrane structures (316). The four different isoforms of PI4K localize to distinct membrane compartments and have specific roles within the cell, however little is known about how their specific protein localization contributes to the PI function (Figure 1.5) (15, 307).

Type II PI4K enzymes are poorly characterized, both because this type was only recently cloned, as well as there is only one type II isoform in yeast (15). The type II PI4Ks are tightly bound to membranes due to palmitoylation on conserved cysteine residues (24).

Figure 1.3: The role of Phosphatidylinositol 4-Kinase.

Phosphatidylinositol 4-kinase (PI4K) enzyme catalyzes the phosphorylation of phosphoinositol (PI) at the D-4 position of the inositol ring to produce phosphatidylinositol 4-phosphate (PI(4)P). Adapted from (159).

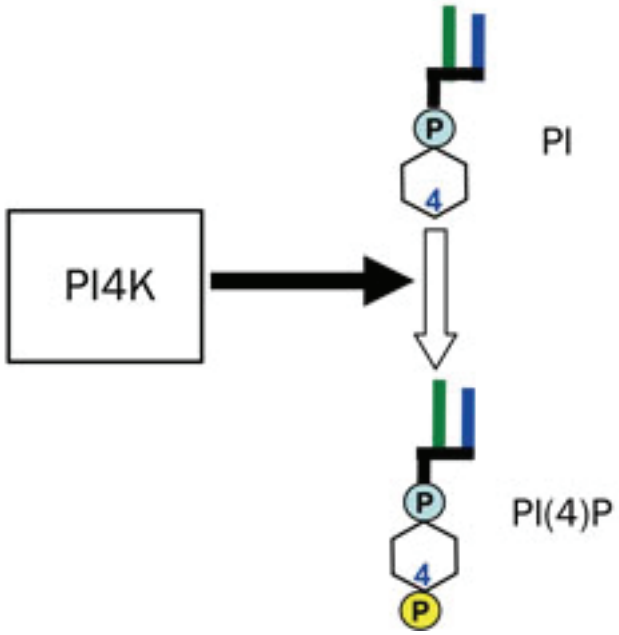

PI4K
P
4
PI
P
4
P
PI(4)P

Figure 1.4: The domain structure of the PI4K isofoms.

The domains of the four different mammalian isoforms of PI4K are illustrated. The four isoforms are classified in to two classes (II and III) and each class has two isoforms: PI4KIIIα, PI4KIIIβ, PI4KIIα and PI4KIIβ. The isoforms have the same function but differ in their localization, amino acid and protein domains. Adapted from (129).

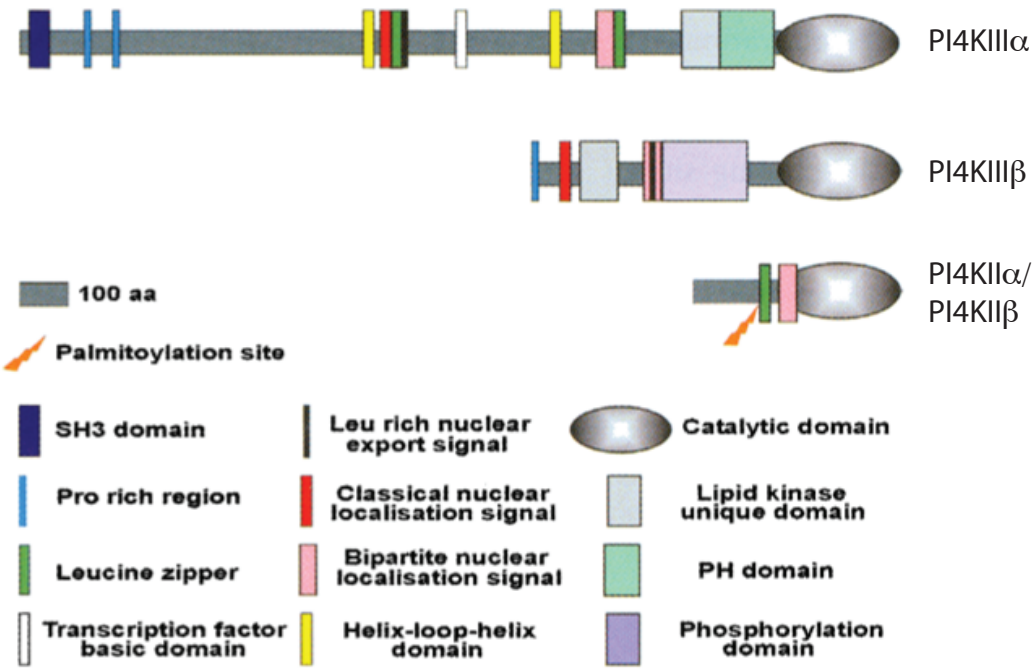
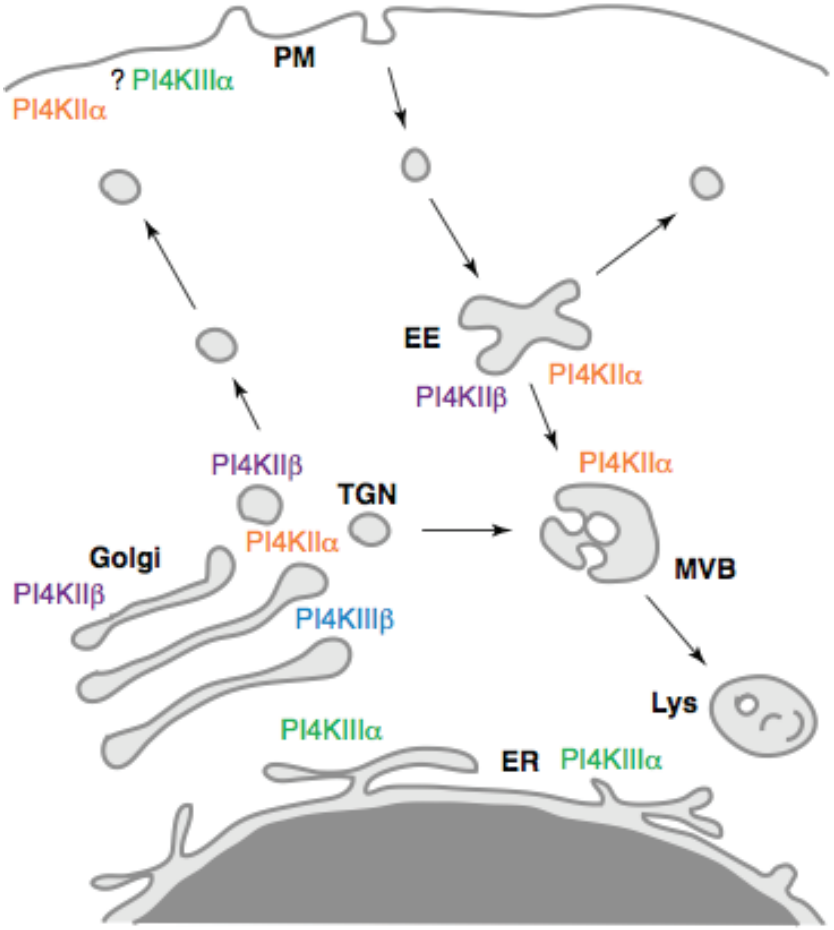

PI4KIIIα
PI4KIIIβ
PI4KIIα/
PI4KIIβ
100 aa
Palmitoylation site
SH3 domain
Leu rich nuclear export signal
Catalytic domain
Pro rich region
Classical nuclear localisation signal
Lipid kinase unique domain
Leucine zipper
Bipartite nuclear localisation signal
PH domain
Transcription factor basic domain
Helix-loop-helix domain
Phosphorylation domain

Figure 1.5: Cellular localization of PI4K enzymes.

The localization of the four different isoforms of PI4K in the cell and major routes of vesicular transport. Abbreviations: EE: early endosome; ER: endoplasmic reticulum, Lys: lysosome; MVB: multi-vessicular body; PM: plasma membrane; TGN: trans-Golgi network. Adapted from (15).


PM
? PI4KIIIα
PI4KIIα
EE
PI4KIIα
PI4KIIβ
PI4KIIβ
PI4KIIα
TGN
PI4KIIα
Golgi
PI4KIIβ
MVB
PI4KIIIβ
Lys
PI4KIIIα
ER
PI4KIIIα

Early studies of the type II enzymes revealed activity at the plasma membrane and the enzyme was shown to associate with epidermal growth factor receptors, which led to the assumption that this type of PI4K was responsible for generating membrane pools of phosphoinositol 4,5 bis-phosphate ($PI(4,5)P_2$) (15, 238). More recently type II PI4Ks have been shown to localize to intracellular membranes, which include the Golgi and endosomes (16, 305). Functionally, the type II kinases have been implicated in intracellular trafficking events and possibly have a role in transferrin receptor endocytosis and recycling (15, 16). While the mechanism of regulation of these kinases is poorly understood, calcium is known to inhibit, and association with a membrane increases activity (309). Importantly, type II PI4Ks are responsible for generating 50% of the total $PI(4,5,)P_2$ pools in the cell (297).

There is more information available as to the differences in alpha and beta isoforms of the type III PI4Ks. PI4KIIIα is mainly localized to the endoplasmic reticulum (ER) in mammalian cells but is also detected in the pericentriolar area over the Golgi and in the nucleolus (15, 221, 316). Also, PI4KIIIα shows the highest expression in the nervous systems and examination of localization in these areas showed that within the cytoplasm there are unique membrane clusters associated with organelles such as the mitochondria and ER (17, 109, 221). Although there is much localization data of PI4KIIIα, there remains much to be elucidated as to what the role of PI4KIIIα is, especially at the ER. The yeast orthologue of PI4KIIIα, Stt4p, is an essential gene in most yeast strains (14, 318). Stt4p is important for maintaining cell wall integrity and organization of the actin cytoskeleton and temperature sensitive mutants display fused and collapsed large vacuoles (14). Unlike its mammalian counterpart, the yeast orthologue is localized to the plasma membrane, but how it is targeted to the membrane is still not understood (13).

1.11 PI4KIIIβ

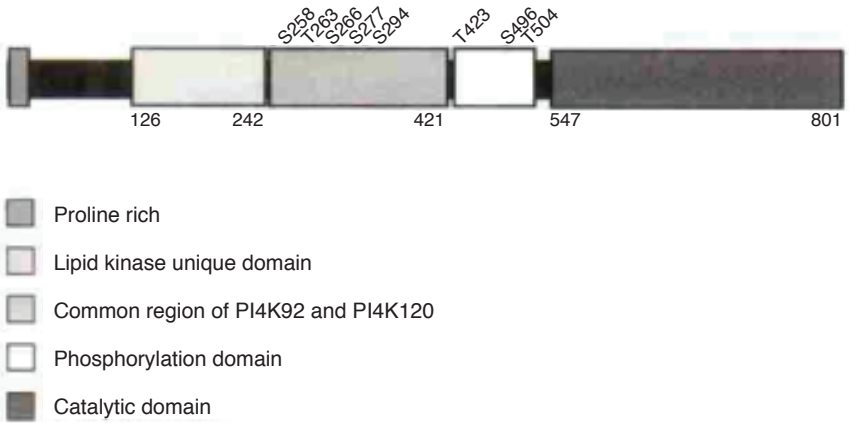
In mammals, PI4KIIIβ is reported to primarily localize to the Golgi, but has also been found to localize to the nucleus, outer membrane of the mitochondria and the membranes of the rough ER (75, 113, 276, 316). Recruitment of PI4KIIIβ to the Golgi is thought to be mediated via the small GTP-binding ADP Ribosylation factor (Arf) 1 (113). PI4KIIIβ is also thought to be recruited to the Golgi through phosphorylation at Ser258 and Ser266 (129, 276). These phosphorylation sites were shown to affect PI4KIIIβ recruitment to the Golgi following brefeldin A treatment (Figure 1.6) (129, 276). In many cell lines, PI4KIIIβ has been shown to regulate Golgi to plasma membrane trafficking and kinase-inactive forms of PI4KIIIβ inhibits this trafficking (43, 112). PI4KIIIβ is phosphorylated by protein kinase D (PKD)-1 and -2 at a motif which is then recognized by 14-3-3 proteins, which stabilizes the active form of PI4KIIIβ (127, 207). Due to PKD's role in regulating vesicle fission from the Golgi, PI4KIIIβ is hypothesized to contribute to this mechanism (207).

While PI4Ks in general are associated with secretory granules and synaptic vesicles, it is unknown which isoforms are present in these structures. However, some research has shown that PI4KIIIβ is important for exocytosis (15). Neuronal calcium sensor 1 (NCS-1) has been shown to increase glucose-induced secretion in the vesicular pool in pancreatic β cells, which requires PI4KIIIβ (122). The PI4KIIIβ enzyme interacts with NSC-1, although it is not known if this interaction contributes to the recruitment of PI4KIIIβ to the Golgi (130).

The yeast orthologue of PI4KIIIβ is *PIK1* and it is an essential for viability and growth (14, 106). Temperature sensitive *pik1* mutants may provide additional insight on the

Figure 1.6: Position of phosphorylated residues on PI4KIIIβ.

The functional domains of PI4KIIIβ are displayed in the figure. The phosphorylation domain is made of 81 amino acids and contains three of the eight determined phosphorylation sites. Recruitment of PI4KIIIβ to the Golgi is affected by phosphorylation of Ser258 and Ser266. Adapted from (276).


S258
T263
S266
S277
S294
T423
S496
T504
126
242
421
547
801
Proline rich
Lipid kinase unique domain
Common region of PI4K92 and PI4K120
Phosphorylation domain
Catalytic domain

localization and function of PI4KIIIβ. The *pik1* temperature sensitive mutants have exaggerated Golgi membranes, show fragmentation of vacuoles, display a defect of polarization of actin at the budding pole of cells, and display multi-nucleated cells indicating a defect in cytokinesis (15, 304). Interestingly, Pik1 also localizes to the Golgi and nucleus and is a regulator of vesicular trafficking in the late secretory pathway in yeast (14, 274, 304). Additionally, *Drosophila* lacking a PI4KIIIβ gene, *four wheel drive*, results in male infertility due to defects in cytokinesis (41).

Functionally, PI4KIIIβ catalyzes the phosphorylation of the D4 position of the inositol ring which is the initiating reaction in the phosphoinositide signalling pathway (129). The activity of this kinase is activated by phosphorylation by protein kinase D (PDK) and PDK is targeted to the Golgi when bound to Arf1 (127, 248). Studies have implicated PI4KIIIβ in the regulation of Golgi disintegration and reintegration during mitosis (113). PI4KIIIβ is a protein that resides in the cytoplasm that is recruited to the Golgi by Arf1 where it is implicated in transporting newly synthesized proteins from the Golgi to the plasma membrane (313).

In addition to localization at the Golgi, PI4KIIIβ has also been reported to shuttle through the nucleus, however, the function of the kinase at this location remains to be elucidated (75, 274, 304). The yeast version of PI4KIIIβ, Pik1, which has been engineered to be expressed solely in the nucleus or cytoplasm does not support cell viability, indicating essential functions for the enzyme in both locations (116). Furthermore, studies in yeast indicate that nuclear accumulation of Pik1 occurs with nutrient deprivation which also causes the release of the kinases effectors from the Golgi (116).

1.12 Phosphoinositides

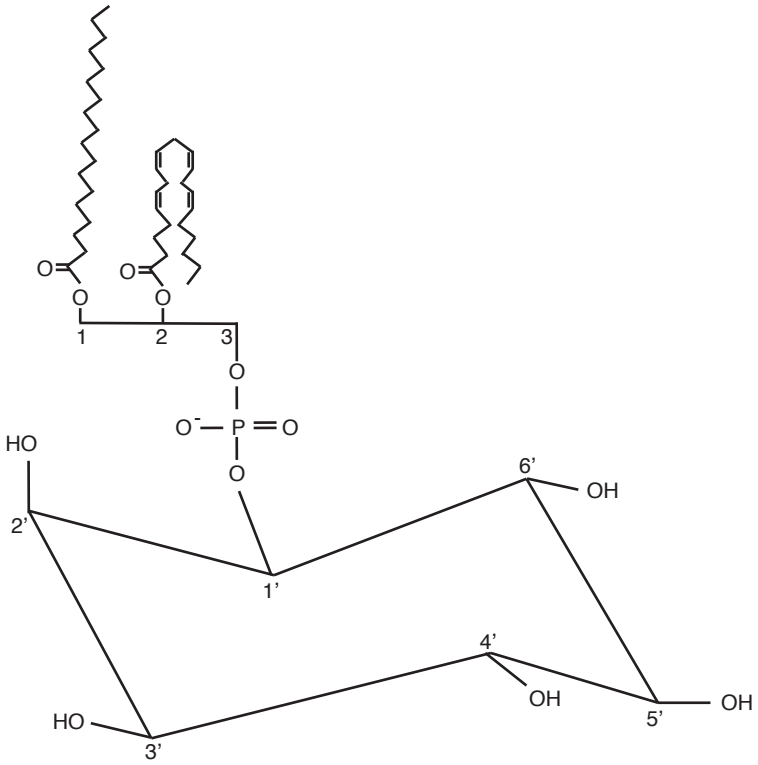
Phosphatidylinositol and its phosphorylated derivatives are classified as glycerophospholipids. Their structure consists of glycerol backbone, two non-polar fatty acid tails connected to an inositol head group (hexahyroxycyclohexane) by a phosphodiester bond (Figure 1.7) (212).

Phosphorylated derivatives of phosphatidylinositols regulate many cell processes by serving as signalling molecules that deliver spatial and temporal information to downstream effectors (116, 160). Phosphoinositide (PI) species are used in signalling in two different manners. First of all, stimulation of signalling results in rapid and localized production of PIs that are utilized as second messengers (116). The second means of PI signalling is through localizing specific PIs to a particular organelle, enriching the area with a certain PI where they constitute a defining feature (116). The advantage of this signalling method is that cellular signalling and trafficking events are activated independently and are under tight spatial and temporal control (313). Inositol phospholipids act as messengers in the signal transduction which is initiated through stimulation of cell surface receptors (221). The synthesis, degradation and re-synthesis constitute the inositol phospholipid cycle (221).

Phosphorylations at the D3, D4 and D5 position of the inositol ring of the phospholipid produce lipophilic effectors and can potentially anchor signalling complexes to membranes (164, 276). Conversely, splitting of the head group by phospholipase C generates diacylglycerol and polyphosphorylated inositol derivatives that are soluble cytosolic messengers (276). In addition to kinases, phosphatases are also important for the distribution of specific lipids (25).

Figure 1.7: The structure of phosphatidylinositol.

Phosphatidylinositol is classified as a glycerophospholipid consisting of a glycerol backbone, two non-polar fatty acid tails connected to an inositol head group by a phosphodiester bond. Adapted from (156).


O
O
O
O
1
2
3
O
O⁻
P
O
O
HO
2'
6'
OH
1'
4'
OH
HO
3'
5'
OH

The ability to reversibly phosphorylate the inositol ring of phosphoinositide at the 3, 4 or 5 positions either once, twice or three times leads to the formation of seven different phosphoinositides (Figure 1.8) (230). Each of these PIs have a unique stereochemistry, bind cytosolic and membrane proteins with different affinities and specificities, and elicits a unique biological response (71, 230). Phosphorylation and dephosphorylation by lipid kinases and phosphatases can rapidly interconvert the phosphoinositide species and result in their dynamic production (45). This also results in short-term alterations of PIs and their binding protein properties and functions of the membrane domain (161). While the PI pathway is a complex signalling network (Figure 1.9A), the focus will remain on a commonly used version of the pathway (Figure 1.9B). Interestingly, there is a strong relationship between PIs, some must exist before others can be generated, as well as degradation of one can generate another (25). This means that a single lipid species have multiple synthetic routes.

Imaging PIs has previously been frustrating with antibodies as the fixation techniques, accessibility of the antibody to the lipid, and sensitivity and variability in antibody lots have been a difficulty (20, 297). Recently, the ability of the phosphorylated head group of PIs, which bind to various protein modules or unique protein surfaces with variable affinity and specificity, has been exploited (45). Visualization of PIs has been achieved through the use of protein domains that naturally recognize PIs and when they are fused to GFP can be used to visualize lipids in cells (20, 21, 297). In most cases, binding occurs through electrostatic interactions between the negatively charged headgroups and the positive charges of the target protein (161). The PI binding site can be found in unstructured regions with clusters of basic residues or within folded domains that recognize distinct

Figure 1.8: Phosphoinositol and the seven distinct phosphoinositide species.

Phosphoinositol is reversibly phosphorylated on the inositol ring at the 3, 4, or 5 positions either once, twice or three times leading to the formation of seven distinct phosphoinositides. Phosphorylation and dephosphorylation by lipid kinases and phosphatases can rapidly interconvert phosphoinositide species. Adapted from (167).

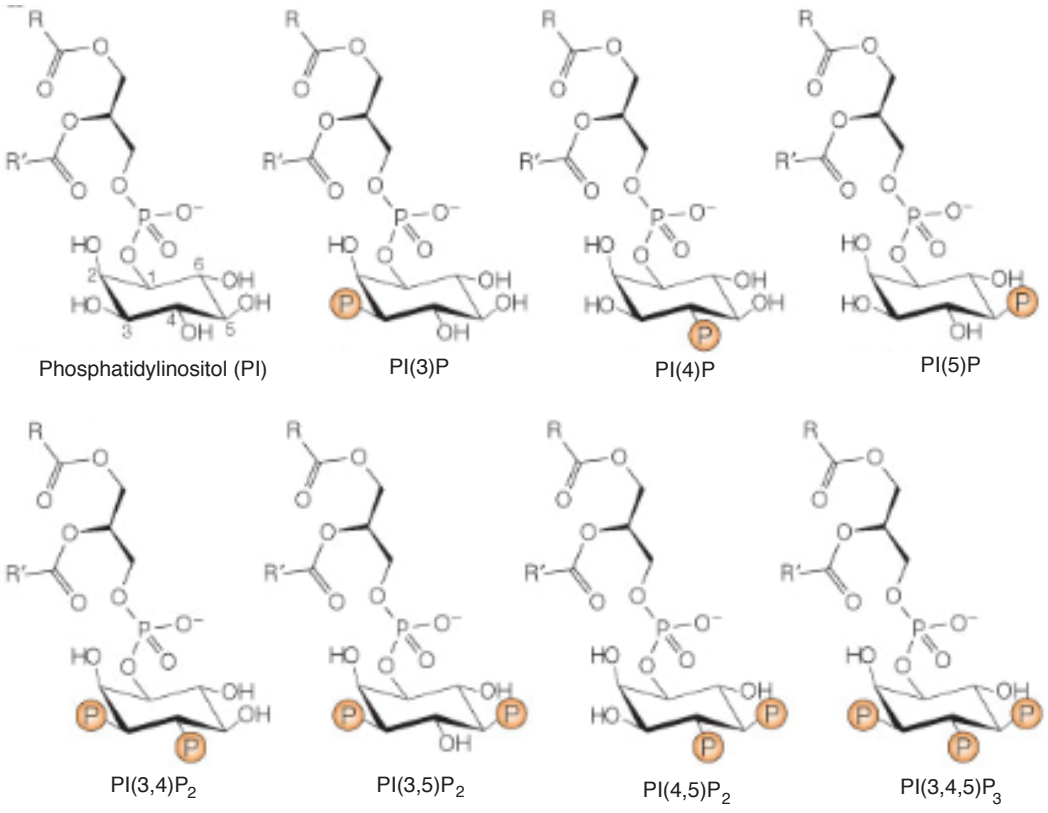
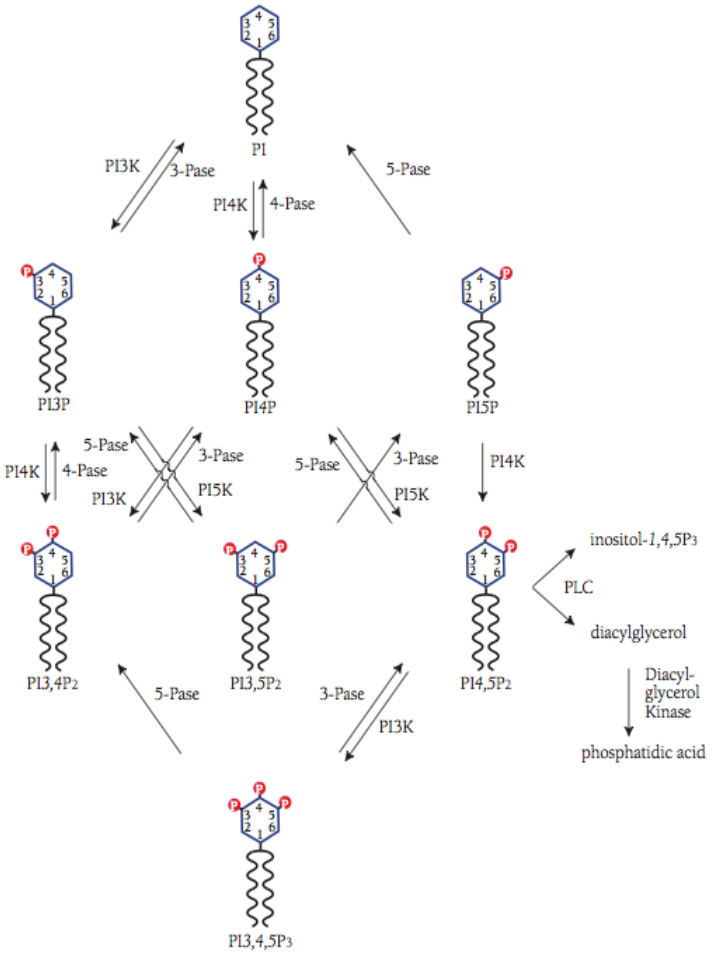

Phosphatidylinositol (PI)
PI(3)P
PI(4)P
PI(5)P
PI(3,4)P2
PI(3,5)P2
PI(4,5)P2
PI(3,4,5)P3

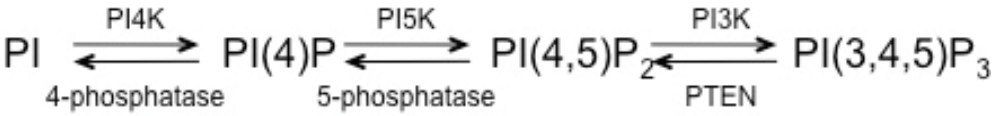
Figure 1.9: The phosphoinositide signalling pathway.

The phosphoinositide signalling pathway. **A.** The PI signalling pathway is a complex network with a strong relationship between PIs, some must exist before others can be generated, as well as degradation of one can generate another, indicating that one lipid may be generated through different routes. **B.** A simplified version of the pathways commonly used within the cell.

A


PI
PI3K
3-Pase
PI4K
4-Pase
5-Pase
PI3P
PI4P
PI5P
5-Pase
3-Pase
PI4K
4-Pase
PI3K
PI5K
5-Pase
3-Pase
PI5K
PI4K
PI3,4P2
PI3,5P2
PI4,5P2
inositol-1,4,5P3
PLC
diacylglycerol
Diacyl-
glycerol
Kinase
phosphatidic acid
5-Pase
3-Pase
PI3K
PI3,4,5P3

B


PI
PI4K
PI(4)P
PI5K
PI(4,5)P2
PI3K
PI(3,4,5)P3
4-phosphatase
5-phosphatase
PTEN

arrangements of binding grooves or surface exposed fingers (18, 54, 161). Many structurally different PI binding domains have been identified and these display a marked difference in specificity and affinity, while some exclusively bind one type of PI exclusively, others will recognize more or less closely related PI species (Figure 1.10) (18, 161). The PI-binding modules include: FYVE, PHOX homology, pleckstrin homology (PH), ENTH and ANTH domains (79). Sequence homology of the most commonly used PI-binding modules, PH domain family members, is fairly low, but there appears to be a tertiary structure of 120 amino acids that is highly conserved (161). This method of imaging PIs does have limitations, as the observed pattern of GFP-tagged PI-binding modules only reflect the free available PIs and not those already engaged in interactions (79). Moreover, long term expression of reporter constructs is toxic to the cells.

PIs are not only associated with signalling in the cell, they are also associated with organelle identity (Figure 1.11) (160). Proteins and lipids move within the cell using transport vesicles and in order to perform this accurately it requires organelles to be correctly recognized (25). Organelles are identified by their activated GTPases and the lipid species they display, which provides the organelles with a unique and flexible identity (25). For example, the inner leaflet of the plasma membrane shows an enrichment of PI(4,5)P_2, while PI(3)P and PI(3,5)P_2 confer identity to endosomal membranes (160). Furthermore, while PI(4)P is associated with the Golgi and plasma membrane, a PI(4)P phosphatase is localized to the ER to prevent PI(4)P from accumulating at this organelle (25).

1.13 Phosphatidylinositol 4-Phosphate - PI4P

Phosphatidylinositol 4-phosphate (PI(4)P) is the most abundant monophosphorylated phosphoinositide (230). PI is synthesized at the ER and transported from there to the other

Figure 1.10: Phosphoinositide recognition domains.

Structurally different phosphoinositide binding domains have been identified and these have a marked difference in specificity and affinity. Domains that bind phosphoinositides are shown as coloured shapes with a schematized binding site. Arrows indicate the preferred ligand of the domain and proteins that contain the domain are listed above each corresponding domain. Adapted from (230).

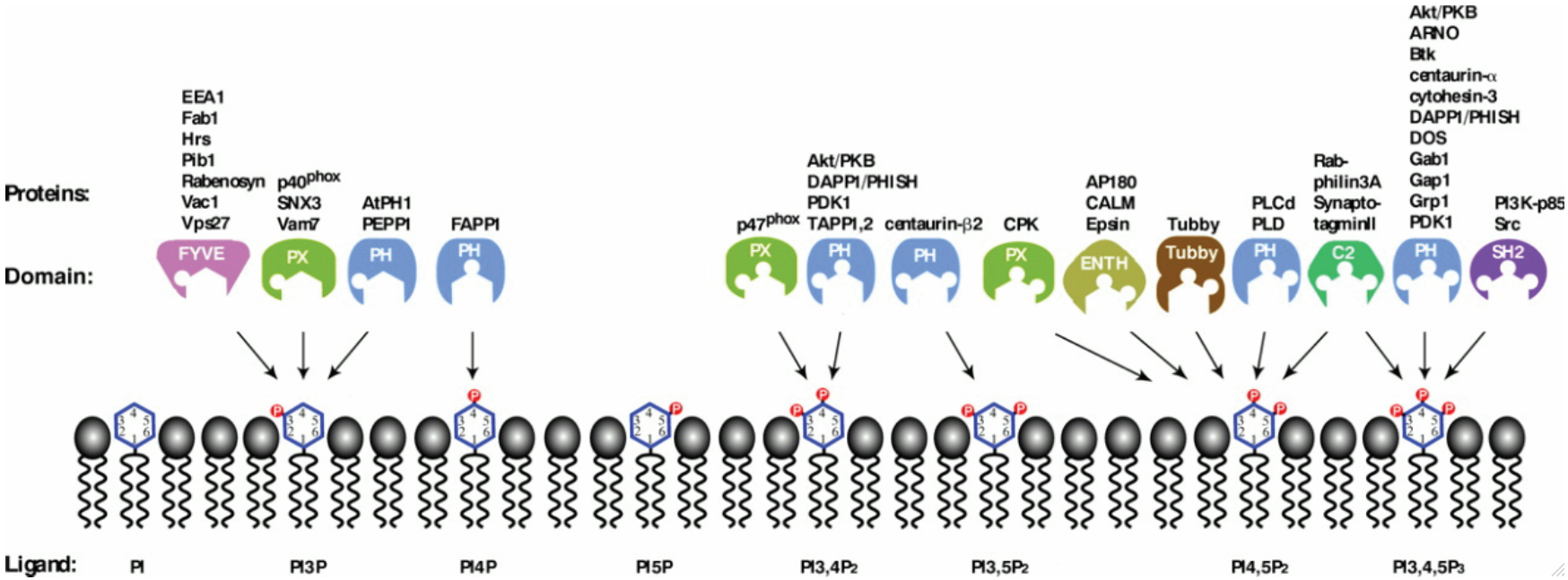
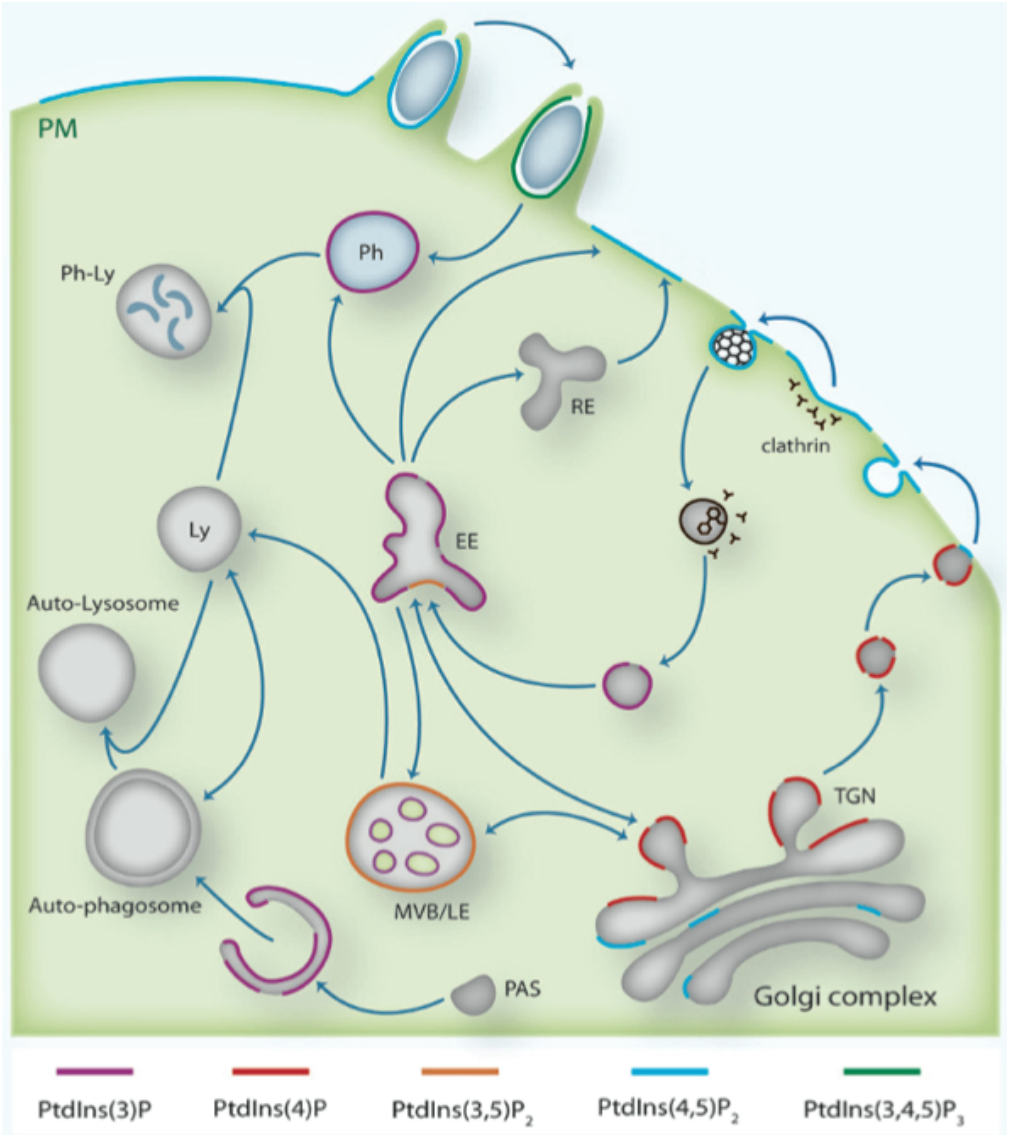

Proteins:
Domain:
Ligand:
EEA1
Fab1
Hrs
Pib1
Rabenosyn
Vac1
Vps27
FYVE
p40phox
SNX3
Vam7
PX
AtPH1
PEPP1
PH
FAPP1
PH
p47phox
PX
Akt/PKB
DAPP1/PHISH
PDK1
TAPP1,2
PH
centaurin-β2
PH
CPK
PX
AP180
CALM
Epsin
ENTH
Tubby
Tubby
PLCd
PLD
PH
Rab-
philin3A
Synapto-
tagminII
C2
Akt/PKB
ARNO
Btk
centaurin-α
cytohesin-3
DAPP1/PHISH
DOS
Gab1
Gap1
Grp1
PDK1
PH
PI3K-p85
Src
SH2
PI
PI3P
PI4P
PI5P
PI3,4P2
PI3,5P2
PI4,5P2
PI3,4,5P3

Figure 1.11: Phosphoinositide localization.

Phosphoinositides are associated with signalling within the cell and organelle identity. Displayed are the common location of each phosphoinositide. Abbreviations: EE: early endosome; LE: late endosome; Lys: lysosome; MVB: multi-vessicular body; PAS: pre-autophagosomal structure; Ph: phagosome; Ph-Ly: phago-lysosome; RE: recycling endosome. Adapted from (299).


PM
Ph
Ph-Ly
RE
clathrin
Ly
EE
Auto-Lysosome
Auto-phagosome
MVB/LE
TGN
PAS
Golgi complex
PtdIns(3)P
PtdIns(4)P
PtdIns(3,5)P2
PtdIns(4,5)P2
PtdIns(3,4,5)P3

organelles (45, 88). The phosphorylation of PI by PI4K is thought to mainly occur in two cellular compartments: the Golgi and the plasma membrane (45). While PI(4)P is abundant within the cell, it was originally viewed solely as a synthetic intermediate for bis- and tris-phosphorylated PI effectors. While PI(4)P generation at the Golgi is associated with the genesis of transport vesicles, the major function of the plasma membrane PI(4)P pools is to be phosphorylated to create phosphatidylinositol 4,5-bisphosphate (45).

The majority of PI(4)P is generated at the Golgi and is essential for Golgi function (67, 207). At the Golgi, two distinct roles for PI(4)P have been identified, functioning as clathrin adaptors and lipid transfer proteins (207). Clatherin-mediated transport from the Golgi is essential to sort proteins to endosomes and lysosomes (306). Lipid transfer proteins are characterized by specific lipid-binding sites and many contain specific PH domains that are required for Golgi targeting (207). It is speculated that these proteins provide a means of non-vesicular transport and exchange of lipids between membranes and may be involved in lipid-sensing and extracting lipids from bilayers (72).

Several Golgi-associated proteins bind PI(4)P, these include adapter protein (AP)-1 and its assessory protein epsinR, along with four adaptor protein (FAPP) -1 and -2, and oxysterol-binding protein (OSBP) (77, 78). FAPP1 and FAPP2 bind PI(4)P through their PH domain, which at the Golgi, binds PI(4)P and the small GTPase, Arf-1 (77, 78). Decreasing or displacing FAPP inhibits the transfer of cargo to the plasma membrane, indicating a role for Golgi to plasma membrane trafficking (77, 78). It still remains to be fully understood how PI(4)P cooperates in the recruitment of effector proteins and how these effectors participate in sorting at the Golgi (264). One possibility is that PI(4)P cooperates

with Arf1 to recruit the FAPP proteins to the Golgi and that the FAPPs are implicated in post-Golgi trafficking (112, 264).

Although PI4Ks localize to the Golgi cisternae, most PI(4)P effectors are enriched in late Golgi compartments, suggesting a PI(4)P gradient across the Golgi where the cis-Golgi containing the lowest levels and the trans-Golgi the highest (116). A major role of PI(4)P at the Golgi is to control sphingolipid biogenesis through recruitment of its binding proteins which modulates this pathway (116). Depletion of PI(4)P at the Golgi prevents the exit of cargo from the Golgi that is destined for the plasma membrane and late endosomes (278). Furthermore, the PI(4)P depletion in the Golgi impaired, but did not abolish, the replenishment of downstream effector PI(4,5)-bisphosphate at the plasma membrane, indicating a novel role for Golgi pools of PI(4)P in this process (278).

While much research has focused on the role of PI(4)P at the Golgi, more recent studies are showing roles at other organelles. For example, PI(4)P has been shown to play a role at ER exit sites. PI(4)P has been shown to be required to promote COPII-mediated ER export (37). Furthermore, there is a localized formation of PI(4)P at the ER exit sites (37). As PI(4)P is displaying a wider subcellular distributions, this initiates the search for roles for PI(4)P outside of the Golgi.

1.14 Phosphatidylinositol 4,5-bisphosphate - PI(4,5)P_2

Phoshphatidylinositol 4,5-bisphosphate (PI(4,5)P_2) accounts for 1% lipids found in the plasma membrane and is the most abundant of PIs which are known to bind cellular proteins (45). Most PI(4,5)P_2 is produced by the phosphorylation of PI(4)P by PI5K,

however it may also be produced through PI4K phosphorylation of PI(5)P as well (88, 165). $PI(4,5)P_2$ is produced by the family of phophatidylinositol phosphate kinases, a family of distinct lipid kinases that all contain a kinase core with conserved catalytic residues that bind both ATP and magnesium ions (91). $PI(4,5)P_2$ is a second messenger and is synthesized in response to external signals (25).

Originally, $PI(4,5)P_2$ was regarded as an unusual resident of the plasma membrane due to the fact that they posses a large head group and an electrostatic charge, and also because they are metabolized quickly at both resting and stimulated states (45). There are two possible fates for $PI(4,5)P_2$ at the plasma membrane. First, it may be phosphorylated by PI3K to create $PI(3,4,5)P_3$, which is also a signalling lipid by serving as a membrane anchor for several proteins (25, 50, 210). Secondly, it may be cleaved to generate two second messengers diacylglycerol (DAG) and inositol (1,4,5)-trisphosphate (Figure 1.12) (25). When $PI(4,5)P_2$ is hydrolyzed by phosphoinositide-specific phospholipase C (PLC) to produce the second messenger, inositol $(1,4,5)P_3$, this releases calcium ions from internal stores, and DAG which acts with calcium ions to activate protein kinase C (210).

Within the cell, there are many functions associated with $PI(4,5)P_2$ (Figure 1.13). $PI(4,5)P_2$ is associated with the production of the above mentioned second messengers, ion-channel and enzyme activation, cytoskeletal rearrangements, recruiting actin-binding proteins, and regulating endocytosis, exocytosis and focal adhesions (91, 210). $PI(4,5)P_2$ anchors to the plasma membrane through several domains including the pleckstrin homology (PH) domains (63, 90, 181). While at the plasma membrane, $PI(4,5)P_2$ can activate dozens of ion channels (131, 277). Additionally, $PI(4,5)P_2$ plays a large role in the transport of cellular components, it is required for clathrin-mediated endocytosis, involved in

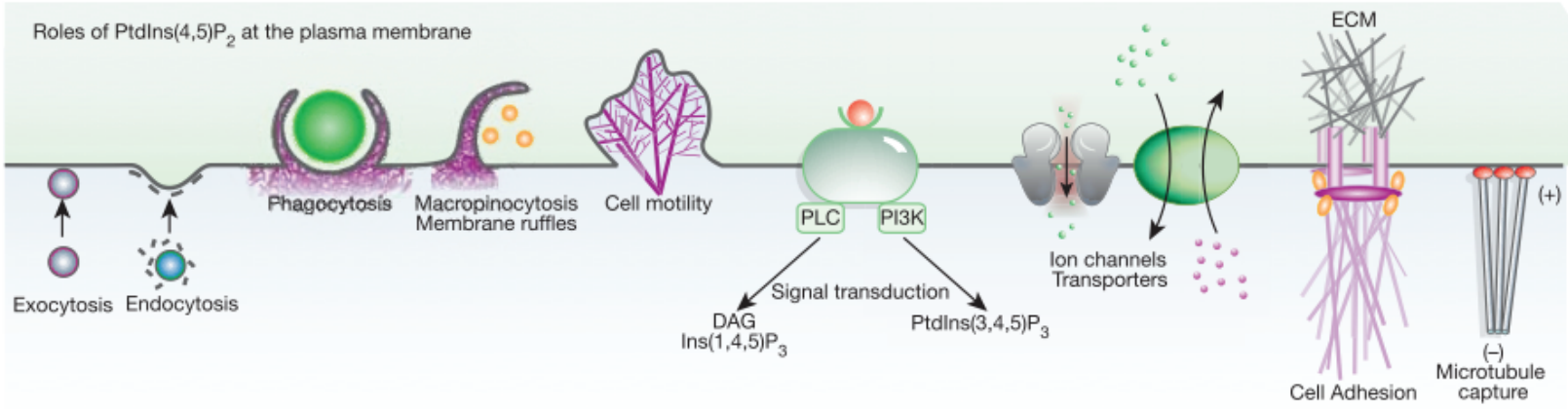
Figure 1.12: The generation and fates of PI(4,5)P_2.

PI(4,5)P_2 is commonly generated through the phosphorylation of PI(4)P by PI5K. The resulting PI(4,5)P_2 may be further phosphorylated by PI3K to form PI(3,4,5)P_3 which is a signalling lipid. Conversely, PI(4,5)P_2 may be cleaved by phospholipase C (PLC) to produce diacylglycerol (DAG) and inositol (1,4,5)-trisphosphate which are both second messengers. Adapted from (295).

Extracellular
Ca 2+
Cadherins
and catenins
PI5K
PI(4)P
PI(4,5)P2
PI3K
PI(3,4,5)P3
PLCδ1
Ins(1,4,5)P3
DAG
Intracellular
Ca 2+
Keratinocyte
differentiation

Figure 1.13: Processes regulated by PI(4,5)P_2 at the plasma membrane.

There are many functions associated with PI(4,5)P_2 at the plasma membrane. It is involved in the production of second messengers, ion-channel and enzyme activation, cytoskeletal rearrangements, actin-binding proteins, endocytosis, exocytosis and focal adhesions. Abbreviations: ECM: extracellular matrix. Adapted from (88).


Roles of PtdIns(4,5)P2 at the plasma membrane
Exocytosis
Endocytosis
Phagocytosis
Macropinocytosis
Membrane ruffles
Cell motility
PLC
PI3K
Signal transduction
DAG
Ins(1,4,5)P3
PtdIns(3,4,5)P3
Ion channels
Transporters
ECM
Cell Adhesion
(+)
(–)
Microtubule
capture

phagocytosis and several stages of exocytosis and synaptic vesicle trafficking (40, 134, 210, 268).

$PI(4,5)P_2$ regulates the adhesion energy between the cytoskeleton and the plasma membrane by acting as a second messenger. Receptors stimulated to hydrolyze $PI(4,5)P_2$ lead to a decrease in the adhesion energy as well as sequestration of $PI(4,5)P_2$ through PH domains that sequestered the lipid (252). Cytoskeleton and membrane interactions drive the formation of filopodia, lamellopodia and other membrane processes in response to stimuli (252). Raucher et al. suggest that plasma membrane localized $PI(4,5)P_2$ controls the dynamic function of the membrane and cell shape by locally increasing and decreasing the adhesion between the actin cytoskeleton and the plasma membrane (252). They indicated that when $PI(4,5)P_2$ levels decrease that the adhesion energy becomes smaller, leading to the formation of membrane blebs (233, 252). Additionally, $PI(4,5)P_2$ enriched domains within the plasma membrane are involved in regulating microtubule plus-end capture and stabilization, which is required for polarized motility of the cell (88, 114).

There are many proteins that bind to $PI(4,5)P_2$ with different specificity and affinity (45). This may be due to the fact that the domains that $PI(4,5)P_2$ binds are diverse and less stringent than most PIs (45). The proteins which bind $PI(4,5)P_2$ have important roles in endocytosis, exocytosis and cytoskeletal rearrangements (45, 88). For example, the clatherin adaptor complex AP-2 is selectively recruited to the plasma membrane due to its ability to bind $PI(4,5)P_2$. The initial contact of the lipid and protein complex triggers a conformational change in the AP-2 complex, exposing additional binding sites for $PI(4,5)P_2$ and membrane protein co-receptors such as cargo proteins with tyrosine-based motifs (104, 135).

While many functions of $PI(4,5)P_2$ have been elucidated, the mechanism for the lipid's ability to participate in several cellular functions remains unknown. It is hypothesized that the multi-functionality of the protein may be attributed to the cells ability to produce the lipid under tight spatial and temporal control (148).

1.15 Phosphatidylinositol 3,4,5-trisphosphate - $PI(3,4,5)P_3$

$PI(4,5)P_2$ can be phosphorylated by PI3K to generate phosphatidylinositol tris-phosphate ($PI(3,4,5)P_3$) (88). In resting cells this lipid is present at very small amounts, but can dramatically increase in response to growth factor stimulation, resulting in dramatic fluctuations of cellular levels (88, 164, 187). A confluent well of a 6-well plate of Swiss 3T3 rodent fibroblast cells had approximately 2.5 pmol of $PI(3,4,5)P_3$ and upon stimulation with 100 ng/ml insulin-like growth factor, these levels increased to approximately 6 pmol (296). Furthermore, when the same cell line $PI(3,4,5)P_3$ levels were analyzed through tritium labelling, the control cells had 185 disintegrations per minute (d.c.m.) while cells stimulated with platelet derived growth factor for 10 minutes showed 1934 d.c.m., indicating the dramatic fluctuations of $PI(3,4,5)P_3$ possible in a short period of time (118, 119).

$PI(3,4,5)P_3$ has a fundamental role in eukaryotes as it mediates many cellular processes, such as cell proliferation, chemotaxis, phago- and micro-pinocytosis, differentiation, survival and metabolic changes (50, 88). $PI(3,4,5)P_3$ acts to recruit effectors and to activate signalling pathways. Prominent targets include the protein kinases 3-phosphoinositide-dependent kinase (PDK) and Akt/PKB which cooperate to activate important signalling cascades, which include the Tor pathway (50, 88, 149). Following the

activation by PI3K, $PI(3,4,5)P_3$ recruits Akt to plasma membrane through interactions with its PH domain. PDK-1, which has an affinity for $PI(3,4,5)P_3$, is targeted to the same area and phosphorylates Akt at Threonine 308. An additional phosphorylation of Akt at Serine 473 by the rictor-mammalian target of rapamycin (rictor-mTOR) complex allows for full activation of Akt which is involved in regulating many processes, including growth, proliferation and apoptosis (50, 233, 259, 315).

$PI(3,4,5)P_3$ is also found in the inner leaflet of the plasma membrane and following growth factor activation may also be found to accumulate on endomembranes (161). $PI(3,4,5)P_3$ also provides a link to the cell cytoskeleton, and upon activation by growth factors, mediates the formation in peripheral ruffles implicated in cell migration (88).

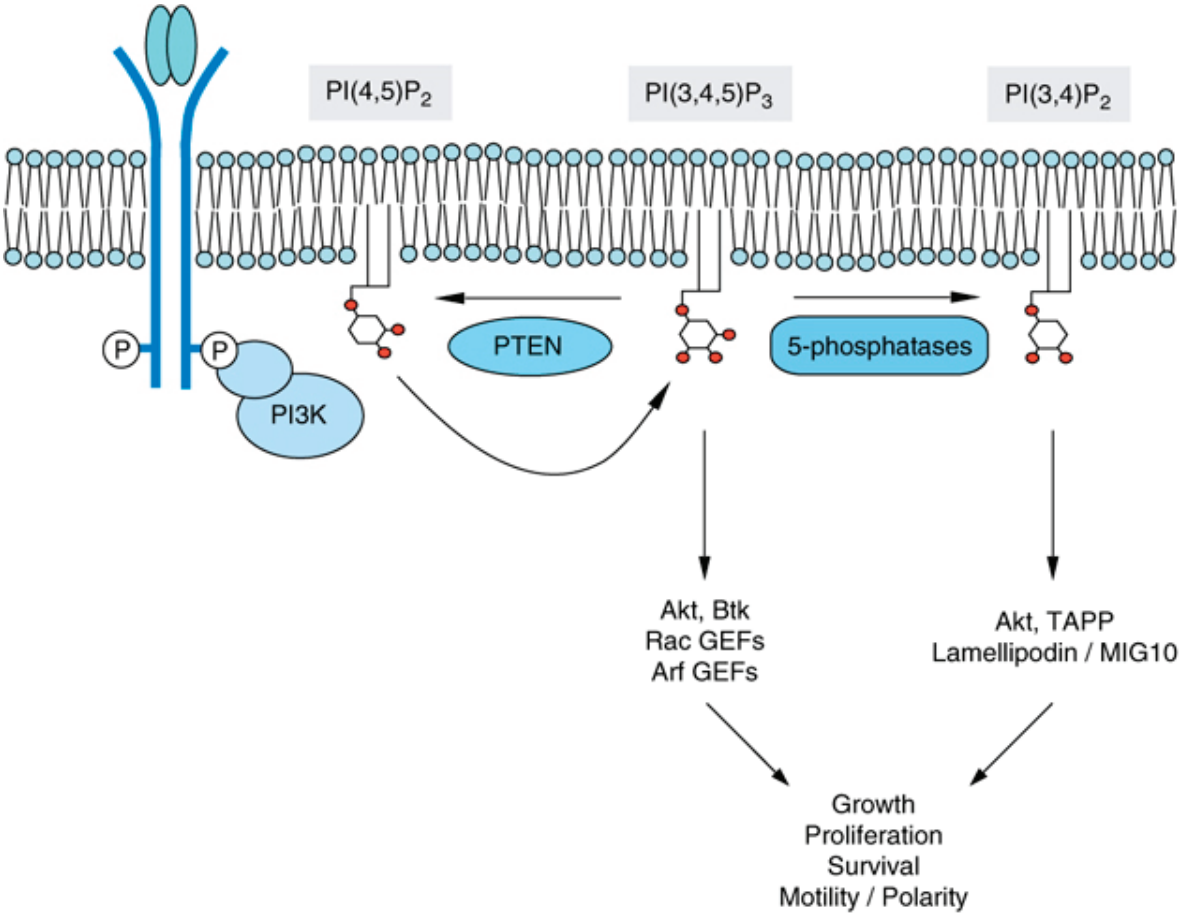
Recently a new role for $PI(3,4,5)P_3$ in mitotic spindle formation has been elucidated (218). Intregrin signalling regulates PI3K and $PI(3,4,5)P_3$ production, where $PI(3,4,5)P_3$ accumulates in the cortical midsection, which is the location in which the spindle subsequently forms during metaphase (218, 289). Inhibition of PI3K lead to misorientation of the spindle in HeLa cells (218, 289).

$PI(3,4,5)P_3$ is dephosphorylated by two enzymes, each with different outcomes (Figure 1.14) (88). Dephosphorylation at the D3 carbon by the PTEN (phosphatase and tensin homolog deleted on chromosome ten) signals to turn off the $PI(3,4,5)P_3$ signalling pathway (88, 233). Dephosphorylation also occurs at the D5 position by 5-phosphatases, including SHIP-1 and -2, to generate $PI(3,4)P_2$ which also has signalling properties (88).

Numerous oncogenes activate PI3K and the Akt pathway, implicating it in a wide range of cancer, including breast, colon, pancreatic, lymphoid and prostate cancer (233, 302). Upon examination of 450 tumours from eight different tumour types, Akt was

Figure 1.14: The role of PI(3,4,5)P_3 at the plasma membrane.

PI(3,4,5)P_3 is a second messenger within the cell and may activate the Akt signalling pathway. Conversely, PI(3,4,5)P_3 may be dephosphorylated by two enzymes, each with different outcomes. Dephosphorylation by phosphatase and tensin homolog deleted on chromosome 10 (PTEN) at the D3 carbon turns off the PI(3,4,5)P_3 signalling pathway. Dephosphorylation at the D5 position by 5-phosphatases generates PI(3,4)P_2 which also has signalling properties. Adapted from (182).


PI(4,5)P2
PI(3,4,5)P3
PI(3,4)P2
P
P
PI3K
PTEN
5-phosphatases
Akt, Btk
Rac GEFs
Arf GEFs
Akt, TAPP
Lamellipodin / MIG10
Growth
Proliferation
Survival
Motility / Polarity

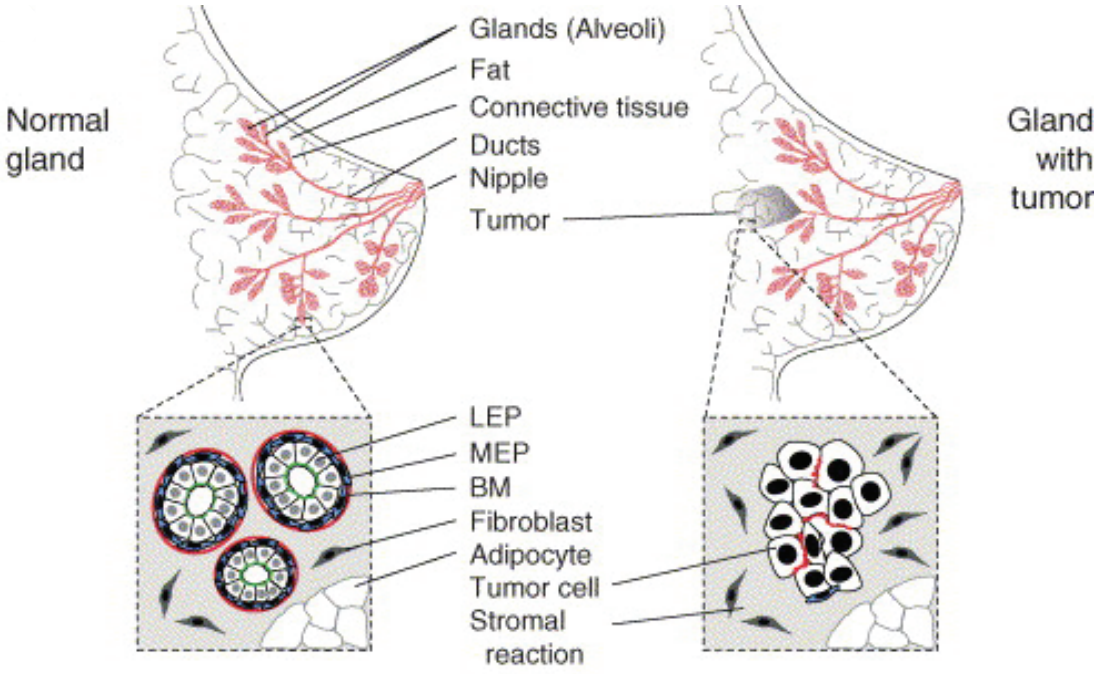
activated in 55% (314). The loss of the PTEN phosphatase is also associated with many cancers and was first identified as a tumour suppressor gene (233). Furthermore, PTEN mutations are also associated with Cowden disease, Lhermitte-Duclos and Bannayan-Zonana syndromes, where patients not only have increased rates of cancer but a variety of hyperplastic, disorganized and non-malignant growths (315). PTEN is pivotal in the control of the Akt pathway, as PTEN mutations abolish its phosphatase activity, this leads to an accumulation of $PI(3,4,5)P_3$ which promotes cell growth, proliferation and survival (45, 187). Regulation of PTEN helps to modulate the spatial and temporal availability of $PI(3,4,5)P_3$ and $PI(3,4,5)P_3$ also provides an alternate route for the metabolism to $PI(3,4)P_2$ by 5-phosphatases (182).

1.16 Breast Cancer and In Vitro Organogenesis

Most human cancers arise from epithelial cells and breast cancer is one of the most common types of epithelial cancer (80). Epithelial cells have several distinguishing features: a polarized morphology, specialized cell-cell contacts and an attachment to the underlying basement membrane, which are required for proper control of proliferation, survival and differentiation (80). Specifically, the mammary gland *in vivo* has a distinct morphological architecture; mammary epithelial cells are the fundamental functional unit of the gland and form the polarized secretory network of hollow ducts and alveolar units (82, 263). The functional unit of the mammary gland is organized into a branched ductal network of epithelial cells surrounded by myoepithelial cells and basement membrane (Figure 1.15) (223). The mammary gland is unique as an organ in that most development occurs after birth. Most branching morphogenesis occurs throughout puberty, the fully functional gland

Figure 1.15: The structure and three-dimensional model of the mammary gland.

The mammary gland morphological architecture. *Left*. Mammary epithelial cells are the fundamental functional unit of the gland and form the polarized secretory network of hollow ducts and alveolar units. The functional unit of the mammary gland is organized into a branched ductal network of luminal epithelial cells (LEP) surrounded by myoepithelial cells (MEP) and basement membrane (BM). *Right*. The highly ordered structure of the mammary gland is disrupted in tumours. Adapted from (223).


Normal gland
Glands (Alveoli)
Fat
Connective tissue
Ducts
Nipple
Tumor
Gland with tumor
LEP
MEP
BM
Fibroblast
Adipocyte
Tumor cell
Stromal reaction

develops during pregnancy and lactation and after weaning, the gland undergoes involution where the mammary cells die and are replaced by adipose tissue and mesenchyme (29, 33, 223).

While certain gene expression is being associated with cancer, very little information is known as to how genetic abnormalities associated with cancer actually elicit the phenotypic changes observed (80). Insight into the mechanism of cancer progression may lead to the development of novel diagnostic markers and therapies. While primary tumour studies and *in vivo* mouse models have been critical in understanding epithelial tumours, it is difficult to garner information as to the biological and cellular processes involved in tumour formation (80). Although traditional cell culture methods are able to study cell processes and signalling pathways, they do not recapitulate the structural organization or functional differentiation of the epithelium *in vivo* (80, 139, 151). Epithelial cells require attachment to a basement membrane, where integration of both cell-matrix and cell-cell interactions are required to regulate cell behaviour, cell polarity, proliferation, adhesion and survival (263).

Epithelial cells cultured on basement membranes that mimic the composition of normal basement membranes, recapitulate many features of *in vivo* organogenesis (29, 80, 82, 263). Commercially available Matrigel is a mixture of basement membrane proteins, rich in collagen and laminin, derived from the Engelbreth-Holm-Swarm (EHS) murine tumour (82, 263). When grown on Matrigel, epithelial cells are considered to be cultured in three-dimension as the presence of extracellular matrix (ECM) promotes the formation of multi-cellular structures in the x,y and z axis (242). Under three-dimensional overlay culture conditions, a basement membrane, measuring 1 – 2 mm in thickness, is laid down and cells are seeded as single cells (82, 263). Cells then proliferate in the ECM, by day 5 – 8 two

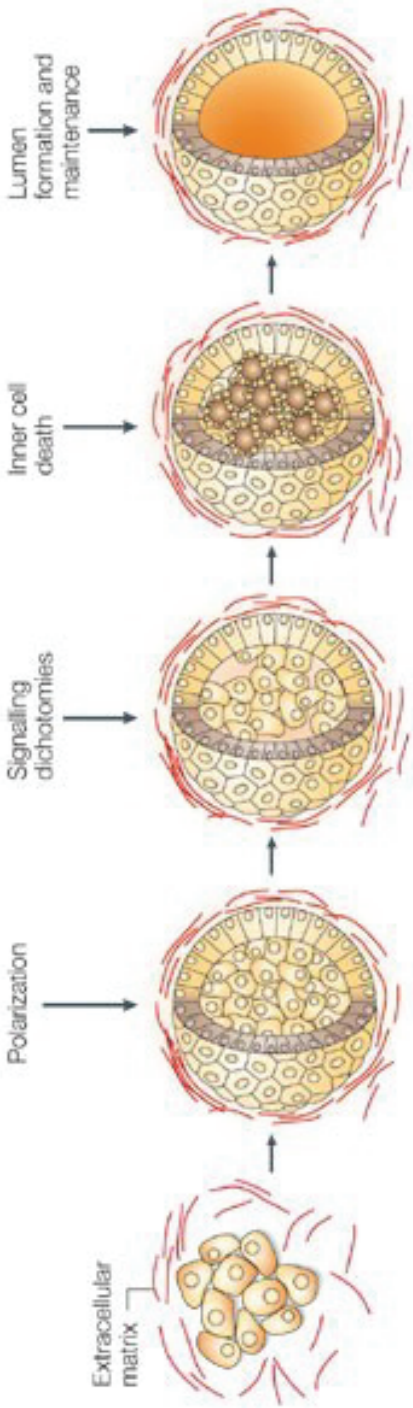
distinct populations of cells becomes evident: cells in direct contact with the ECM form a polarized outer layer, while the inner cells that lack contact with the ECM are poorly polarized start to die by apoptosis to form the hollow lumen (Figure 1.16) (82, 198). It takes 10 – 15 days to create fully formed acini but once they are formed they exhibit low levels of proliferation and remain stable with respect to acini size and cell number (29, 82). The term 'acini' refers to the spheroid structure formed by epithelial cells in three-dimensional culture and is an operational term with no anatomical connotation (80). The acini have a well-defined structure that recapitulates the features of epithelial cells found *in vivo*, including the formation of a spheroid with a centrally localized, hollow lumen, a layer of polarized cells surrounding the lumen and may be induced to secrete milk proteins (80, 223). The benefit of three-dimensional culture is that processes responsible for creating glandular epithelia structures are amenable to experimental manipulation and analysis (80, 82). The similarities to *in vivo* breast biology have made the three-dimensional culture system important to elucidate the mechanism of oncogenes within the context of a tissue (292).

1.17 Breast Morphogenesis and Cancer

There have been six features of malignant growth characterized: 1) enhanced proliferative potential, 2) decreased sensitivity to apoptosis, 3) autonomous production of growth signals, 4) insensitivity to growth inhibition signals, 5) angiogenic potential and 6) migratory and invasive capability (126, 263). Epithelial tumours require the disruption of the well-ordered architecture by an increased proliferation of epithelial cells, a loss of acinar organization and filling of the luminal space (82, 174, 220). When cultured in monolayer, normal and breast tumour cell lines display subtle phenotypic differences but when cultured

Figure 1.16: *In vitro* formation of mammary acini in three-dimensional culture.

Breast epithelial cells are seeded on a basement membrane, composed of Matrigel, as single cells. Cells then proliferate in the extracellular matrix. By day 5 – 8 two distinct populations of cells becomes evident: cells in direct contact with the ECM form a polarized outer layer, while the inner cells that lack contact with the ECM are poorly polarized start to die by apoptosis to form the hollow lumen. It takes 10 – 15 days to create fully formed acini but once they are formed they exhibit low levels of proliferation and remain stable with respect to acini size and cell number. Adapted from (80).


Lumen formation and maintenance
Inner cell death
Signalling dichotomies
Polarization
Extracellular matrix

in three-dimension, the two cell types display a marked difference (80). Expression of an oncogene in three-dimensional culture leads to a diverse array of morphological phenotypes.

First of all, the expression of an oncogene may lead to an escape from proliferative arrest. When breast epithelial cells, MCF10A, that overexpress cyclin D1 or have an inactive retinoblastoma (Rb) protein with human papillomavirus E7, are cultured in Matrigel, the acini undergo excessive proliferation (81, 82). Although there is increased proliferation, generating acini that are 30% larger than their wildtype counterparts, the structures retain a hollow morphology as the cells not in direct contact with the basement membrane still undergo apoptosis (81, 82). The constitutive proliferation by cyclin D1 is dependent on growth factors to stimulate the signalling pathway (80). The downstream effector of PI3K, Akt, is often activated in epithelial cancers and increases the proliferation during early morphogenesis but cannot overcome the suppression of proliferation at later stages leading to arrested structures that have an increase in cell number over controls (80, 83). While activation of Akt leads to an escape from proliferative arrest, the structures are large but also misshapen, leading to a second observed phenotype: the multi-acinar structure (80, 83). A multi-acinar structure is a large, misshapen, irregular structure that still displays a lumen, however, the lumen formed may spontaneously occur within small clusters of the large structure (80, 82, 83, 263).

A third morphology observed is a filled multi-acinar structure. The filled multi-acinar structure results from several disregulations of pathways, the oncogene expressed not only leads to an escape from proliferative arrest but leads to the disruption of cell polarity (80). The expression of a constitutively active member of the epidermal growth factor receptor, ErbB2, leads to the formation of a filled multi-acinar structure (82, 220, 263).

Interestingly, the overexpression of ErbB2 is seen in 20-30% of breast cancers and is associated with poor clinical prognosis (188). This ErbB2-induced multi-acinar structure is formed through excess proliferation, protection from apoptosis and changes in apico-basal polarization (82). When only proliferation was enhanced or apoptosis was inhibited the resulting phenotype did not show luminal filling, indicating that both proliferation enhancement and inhibition of apoptosis is required (80, 81). Although the inhibition of apoptosis alone may delay lumen formation, it does not prevent it suggesting that other pathways, perhaps autophagy, is contributing to lumen formation (253).

1.18 Phosphoinsitides and Three-Dimensional Morphogenesis

The three-dimensional structures formed have three distinct plasma membrane surfaces in the polarized structure. The apical surface is a free surface that borders the lumen and is specialized to regulate the exchange of materials, the basal surface is in contact with the underlying basement membrane, ECM and underlying blood vessels, and the lateral surface that adheres neighbouring cells (44, 180, 226). While the basal and lateral surfaces have a similar composition and is often referred to as the basolateral surface, the apical and basolateral surfaces have very different compositions (44, 203). Tight junctions composed primarily of zonula occluden (ZO) proteins, separate the apical and basal surfaces and are credited with preventing the diffusion of cell components between the two surfaces (180, 272). These specialized surfaces in polarized tissues determine cell orientation, function and fate (211).

In addition to creating the three distinct membrane surfaces, cells must transport specialized proteins and structures to the appropriate surface (226). Each cell must be polarized and this must be coupled to the overall organization of the tissue (226). The organization of the cells within a tissue requires the cells to sense their environment, including where they are in relation to their neighbours, and this may be mediated through interaction with the ECM through receptors (44). Furthermore, cells must asymmetrically distribute polarity complexes (44).

Asymmetries in the phospholipid content of the plasma membrane have also been shown to affect polarity (211). Much of the work done on phospholipids and polarity have been explored by Dr. Mostov's laboratory using the Madin-Darby canine kidney (MDCK) epithelial cell line cultured in three-dimension. When cultured in three-dimension, MDCK cells also form cysts of a polarized outer layer but possess a fluid-filled lumen (202, 204, 214). Phosphoinositides are viewed as general determinants of membrane identity and more recently as key determinants of the different surface types in epithelial cells (202).

In the MDCK three-dimensional culture system, it was found that $PI(4,5)P_2$ and $PI(3,4,5)P_3$ are key determinants of the apical and basolateral surfaces respectively (107, 201, 202). Although $PI(4,5)P_2$ was found at the basolateral surface, it was enriched at the apical surface (201, 202). Furthermore, $PI(3,4,5)P_3$ was found exclusively at the basolateral surface (107). The PTEN phosphatase which converts $PI(3,4,5)P_3$ to $PI(4,5)P_2$ localizes to the apical domain early in development to segregate the two lipids (201). Insertion of exogenous $PI(4,5)P_2$ into the basolateral surface of mature MDCK cysts was sufficient to relocalize apical proteins to the basolateral surface indicating that the lipid composition specifies the membrane identity (68, 201, 202). The inverse is also true, upon ectopic

insertion of PI(3,4,5)P_3 to the apical plasma membrane, the apical membrane quickly transformed to the basolateral surface as detected by the presence of basolateral proteins and the exclusion of apical proteins (107).

Interestingly, it appears that different organs and tissues use different *modus operandi* when determining the apical and basolateral surfaces lipid composition. While the MDCK cells have a basolateral localization of PI(3,4,5)P_3, the *Drosophila* photoreceptor, a specialized epithelial cell, specifically accumulates the lipid to the apical surface and this polarization is essential for proper cell function (239).

While the disruption of polarity is a hallmark of malignant epithelial cells there are many other diseases and genetic disorders associated with improper sorting and polarity (211). Furthermore, more recent studies suggest that the polarity of the tissue may function as a tumour suppressor, as it relies on the symbiotic relationship of the cells within it to suppress the malignant phenotype of the individual mutant cells in order for the tissue to survive (174). As research continues to examine oncogenes within the context of a tissue or organelle hopefully novel modes of action will be elucidated for these genes and more effective treatment options will be developed.

1.19 Research Hypotheses and Objectives

There are two parts to my thesis. In the first part I aim to examine the role of eEF1A2 in ovarian cancer while the second aim of my project is to investigate the role of eEF1A2 in three-dimensional *in vitro* mammary morphogenesis.

1.19.1 Aim 1: The role of eEF1A2 in ovarian cancer

This aim has two central objectives. The first objective was to determine whether or not eEF1A2 expression had prognostic significance in ovarian cancer. In the second objective, the effect of eEF1A2 expression in a SK-OV-3 ovarian cancer cell line was studied, with the goal of understanding a mechanism of function for this oncogenic translation factor.

Objective 1: The prognostic significance of eEF1A2

While our lab had previously shown that eEF1A2 was expressed in 20 – 30% of ovarian tumours we were unsure of the prognostic significance of the oncogene. Upon a large scale examination of breast tumours, our laboratory had shown that eEF1A2 was expressed in approximately 50% of breast tumours. Expression was associated with increased 20 year survival of patients (162). Thus, a collaboration was established with Dr. David Huntsman to investigate the prognostic significance of eEF1A2 in ovarian cancer, using 500 ovarian tumour samples.

Objective 2: The mechanism of eEF1A2-induced oncogenesis in ovarian cancer cell lines.

In order to determine the mechanism of eEF1A2-induced oncogenesis, several different parameters that are associated with malignancy were examined. First, the effect of eEF1A2 on cell proliferation and apoptosis was studied. Additionally, due to eEF1A2's ability to bind actin and alter the actin cytoskeleton, the effect of eEF1A2 expression on the actin cytoskeleton and cell-cell junctions was then examined. Finally, to model avascular tumour development, ovarian cancer cell lines were cultured in a hanging-drop method and the effect of eEF1A2 expression was analyzed.

1.19.2 Aim 2: The role of eEF1A2 in *in vitro* three-dimensional breast morphogenesis

eEF1A2 has been shown to be highly expressed in 50% of primary breast tumours and preliminary data from our laboratory indicated that eEF1A2 expression had some physiological effects on the morphology of *in vivo* mammary tissues. Additionally, our laboratory has shown that eEF1A2 binds and activates PI4KIIIβ. PI4KIIIβ is a lipid kinase that phosphorylates phosphoinositol (PI) at the 4th carbon to generate phosphoinositide 4-phosphate (PI(4)P). In order to examine the effect of eEF1A2 on *in vitro* breast morphogenesis, MCF10A cells were cultured in Matrigel. When cultured in Matrigel, wild-type MCF10A cell lines form a single polarized, growth arrested, highly structured spherical acinus. Parental MCF10A cells do not endogenously express eEF1A2. Upon ectopic expression of eEF1A2, MCF10A cells form large multi-acinar structures. *The overall hypothesis of this aspect of the work is that eEF1A2 regulates breast morphogenesis by controlling the PI levels through its interaction with PI4KIIIβ.*

In order to investigate this hypothesis, I had two objectives:

Objective 1: The role of eEF1A2's interaction with PI4KIIIβ in the altered acini structure.

My research was based on the hypothesis that the interaction between eEF1A2 and PI4KIIIβ was directly leading to altered acini morphology. Based on this hypothesis, three predictions were made to investigate experimentally:

1. That over-expression of PI4KIIIβ in MCF10A cells will result in multi-acinar structures.

2. Acini expressing eEF1A2 will show co-localization with PI4KIIIβ.

3. Multi-acinar structures that are formed due to eEF1A2 expression will form normal structures when PI4KIIIβ levels are decreased through siRNA treatement.

Objective 2: To determine the role of eEF1A2-dependent phosphoinositide metabolism in breast oncogenesis.

As PI4KIIIβ phosphorylates PI to create PI(4)P, I wanted to determine the role of phosphoinositide lipids on morphogenesis. Previous work in our lab has shown that expression of eEF1A2 or over-expression of PI4KIIIβ leads to increased levels of $PI(4,5)P_2$ at the plasma membrane which leads to cytoskeletal rearrangements (6, 142, 144). Previous work performed in Keith Mostov's laboratory (UCSF) in Madin-Darby canine kidney (MDCK) cells in three-dimensional culture has shown that different surfaces of acini have different PI compositions. As it has been shown that the lipid composition of surfaces are specific to the location within the acini, I wanted to examine how the phosphoinositides, PI(4)P, $PI(4,5)P_2$ and $PI(3,4,5)P_3$ are distributed throughout MCF10A acini, as well as how their distribution is affected upon ectopic expression of eEF1A2 and over-expression of PI4KIIIβ.

1.20 Summary of Thesis

In the second chapter of my thesis I have shown that eEF1A2 has high expression levels in ~30% of all primary ovarian tumours. 50% of serous tumours, 30% of endometrioid, 19% of mucinous and 8% of clear cell tumours highly express eEF1A2. Ectopic expression of eEF1A2 in the SK-OV-3 clear cell carcinoma line enhances their *in*

vitro proliferative capacity and ability to form tumour-like spheroids in hanging drop culture. Expression of eEF1A2 leads to an increase in stress fiber formation along with tighter cell-cell interfaces. Expression of eEF1A2 did not alter sensitivity to anoikis, cisplatin, or taxol. In serous cancer, eEF1A2 is an independent prognostic marker for survival and high eEF1A2 protein expression was associated with increased probability of 20-year survival. Therefore, eEF1A2 is highly expressed in ovarian carcinomas. Its expression enhances *in vitro* cell growth, and eEF1A2 expression is likely to be a useful ovarian cancer prognostic factor in ovarian cancer patients with serous tumours.

In the third chapter of my thesis I examine the role of PI4KIIIβ and its activator, eEF1A2 on three-dimensional morphogenesis. In this chapter, I found that PI4KIIIβ has an important role in acinar morphogenesis. The PI4KIIIβ protein specifically accumulates at the basolateral surface and its ectopic expression induces multi-acinar formation in MCF10A cells. Moreover, an oncogenic activator of PI4KIIIβ, eEF1A2, stimulates multi-acinar formation in a manner dependent on PI4KIIIβ. Expression of eEF1A2 or PI4KIIIβ disrupts the polarization of PI(4)P and PI(4,5)P_2. My work shows that PI4KIIIβ, and therefore PI(4)P, are key regulators of three-dimensional breast development. I also believe that PI4KIIIβ plays a role in regulating an oncogenic pathway through the oncogene eEF1A2.

2: THE PROGNOSTIC SIGNIFICANCE OF ELONGATION FACTOR EEF1A2 IN OVARIAN CANCER

2.1 Abstract

Our goal was to determine whether eukaryotic elongation factor 1 alpha 2 (eEF1A2), a transforming gene previously shown to be highly expressed in primary human ovarian tumours, is a prognostic marker. We have used an antibody specific for eEF1A2 to measure eEF1A2 protein expression in 500 primary ovarian tumours in a tissue microarray. We have also ectopically expressed eEF1A2 in SK-OV-3 cells, a clear cell carcinoma line that does not normally express eEF1A2. We have shown that eEF1A2 has high expression levels in ~30% of all primary ovarian tumours. 50% of serous tumours, 30% of endometriod, 19% of mucinous and 8% of clear cell tumours highly express eEF1A2. Ectopic expression of eEF1A2 in the SK-OV-3 clear cell carcinoma line enhances their *in vitro* proliferative capacity and ability to form tumour-like spheroids in hanging drop culture. Expression of eEF1A2 did not alter sensitivity to anoikis, cisplatin, or taxol. In serous cancer, eEF1A2 is an independent prognostic marker for survival and high eEF1A2 protein expression was associated with increased probability of 20-year survival. eEF1A2 is highly expressed in ovarian carcinomas. Its expression enhances cell growth *in vitro,* and eEF1A2 expression is likely to be a useful ovarian cancer prognostic factor in ovarian cancer patients with serous tumours.

2.2 Introduction

The identification of genetic abnormalities that occur during ovarian cancer development is a necessary part of understanding its root causes. We have previously identified eEF1A2 (eukaryotic elongation factor 1 alpha 2) as a putative ovarian cancer oncogene by virtue of its high expression and gene amplification in primary human ovarian tumours (9, 283). In addition, the eEF1A2 gene is transforming: ectopic eEF1A2 expression in rodent fibroblast cells allows these cells to grow in an anchorage independent fashion and enhances their tumourigenicity when xenografted in nude mice (9).

eEF1A2 is one of two isoforms of the eukaryotic elongation factor 1 alpha: eEF1A1 and eEF1A2 (208, 224). eEF1A proteins bind amino-acylated tRNAs and recruit them to the ribosome during the elongation phase of protein translation (92, 283). In addition to its role in protein elongation, eEF1A2 regulates a multitude of other cellular processes (7, 9, 143, 283). For example, eEF1A2 is reported to be an inhibitor of caspase 3-dependent apoptosis (258) and deletion of the mouse eEF1A2 homologue, *Eef1a2*, results in immunodeficiency, elevated lymphoid apoptosis and death by 30 days of age (246). eEF1A2 also regulates cell signalling and we have previously reported that eEF1A2 binds and activates the PI4KIIIβ lipid kinase (143). eEF1A2 also stimulates the Akt serine/threonine protein kinase and activates Akt-dependent cell migration and actin remodeling (7).

The two eEF1A isoforms have markedly different tissue-specific expression patterns: eEF1A1 is expressed ubiquitously while eEF1A2 expression is restricted to the brain, heart and skeletal muscle (208, 224, 283). Moreover, eEF1A2 is highly expressed in a subset of ovarian, lung and breast tumours, suggesting an important role for eEF1A2 in oncogenesis, (9, 162, 184, 288). eEF1A2 expression predicts poor survival in lung cancer (300).

However, in breast cancer, eEF1A2 expression is a marker for good outcome (162). It is not yet known whether eEF1A2 expression has prognostic significance in ovarian cancer.

In this report, we show that ~30% of primary human ovarian tumours have high eEF1A2 protein expression. eEF1A2 is highly expressed in 50% of serous and 30% of endometroid tumours but in a much smaller fraction of clear cell and mucinous carcinomas. Expression of eEF1A2 in ovarian clear cell carcinoma cells enhances *in vitro* proliferation and their ability to form tumour-like spheroid cultures. eEF1A2 expression is also a significant predictor of 20-year survival in ovarian cancer of the serous type.

2.3 Materials and Methods

Ovarian tumour tissue microarray (TMA). Tissue arrays were constructed from 500 archival formalin-fixed, paraffin-embedded ovarian tumour samples from Vancouver General Hospital. These included a variety of high and low grade tumours. The tissue microarrays (TMA) were assembled using a tissue-arraying instrument (Beecher Instruments, Silver Springs, MD) as described previously (162, 232). Briefly, the recipient block had holes created by the instrument with defined array coordinates and a stylet was used to transfer the tissue cores to the recipient block.

Immunohistochemistry. The TMA sections underwent immunostaining on a Ventana Discovery Instrument (Ventana Medical systems, Tuscon, Arizona) using a DAB Map Kit (HRP labeled Biotin-Streptavidin System). The staining was done as outlined in (163). Briefly, the staining steps were: deparaffinization, heat induced antigen retrieval with EDTA pH 8.0 (24 min), hydrogen peroxide quenching 3% H_2O_2 (8 min), eEF1A2 antibody (1:100 dilution) (32 min), biotynilated universal secondary antibody (32 min), streptavidin-biotin peroxidase complex (16 min), DAB (8 min), counterstain with hematoxylin (4 min).

Evaluation of eEF1A2 protein expression. The staining levels of eEF1A2 were evaluated as previously described (162, 290). Briefly, the images of the tissue cores were scanned using a Bacus Laboratories Inc. Slide Scanner (BLISS) (Bacus Laboratories, Inc., Lomabard, IL). WebSlide Browser v.3.98 (Bacus Laboratories, Inc., Lombard, IL) was used to view the images of the arrays and to assess the individual core images. H&E slides were scanned along with immunohistochemical ones and used as a reference to determine the expression

of protein to the specific breast carcinoma structures. Images are available through http://www.gpecimage.ubc.ca/tma/webviewer.php.

Scoring of the eEF1A2 immunostaining was performed semi-quantitatively, using digital images and 22-inch monitor with hardware color calibration capabilities. Staining was considered to be negative (0) if no staining was seen in the tumour, weak positive (1+), moderate (2+) and strong (3+), depending on the intensity of the staining in the cytoplasm. Scores were entered into a standard Excel worksheet (Microsoft Excel, Microsoft, Redmond, WA) with a sector map matching each TMA section. Scores for duplicate cores were consolidated to a single value per case using an Excel macro developed by DT. If there were discrepant scores for the two the high value was excepted for the case. Cases were not included if there was no tumour tissues in the core or if the core was cut through. Original scoring tables were deconvoluted together with the core identification file using Deconvoluter 1.10 (189), and the resulting table files imported into SPSS 11.0 for Windows (SPSS Inc., Chicago, IL).

Cell culture and western blotting. SK-OV-3 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and grown according to their instructions. Cells were transfected with 5 μg *EEF1A2* (with C-terminal V5 tag) plasmid and 15 μl Super-Fect (Qiagen) per 60 mm dish and 0.4 mg ml^{-1} Zeocin (Invitrogen) was used for selection. Cells were then lysed using radioimmunoprecipitation assay (RIPA) buffer (50mM Tris-Cl; pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1mM ethylenediaminetetraacetic acid (EDTA); pH 7.0, 150 mM NaCl) supplemented with 1% aprotonin, 1 mg/ml leupeptin, 50 mM NaF, 1 mM Na_3VO_4, 10 μg/ml pepstatin in

ethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF) in dimethylsulfoxide (DMSO). Protein was quantified using Bradford protein assay (Pierce, Rockford, IL, USA) as per the manufacturer's instructions. Approximately 20 μg of protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane. Anti-V5-horseradish peroxide (HRP) (Invitrogen) and β-actin (Sigma) antibodies were used according to manufacturers' instructions.

Cell Proliferation Assay and Multi-cellular Spheroid Culture. Cell proliferation assays were performed by culturing cells as mentioned previously. At indicated time points cells were trypsinized and counted by typan blue exclusion. This was performed in triplicate. The multi-cellular spheroids were cultured as outlined by Kelm *et al.* (150). Briefly, 15 μl droplets containing 1000 SK-OV-3 cells were placed on the lids of non-adherent, bacterial grade polystyrene Petri dishes (Starstead). Lids were then inverted over Petri dishes filled with 10 ml 1X phosphate buffered saline (PBS).

Anoikis and Cell Death. To induce anoikis, cells were shaken at 40 rpm in standard tissue culture dishes on an S-500 orbital shaker (VWR) in a 37^{o}C, 5% CO_2 incubator. Cells were counted using a Z2 Coulter Counter (Beckman Coulter, Fullerton, CA) at set time points. Sensitivity of cells to cisplatin and paclitaxel were performed by adding serial dilutions of either cisplatin or taxol to cells and determining cell number using trypan blue exclusion counting. The IC_{50} was calculated as the drug dose that reduced surviving cell abundance by 50%.

Flow Cytometry. In order to analyze the levels of apoptosis by flow cytometry, 500 000 SK-OV-3 cells were shaken at 40 rpm in standard tissue culture dishes on an S-500 orbital shaker (VWR) in a 37^{o}C, 5% CO_2 incubator. Cells were then removed from the culture dishes by pipetting. Control SK-OV-3 cells were trypsinized from standard tissue culture dishes. Cells were washed with 1X PBS then resuspended in 500µl 1X Binding Buffer (Sigma). Cells were then stained with 2.5µl of a 50µg/mL Annexin V FITC conjugate (Sigma) and analyzed by flow cytometer, FC500 Flow Cytometer Beckman Coutler (Beckman Coulter, Fullerton, CA).

Statistical Analysis. Univariable survival analysis was performed using the Kaplan-Meier method and log-rank test. Multivariable survival analysis was performed using the proportional-hazards model. The level of significance for all statistical tests was defined as $p<0.05$. All survival analyses were performed using JMP v6.0.3 (SAS Institute, Cary, NC, U.S.A.).

2.4 Results

2.4.1 eEF1A2 expression in ovarian cancer

We have previously reported that eEF1A2 mRNA is not detectable in normal ovarian epithelium but is expressed in a fraction of human ovarian tumours (9, 283). To further characterize the expression of eEF1A2 in ovarian cancer, we analyzed eEF1A2 protein expression in 500 ovarian tumours on a tumour microarray. Of the tumours sampled, 42% of those were in stage I, 41% were stage II and 17% were stage III and the entire cohort is defined as having no macroscopic residual disease after the surgery. The distribution of the subtypes of the tumour samples are as follows: 42% Serous, 26% Clear cell, 25% Endometeroid and 7% Mucinous. The age of the patients assayed range from 9258 to 32502 days with a mean age of 21197 ± 4665 days. The creation and validation of this antibody is described elsewhere (163). Staining was categorized as negative, weak, moderate or high. Representative photographs of the 4 categories are shown in Figure 2.1. Overall, 32% of the tumours (159/500) tested have high expression of eEF1A2. As shown in Table 2.1, the tumour types with the most frequent expression of eEF1A2 were serous and endometriod tumours, with 50% and 30% of these tumours having high levels of eEF1A2 expression respectively. 19% of the mucinous tumours and 8% of clear cell tumours had high expression of eEF1A2.

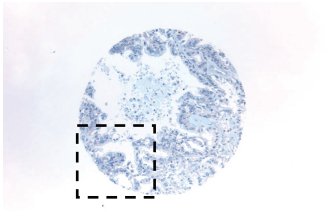
2.4.2 eEF1A2 increases cell proliferation in vitro

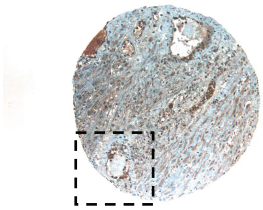
In order to determine the role that eEF1A2 might have in ovarian tumour development, we ectopically expressed eEF1A2 in SK-OV-3 cells. SK-OV-3 cells are

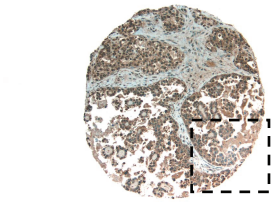
Figure 2.1: eEF1A2 expression in primary ovarian tumours in a tissue microarray.

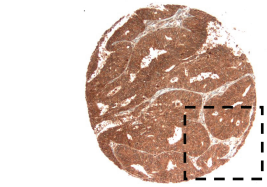
Representative examples of eEF1A2 immunostaining in tumours classified as showing negative, weak, moderate and strong expression of eEF1A2. The right column is a higher magnification view of the boxed square of the left column.

eEF1A2

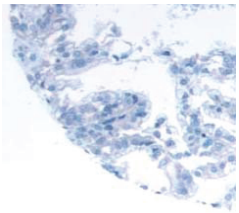


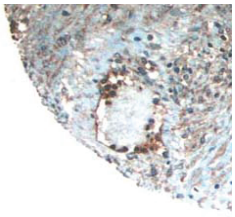


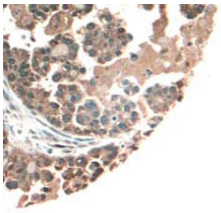


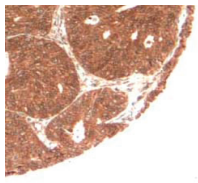


eEF1A2









negative

weak

moderate

strong

Table 2.1: Evaluation of eEF1A2 expression in ovarian cancer.

Tumours were separated based on their histological type: serous, endometriod, mucinous and clear cell and then subdivided based on their expression level of eEF1A2. The *n* value indicates the number of tumours assayed for each histological type.

Table 2.1. Evaluation of eEF1A2 expression in ovarian cancer

Histological type	eEF1A2 expression	Percentage of tumours (%)
Serous (n=212)	High	50
	None, low, moderate	50
Endometriod (n=125)	High	30
	None, low, moderate	70
Mucinous (n=31)	High	19
	None, low, moderate	81
Clear cell (n=132)	High	8
	None, low, moderate	92

derived from a human clear cell ovarian adenocarcinoma and do not endogenously express eEF1A2 (Figure 2.2A). eEF1A2 protein expression in three independent SK-OV-3 clones is shown in Figure 2.2A. As shown in Figure 2.2B, SK-OV-3 variants that express eEF1A2 proliferate at a faster rate than their wild type and vector counterparts (Figure 2.2B). eEF1A2-negative parental and empty vector controls had a doubling time of between 53 to 61 hours. On the other hand, the doubling time for eEF1A2-expressors was substantially less, between 22 to 39 hours depending on the cell line. This indicates that expression of eEF1A2 increases the cells *in vitro* proliferative capacity.

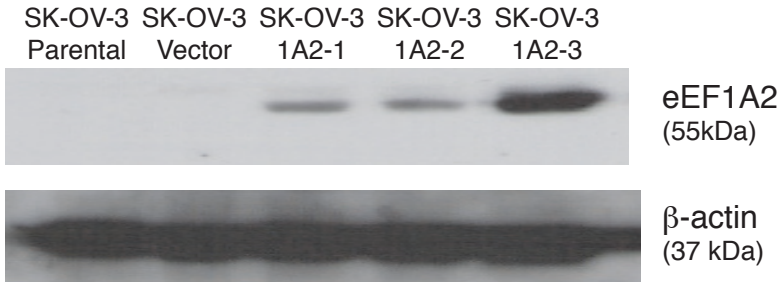
2.4.3 Expression of eEF1A2 increases the rate of spheroid formation

We next wanted to determine whether eEF1A2 could affect other aspects of *in vitro* cell growth. Multi-cellular spheroids (MCS) have been used as an *in vitro* model system of avascular tumour development (84, 124, 125, 150, 217, 284). When SK-OV-3 cells were grown as MCS by the hanging drop method, they form a three-dimensional sphere approximately 2-3 days after culture initiation. As shown in Figure 2.3A, SK-OV-3 cells that express eEF1A2 form MCS more rapidly than vector counter parts (Figure 2.3A). To quantitate this difference, we estimated the size of each spheroid by counting the number of pixels in photographs of each spheroid. The vector controls formed spheroids which were approximately 104 000 pixels in size (Figure 2.3B). On the other hand, eEF1A2-expressors have significantly ($p < 0.001$, ANOVA) smaller spheroids, 65 000 to 74 000 pixels in size. This indicates that the expression of eEF1A2 in these cell lines is enhancing the adhesive interactions responsible for spheroid formation (84, 217).

Figure 2.2: Expression of eEF1A2 enhances cell proliferation.

A. Western blot of eEF1A2 expression. Parental and Vector controls do not express eEF1A2 while SK-OV-3 cell lines 1A2-1, 1A2-2 and 1A2-3 show detectable eEF1A2 protein expression. **B.** eEF1A2-expressing cells (closed symbols) proliferate at a faster rate than the Parental and Vector controls (open symbols). Error bars represent the standard deviation of three independent samples.

A


SK-OV-3 Parental
SK-OV-3 Vector
SK-OV-3 1A2-1
SK-OV-3 1A2-2
SK-OV-3 1A2-3
eEF1A2
(55kDa)
β-actin
(37 kDa)

B

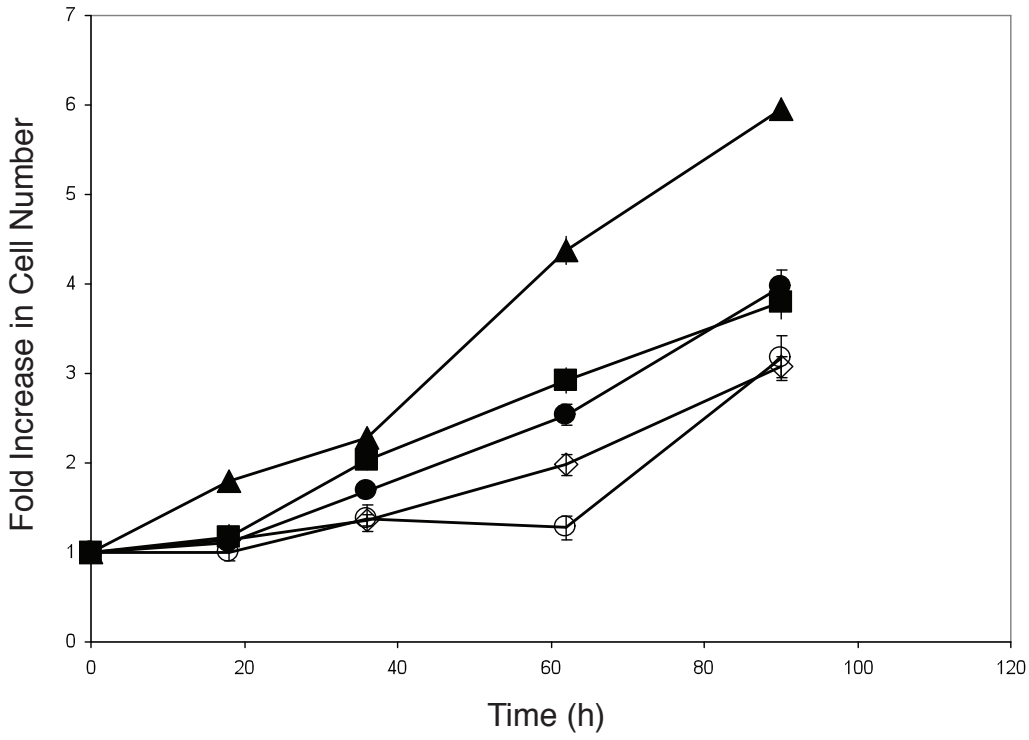
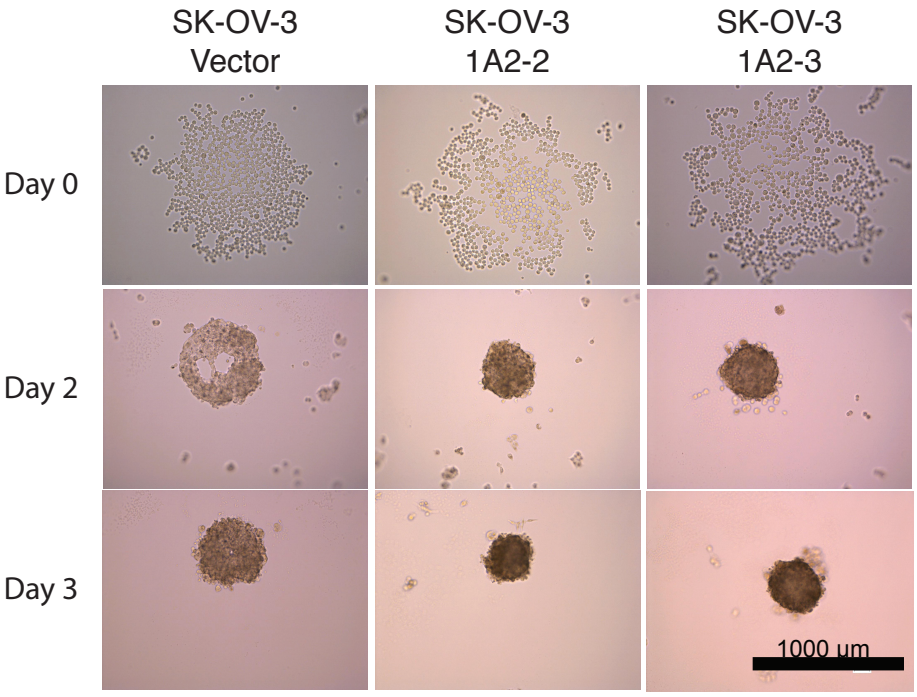

Fold Increase in Cell Number
Time (h)
0
1
2
3
4
5
6
7
0
20
40
60
80
100
120

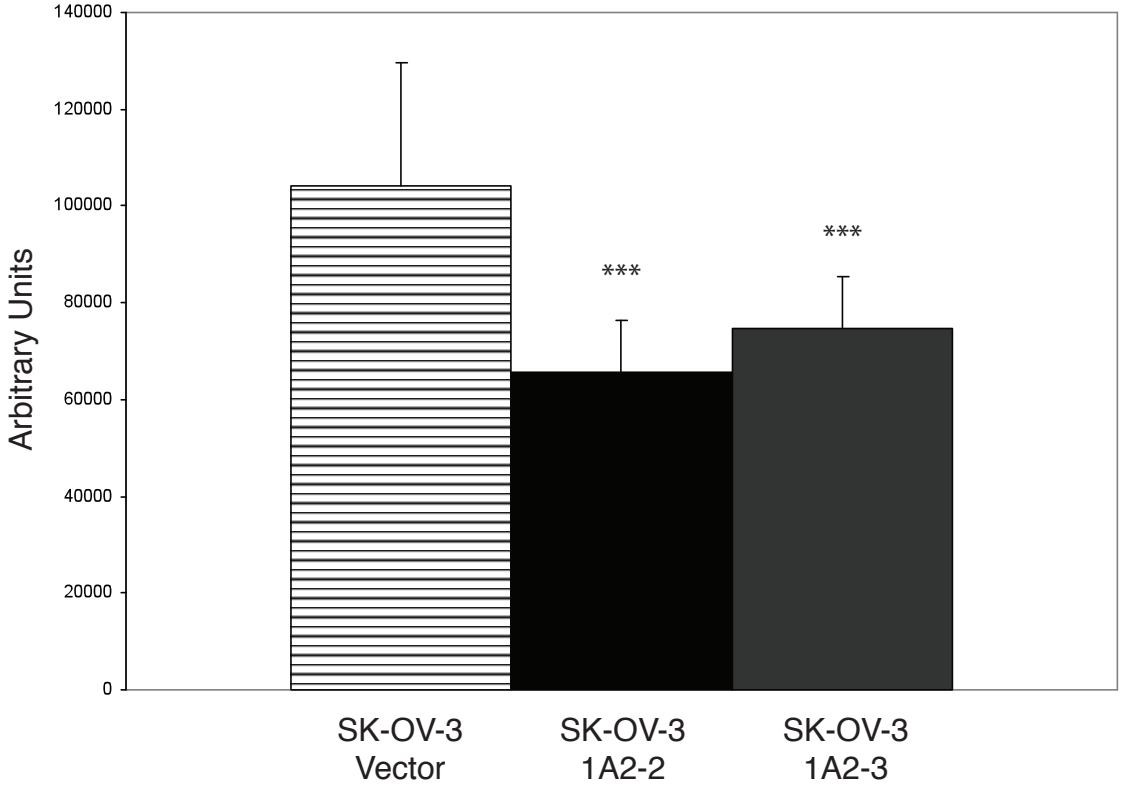
Figure 2.3: Expression of eEF1A2 enhances spheroid formation.

A. Representative photographs of SK-OV-3 cells forming tumour-like spheroids in hanging drop culture. Cells that express eEF1A2 (1A2-2 and 1A2-3) form spheroids more rapidly than their vector counterparts. **B.** Aggregation was quantified after 2 days by quantifying the approximate cross sectional area of the spheroids. Data is the mean and standard deviation of three independent experiments with triplicates measurements. 1A2-2 and 1A2-3 spheroids are significantly smaller than the vector controls. Significance is indicated by *** ($p < 0.001$, ANOVA).

A


SK-OV-3
Vector
SK-OV-3
1A2-2
SK-OV-3
1A2-3
Day 0
Day 2
Day 3
1000 um

B


140000
120000
100000
80000
60000
40000
20000
0
Arbitrary Units
***
***
SK-OV-3
Vector
SK-OV-3
1A2-2
SK-OV-3
1A2-3

2.4.4 Expression of eEF1A2 does not affect resistance to anoikis or chemotherapeutics.

As eEF1A2 has previously been linked to an increased resistance to apoptosis (246, 258), we next determined whether or not expression of eEF1A2 altered cellular susceptibility to anoikis. Anoikis is a specific type of apoptosis that occurs when cells lose cell-matrix adhesion (26, 133, 140). We used shaking culture to induce anoikis and as shown in Figure 2.4A, SK-OV-3 cells that express eEF1A2 do not show a substantial differences in overall survival in shaking culture relative to controls. We also measured apoptosis in these cells using Annexin V and find no substantial difference in apoptosis between eEF1A2 expressing and controls SK-OV-3 cells (Figure 2.4B). We also investigated whether eEF1A2 might alter the cytotoxicity of cisplatin or taxol. As with anoikis, eEF1A2 expression does not detectably affect the cytotoxicity of either cisplatin or taxol (Table 2.2).

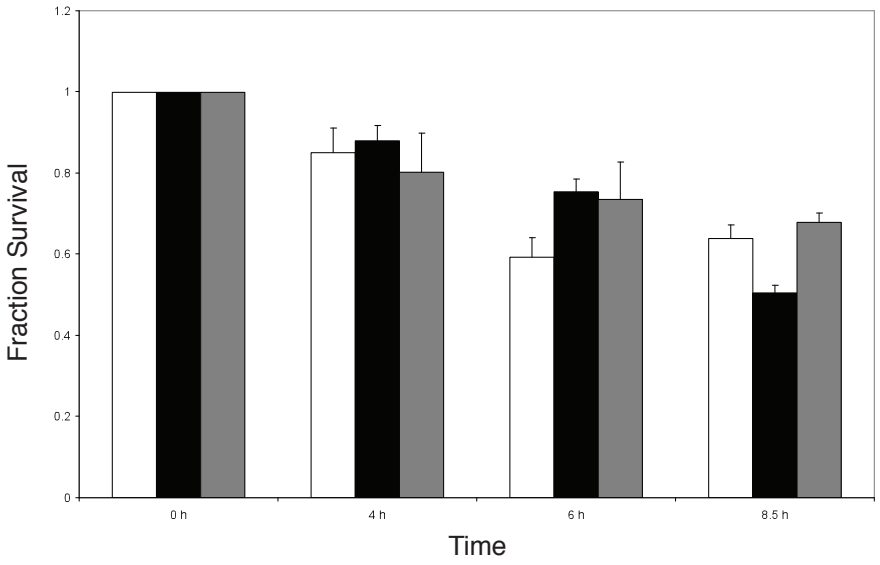
2.4.5 Expression of eEF1A2 alters the actin cytoskelton of cells.

There has been evidence that indicates that eEF1A interacts with the cytoskeleton. Originally, eEF1A was first isolated as an actin binding protein in *Dictostelium discodieum* and was given the moniker actin binding protein 50 (ABP-50) in part due to its molecular weight (85). Co-precipitation assays in whole cell extracts indicate that the two precipitate in a 1:2 molar ratio of eEF1A:actin (219). It is hypothesized that through the interaction with the cytoskeleton, eEF1A binds and bundles actin and stabilizes microtubules (219, 269). Therefore, we wanted to examine the effect of eEF1A2 on the actin cytoskeleton. When the actin cytoskeleton of the eEF1A2-expressing cell line was examined, they had more stress fibers and appeared to have closer cell-cell junctions (Figure 2.5A). The cell-cell

Figure 2.4: Expression of eEF1A2 does not detectably affect anoikis.

A. SK-OV-3 Vector only cells (white bar) and eEF1A2 expressors, 1A2-2 (black) and 1A2-3 cells (gray), were induced to undergo anoikis and cell number counted at set time points. Data is the mean and standard deviation of triplicate cell counts. **B.** Annexin V staining was measured in SK-OV-3 vector and eEF1A2-expressors after 20h in shaking culture. Figure is representative of three independent experiments.

A


Fraction Survival
1.2
1
0.8
0.6
0.4
0.2
0
0 h
4 h
6 h
8.5 h
Time

B

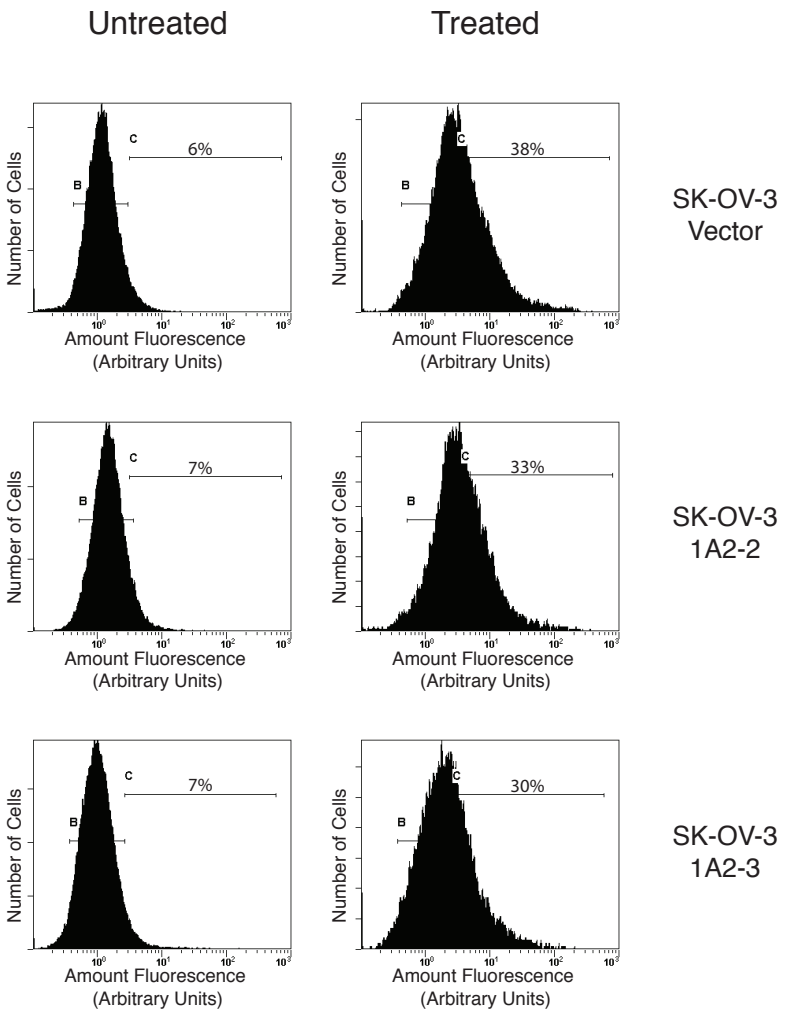

Untreated
Treated
Number of Cells
Amount Fluorescence
(Arbitrary Units)
B
C
6%
38%
7%
33%
7%
30%
SK-OV-3
Vector
SK-OV-3
1A2-2
SK-OV-3
1A2-3

Table 2.2: Effect of eEF1A2 on cisplatin and taxol sensitivity.

The SK-OV-3 parental, vector and eEF1A2 expressors (1A2-1, 1A2-2 and 1A2-3) were assayed for the cytotoxicity of cisplatin and taxol. The half maximal inhibitory concentration (IC_{50}) for each cell line is shown.

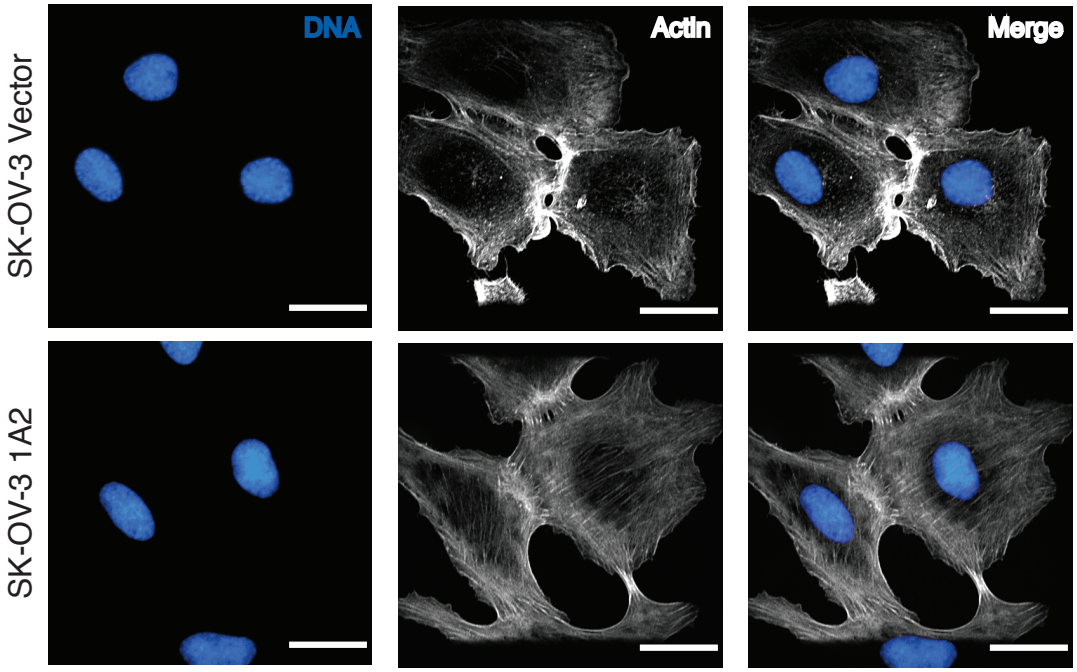
Table 2.2. Effect of eEF1A2 on cisplatin and taxol sensitivity

Cell Line	Cisplatin IC_{50} (mM)	Taxol IC_{50} (mM)
SK-OV-3-Parental	82.5	35.3
SK-OV-3-Vector	78.8	38.6
SK-OV-3-1A2	75.2	42.8
SK-OV-3-1A2	83.4	35.6
SK-OV-3-1A2	80.5	39.6

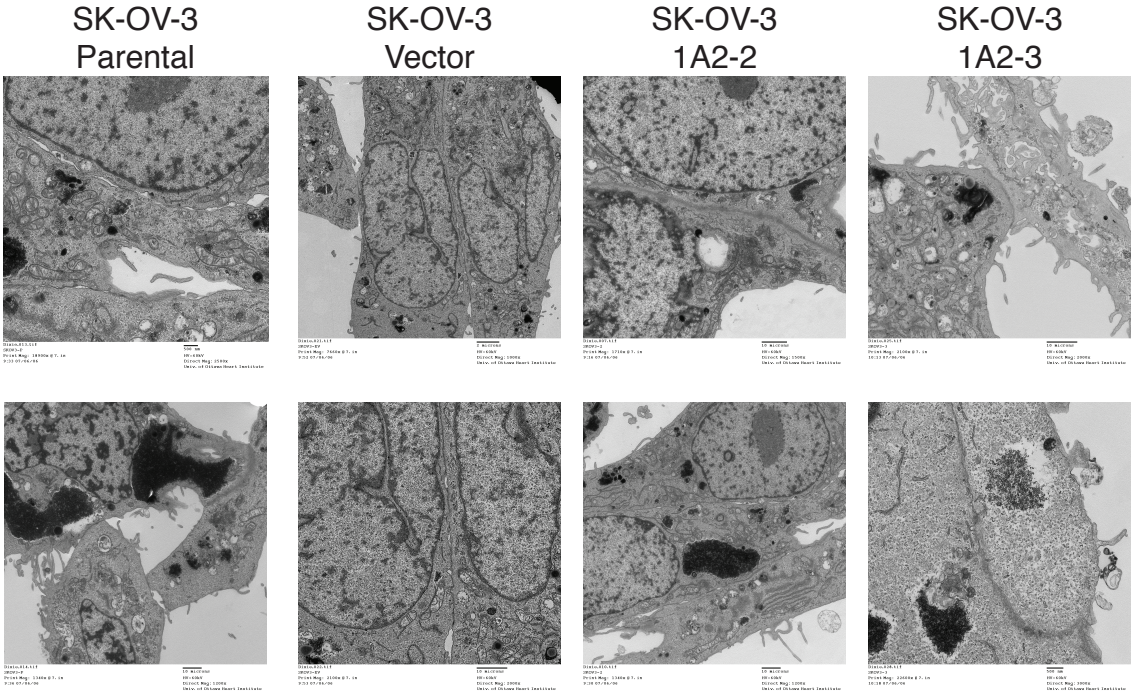
Figure 2.5: Expression of eEF1A2 alters the cells actin cytoskeleton.

A. The SK-OV-3 vector and eEF1A2 expressing cells were stained with phalloidin to show the actin cytoskeleton (white) and the nuclei were stained with DAPI (blue). Scale bars represents 190 μm. **B.** Electron microscopy pictures of parental, vector and eEF1A2-expressing (1A2-2 and 1A2-3) SK-OV-3 cell lines. The scale bar indicates 10 μm, with the exception of SK-OV-3 Parental *top* and SK-OV-3 *bottom* are 500 nm; and SK-OV-3 vector *top* is 2 μm.

A


DNA
Actin
Merge
SK-OV-3 Vector
SK-OV-3 1A2

B


SK-OV-3
Parental
SK-OV-3
Vector
SK-OV-3
1A2-2
SK-OV-3
1A2-3

junctions of the SK-OV-3 cell lines were further analyzed through electron microscopy. The eEF1A2-expressing cell lines appear to have closer and tighter cell-cell junctions when compared to the parental and vector controls (Figure 2.5B)

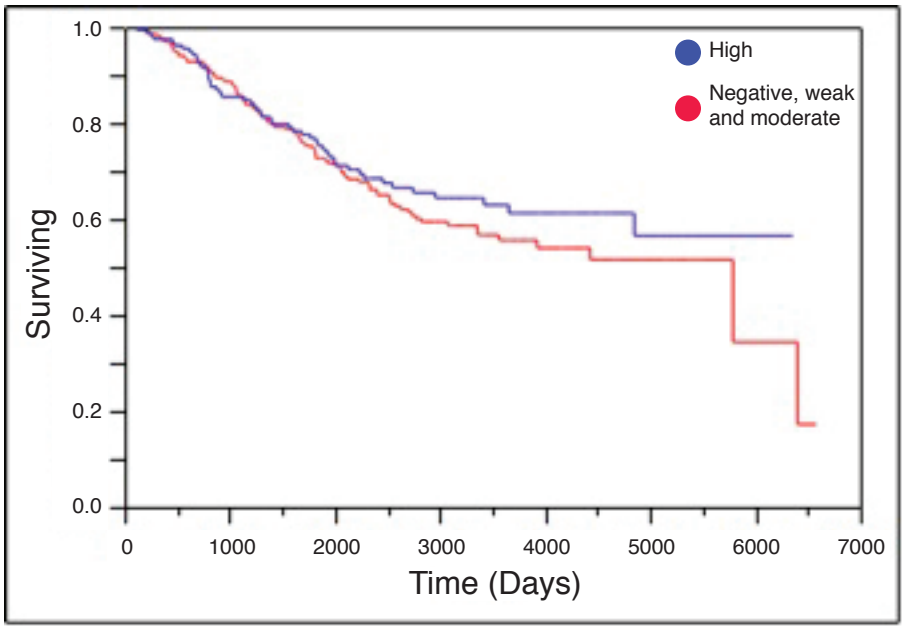
2.4.6 Expression of eEF1A2 indicates good long term prognosis in serous tumour types.

We next investigated whether eEF1A2 expression had prognostic significance for long-term survival in ovarian cancer. Based on the assumption that eEF1A2 is an intensity-based marker, we used recursive partitioning, an unsupervised procedure, to divide the tumour cohort into two groups to maximize observable differences in mean disease specific survival time. We portioned the tumours cohort in the high expressors (3) and grouped the negative to moderate staining (0-2) tumours together. When not separated by tumour type, patients whose ovarian tumours had high expression of eEF1A2 had approximately the same 20-year survival outcome as their eEF1A2 low or negative group (Figure 2.6A), 50% survival in both groups is approximately 12 years. However, in women with serous tumours, high expression of eEF1A2 was associated with significantly ($p<0.01$, log rank test) increased 20-year survival probability (Figure 2.6B). For example, at 10 years after diagnosis, the surviving fraction of women with $eEF1A2^{high}$ tumours was ~50%, compared to ~30% in the others. eEF1A2 was not a significant prognostic marker for endometriod, clear cell or mucinous cancers (Figure 2.7). Proportional hazards analysis shows that eEF1A2 is an independent prognostic marker in the serous subtype when age, stage and Silverberg grade are included in the model (Table 2.3). Other groupings do not lead to statistically significant differences in survival probability (not shown).

Figure 2.6: Prognostic significance of eEF1A2.

Fractional survival comparison of patients whose ovarian tumours showed negative, weak and moderate expression of eEF1A2 (red line) and with high expression (blue line) as a function of time. **A.** Fractional survival is similar between the two populations when all ovarian tumour types are examined. **B.** Long term fractional survival is increased in serous tumours when they highly express eEF1A2.

A


1.0
0.8
0.6
0.4
0.2
0.0
Surviving
0
1000
2000
3000
4000
5000
6000
7000
Time (Days)
High
Negative, weak and moderate

B

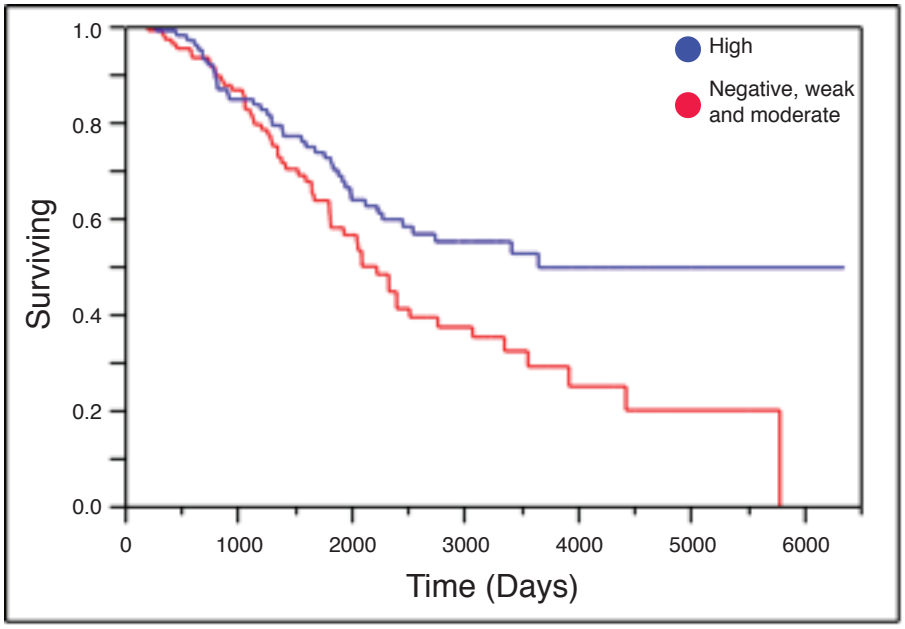
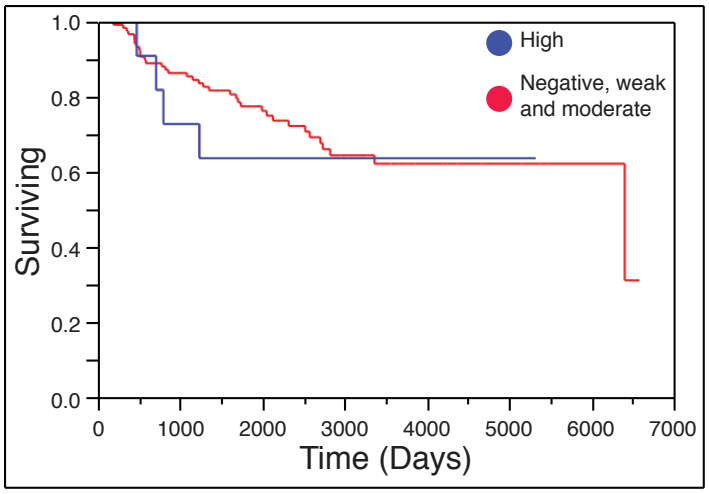

1.0
0.8
0.6
0.4
0.2
0.0
Surviving
0
1000
2000
3000
4000
5000
6000
Time (Days)
High
Negative, weak and moderate

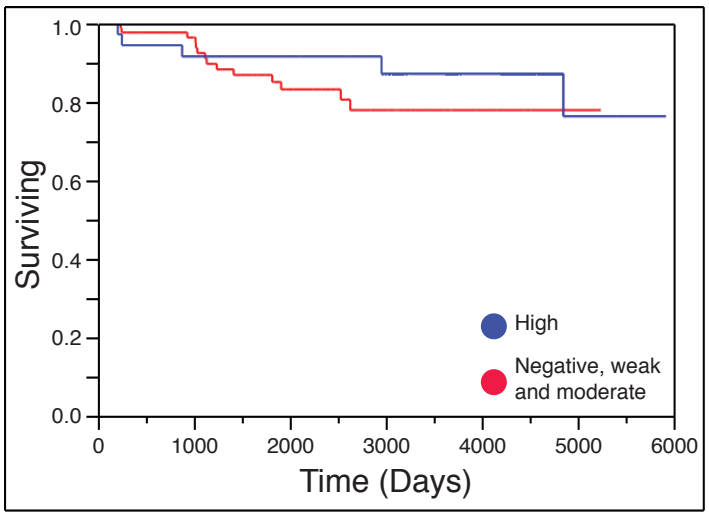
Figure 2.7: eEF1A2 does not have prognostic significance in clear cell, endometriod and mucinous tumours.

Fractional survival comparison of patients whose ovarian tumours showed negative, weak and moderate expression of eEF1A2 (red line) and with high expression (blue line) as a function of time. **A.** Fractional survival is similar between the two populations when all clear cell tumour types are examined. **B.** Fractional survival is similar between the two populations when all endometriod tumour types are examined. **C.** Fractional survival is similar between the two populations when the mucinous tumour types are examined.

A


1.0
0.8
0.6
0.4
0.2
0.0
Surviving
0
1000
2000
3000
4000
5000
6000
7000
Time (Days)
High
Negative, weak
and moderate

B


1.0
0.8
0.6
0.4
0.2
0.0
Surviving
0
1000
2000
3000
4000
5000
6000
Time (Days)
High
Negative, weak
and moderate

C

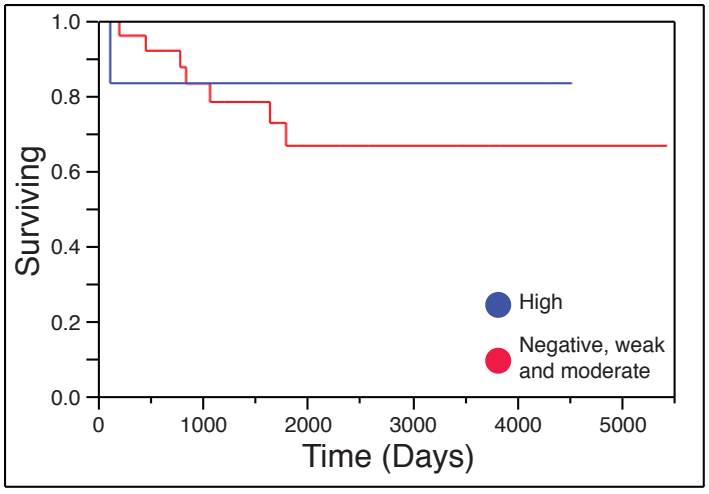

1.0
0.8
0.6
0.4
0.2
0.0
Surviving
0
1000
2000
3000
4000
5000
Time (Days)
High
Negative, weak
and moderate

Table 2.3: Multivariable analysis of eEF1A2.

A proportional analysis of eEF1A2 as a prognostic marker in the serous subtype of tumours when age, stage and Silverberg grade are included.

Table 2.3. Multivariable analysis of eEF1A2

Term	Levels	Risk Ratio	P-value
eEF1A2			0.0210
	0	1.0 [1.0-1.0]	
	1	0.62 [0.41-0.93]	
Stage			0.0002
	I	0.81 [0.57-1.13]	
	II	0.68 [0.50-0.92]	
	III	1.0 [1.0-1.0]	
Silverberg Grade			0.6880
	1	0.92 [0.45-1.59]	
	2	1.15 [0.78-1.78]	
	3	1.0 [1.0-1.0]	
Age	-	NR	0.2414

Discussion

In this report we show that *EEF1A2*, a gene not expressed in normal ovary, is highly expressed in ~30% of human ovarian tumours. 50% and 30% of the serous and endometroid tumours have high expression of eEF1A2, while only 19% and 8% of mucinous and clear cell tumours, respectively, express eEF1A2. This high expression of eEF1A2 in ovarian tumours implies that eEF1A2 expression has some causal role in neoplastic development. We have previously reported that eEF1A2 transforms rodent fibroblasts and enhances their tumorigenicity in nude mice. Consistent with these observations, we find that eEF1A2 expression in SK-OV-3 clear cell carcinoma cells increases their *in vitro* growth rate. eEF1A2 also enhances the ability of SK-OV-3 cells to form *in vitro* spheroids in hanging drop culture. The hanging drop spheroid culture provides a model for studying avascular tumour regions *in vitro* (284). eEF1A2 expression causes cells to aggregate into spheroids more quickly than their vector control, suggesting that eEF1A2 may be contributing to primary tumour formation *in vivo*.

Given the ability of eEF1A2 to enhance cell growth *in vitro*, it is therefore somewhat of a paradox that eEF1A2 expression predicts good survival probability in serous cancers. We have previously reported that eEF1A2 expression similarly marks good survival probability in breast cancer (163). There are several possible explanations for the ability of eEF1A2 to mark good survival. eEF1A2 could affect chemotherapy resistance or alter primary tumour dissemination, both of which have important roles in controlling ultimate clinical outcome (3, 222). However, we do not find that eEF1A2 expression has a detectable effect on anoikis or sensitivity to cisplatin or taxol. This was a surprise to us since it has been previously been reported that ectopic expression of S1, the rat eEF1A2 homologue, inhibits

caspase 3-dependent apoptosis (246, 258). In addition, homozygous deletion of the mouse eEF1A2 gene, *Eef1a2*, increases lymphoid apoptosis (56). While these reports indicate that eEF1A2 inhibits apoptosis, we find no effect on apoptosis in SK-OV-3 cells. With respect to tumour dissemination, we have previously reported that eEF1A2 can enhance *in vitro* cell migration and invasion of breast cancer cells (7). At first glance it would therefore seem probable that eEF1A2 should enhance *in vivo* dissemination of the primary tumour in the peritoneal cavity. However, successful tumour dissemination is a balance between two opposing requirements: sufficient mobility to leave the primary tumor but sufficient "immobility" to colonize secondary sites (173, 260, 280). We postulate that eEF1A2 might make cells too migratory to successfully colonize secondary sites. Alternatively, eEF1A2 may enhance prognosis by altering other oncogenic process, perhaps tumor self-renewal potential or angiogenic capacity. Further investigation will be necessary to clarify the mechanism by which eEF1A2 leads to increased long term survival.

eEF1A2 does not predict good survival probability in all cancers. For example, others have reported that eEF1A2 expression is a marker for poor survival in lung cancer (300). This disparity in prognostic significance of eEF1A2 between ovary, breast and lung cancer implies that eEF1A2 is having markedly different biological effects in the three distinct tissues. An oncogene undertaking different roles and partaking in different signaling pathways often differs as it is expressed in different tumour types (136, 228).

The reason why eEF1A2 might be highly expressed in one ovarian tumour sub-type remains unclear. The gene is not detectably expressed in normal mammalian ovary (9) so tumour-specific expression is unlikely to be related to any unique cell type in normal ovary. Similarly, the reason why eEF1A2 predicts long-term survival in only serous tumours is

unknown. The origin and developmental history of ovarian tumours is not well characterized, since most tumours are identified only in more advanced stages. Thus, further investigation is necessary to determine the role for eEF1A2 in controlling the development of specific gynecological cancers. .

Tomlinson et al. have previously reported that expression of eEF1A2 is associated with the clear cell histology, whereas we have found that high expression of eEF1A2 is only found in 8% of these tumour types as opposed to 75% of those they examined (285). The discrepancies between the results can be attributed to two possible differences. Firstly, Tomlinson et al examined eEF1A2 protein expression in a total of 44 tumours, only 5 of which were of the clear cell type. We have used 500 ovarian tumours, of which 132 were clear cell carcinomas. Secondly, Tomlinson et al. have grouped the tumours examined as either eEF1A2 negative or positive whereas the grouping used here is eEF1A2 negative to moderate expression and high expression of eEF1A2. Therefore, the different sample sizes, as well as the different method of categorizing the expression of eEF1A2 could explain the difference between our two studies.

In summary, we report here that eEF1A2 is highly expressed in ovarian tumours and enhances *in vitro* properties of ovarian cancer cells. Thus, eEF1A2 likely has some causal role in the ovarian neoplastic process. Consistent with this idea, we find that eEF1A2 expression predicts increased survival probability in serous ovarian cancer.

3: CONTROL OF THREE-DIMENSIONAL MAMMARY MORPHOGENESIS BY CO-OPERATION BETWEEN THE LIPID KINASE PI4KIIIβ AND THE EEF1A2 ONCOGENE

3.1 Abstract

The study of *in vitro* morphogenesis is fundamental to understanding neoplasia since the dysregulation of processes that creates multi-cellular organisms is a common hallmark of oncogenesis. The *in vitro* culture of human breast epithelial cells on reconstituted basement membranes recapitulate some features of *in vivo* breast development, including the formation of a three-dimensional structure termed an acinus. Importantly, the capacity to disrupt *in vitro* acinar morphogenesis is a common property of human breast oncogenes. In this report, we find that phosphatidylinositol 4-kinase IIIβ (PI4KIIIβ), a lipid kinase that phosphorylates phosphatidylinositol (PI) to PI(4)P, disrupts *in vitro* mammary acinar formation. The PI4KIIIβ protein localizes to the basal surface of acini created by human MCF10A cells and ectopic expression of PI4KIIIβ induces multi-acinar devlopment. Furthermore, expression of an oncogenic PI4KIIIβ activator, eEF1A2 (eukaryotic elongation factor 1 alpha 2), phenocopies the PI4KIIIβ multi-acinar phenotype. Ectopic expression of PI4KIIIβ or eEF1A2 alters the localization of PI(4)P and PI(4,5)P_2 within acini, indicating the importance of these lipids in acinar development. Our work shows that PI4KIIIβ, eEF1A2 and PI(4)P likely play an important role in mammary neoplasia and acinar development.

3.2 Introduction

PI4KIIIβ is one of the four isoforms of the PI4K family (PI4KIIα, PI4KIIβ, PI4KIIIα, PI4KIIIβ). These kinases selectively catalyze the production of PI(4)P from PI. The four isoforms of PI4K differ primarily in their intracellular localization. In many eukaryotic cell types, PI4KIIIβ, a cytoplasmic protein, localizes to the Golgi and regulates Golgi-to-plasma membrane trafficking (15). However, PI4KIIIβ protein can also be found in non-Golgi locations, indicating functional importance at other intracellular locations. PI(4)P is a signalling moiety and regulates vesicular trafficking through its interaction with ADP ribosylation factor (Arf) 1 and four-adaptor-proteins (FAPP) 1 and 2 (12, 112). PI(4)P pools have also been visualized to accumulate in cytoplasmic vesicles and filamentous structures (17). Importantly, PI(4)P is a synthetic precursor for both $PI(4,5)P_2$ and $PI(3,4,5)P_3$ (15). While PI(4)P is the most abundant of the phosphorylated PIs, its localized abundance can affect the accumulation of $PI(4,5)P_2$. For example, we have previously shown that PI4KIIIβ can control filopodia generation by co-ordinately stimulating the membrane accumulation of both PI(4)P and $PI(4,5)P_2$ (144).

Due to the importance of $PI(4,5)P_2$ and $PI(3,4,5)P_3$ in controlling *in vitro* morphogenesis in three-dimensional canine kidney cell morphogenesis (201-203), we investigated whether PI4KIIIβ and PI(4)P might be involved in regulating the formation of three-dimensional structures. We utilized the established MCF10A breast acinus system (80, 82). MCF10A is an immortalized, non-transformed human breast epithelial cell line that forms a spherical structure, termed an acinus, when cultured on Matrigel. MCF10A acini recapitulate some features of breast node development *in vivo* in that they have a hollow lumen and show apico-basal polarization (32, 80, 137, 170, 226, 242, 275). The MCF10A

acinar morphogenesis has particular relevance to breast cancer in that many genes that have a causal role in breast oncogenesis disrupt *in vitro* MCF10A morphogenesis. The activation of oncogenic pathways in three-dimensional culture disrupts the specialized cell-cell contacts by enhancing proliferation and increasing cell size, as well the individual cells within the structure can vary in size and shape (80, 83, 263). The analogous features between *in vivo* breast biology and three-dimensional culture systems make it an important system to elucidate the mechanisms of oncogenes in breast cancer within the context of a tissue (292).

In this report, we find that PI4KIIIβ has an important role in acinar morphogenesis. The PI4KIIIβ protein specifically accumulates at the basal surface and its ectopic expression induces multi-acinar formation in MCF10A cells. Moreover, an oncogenic activator of PI4KIIIβ, eEF1A2, stimulates multi-acinar formation in a manner dependent on PI4KIIIβ. Expression of eEF1A2 or PI4KIIIβ disrupts the polarization of PI(4)P and PI(4,5)P_2. Our work shows that dysregulation of PI4KIIIβ, and therefore PI(4)P, leads to the formation of multi-acinar structures. We believe that PI4KIIIβ plays a role in regulating a neoplastic pathway pathway through the oncogene eEF1A2.

3.3 Materials and Methods

Cell Culture. MCF10A cells were purchased from the American Type Culture Collection (Manassas, VA) and maintained at 37°C, 5% CO_2 in DMEM/F12 (Invitrogen) supplemented with 5% horse serum (Invitrogen); 20 ng/ml EGF (Peprotech); 0.5 μg/ml hydrocortisone (Sigma); 100 ng/ml cholera toxin (Sigma); 10 μg/ml insulin (Sigma); and 1% antibiotic antimycotic (Invitrogen). PI4KIIIβ-expressing and eEF1A2-expressing stable cell lines were generated using a retrovirus as described by Debnath et al. (82). The PI4KIIIβ-pLXSN vector is described by Jeganathan et al. (144) and eEF1A2-pLXSN by Amiri et al. (6). Cells were selected with 300 μg/ml G418 (Bioshop).

Morphogenesis assays. The three-dimensional culture of MCF10A cells is described by Debnath et al. (82). Briefly, cells were cultured on 100% Matrigel (BD Biosciences) in assay media (DMEM/F12) (Invitrogen) supplemented with 2% horse serum (Invitrogen); 5 ng/ml EGF; 10 μg/ml insulin (Sigma); 100 ng/ml cholera toxin (Sigma); 1% antibiotic antimycotic (Invitrogen); and 2% Matrigel (BD Biosciences).

siRNA and transfections. PI4KIIIβ siRNA sequence (5'-GGAGGUGUUGGA-GAAAGUCtt-3') (catalogue no. AM51331) and negative control siRNA (catalogue no. AM4611) were both obtained from Ambion. siRNA transfections were performed with Lipofectamine RNAiMAX (Invitrogen) according to manufacturer's instructions. Cells were transfected with siRNA in 2D and the following day were washed, trypsinized and re-plated in Matrigel for morphogenesis assays.

Western Blotting and Immunoprecipitation. Cells were lysed using radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-Cl, pH 7.4; 1% Triton X-100; 1% sodium deoxycholate; 0.1% sodium dodecyl sulfate (SDS); 1 mM ethylenediaminetetracetic acid (EDTA), pH 7.0; 150 mM NaCl; 1% aprotonin; 1 mg/ml leupeptin; 50 mM NaF; 1 mM Na_3VO_4; 10 mg/ml pepstatin in ethanol; 1 mM phenylmethylsulfonyl fluoride (PMSF) in dimethylsulfoxide (DMSO)). Protein was quantified using Bradford protein assay (Biorad) according to manufacturer's instructions. Approximately 10 μg of protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore). Anti-V5-horseradish peroxide (HRP) (Invitrogen), PI4KIIIβ (BD Biosciences), and β-actin (Sigma) antibodies were used according to manufacturer's instructions.

Immunofluorescence. Immunostaining in 2D was performed on cells plated in six-well plates (Corning) containing coverslips. Immunostaining in three-dimension was performed on cells plated in 8 well plates (BD Biosciences) as described previously by Debnath et al. (82). Cells in 2D were fixed with 3.7% paraformaldehyde for 20 min at room temperature (RT), permeabilized with 0.1% Triton X-100 for 20 min and blocked with IF buffer (130 nM NaCl, 7 mM Na_2HPO_4, 3.5 mM NaH_2PO_4, 7.7 mM NaN_3, 0.1% bovine serum albumin, 0.2% Triton X-100, 0.05% Tween-20, 10% goat serum) for 1h. Primary antibodies used in staining: PI4KIIIβ (Millipore), V5 (Sigma) GM130 (BD Biosciences), were allowed to bind overnight at 4°C. Secondary antibodies used: Alexa fluor 515 goat anti-mouse IgG, Alexa fluor 647 goat anti-rabbit IgG (1:200, 1h at RT) were all obtained from Invitrogen. Nuclei

were stained with Hoescht 33258 at 5 μg/ml (Sigma) for 20 min at RT and then cells were mounted on slides using fluorescence mounting medium (Dako). Slides were imaged with a 100X NA 1.4 oil immersion objective (Olympus) at 1 airy U on a laser-scanning confocal microscope (IX80, Olympus) with Olympus FluoView FV1000 software (Olympus).

Phosphoinositide identification. FAPP1-PH, PLCδ-PH and BTK-PH GFP-tagged reporter constructs (gift from T. Balla) were cloned into pLXSN retrovirus vector at the HpaI site. Retroviruses were created as described previously (120) with the following modifications. Briefly, Phoenix cells were transfected with 7.5 μg of reporter construct DNA. Following 2 days of virus production, the resulting retrovirus was used to infect day 1 Matrigel cultures. Each well was infected with 250 μl of retrovirus supernatant for one day. The following day, 250 μl of 2X assay media was added to the cultures and cells were allowed to propagate for an additional 2 days prior to fixation.

3.4 Results

3.4.1 PI4KIIIβ is localized to the basolateral surface of acinar cultures.

In experiments designed to identify proteins that might control 3D morphogenesis in human mammary epithelia, we observed that PI4KIIIβ accumulates at the basolateral surface in acini created by the MCF10A cell line. When grown in Matrigel suspension, basolateral localization of PI4KIIIβ protein is observed on day 5, as well at day 13 (Figure 3.1A and Movie 3.1). These time points represent mid and late stages of development. This localization is visibly different from that of MCF10A growing in monolayer culture. There, PI4KIIIβ is found diffusely throughout the cytoplasm with little to no staining in the nucleus (Figure 3.1A). While the levels of PI4KIIIβ can vary in monolayer culture depending on the stage of the cell cycle and cell density, PI4KIIIβ levels increase from day 5 to 13 as acini differentiate (Figure 3.1B). The enrichment of PI4KIIIβ at the acinar basolateral surface suggests an important role for the protein in acinar development.

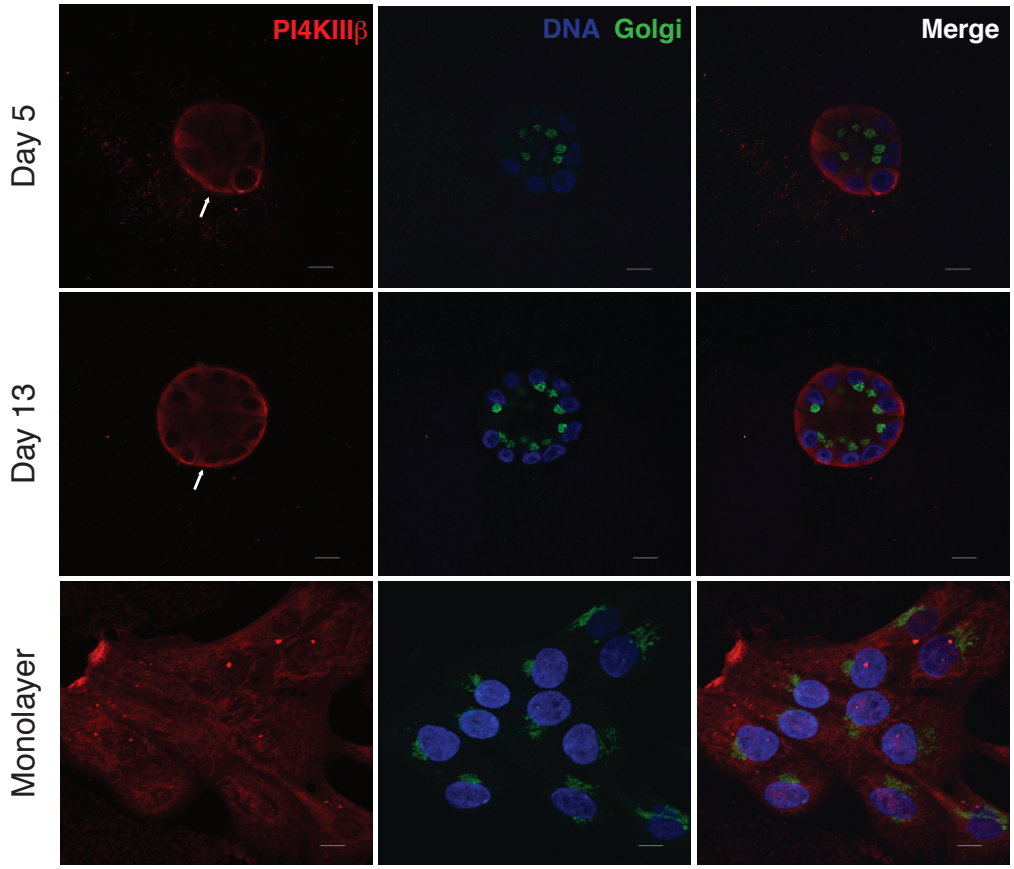
3.4.2 Ectopic expression of PI4KIIIβ leads to the formation of multi-acinar structures.

To investigate a functional role for PI4KIIIβ in acinar morphogenesis, we generated MCF10A cells that ectopically express PI4KIIIβ (Figure 3.2B). Approximately 56% of MCF10A cells ectopically expressing PI4KIIIβ form large multi-acinar structures in Matrigel and are significantly different than the hollow polarized cysts of vector controls (t-test, $p < 0.0001$) (Figure 3.2A and 3.2C). The multi-acinar structures retain a hollow lumen, although each acinus has several distinct lumen. Multi-acinar structures are a hallmark of

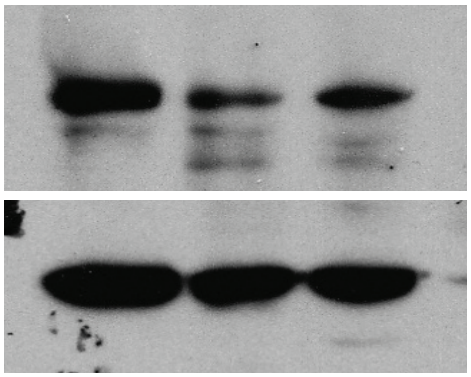
Figure 3.1: The PI4KIIIβ protein localizes to the basolateral surface of breast acini.

A. Confocal microscopy images of MCF10A acini at day 5, 13 in Matrigel and MCF10A cells in monolayer culture. PI4KIIIβ protein is shown in red and arrows indicate localization to basolateral surface of acini. The Golgi (GM130) is shown in green while DNA (Hoechst 33258) is shown in blue. Scale bar indicates 10 μm. **B.** Western blot analysis showing PI4KIIIβ protein levels in monolayer culture (2D) and at day 5 and 13 of Matrigel culture. β-actin is the loading control.

A


PI4KIIIβ
DNA Golgi
Merge
Day 5
Day 13
Monolayer

B


2D
Day 5
Day 13
PI4KIIIβ
(110 kDa)
β-actin
(37 kDa)

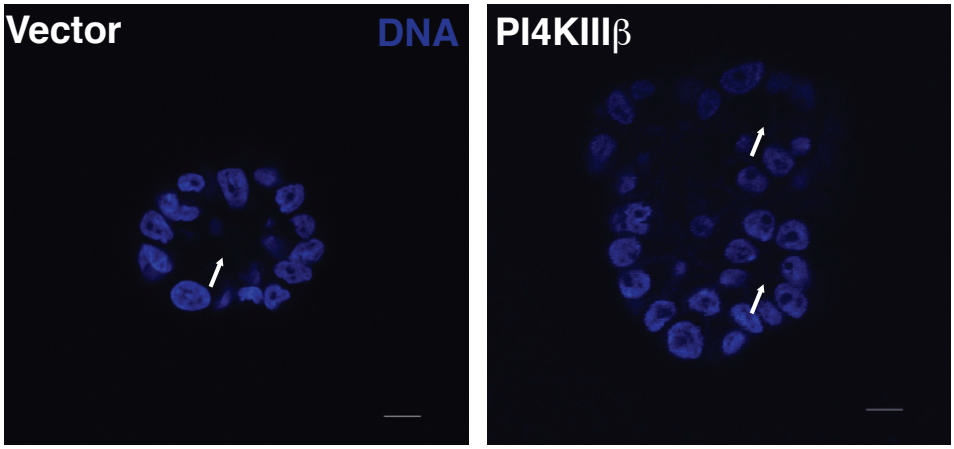
Movie 3.1: Basal localization of PI4KIIIβ in MCF10A cells.

Wild-type MCF10A cells were stained for PI4KIIIβ (red). Movie was created as a compilation of z-slices taken through an acini. Scale bar represents 10 μm.

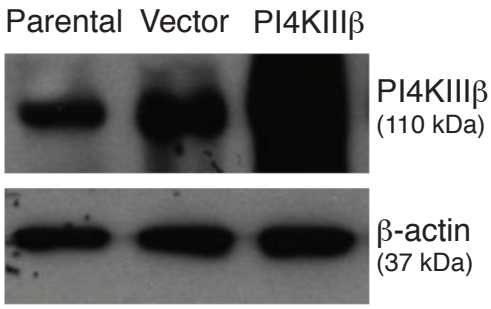
Figure 3.2: Ectopic expression of PI4KIIIβ disrupts acinar morphogenesis.

A. Confocal image of MCF10A vector and stable PI4KIIIβ expressors. DNA is shown in blue while arrows indicate lumen. Scale bar indicates 10 μm. **B.** Western blot of PI4KIIIβ expression levels in MCF10A cell lines in monolayer culture. β-actin is the loading control. **C.** Histogram quantifies the percent of cells forming multi-acinar structures at day 13, with *n* values indicated. Error bars show standard deviation and (*) indicates statistical significance ($p < 0.0001$).

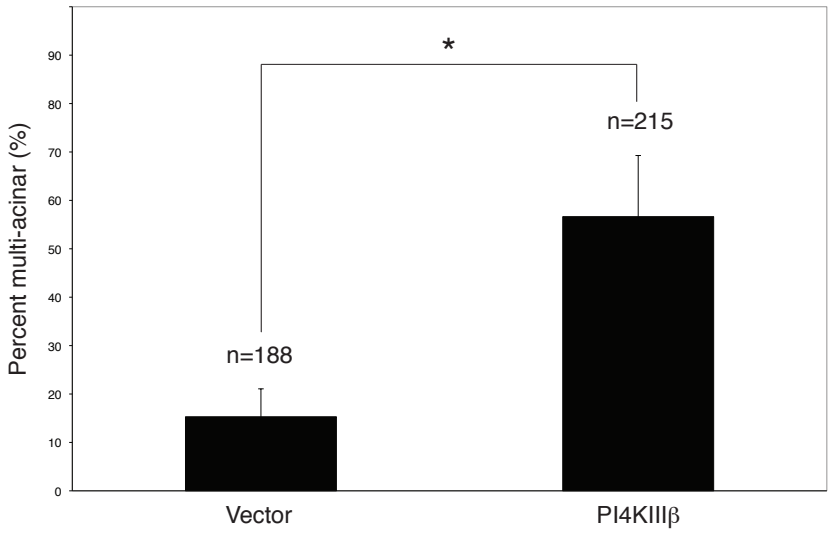
A


Vector
DNA
PI4KIIIβ

B


Parental
Vector
PI4KIIIβ
PI4KIIIβ
(110 kDa)
β-actin
(37 kDa)

C


Percent multi-acinar (%)
0
10
20
30
40
50
60
70
80
90
*
n=215
n=188
Vector
PI4KIIIβ

disruption of the morphogenesis process and are a common characteristic of multiple breast cancer oncogenes (32, 80). This suggests that PI4KIIIβ might participate in some pathways of breast oncogenesis (see below).

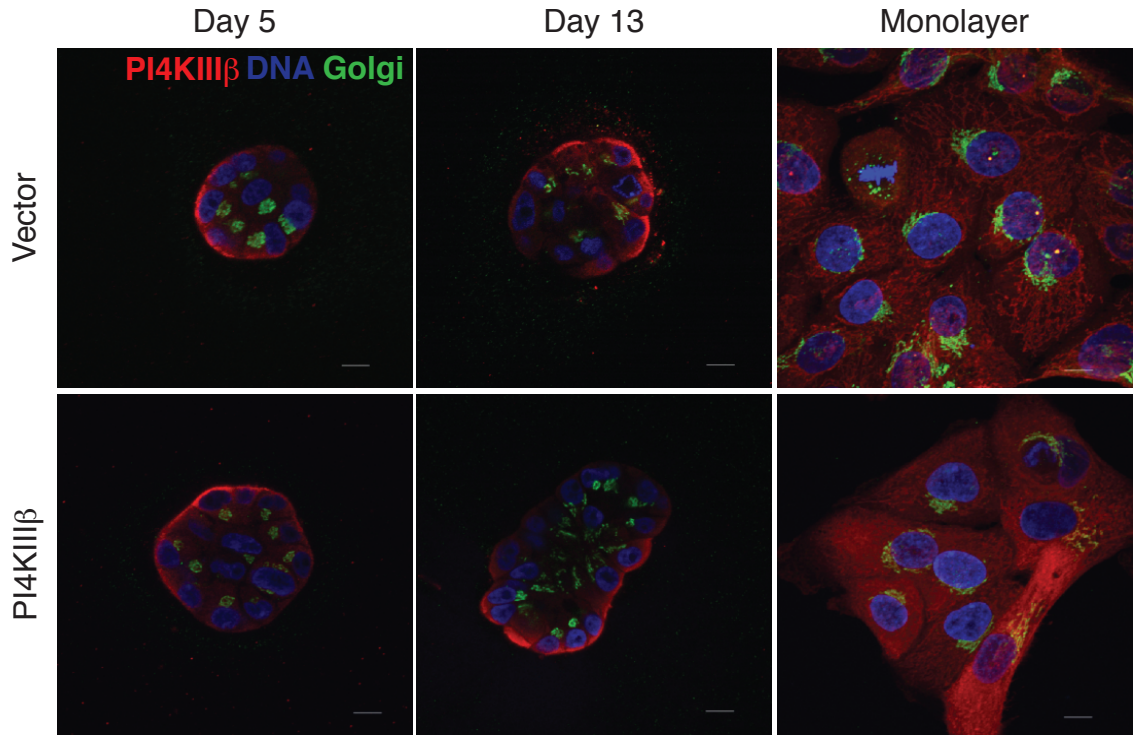
In cells with ectopic expression of PI4KIIIβ, the PI4KIIIβ protein localization is not altered and remains at the basolateral surface while the Golgi remains oriented to the apical surface (Figure 3.3A). While the levels of PI4KIIIβ can vary in monolayer culture, depending on the stage of cell cycle and cell density, the PI4KIIIβ ectopic expressors have increased levels of PI4KIIIβ when compared to wild-type and vector controls (Figure 3.2B and 3.3B). Furthermore, as the acini differentiate from day 5 to day 13 in both the vector and the PI4KIIIβ expressing cell lines, the expression of PI4KIIIβ also increases, indicating a functional role for the kinase (Figure 3.3B).

To confirm that this multi-acinar phenotype was dependent on PI4KIIIβ, we transiently decreased PI4KIIIβ expression with siRNA and then cultured the cells in Matrigel. As expected, PI4KIIIβ over-expressors reverted to more of a wild-type phenotype (Figure 3.4A). Of the PI4KIIIβ over-expressors treated with PI4KIIIβ-targeted siRNA 11% were multi-acinar, significantly less than the 75% mult-acinar phenotype observed in the control treated cells (t-test, $p < 0.0001$) (Figure 3.4B). The decrease in PI4KIIIβ protein levels is lower than controls until at least day 5 and return to levels that are comparable with controls by day 13 (Figure 3.4C). Therefore, PI4KIIIβ likely has a functional role in 3D acinar morphogenesis and increased expression disrupts normal morphogenic progression.

Figure 3.3: Localization and expression of PI4KIIIβ.

A. Localization of PI4KIIIβ is shown in red at day 5, 13 of Matrigel culture and also in monolayer (2D). Golgi is seen in green and DNA is blue. Scale bar indicates 10 μm. **B.** Western blot analysis showing PI4KIIIβ protein levels in monolayer culture and duplicate samples at day 5 and 13 of Matrigel culture for both the vector and PI4KIIIβ MCF10A cell lines. β-actin is the loading control.

A


Day 5
Day 13
Monolayer
PI4KIIIβ DNA Golgi
Vector
PI4KIIIβ

B

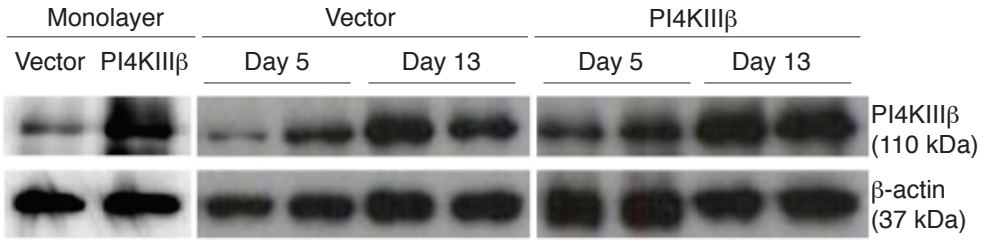
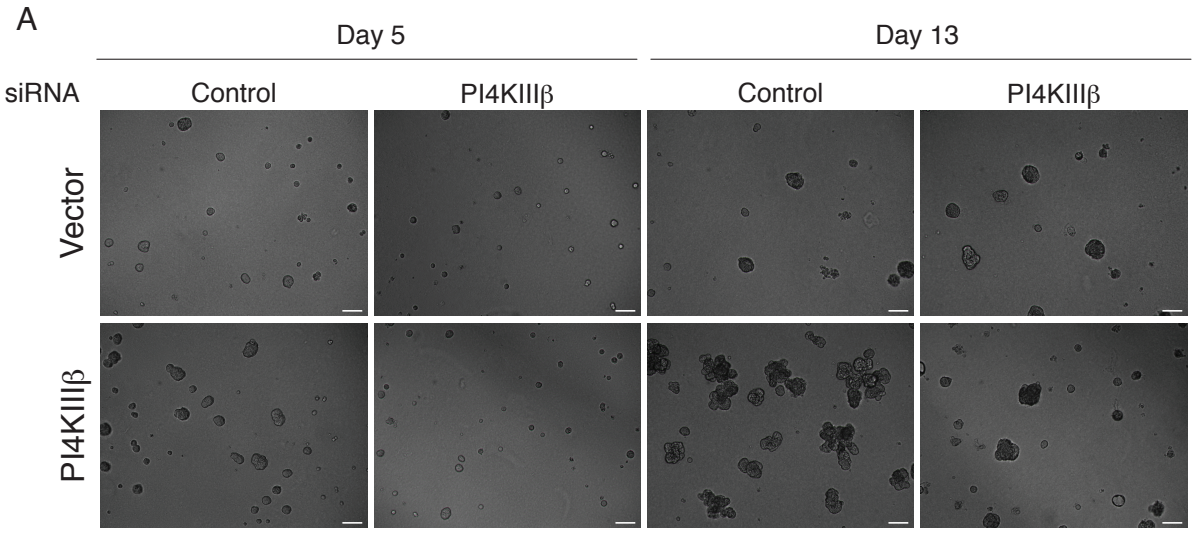
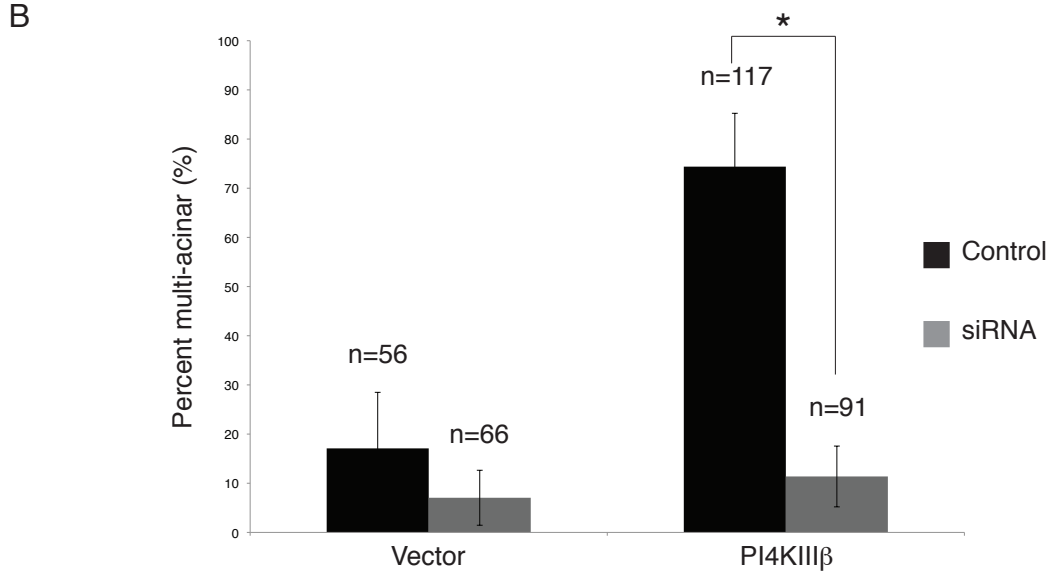
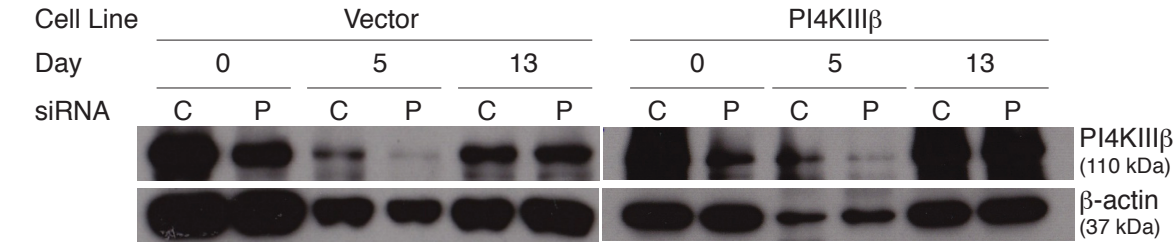

Monolayer
Vector
PI4KIIIβ
Vector
PI4KIIIβ
Day 5
Day 13
Day 5
Day 13
PI4KIIIβ
(110 kDa)
β-actin
(37 kDa)

Figure 3.4: Effect of PI4KIIIβ targeted siRNA treatment.

A. Phase contrast images of acinar cultures treated with control and PI4KIIIβ targeted siRNA. Scale bar indicates 190 μm. **B.** Multi-acinar phenotype is quantified in the histogram with *n* values indicated, error bars showing standard deviation and (*) indicates statistical significance ($p < 0.0001$). **C.** Western blots shows PI4KIIIβ and β-actin protein levels for cells treated with control siRNA (C) and PI4KIIIβ targeted siRNA (P).


A
Day 5
Day 13
siRNA
Control
PI4KIIIβ
Control
PI4KIIIβ
Vector
PI4KIIIβ


B
Percent multi-acinar (%)
100
90
80
70
60
50
40
30
20
10
0
n=56
n=66
n=117
n=91
*
Control
siRNA
Vector
PI4KIIIβ


C
Cell Line
Vector
PI4KIIIβ
Day
0
5
13
0
5
13
siRNA
C
P
C
P
C
P
C
P
C
P
C
P
PI4KIIIβ
(110 kDa)
β-actin
(37 kDa)

3.4.3 Activation of PI4KIIIβ by eEF1A2 induces the formation of multi-acinar structures.

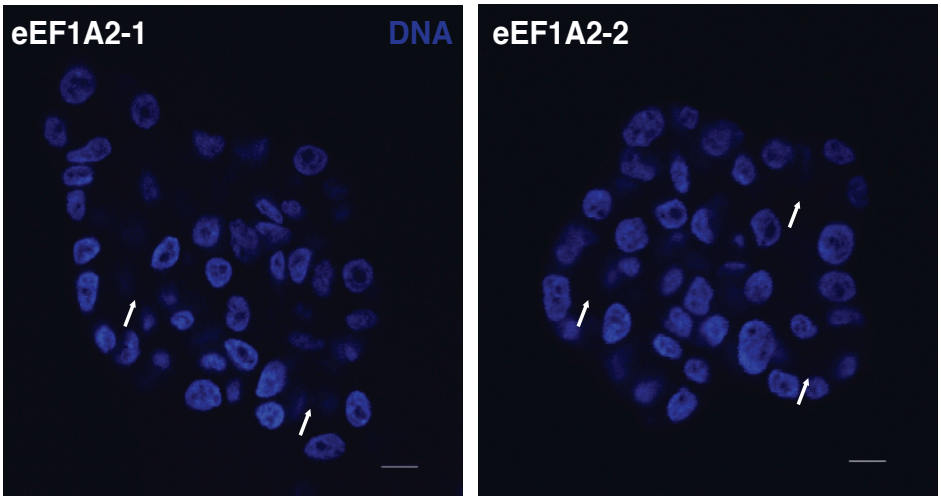
To determine whether a known biochemical activator of PI4KIIIβ could similarly affect acinar development, we examined the role of eukaryotic elongation factor, eEF1A2, in acinar formation. The best characterized function of eEF1A2 is to recruit an amino acylated tRNA to the ribosome during translation elongation but we have found that a small percentage of cellular eEF1A2 proteins bind to PI4KIIIβ with a 1:1 stoichiometry and doubles the enzyme's V_{max} (142, 144). Importantly, our laboratory has previously shown that eEF1A2 is a breast, ovarian and lung cancer oncogene based on its high expression and genetic amplification in these cancers (6, 8, 162, 240). MCF10A cells do not endogenously express eEF1A2 and we generated cell lines that ectopically express it (Figure 3.5A and 3.5B). Upon expression of eEF1A2, approximately 61% of acini were multi-acinar while the vector controls showed a significantly less 8% multi-acinar (t-test, $p < 0.0001$) (Figure 3.5C)

To confirm the interaction of eEF1A2 and PI4KIIIβ in MCF10A cells, as previous interactions were observed in the breast cancer cell line MCF7, a co-immunoprecipitation was performed and eEF1A2 and PI4KIIIβ interacted in the MCF10A cell line (Figure 3.6A). When eEF1A2 localization was examined in cells that ectopically express eEF1A2, the protein was found throughout the acini at both day 5 and day 13 in Matrigel. There was visible co-localization of PI4KIIIβ and eEF1A2 at the basal surface (Figure 3.6B). MCF10A-expressing eEF1A2, phenocopy PI4KIIIβ expression and form multi-acinar structures (Figure 3.5A). In eEF1A2-expressing cells, PI4KIIIβ is found at the basal surface from day 5 to day 13 and the Golgi remains oriented to the apical surface (Figure 3.6C).

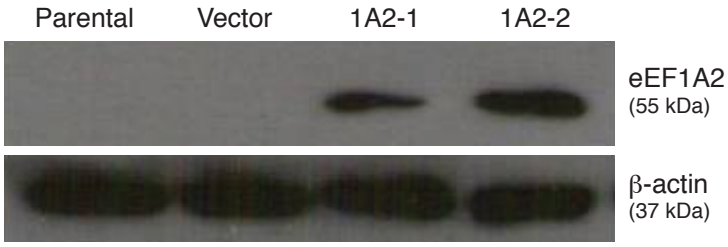
Figure 3.5: Expression of eEF1A2 induces multi-acinar structure formation.

A. Confocal images of MCF10A acini expressing eEF1A2 at day 13 with DNA shown in blue. Arrows indicate hollow lumens and scale bar represents 10 μm. **B.** eEF1A2 expression in MCF10A cells is shown by Western blot with β-actin as a loading control. **C.** Multi-acinar phenotype is quantified in the histogram with *n* values indicated, error bars represent standard deviation and (*) indicates statistical significance where $p < 0.0001$.

A


eEF1A2-1
DNA
eEF1A2-2

B


Parental
Vector
1A2-1
1A2-2
eEF1A2
(55 kDa)
β-actin
(37 kDa)

C

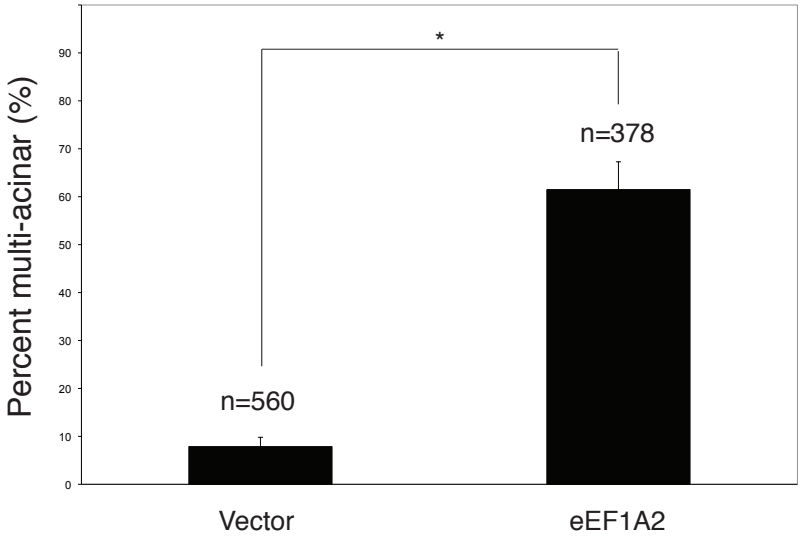
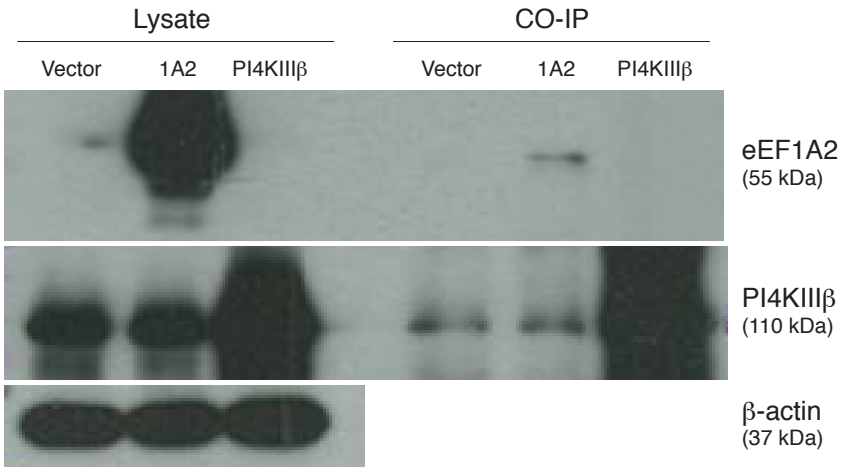

Percent multi-acinar (%)
0
10
20
30
40
50
60
70
80
90
*
n=560
n=378
Vector
eEF1A2

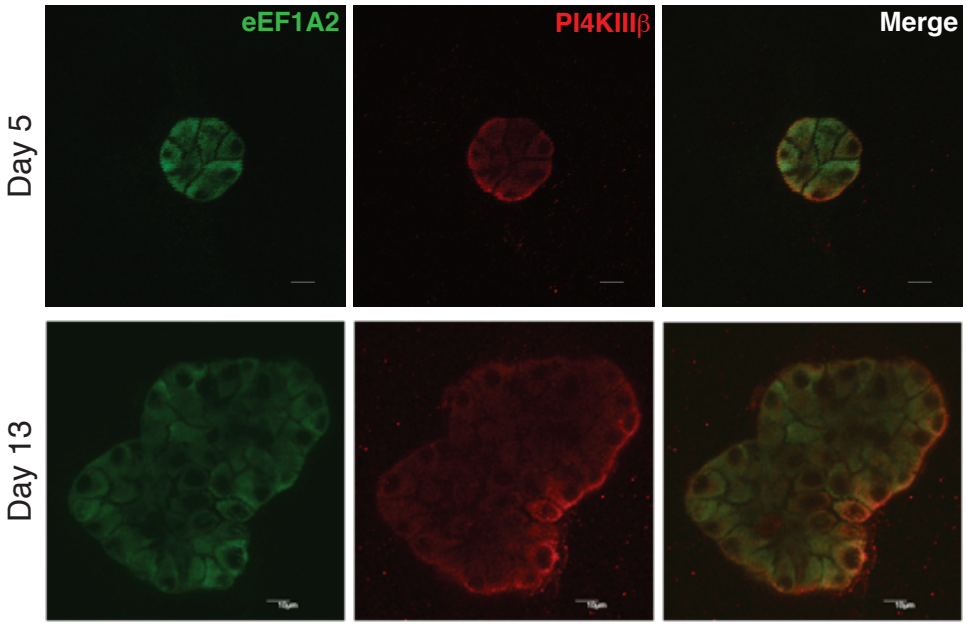
Figure 3.6: The interaction between eEF1A2 and PI4KIIIβ in MCF10A cells.

A. Western blot and co-immunoprecipitation was performed on cell lysates. Lysate lanes show PI4KIIIβ, eEF1A2 and β-actin protein levels in 20 μg of total protein when cultured in monolayer. Co-immunoprecipitation (CO-IP) was performed on membrane-free whole cell lysates of the MCF10A cell lines by using a PI4KIIIβ specific antibody. Interacting proteins were detected by Western blot analysis using PI4KIIIβ and eEF1A2 antibodies. **B.** Localization of eEF1A2 (green) and PI4KIIIβ (red) are shown at day 5 and 13 of acinar cultures. DNA is shown in blue and scale bars represent 10 μm. **C.** PI4KIIIβ (red), Golgi (green) and DNA (blue) are shown for day 5 and day 13 in three-dimension culture as well as in monolayer culture (2D).

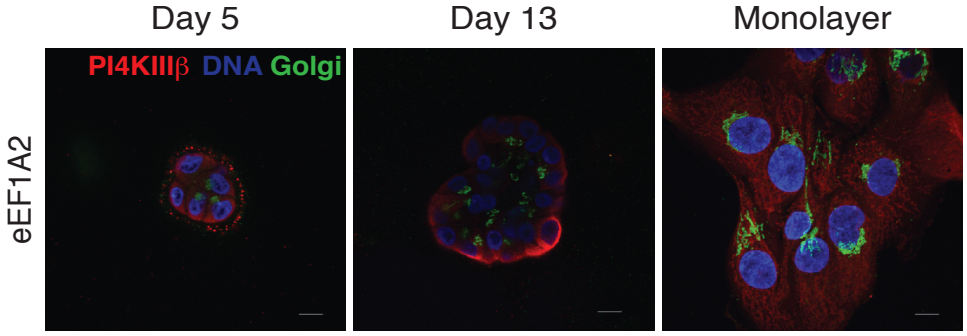
A


Lysate
CO-IP
Vector
1A2
PI4KIIIβ
Vector
1A2
PI4KIIIβ
eEF1A2
(55 kDa)
PI4KIIIβ
(110 kDa)
β-actin
(37 kDa)

B


eEF1A2
PI4KIIIβ
Merge
Day 5
Day 13

C


Day 5
Day 13
Monolayer
PI4KIIIβ DNA Golgi
eEF1A2

In order to determine if the multi-acinar phenotype displayed by eEF1A2 was dependent on its interaction with PI4KIIIβ, we transiently decreased PI4KIIIβ expression with siRNA treatment. Upon reduction of PI4KIIIβ, the eEF1A2-expressing cell lines closely resemble wild-type and control cells (Figure 3.7A and Figure 3.7B). At day 13 the eEF1A2 control siRNA cell lines showed approximately 67% multi-acinar colonies, significantly more than the PI4KIIIβ-targetted siRNA which showed 22% multi-acinar cells (t-test, $p < 0.0001$) (Figure 3.7C). The siRNA experiments indicate that the eEF1A2-dependent morphogenic disruption is mediated by its interaction with PI4KIIIβ. This suggests that PI4KIIIβ might participate in some pathways of breast oncogensis.

3.4.4 Ectopic expression of PI4KIIIβ or eEF1A2 alters the distribution of phosphoinositides.

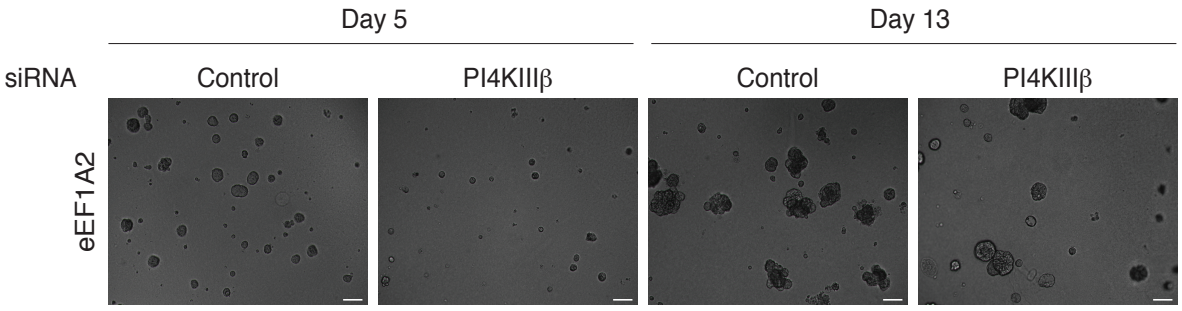
We next chose to examine how PI4KIIIβ might control acinar morphogenesis. PI4KIIIβ generates PI(4)P from PI, therefore we hypothesized that PI4KIIIβ controls acinar morphogenesis by regulating PI localization. To visualize PI moieties, we generated retroviruses that encode fluorescently tagged reporter constructs that bind to a target lipid. Retroviruses were then used to infect MCF10A cells in Matrigel. We utilized the virus due to the toxicity of long-term reporter expression and our inability to transiently transfect MCF10A cells cultured in Matrigel with plasmid-based reporters. Retrovirus reporter constructs have the same fluorescent staining profile as the original plasmid based constructs (Figure 3.8).

Initially, we examined the localization of PI(4)P with the FAPP1-PH reporter construct. Upon infection of the Matrigel cultures at day 5, FAPP1-PH was recruited to the basal surface in the parental and vector cell lines (Figure 3.9A and Movie 3.2). In the

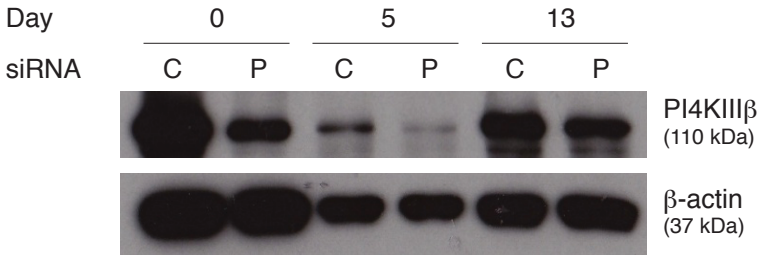
Figure 3.7: PI4KIIIβ targeted siRNA return eEF1A2 expressors to a control phenotype.

A. Phase contrast images of control and PI4KIIIβ siRNA treated acini. Scale bar represents 190 μm. **B.** Western blot analysis shows PI4KIIIβ and β-actin protein levels for the control siRNA (C) and PI4KIIIβ (P) targeted siRNA in eEF1A2-expressing cells. **C.** Histograms quantify the percent of multi-acinar structures in each condition with *n* values indicated, error bars represent standard deviation and (*) indicates statistical significance ($p < 0.0001$).

A


Day 5
Day 13
siRNA
Control
PI4KIIIβ
Control
PI4KIIIβ
eEF1A2

B


Day
0
5
13
siRNA
C
P
C
P
C
P
PI4KIIIβ
(110 kDa)
β-actin
(37 kDa)

C

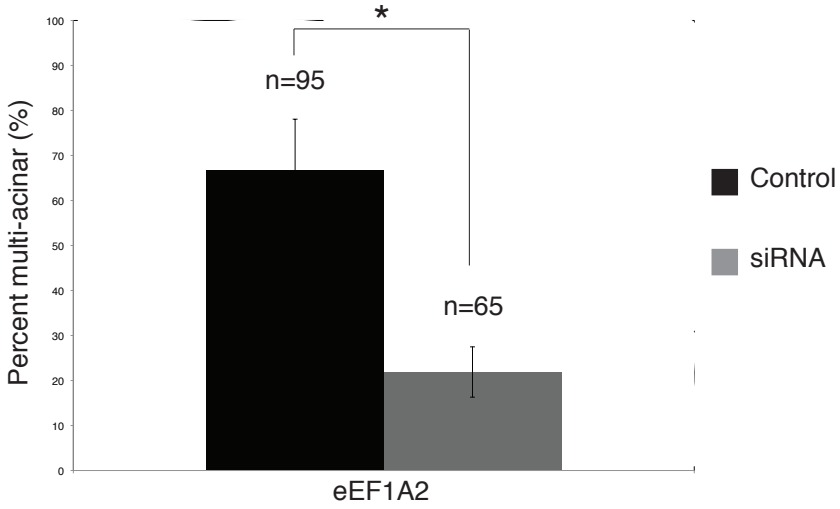
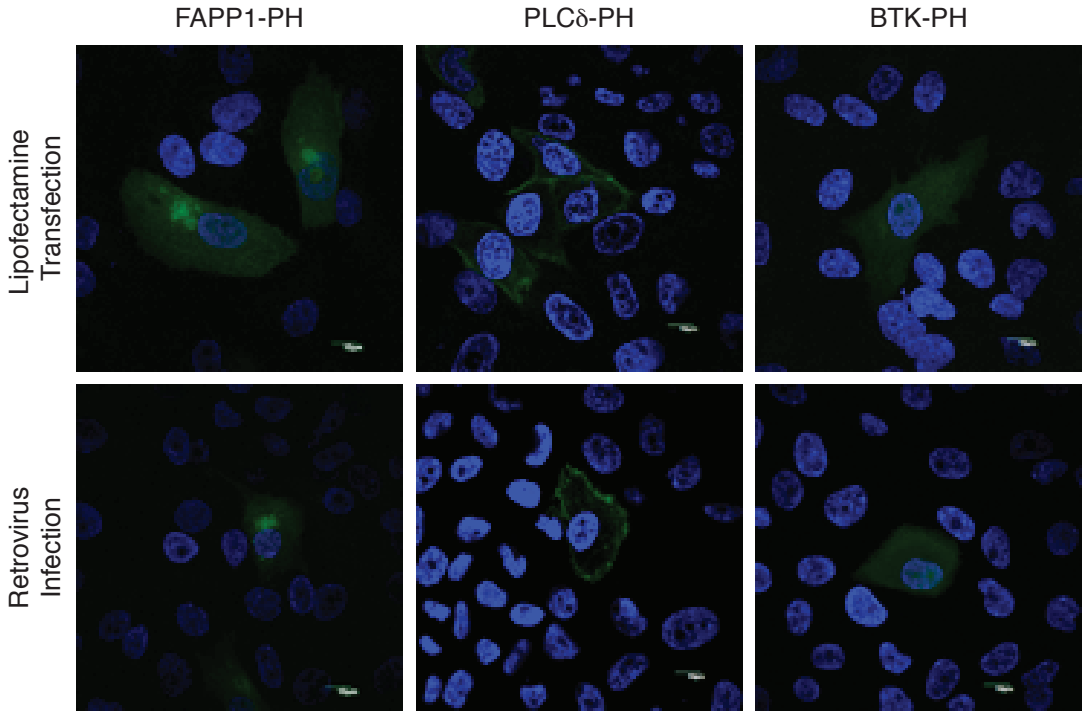

*
n=95
n=65
Percent multi-acinar (%)
100
90
80
70
60
50
40
30
20
10
0
eEF1A2
Control
siRNA

Figure 3.8: Comparison of reporter construct infection and transfection.

Left. FAPP1-PH-GFP reporter (green) was transfected into MCF10A cells while in the pEGFP vector (top) and infected from the pLXSN retroviral vector (bottom). *Middle.* PLCδ-PH-GFP reporter (green) was transfected (top) and retrovirally infected into cells (bottom). *Right.* BTK-PH-GFP reporter (green) was transfected from original vector (top) and infected with pLXSN reporter constructs (bottom). DNA is shown in blue and scale bars represent 10 μm.


FAPP1-PH
PLCδ-PH
BTK-PH
Lipofectamine Transfection
Retrovirus Infection

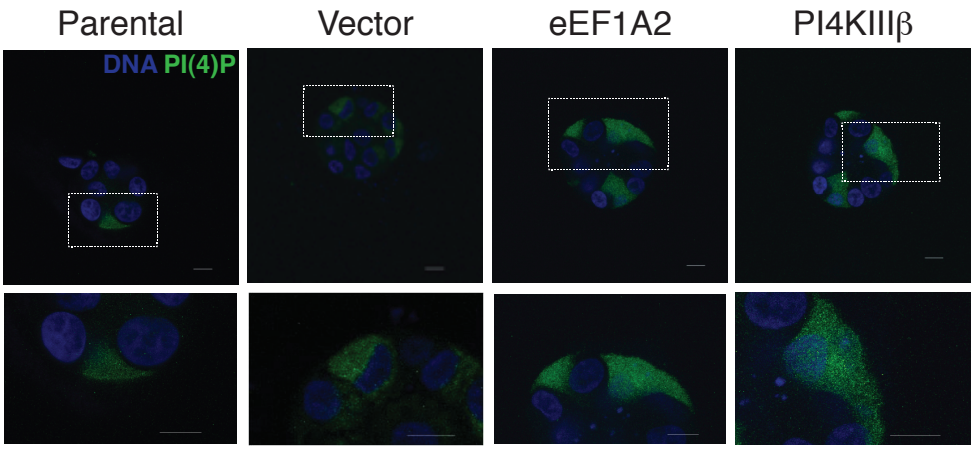
Movie 3.2: PI(4)P is localized to the basolateral surface in MCF10A parental cell lines.

MCF10A parental cells infected with the PI(4)P reporter construct FAPP1-PH-GFP. Movie is a three-dimensional rendering of a compilation of z-stacks rotated about the y-axis. Scale bar represents 10 μm.

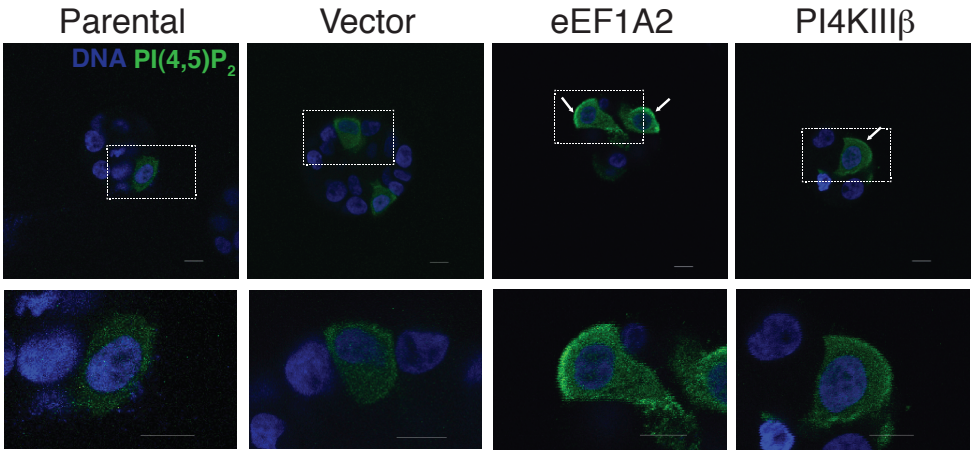
Figure 3.9: Ectopic expression or activation of PI4KIIIβ alters phosphoinositide distribution.

A. PI(4)P (green) and DNA (blue) are shown in day 5 three-dimension cultures through the use of the FAPP1-PH-GFP reporter construct. Below, areas enclosed in the boxes are shown at a higher magnification. **B.** Localization of $PI(4,5)P_2$ (green) and DNA (blue) is seen in day 5 three-dimension cultures. Increased accumulation of the PLCδ-PH-GFP domain is indicated with arrows. Below, images of enclosed area is shown at a higher magnification. **C.** Visualization of $PI(3,4,5)P_3$ (green) is seen with the BTK-PH-GFP reporter. DNA is shown in blue and removed for the lower panels. Scale bar represents 10 μm.

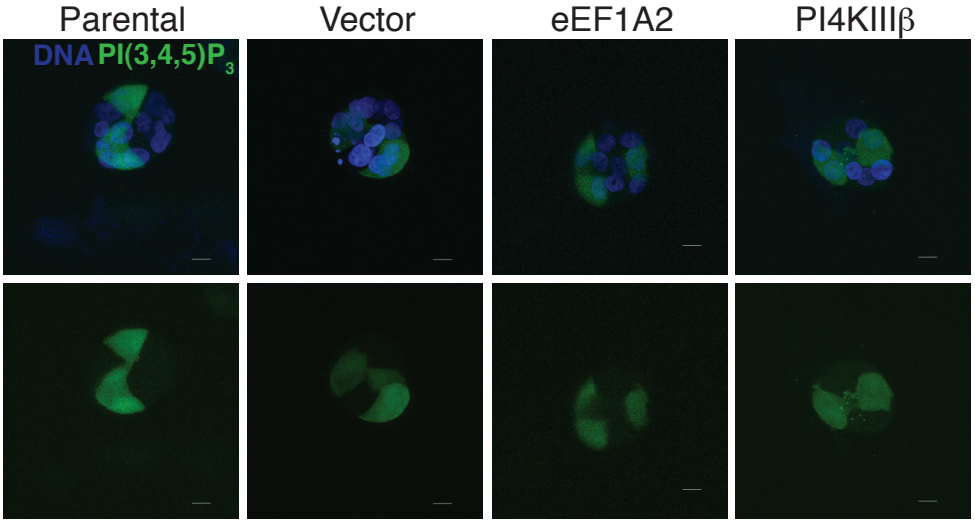
A


Parental
Vector
eEF1A2
PI4KIIIβ
DNA PI(4)P

B


Parental
Vector
eEF1A2
PI4KIIIβ
DNA PI(4,5)P2

C


Parental
Vector
eEF1A2
PI4KIIIβ
DNA PI(3,4,5)P3

PI4KIIIβ and eEF1A2 expressing cell lines we still see a recruitment of FAPP1-PH to the basal surface, however it appears to encroach further into the acini (Figure 3.9A).

We then examined the localization of $PI(4,5)P_2$ with the PLCδ-PH domain. PI4KIIIβ-expressing and eEF1A2-expressing cell lines show an enhanced recruitment to the basolateral surface that appears to delimit the edge of the basal surface, as compared to vector and parental controls (Figure 3.9B). We then examined the localization of $PI(3,4,5)P_3$ through the use of the BTK-PH reporter construct. In all of the cell lines, parental, vector, eEF1A2 or PI4KIIIβ expressing, we do not see any specific recruitment or localization of the reporter construct (Figure 3.9C). Thus, we propose a model of PI4KIIIβ control of acinar morphogenesis where PI4KIIIβ is required for basolateral surface identity through its control of PI(4)P and $PI(4,5)P_2$ localization.

3.5 Discussion

The hollow spherical structure of an acinus is a common motif from which vertebrate develop into complex organisms. In this report, we demonstrate that the lipid kinase PI4KIIIβ has a key role in regulating *in vitro* acinar formation of human breast epithelial cells. We have found that activation or over-expression of the kinase alters the architectural development of breast acini, causing multi-acinar morphogenesis. Our working model is that PI4KIIIβ regulates morphogenesis by altering the abundance and localization of PI(4)P and PI(4,5)P_2.

Importantly, MCF10A cells are an immortalized breast epithelial cell line that form spherical acini when cultured in Matrigel (80). In three-dimensional culture, MCF10A acini consist of a polarized outer layer of cells surrounding a hollow lumen. It is important to note however, that while an MCF10A acinus physically resembles a breast node, the term acinus has no anatomical connotation and *in vitro* MCF10A morphogenesis does not completely recapitulate the histological complexity of the normal breast (310).

Phosphoinositides have a well-documented role as spatial and temporal landmarks for compartmentalizing cell components (299). PI(4,5)P_2 and PI(3,4,5)P_3 have long been thought to be the primary regulators of this process. Our report is the first to suggest that PI(4)P might also be important. In breast acinar culture, we find that PI(4)P accumulates at the basal surface and is not readily detectable at the apical side. Ectopic PI4KIIIβ expression and concomitant multi-acinar development does not alter the overall basal localization of PI(4)P, but the PI(4)P pattern is visibly thicker and encroaches further towards the acinar apical surface. Upon ectopic PI4KIIIβ expression, PI(4,5)P_2 is readily detected at the basal surface. Our data is consistent with the idea that increased accumulation of basal PI(4)P,

induced by PI4KIIIβ expression, disrupts morphogenesis by causing an increase in PI(4,5)P_2 polarization there. The ability of PI4KIIIβ to affect both PI(4)P and PI(4,5)P_2 is consistent with our previous work that shows that PI4KIIIβ can control filopodia generation by co-ordinately stimulating the membrane accumulation of these two lipids (144). PI(4,5)P_2 has previously been reported to localize to the apical surface duing acinar formation in MDCK, kidney epithelial cells cultured in 3D, so it is likely that MDCK and MCF10A cells use different lipid-dependent strategies to shape acini (132, 198, 201, 202).

It is a surprise to us that PI(3,4,5)P_3 is neither substantially polarized during breast morphogenesis nor does its localization change following PI4KIIIβ expression. Previous studies in 3D culture using MDCK and HMT-3522 S-1 cells indicated that PI(3,4,5)P_3 localizes to the basolateral surface in the canine kidney and breast epithelial cells respectively. Using a BTK-PH reporter construct (107, 191, 203), we did not detect any specific basal or apical recruitment of PI(3,4,5)P_3 in wild type MCF10A cells or those with ectopic PI4KIIIβ expression. One possible explanation for the discrepancy between our work and the study using MDCK cell lines, is the use of an Akt-PH reporter construct in the MDCK work. It has recently been shown that the Akt-PH domain binds PI(3,4)P_2 as well as PI(3,4,5)P_3. On the other hand, the BTK-PH reporter is believed to bind only to PI(3,4,5)P_3 (107, 293). Moreover, the lack of specific recruitment of PI(3,4,5)P_3 during acinar morphogenesis is further consistent with reports that only cell lines lacking the PTEN phosphatase or having constitutively active PI3K display an increase in PI(3,4,5)P_3 (19, 142, 144). Overall, our work indicates that PI4KIIIβ and the lipids PI(4)P and PI(4,5)P_2 are key to acinar breast morphogenesis and that PI(3,4,5)P_3 polarization is likely to have a minor role in MCF10A cell 3D morphology.

We propose that the accumulation of PI(4)P at the basal surface results from the specific localization of PI4KIIIβ protein there throughout the acinar development. The PI4KIIIβ protein polarization in three-dimensional Matrigel culture stands in marked contrast to its diffusely cytoplasmic localization in MCF10A and other cell lines grown in monolayer culture (15, 75, 316). Moreover, in many cell types, PI4KIIIβ is found concentrated in the Golgi, whereas we find little Golgi localization in 3D acinar culture. The spatial reorganization of PI4KIIIβ localization during morphogenesis further suggests an important role for PI4KIIIβ in controlling acinar development. PI(4)P is the most abundant of the monophosphorylated PIs, and it is often regarded as simply an intermediate along the $PI(4,5)P_2$ and $PI(3,4,5)P_3$ synthesis pathway (72, 298). However, PI(4)P is a signalling intermediary in its own right and regulates process such as Golgi tubulation (17, 21). While much work has focused on the role of PI(4)P in the Golgi, closer investigation into its role has revealed a broader subcellular distribution outside of the Golgi (72). Previous work in MDCK T23 cell lines cultured in monolayer suggests an effector role for PI4KIIIβ in regulating basolateral traffic (23, 43). In this previous work, the basolateral localization of PI4KIIIβ appears to be mediated via effector molecules that interact with the kinase, rather than the kinase itself (43). It is likely that effector molecules are performing a similar role in acini. PI4KIIIβ-targeting RNAi was applied while MCF10A was in monolayer culture and achieved a decrease in PI4KIIIβ for the first five days of development in Matrigel. Protein levels return to that of control cells by day 13. When PI4KIIIβ levels were decreased in cell lines with activated or increased levels of PI4KIIIβ we saw reversion to a wild-type single acinus phenotype. Thus, proper PI4KIIIβ and PI(4)P expression and localization are required for early morphogenic progression.

The morphogenic disruption induced by ectopic expression of PI4KIIIβ is a common attribute of genes functionally involved in breast cancer. This suggests that PI4KIIIβ might participate in some pathways of breast oncogenesis. Our lab has previously shown that one PI4KIIIβ activator, eEF1A2, is a breast cancer oncogene based on its transforming properties and its amplification and overexpression in breast cancer (10, 162, 171, 240). eEF1A2, which is not expressed in normal breast epithelia, enables anchorage independent growth in mouse and human cells and enhances their tumorigenicity in xenograft models. The eEF1A2 protein binds PI4KIIIβ with a 1:1 stoichiometry and doubles the maximum velocity of the enzyme (142, 144). We hypothesize that one mechanism by which eEF1A2 is oncogenic is through PI4KIIIβ activation. Consistent with this idea, eEF1A2 expression in MCF10A phenocopies PI4KIIIβ ectopic expression in that it induces multi-acinar formation in Matrigel and induces similar changes in PI(4)P and PI(4,5)P_2 polarization. Moreover, eEF1A2-expressing cells treated with siRNA targeted to PI4KIIIβ showed a reversion to wild-type structures, indicating that eEF1A2-dependent morphogenic disruption is mediated by its interaction with PI4KIIIβ. Taken together, our results show that PI4KIIIβ likely cooperates with eEF1A2 during breast oncogenesis and increases in PI(4)P abundance may be part of the pathology of breast cancer. This suggests that specific inhibitors of PI4KIIIβ may be useful anti-cancer agents. Some small molecule PI4K inhibitors have been developed, but none, as of yet have shown clinical utility (271, 278).

In this report we used retrovirus expressing PI reporter proteins to visualize their cellular localization. To the best of our knowledge, this is the first time that retroviral reporters have been used in Matrigel culture. We utilized the virus due to the toxicity of long-term reporter expression and our inability to transiently transfect MCF10A cells

cultured in Matrigel with plasmid-based reporters. In addition, we have been unsuccessful at using antibodies specific to PIs for their detection. The use of reporters is not without its limitations since these reporters only bind to a specific PI in the context of PI-binding proteins. For example, we used the FAPP1-PH domain to detect PI(4)P, and FAPP1 recognizes plasma membrane and Golgi pools of PI(4)P in the context of Arf-1 but interestingly, PI(4)P localization at extra-Golgi sites does not require Arf1 (17, 19, 293).

In conclusion, this study indicates an important role for PI4KIIIβ in 3D morphogenesis. This alteration in morphology appears to be mediated by PI(4)P and $PI(4,5)P_2$ and we believe that PI4KIIIβ plays an important role in regulating a breast neoplastic pathway activated by the eEF1A2 oncogene.

4: DISCUSSION

4.1 Summary of thesis

The focus of my research has been on the oncogene eEF1A2 and its role in ovarian and breast cancer. The eEF1A2 protein, normally expressed only in the brain, heart and skeletal muscle, is amplified and hyper-expressed in breast and ovarian tumours (2, 6, 8, 162). More recent studies have further implicated eEF1A2 in pancreatic, hepatocellular, colon and lung carcinomas as well as multiple myeloma (51, 117, 145, 185, 186). Thus, understanding the mechanism of oncogenesis of eEF1A2 was the thread of my thesis.

As previously reported by our laboratory, eEF1A2 is an oncogene expressed in 20 – 30% of ovarian tumours (8). The first aim of my research was to determine the prognostic significance of eEF1A2 in ovarian cancer and to determine the mechanisms by which eEF1A2 might lead to oncogenesis. I have found that eEF1A2 has high expression in approximately 30% of all ovarian tumours. When divided by the histological classification of ovarian tumours, 50% of serous tumours, 30% of endometroid tumours, 19% of mucinous tumours and 8% of clear cell tumours highly express eEF1A2. In serous cancer, eEF1A2 is an independent prognostic marker for survival, and high eEF1A2 expression was associated with an increased probability of 20-year survival. Furthermore, ectopic expression of eEF1A2 in the clear cell carcinoma cell line, SK-OV-3, enhances their *in vitro* proliferative capacity, increases their ability to form tumour-like spheroids in hanging drop culture, increases stress fiber formation and causes the formation of tighter cell-cell interfaces. Therefore, eEF1A2 is highly expressed in ovarian cancers and its expression enhances *in*

vitro cell growth. Furthermore, eEF1A2 expression is likely to be a useful ovarian cancer prognostic factor for ovarian cancer patients with serous tumours.

The second aim of the thesis examined the role of eEF1A2 in *in vitro* breast morphogenesis. Previous work from our laboratory shows that eEF1A2 is highly expressed in approximately 50% of breast tumours and its expression is associated with an increased probability of 20-year survival in patients (162). Furthermore, Dr. Lee's laboratory has established that eEF1A2 binds and activates the lipid kinase, PI4KIIIβ (142, 144). I determined that both PI4KIIIβ and eEF1A2 have an important role in acinar morphogenesis. The PI4KIIIβ protein accumulates at the basal surface of acini and ectopic expression of the protein leads to the formation of multi-acinar structures. Furthermore, expression of eEF1A2 stimulates multi-acinar formation in a PI4KIIIβ-dependent fashion. The ectopic expression of PI4KIIIβ or eEF1A2 also disrupts the localization of PI(4)P and PI(4,5)P_2. This work demonstrates that PI4KIIIβ and the lipids PI(4)P and PI(4,5)P_2 are key regulators of three-dimensional *in vitro* breast morphogenesis. Furthermore, these pathways likely have a role in neoplastic development.

4.2 Factors impacting cancer prognosis

Tumours are malignant when they grow to invade surrounding tissues or when they metastasize (199). While surgeries and treatments are often effective to control cancer at the primary site, the development of metastatic disease signals poor prognosis and is the primary cause of death for most patients (157, 273). Mortality arising from the metastatic disease can result from organ damage by the growing lesion, paraneoplastic syndromes or due to

complications associated with the treatments (273). Metastasis is a multi-step process of invasion, engaging developmental programs that bring cancer cells to lymph nodes and distant organs where they establish secondary tumours (199, 311). There have been many genes associated with invasion and metastasis. While there has been much focus on protein transcription factors and regulators and their role in metastasis, the eukaryotic translation initiation factor 4E (eIF4E) is the only protein translation factor to be implicated in invasion and metastasis (251). As might be expected, expression of eIF4E is associated with poor prognosis (236).

On the other hand, eEF1A2 has been reported to activate cell migration and invasion *in vitro* and is associated with good prognosis (6, 162, 240). At first glance these findings may appear to be counter-intuitive. However, upon a large scale analysis of genes described as promoters of invasion, only a few of these genes were found to be a marker for poor prognosis (199). It is currently a challenge to explain how an increase in invasiveness is not correlated with poor prognosis. One hypothesis suggests that while these invasion promoting genes, such as eEF1A2, are sufficient to mobilize cells from the primary tumour site, it renders them too migratory to successfully colonize a secondary site (172). Furthermore, while eEF1A2 expression may cause the cell to be more mobile, in order to colonize a secondary site the migrating cells must be able to survive the shear stress of transport, resist anoikis and evade immune response (38). As eEF1A2 has not been associated with an increase in resistance to apoptosis or anoikis in ovarian cells, this could be a potential mechanism by which eEF1A2 expression may lead to increased survival.

Alternatively, eEF1A2 could affect the timing of when the cells associated with the primary tumour disseminate for metastasis. Perhaps due to the increase in invasiveness from

eEF1A2 expression, tumour cells are more likely to depart from the primary tumour prior to accumulating additional mutations. Thus, the cells that are colonizing secondary sites are not as transformed or malignant as they would be at a later time.

On the other hand, eEF1A2 may enhance prognosis by inhibiting other oncogenic process, perhaps tumour self-renewal potential or angiogenic capacity. Further investigation will be necessary to clarify the mechanism by which eEF1A2 leads to increased long term survival.

Another possible reason to explain the expression of an invasion-promoting gene lending to good prognosis may be due to the manner in which we study cancer. While it is important to study the eEF1A2 oncogene and the pathology of expression, the tumour microenvironment should also be considered. Tumours are composed of a heterogenous population of cells incorporating several different mutational events (205). Upon studying eEF1A2 independently, we find that its expression increases *in vitro* invasion. While these studies were carried out in a breast cancer cell line, and thus incorporate other breast cancer mutations, they are also studied in a monoclonal pool of cells. While this is the common method to study oncogenes, it does not necessarily represent what is occurring *in vivo*. Thus, we are studying the pathology of the oncogene *in vitro* and comparing the outcomes of prognosis through *in vivo* samples. Although *in vitro* studies are informative to the processes used by cancer cells, they do not recapitulate the complicated tumour microenvironment. While several studies have tried to examine protein co-expression in terms of prognostic significance, these studies are a large undertaking and it appears that as more oncogenes are expressed, the worse the overall survival becomes (35, 294).

Furthermore, it may be interesting to examine how the timing of an oncogene's expression may affect prognosis. This is a difficult parameter to study as the earliest stages of tumourigenesis are challenging to detect *in vivo* and require the formation of a detectable tumour to indicate a diseased state. However, the timing of oncogene expression is important. An oncogene that plays a key role in neoplasia may initiate the neoplastic program but may not be required to sustain the process (100). Additionally, an oncogene activated as a late tumourigenic event may not be as critical to the cancers progression as it would have been if activated as an initiating event (100). Therefore, the timing at which an oncogene is expressed will likely influence the neoplastic properties of the tumour. Thus, it may be advantageous to examine if there is a trend in the percentage of tumours expressing eEF1A2 based on tumour stage, to determine if there is a trend in the onset of eEF1A2 expression. Perhaps the timing of eEF1A2 expression may contribute to its role as a positive prognostic factor.

Another possible hypothesis on how the eEF1A2 oncogene may lead to an increase in survival is through an inhibition of the development of more malignant forms of cancer (172). The expression of oncogenes may prevent the development of secondary mutations by sensitizing cells to senescence, which can occur following DNA damage or the expression of a strong oncogene (172). Oncogene-induced senescence (OIS) is a robust and sustained anti-proliferative response initiated from an activating mutation of an oncogene, which is induced upon unscheduled mitogenic signalling and constitutes a maintained, long-term nonproliferative state (58). This theory is based on the fact that there are many oncogenic mutations occurring daily within our cells without giving rise to cancer, but may also be applicable to oncogenes, such as eEF1A2, associated with a positive prognosis.

Another explanation for why the expression of eEF1A2 correlates with good prognosis is perhaps due to the fact that cancerous cells may not be able to sustain the high expression of eEF1A2 and thus inactivate its expression. There are studies that indicate that the targeted inactivation of oncogenes may reverse tumourigenesis (100). This hypothesis was developed through studying the consequences of oncogene expression in *in vivo* transgenic models with mice that exhibit the conditional expression or activation of an oncogene (100). Many oncogenes, including protein transcriptions factors, Myc, signalling genes, Ras, apoptosis receptors, Met, and DNA repair and/or genomic instability, Abl and T-antigen, have been shown to induce reversible tumourigenesis in these models (100). Furthermore, tumours that are engineered to express more than one oncogene still regress upon inactivation of a single oncogene and the tumours start to regress almost immediately after inactivation (62, 99, 100). Interestingly, these results are also seen in immuno-compromised mice indicating the immune system does not play a role in the reversion of the tumour upon oncogene inactivation. The mechanism by which oncogene inactivation leads to the regression of tumours is still unknown and again there are many hypotheses as to how this happens. An interesting possibility was raised in studies with both the inactivation of Myc and Src as when either is inactivated, even for short periods of time, upon reactivation there is a sustained loss of neoplastic features (39, 141, 216). For example, upon inactivation of Myc, osteogenic sarcomas differentiate into mature osteocytes and upon reactivation of Myc, does not become neoplastic, and in fact it induces apoptosis (100, 141). It would be interesting to investigate whether eEF1A2 could lead to oncogene inactivation, implicating this as the mechanism of good long-term prognosis.

Furthermore, it is hypothesized that the inactivation of the oncogene causes a reversion to a more normal phenotype as the tumour is now cognizant of the processes it is over-activating through the expression of the oncogene (99-101, 141). As was seen in the *in vitro* morphogenesis studies presented in chapter 4, upon decreasing PI4KIIIβ expression in the PI4KIIIβ ectopic expressors, the PI4KIIIβ levels decrease to wild-type levels at the early stages and return to normal by later morphogenic stages, still maintain a normal phenotype. While the acini may simply require proper PI4KIIIβ signalling at early morphogenic stages in order to create normal shaped acini, this could also be an example of the inactivation of an oncogene leading to the restoration of a non-malignant phenotype. This is supported by studies in which the suppression of an over-expressed oncogene, in lieu of a complete inactivation, is sufficient to induce tumour suppression (99). Thus, if increased PI4KIIIβ activation through eEF1A2 expression is leading to oncogenesis, we would predict that the inactivation of eEF1A2 at later tumour developmental stages would also lead to the restoration of a normal phenotype and this would also indicate that this could be the manner in which eEF1A2 leads to good long term prognosis.

Another version of the oncogene inactivation theory by which eEF1A2 may lead to good prognosis is through oncogene addiction. Oncogene addiction is a term used to describe the observation that tumour maintenance often depends on the continued activity of certain oncogenes (196, 262). Again, if the cancerous cells are unable to sustain extended periods of eEF1A2 expression and it is inactivated, perhaps the tumour cells have become addicted to eEF1A2 signalling. Studies of human cancers has shown that while cancer cells are aneuploid and carry several genetic and epigenetic abnormalities, they may also be dependent on the activity of a single oncogene for continued proliferation and survival (303,

312). It is thought that a cancer cell becomes "addicted" to an oncogene in part due to the activation of several oncogenes and inactivation of tumour suppressors. Due to these genetic modifications, the intracellular circuits that regulate signal transduction and gene expression are significantly modified in cancer cells and thus, an oncogene may play a more essential role than it normally would (312). Furthermore, cancer cells may be more dependent on the oncogene as they are less adaptable due to several inactivated genes (312).

To date there are two classes of oncogene addiction mechanisms: tumour cell intrinsic mechanisms of cell senescence and apoptosis, and an extrinsic host-dependent mechanism that include inhibition of angiogenesis (101). Several cancer therapies have been developed based on the concept of oncogene addiction, with varying degrees of success. The drug imatinib, which binds to tryrosine kinases and prevents their activity, had a great initial response for patients with chronic myeloid leukemia but they later relapsed (209, 312). It would be worthwhile to investigate if eEF1A2 expression causes the tumours to become addicted to eEF1A2 expression. Along the lines of oncogene inactivation, it would be beneficial to examine if decreased eEF1A2 signalling in an eEF1A2-transgenic mouse model would cause a reversion of the tumourigenic phenotype.

While the oncogene addiction theory explains how the inactivation of an oncogene results in tumour and not normal cell death, it does not account for how tumour regression occurs, nor does it account for the mechanism by which an oncogene can initiate and then regress tumourigenesis. Further studies and analysis are needed to specifically elucidate how an oncogene can induce tumourigenesis and to examine the shift in pathways used after the interruption of expression and upon regression of the tumour.

Although understanding how oncogenes may lead to a good prognosis is important for the development of novel and more effective treatments, a key gap in cancer research includes the identification of the cause of oncogenic mutations (267). While it is important to understand how eEF1A2 causes oncogenesis, it would be beneficial to understand what is causing the protein, which is normally only expressed in brain, heart and skeletal muscles, to be expressed in the breast and ovary.

Interestingly, eEF1A2 is highly expressed in approximately 50% of serous tumours, while only 8% of clear cell tumours highly express eEF1A2. Clear cell tumours are the second most common ovarian epithelial cancer and are associated with the poorest prognosis of all the ovarian cancer sub-types (11, 49, 179). While clear cell carcinomas are associated with poor prognosis, 67.3% are discovered in early stages compared to serous tumour types where only 19.2% are discovered at early stages (57). However, the survival rates for patients with serous cancer is greater than those with clear cell carcinoma when survival at each of the four stages of cancer is independently compared (57). It is important to note however, that eEF1A2 is not a good prognostic marker based on the histological type of tumour it is expressed in as it identified good prognosis solely within the serous tumour subtype.

Furthermore, the fact that there is a disparity in the prognostic significance of eEF1A2 between ovary, breast and lung cancer implies that eEF1A2 is having markedly different biological effects in the three distinct tissues. This indicates that the microenvironment, development and pathology of cancer are different in each of these tissue types. The expression of eEF1A2 in breast cancer is associated with good prognosis, expression in ovarian cancer is associated with good prognosis in serous tumour types and

does not have a co-relation in any other ovarian tumour type and is associated with poor prognosis in lung cancer (162, 185, 213, 240). When an oncogene is expressed in different tissues and tumour types it also undertakes different roles and partakes in different signalling pathways (136, 228).

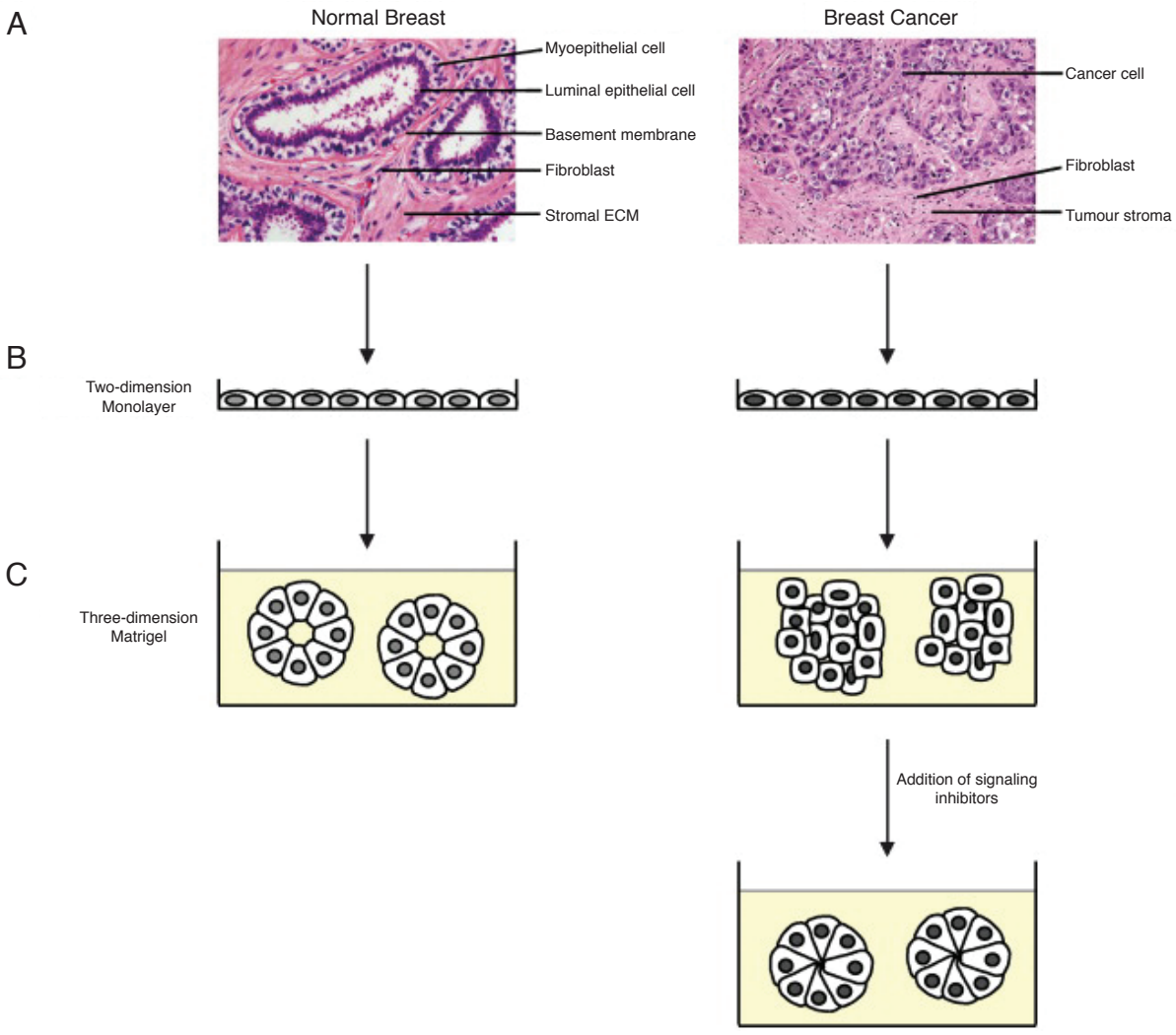
4.3 Morphogenesis and Malignancy

The most visible feature of cancer is the disruption of normal tissue architecture. As mentioned previously, there are six steps that are required for cells to become malignant: 1) enhanced proliferative potential, 2) decreased sensitivity to apoptosis, 3) autonomous production of growth signals, 4) insensitivity to growth inhibition signals, 5) angiogenic potential and 6) migratory and invasive capability (126, 263). Intriguingly, these are properties are also associated with normal cells at some point during their life cycle. While important to the progression of cancer within a cell, these are not properties of the tissue itself, and tissues are where cancer arises, even if it originates from a single cell (244). Furthermore, while the above-mentioned characteristics apply to the individual cell, they do not address one of the most visible aspects of cancer: the permanent and increasing loss of tissue microarchitecture, which is affected by the relationship between cells (31, 243, 244). As such, it is important to study cancer within the context of the tissue.

As previously mentioned, placing cells in culture disrupts tissue architecture and culture in three-dimension on a reconstituted basement membrane allows for the growth of structures that resemble their *in vivo* phenotype (Figure 4.1). There have been specific morphogen-like molecules identified in this system that support the epithelial morphology and microarchitecture (102, 243). Normally, tissues maintain a consistent microarchitecture

Figure 4.1: The advantage of studying cancer in a three-dimensional model.

The normal and malignant breast. (A). Hematoxylin and eosin staining of tissue sections from a normal adult breast and an invasive ductal carcinoma. The normal mammary gland has an organized structure of acini having a central lumen lined by an inner layer of epithelial cells and an outer layer of myoepithelial cells. Breast carcinoma lose the organization of the tissue. (B). Both mammary cell types form indistinctive monolayers when plated in conventional two-dimension culture. (C). Upon plating in three-dimension on a laminin-rich basement membrane, such as Matrigel, normal mammary cells form spherical acini with a hollow lumen. Breast cancer cells form disorganized tumour-like structures. Upon normalizing aberrant signaling pathways in three-dimensional culture, disorganized tumour structures can be reverted to a near-normal morphology. Adapted from (310).


A
Normal Breast
Breast Cancer
Myoepithelial cell
Luminal epithelial cell
Basement membrane
Fibroblast
Stromal ECM
Cancer cell
Fibroblast
Tumour stroma
B
Two-dimension
Monolayer
C
Three-dimension
Matrigel
Addition of signaling
inhibitors

which is disrupted upon neoplasia. It is hypothesized that tissues maintain their microarchitecture through the use of morphostats. A morphostat is thought to maintain tissue microarchitecture in a manner analgous to the role of a morphogen in morphogenesis (244). The morphostat theory is related to the microenvironment and is based on embryogenesis observations (34, 301). Morphogenic fields organize tissues in the embryo, morphostatic fields maintain normal cell behaviour and microarchitecture in the adult (301). Accordingly, cancer occurs frequently when morphostatic influences fail or at the junction of two morphostatic fields (243, 244, 301). For example, cancers occur frequently at the junctions of different epithelial cell types, such as at squamocolumnar junctions in the gastrointestinal and genital tracts (245). A common method of cancer progression is through the precursor of focal proliferative lesions. As research continues it has become apparent that these lesions are dependent on environmental cues from surrounding tissues and alterations in the tissue microenvironment can foster selective growth of focal lesions (166). The maintenance of microarchitecture requires physical conditions, regeneration and repair of malignancy associated structures, and endogenous and exogenous signals (244). Thus, it is important to understand how competing morphostatic fields lead to the generation of cancer.

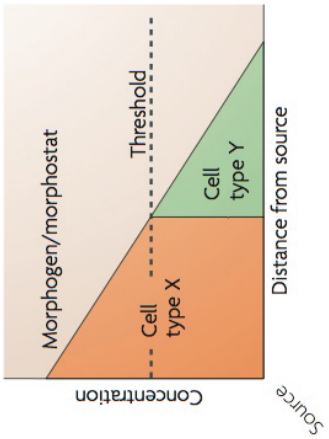
The morphostat hypothesis of morphogen-like molecules as regulators of adult tissue integrity is relatively novel with relatively few molecules explored for maintaining tissue microarchitecture (244). A likely candidate for a morphostat is the Wnt protein. The absence of Wnt results in the destruction of β-catenin, and the presence of Wnt leads to the formation of a stable non-phosphoryalted β-catenin that translocates to the nucleus and regulates gene expression (225, 244). Wnt signalling is primarily associated with

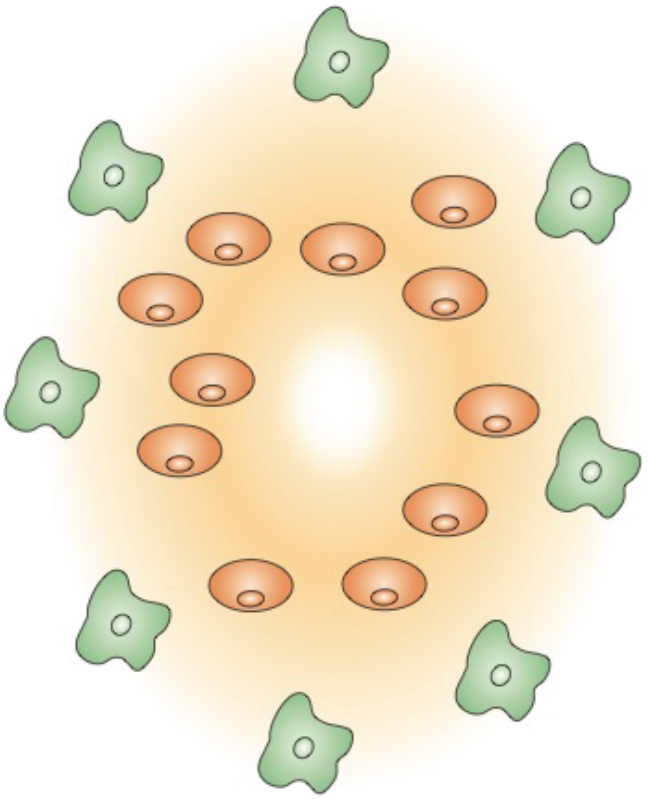
embryogenesis but it also maintains adult tissue structure (66). For example, Wnt maintains proliferation and differentiation in adult epithelial cells of the intestine, hair follicle and bone (244, 254). Furthermore, a disruption of Wnt signalling is also associated with cancer of the colon, stomach, ovary and breast, making it a prime candidate as a morphostat (27, 36, 52, 73, 194, 215).

I hypothesize that the PI4KIIIβ effector PI(4)P functions as a morphostat. The disruption seen in three-dimensional culture upon the expression of eEF1A2, which activates PI4KIIIβ, or the ectopic expression of PI4KIIIβ itself, indicates the potential for PI(4)P to be viewed as a morphostat. In three-dimensional culture of MCF10A, we see PI4KIIIβ localized to the basal surface of acini. Upon increased expression or activity of PI4KIIIβ multi-acinar structures are created. Due to the alteration of PI4KIIIβ levels, which leads to an alteration in PI(4)P levels, this could cause a disruption in the normal concentration gradient seen in wild-type cells (Figure 4.2). When the concentration is under a certain threshold the cell will have a type "Y" and upon the concentration of the morphostat exceeding that threshold concentration, the cell will become type "X". Under normal conditions, I propose that the expression of PI4KIIIβ at the basal surface maintains the polarized structure. If the hypothesis holds true, upon activation with eEF1A2 or ectopic expression of PI4KIIIβ, the increase of the PI(4)P levels to exceed threshold levels to maintain this specific cell type, and lead to the transformation of another variant cell type that can promote tumourigenesis. While this alteration may not alter the cell lineage within the tissue, it alters the structural organization of the cells within the tissue. If this in fact what is occurring in eEF1A2-mediated tumourigenesis, this indicates that the eEF1A2 oncogene activates PI4KIIIβ and

Figure 4.2: Morphogens and morphostats in regulating development and differentiation.

Diffusion gradients provide cells with information. The position of a cell in a morphogenic gradient determines gene activity and path to differentiation. At a higher concentration cells assume one phenotype (orange cells). As the concentration drops below a threshold, a second phenotype emerges (green cells). Adapted from (244).


Morphogen/morphostat
Threshold
Cell type Y
Cell type X
Distance from source
Concentration
Source



alters the environment of the cell which potentially influences the gene activity or differentiation of the cell.

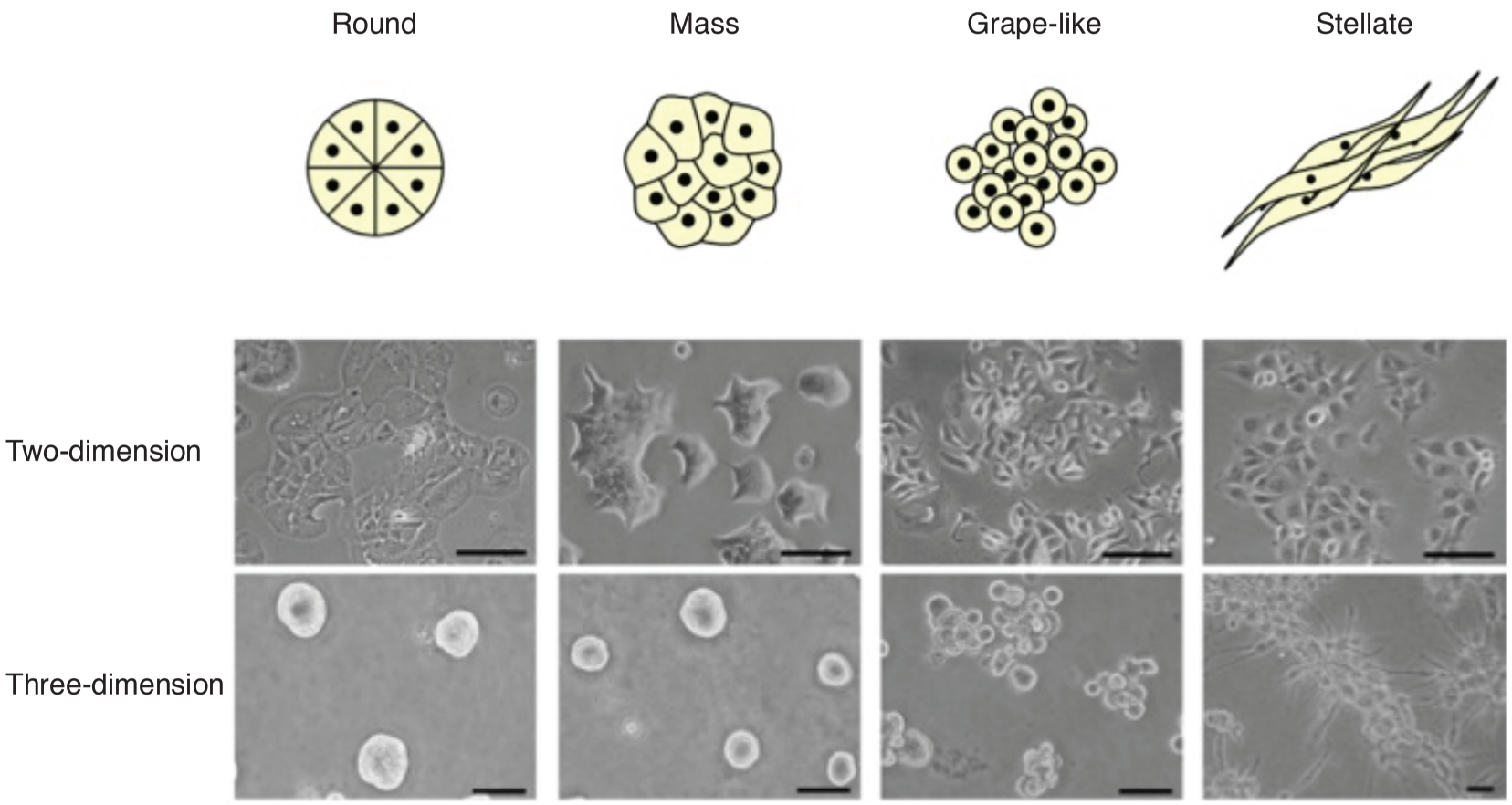
Furthermore, the role of PI(4)P as a morphostat may account for the variability associated with the prognostic value of eEF1A2 depending on the tumour type. As expression of the eEF1A2 oncogene may have a positive or negative prognostic outcome depending on the tissue it is expressed in due to the different morphogenic and morphostatic gradients that occur in these tumour types.

Interestingly, a more recent large scale study indicates that the morphological architecture that is exhibited by breast cancer cells can be attributed to underlying gene expression patterns and functions (310). They grouped the different three-dimensional morphologies of breast cancer cell lines into four classes: Round, Mass, Stellate and Grape-like (Figure 4.3) (151, 310). The cell lines that exhibited the Stellate morphology in three-dimension also lacked E-cadherin and the Grape-like morphology have elevated levels of HER2 (151, 310). This suggests that the three-dimensional morphology displayed by the cell may be indicative of the pathways used by the cancer cells.

The phenotype displayed by MCF10A cells ectopically expressing eEF1A2 or PI4KIIIβ appears to be Grape-like in lieu of Mass due to the fact that the Mass phenotype has disorganized nuclei and usually filled colony centers (151). The Grape-like morphologies, in addition to being associated with elevated levels of HER2, displayed high levels of ErbB2 but were variable in the expression level of estrogen receptor, E-cadherin and phosphorylated Akt (151). Consistent with this data, our laboratory has shown that eEF1A2 expression leads to an increase in phosphorylated Akt (6). However, our laboratory has previously shown that eEF1A2 expression in breast cancer tumours is independent of

Figure 4.3: The four classes of breast cell morphologies in three-dimensional culture.

Upon examination of 25 breast cell lines cultured in three-dimension they were grouped in to four distinct catagories: Round, Mass, Grape-like and Stellate. (Top). A schematic representation of the morphology of each category of morphology. (Middle). Images representative of each category when cultured in two-dimension. (Bottom). Representative images of each category when cultured in three-dimension. Adapted from (151, 310).


Round
Mass
Grape-like
Stellate
Two-dimension
Three-dimension

HER2 and estrogen receptor status (162). Furthermore, in this study the breast cancer cell line MCF7, a breast cancer cell line endogenously expressing eEF1A2, was found to display a Mass phenotype and not the Grape-like morphology seen in the three-dimensional culture of MCF10A cells expressing eEF1A2 (151). This indicates that while the morphology of a breast cancer cell line cultured in three-dimension may provide insight as to the biological pathways occurring in the cell, it is not a definite indicator. Furthermore, the heterogeneity of genes abnormally expressed in breast cancer cells may be too variable for this principle to apply to such a wide range of breast cancer types. Importantly though, microarray studies of polarized cells compared to non-polarized cells revealed that genes down-regulated in organized, growth arrested acini had prognostic value and could be used to discriminate between patients outcome (103), indicating the potential for elucidating important pathways in malignancy through three-dimensional studies.

The architecture formed through the three-dimensional culture of cells appears to have an effect on the behaviours of cells. Normal and malignant breast cells displayed a relatively similar sensitivity to agents inducing apoptosis when cultured in two-dimension (308). Upon culturing in three-dimension, the malignant cells were sensitive to apoptosis while acini formed by non-malignant cells were resistant (308). Furthermore, the eEF1A2-expressing MCF7 breast cancer cell line are less responsive to tamoxifen when cultured in three-dimensions, the cells cultured in two-dimension showed a 50% growth reduction while those cultured in three-dimension only displayed a 25% decrease (87). Based on these findings, it indicates that studies relating to drug responses would benefit to be undertaken in three-dimension. It would be beneficial to revisit the work presented in chapter 2 of this thesis to examine the effect of eEF1A2 on apoptosis in three-dimensions. Due to the fact

that eEF1A2-expressing MCF10A cells form multi-acinar structures that still retain hollow lumens, it appears as though eEF1A2 does not alter apoptotic pathways. However there may be a difference based on it being examined in a different tissue type and although it does not inhibit the apoptosis program of the acini it may render the cells more sensitive to apoptosis-inducing agents.

4.4 The role of the extracellular matrix in malignancy

The cell secreted extra-cellular matrix (ECM) is composed of large molecules, including collagens, fibronectin and laminins, and polysaccharides (4, 111). The ECM was initially viewed as a physical scaffolding to provide mechanical support to the cells and tissues. As research continued it became apparent that the ECM regulated cell shape, proliferation, polarity, differentiation, transcription, synthesis and secretion in a variety of cell types (30, 111). With the ECM regulating so many cell processes, changes in the ECM itself or how cells internalize signals from the ECM can disrupt tissue organization and promote malignancy (111). Whether changes in tissue architecture precedes and/or select for malignant cells or genomic alterations occur first remains unknown, however it is believed there is a dynamic reciprocity between the cell and the ECM, where the ECM influences gene expression in the cell which in turn can remodel the ECM (111).

There is some evidence that disruption in the composition of the ECM can precede tumour formation or trigger genomic alterations (111). Studies have shown that collagen VII, a component of the ECM, is necessary for tumourigenesis of Ras-transformed keratinocytes in a model of squamous cell carcinoma (111, 229). Furthermore, overexpression of MMP-3 stimulates the formation of reactive stroma, characterized by the deposition of collagen I prior to tumour formation, which causes genomic instability in

mammary epithelial cells and stimulates the epithelial to mesenchymal transition (249, 281). Thus, morphogenesis is not only controlled by a genetic program within a cell, but is the result of interaction and feedback between the environment, the constraints of the ECM and the developing epithelium itself (110, 244). While targeting the microenvironment of the tumour is a relatively new concept, there are several drugs in clinical trials that target the microenvironment (31). Hopefully with the new attention being paid to the tumour microenvironment novel mechanisms of cancer may be elucidated. Furthermore, this may be a key aspect in the pathogenesis of cancers where the understanding of disease progression has gaps. Efforts to establish the cellular mechanisms that drive eEF1A2's abnormal expression in breast and ovarian tissues has not yet yielded possible activators. Perhaps it is through signals from the surrounding tissues that leads to the activation of eEF1A2 expression. The activation of eEF1A2 by the ECM will then trigger a tumourigenic response through activation of PI4KIIIβ and increased generation of PI(4)P.

4.5 Phosphoinositides and Cancer

The morphogenic disruption induced by ectopic expression of PI4KIIIβ is a common attribute of genes functionally involved in breast cancer. This suggests that PI4KIIIβ might participate in some pathways of breast oncogenesis. Our results show that PI4KIIIβ likely cooperates with eEF1A2 during breast oncogenesis and increases in PI(4)P abundance may be part of the pathology of breast cancer. PI(4)P is the most abundant of the monophosphorylated PIs and it is often regarded as simply an intermediate along the $PI(4,5)P_2$ and $PI(3,4,5)P_3$ synthesis pathway (72, 298). However, PI(4)P is a signalling

intermediary in its own right and regulates process such as Golgi tubulation (17, 21). To the best of our knowledge, this is the first report implicating a PI4K isoform and PI(4)P in oncogenesis.

While PI4K has not previously been implicated in cancer, other PIs have been extensively studied in neoplastic transformation. $PI(4,5)P_2$ can be phosphorylated by PI3K to generate $PI(3,4,5)P_3$ (88). The phosphorylation by PI3K leads to the recruitment of Akt to the cell membrane, where it becomes activated (76, 317). The PI3K/Akt pathway is activated in cancers by several different mechanisms and it's signalling is crucial in cancer as it promotes cell growth and survival (98, 255, 317). Furthermore, the PTEN phosphatase, which degrades the phosphoinositide products of PI3K is frequently lost in many different cancer types (183, 192, 317).

Additionally, breast cancer metastasis suppressor 1 (BRMS1) was found to affect the cellular levels of $PI(4,5)P_2$. Expression of BRSM1, in breast cancer cell lines deficient in the protein, led to a dramatic reduction of $PI(4,5)P_2$ levels while not affecting its localization (86). Conversely, the expression of PI4KIIIβ in breast acinar studies lead to an increase in $PI(4,5)P_2$ recruitment to the basal surface. It is suggested that the metastasis suppression activity of BRSM1 is due to its regulation of phosphoinositide signalling (86). The hydrolysis of $PI(4,5)P_2$ by phospholipase C (PLC) is also associated with cancer. Enhanced signalling of PLC has been implicated in upregulated cell motility and metastasis (61, 237).

Since eEF1A2 can increase PI4KIIIβ activity and also leads to the formation of multi-acinar structures, it is likely that increasing PI4KIIIβ lipid kinase activity itself has transformative effects. This suggests that PI4KIIIβ might participate in some pathways of breast oncogenesis and that specific inhibitors of PI4KIIIβ may be useful anti-cancer agents.

Some small molecule PI4K inhibitors have been developed, but none, as of yet have shown clinical utility (271, 278). With relevance to breast cancer, the decrease of PI4KIIIβ, through RNAi experiments in the MDA-MB-231 breast cancer cell line, lead to an increase in apoptosis indicating that PI4KIIIβ may play a role in anti-apoptotic signalling and cell survival (64). However, it is not clear how much excess PI(4)P is required for morphogenic acinar disruption or how much a reduction in PI(4)P is necessary for activating apoptosis. This issue is likely to be complex since PI(4)P and other PIs are found in multiple intracellular locations (15, 16).

The PI4KIIIβ gene maps to 1q21, a locus whose amplification has been reported in multiple myeloma, liposarcomas and mucinous adenocarcinoma (47, 105, 291). However, no PI4KIIIβ gene amplification has been reported for breast or ovarian cancer. Since eEF1A2 is overexpressed in at least 50% of human breast tumours (162), PI4KIIIβ amplification may not be required for oncogenic progression in tumours with elevated eEF1A2 expression as they already have increased PI4KIIIβ activity. In the future, it would be important to investigate whether or not PI4KIIIβ overexpression is sufficient for tumour development in xenograft and transgenic models of cancer.

As previously mentioned, this is the first report to implicate PI4K and PI(4)P in cancer. However, changes in the concentrations of phosphoinositides affect cellular processes and phosphoinositide species target proteins to specific subcellular compartments with importance to the control of membrane traffic and cell movement (45, 46, 88). Furthermore, the decreasing PI4KIIIβ, through RNAi experiments, indicate that PI4KIIIβ may play a role in anti-apoptotic signalling and cell survival (64). Additionally, given that PI4K can supply phosphoinositide substrates for PLC and PI3K and is associated with

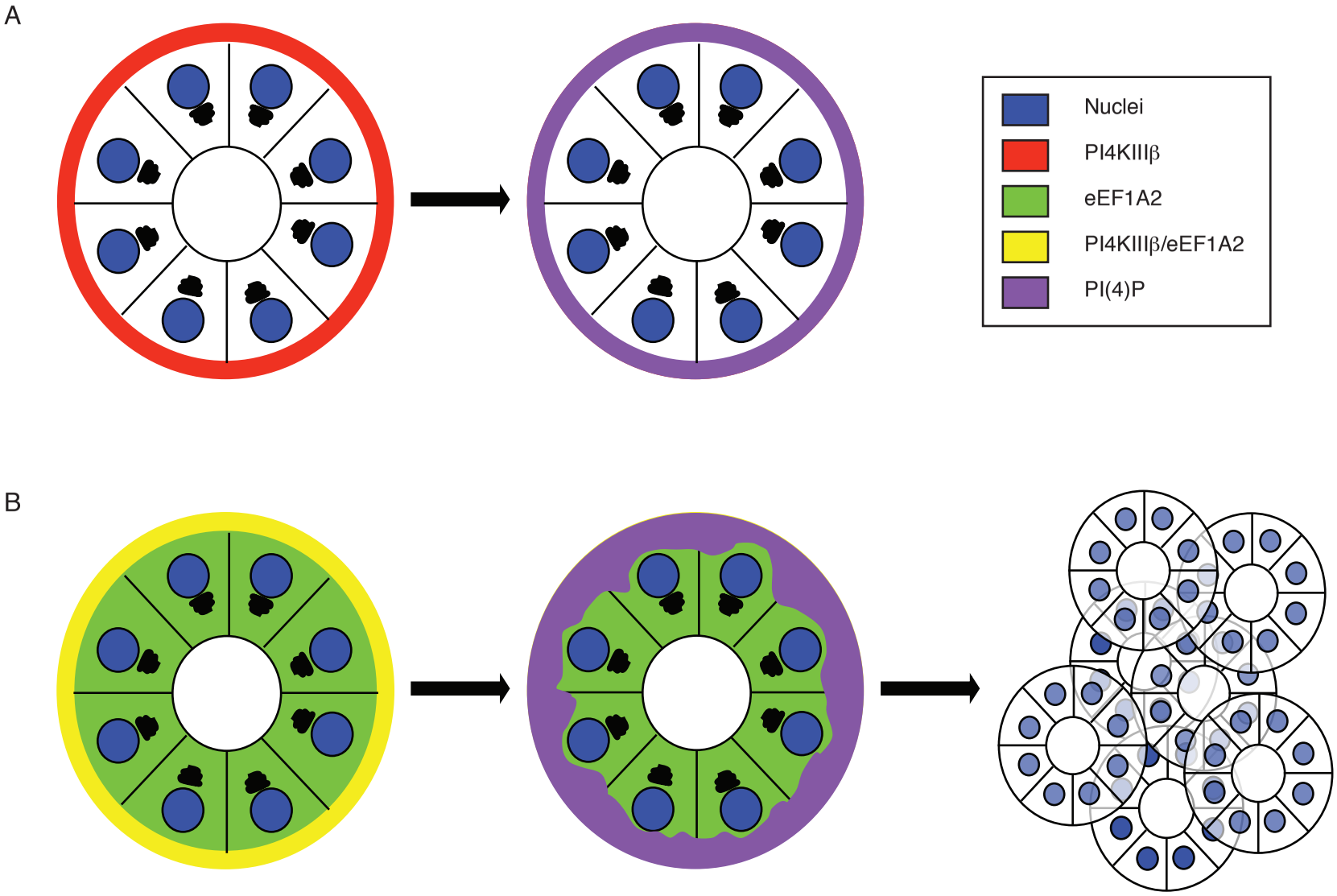
increased cell survival it makes for an interesting and novel target for cancer therapeutics. As research continues to discover the roles of PI4K within the cell more implications for the kinase in cancer may arise.

4.6 Model of eEF1A2 and PI4KIIIβ induced multi-acinar phenotype

In breast acinar culture, we find that PI4KIIIβ localizes to the basal surface, leading to the accumulation of PI(4)P at the basal surface. Expression of eEF1A2, or ectopic PI4KIIIβ expression, does not alter the overall basal localization of PI(4)P, but the PI(4)P pattern is visibly thicker and encroaches further towards the acinar apical surface. Upon ectopic eEF1A2 or PI4KIIIβ expression, $PI(4,5)P_2$ is readily detected at the basal surface. Our data is consistent with the idea that increased accumulation of basal PI(4)P, induced by PI4KIIIβ expression or activation, disrupts morphogenesis by causing an increase in $PI(4,5)P_2$ polarization there, leading to the formation of multi-acinar structures (Figure 4.4). The ability of PI4KIIIβ to affect both PI(4)P and $PI(4,5)P_2$ is consistent with our laboratory's previous work that shows that PI4KIIIβ can control filopodia generation by co-ordinately stimulating the membrane accumulation of these two lipids (144). $PI(4,5)P_2$ has previously been reported to localize to the apical surface duing acinar formation in MDCK, kidney epithelial cells, cultured in 3D, so it is likely that MDCK and MCF10A cells use different lipid-dependent strategies to shape acini (132, 198, 201, 202). While the RNAi studies presented indicate that proper PI4KIIIβ signalling is required at early morphogenic stages, it may also present PI4KIIIβ as a potential morphostat. Furthermore, the RNAi experiments may also implicate PI4KIIIβ in the oncogene inactivation theory, as the reduction of

Figure 4.4: Potential model for eEF1A2 and PI4KIIIβ regulated alteration of breast morphogenesis.

The acini and multi-acinar phenotypes. (A). Normal acini are spherical with a polarized outer layer of cells and a hollow lumen. PI4KIIIβ is shown in red at the basal surface which then generated PI(4)P (purple) at the basal surface. (B). In acini expressing eEF1A2 (green) this increases the activation of PI4KIIIβ where the two co-localize, shown in yellow. The increased PI4KIIIβ activation alters the PI(4)P (purple). While PI(4)P is still localized to the basal surface it appears to encroach further into the acini. These alterations lead to the generation of multi-acinar structures.


A
B
Nuclei
PI4KIIIβ
eEF1A2
PI4KIIIβ/eEF1A2
PI(4)P

PI4KIIIβ causes are return to a wild-type phenotype even though PI4KIIIβ expression is only reduced for a short period of time.

4.7 Future Directions

While this work presents novel roles for eEF1A2, PI4KIIIβ and phosphoinositide signalling in cancer, it also raises more questions. A priority for eEF1A2-induced oncogenesis should be to elucidate the mechanism of expression in tumours and in normal tissue. If the mechanism by which eEF1A2 is abnormally expressed in tissues which leads to oncogenesis could be elucidated, it would provide a novel manner in which to target the cancer. Along those lines, it would also be beneficial to determine if eEF1A2 expression is associated with certain timing or stages of cancer. It would be practical to review the tumour core samples in chapter two to determine if there is a correlation with tumour stage and eEF1A2 expression. Furthermore, it would be valuable to investigate if eEF1A2 is a potential therapeutic target.

Another interesting aspect of cancer is demonstrated in chapter three, where the role of the three-dimensional architecture was shown to affect the localization of proteins and lipids. It would be advantageous to create a three-dimensional culture model that is representative of the ovarian microenvironment *in vivo*, comparable to the three-dimensional culture models created for breast and kidney epithelial cells. Upon the creation of a three-dimensional culture system, it would then be informative to re-examine the effect of eEF1A2 on apoptosis and anoikis. As there were significant differences in survival between two-dimension and three-dimension when studying the same oncogenes, there may be an effect of eEF1A2 on survival that is not detectable or noticeable in two-dimension. If a three-dimensional culture system to represent the ovarian environment could not be generated, it

may be worthwhile to examine the effect of eEF1A2 expression on survival in the three-dimensional breast morphogenesis model already established.

In chapter 3, it appears that the ectopic expression of eEF1A2 or PI4KIIIβ leads to an alteration in PI(4)P and PI(4,5)P_2. The ectopic expression of eEF1A2 or PI4KIIIβ leads to the localization of PI(4)P to the basal surface but it appears to encroach further into the acini than the wild-type and control cells exhibit. Furthermore, the eEF1A2 and PI4KIIIβ expressors exhibit an enhanced recruitment of PI(4,5)P_2 reporter construct to the basal surface, which is not detectable in control cells. It would be worthwhile to examine if the expression of eEF1A2 and PI4KIIIβ is increasing the overall levels of these lipids or if it is simply altering their localization. Attempts were made to quantify PI(4)P and PI(4,5)P_2 levels, however the current quantification techniques are not sensitive enough to reliably detect changes in phospholipid levels on such a small scale. Upon the improvement of lipid detection assays this empirical question would be informative in elucidating the mechanism of disrupted morphogenesis.

Furthermore, due to the reversion of the multi-acinar phenotype upon treatment with PI4KIIIβ-targetted RNAi, experiments investigating the effect of the RNAi treatment on the phosphoinositide distribution and localization would further our understanding of the role these phospholipids play in morphogenesis.

As previously mentioned, PI4KIIIβ, or other PI4Ks, have not been implicated in cancer. However, there are downstream effectors of the kinase that have been implicated in cancer. The role of PLC hydrolysis in PI4KIIIβ-induced multi-aciniar structures should be examined through three-dimensional morphogenesis studies. Futhermore, the role of PI4KIIIβ on PI3K/Akt activation could further provide mechanisms of oncogenesis. In

order to advance and develop the hypothesis that PI4KIIIβ is involved in breast cancer, experiments examining the ability for cells ectopically expressing of PI4KIIIβ to become tumourigenic in mice should be performed.

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CONTRIBUTIONS OF COLLABORATORS

This thesis was written by me, Dixie Pinke, with editing done by Dr. Jonathan Lee and Anne Morrow. The work presented in Chapter 2 was done by myself, with the exception of the tissue microarray which was performed by Dr. David Huntsman's laboratory and the statistical analysis of the prognostic significance of eEF1A2 in serous cancer was performed by Steve Kalloger. Furthermore, the multi-cellular spheroid experiment and the cytotoxicity of cisplatin and taxol were performed by Tania Francetic and Nisha Anand respectively. All of the work presented in Chapter Three was performed by myself. I would like to thank Heidi McBride for the use of her confocal microscope. I would also like to thank Tamas Balla and Nadine Wiper-Bergeron for various plasmid constructs. I am thankful to Anne Morrow, Sandy Szeto, Sahir Sahik, Barbara Vanderhyden, Joan Brugge and Keith Mostov for helpful discussions and critical reviews of manuscripts.

APPENDIX A: CD-ROM DATA

The CD-ROM, attached, forms a part of this work.

Data file can be opened with programs that play movie files (.avi).

APPENDIX B

PUBLISHED PAPERS

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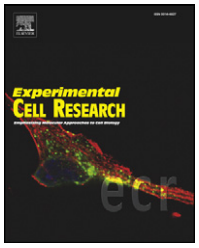

Research Article

The lipid kinase PI4KIIIβ and the eEF1A2 oncogene co-operate to disrupt three-dimensional in vitro acinar morphogenesis☆

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ABSTRACT

The study of in vitro morphogenesis is fundamental to understanding neoplasia since the dysregulation of morphogenic pathways that create multi-cellular organisms is a common hallmark of oncogenesis. The in vitro culture of human breast epithelial cells on reconstituted basement membranes recapitulate some features of in vivo breast development, including the formation of a three-dimensional structure termed an acinus. Importantly, the capacity to disrupt in vitro acinar morphogenesis is a common property of human breast oncogenes. In this report, we find that phosphatidylinositol 4-kinase IIIβ (PI4KIIIβ), a lipid kinase that phosphorylates phosphatidylinositol (PI) to PI(4)P, disrupts in vitro mammary acinar formation. The PI4KIIIβ protein localizes to the basal surface of acini created by human MCF10A cells and ectopic expression of PI4KIIIβ induces multi-acinar devlopment. Furthermore, expression of an oncogenic PI4KIIIβ activator, eEF1A2 (eukaryotic elongation factor 1 alpha 2), phenocopies the PI4KIIIβ multi-acinar phenotype. Ectopic expression of PI4KIIIβ or eEF1A2 alters the localization of PI(4)P and PI(4,5)P_2 within acini, indicating the importance of these lipids in acinar development. Our work shows that PI4KIIIβ, eEF1A2 and PI(4)P likely play an important role in mammary neoplasia and acinar development.

Introduction

PI4KIIIβ is one of four isoforms of the PI4K family (PI4KIIα, PI4KIIβ, PI4KIIIα, PI4KIIIβ). These kinases selectively catalyze the production of PI(4)P from PI. The four isoforms of PI4K differ primarily in their intracellular localization. In many eukaryotic cell types, PI4KIIIβ, a cytoplasmic protein, localizes to the Golgi and regulates Golgi-to-plasma membrane trafficking [1]. However, PI4KIIIβ protein can also be found in non-Golgi locations, indicating functional importance at other intracellular locations. PI(4)P is a signaling moiety and regulates vesicular trafficking through its interaction with ADP ribosylation factor (Arf) 1 and four-adaptor-proteins (FAPP) 1 and 2 [2,3]. PI(4)P pools have also been visualized to accumulate in cytoplasmic vesicles and filamentous structures [4]. Importantly, PI(4)P is a synthetic precursor for both PI(4,5)P_2 and PI(3,4,5)P_3[1]. While PI(4)P is the most abundant of the phosphorylated PIs, its localized abundance can affect the accumulation of PI(4,5)P_2. For example, we have previously shown that PI4KIIIβ can control filopodia generation by co-ordinately stimulating the membrane accumulation of both PI(4)P and PI(4,5)P_2[5].

Due to the importance of PI(4,5)P_2 and PI(3,4,5)P_3 in controlling in vitro morphogenesis in three-dimensional canine kidney cell morphogenesis [6–8], we investigated whether

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PI4KIIIβ and PI(4)P might be involved in regulating the formation of three-dimensional structures in vitro. To this end, we utilized the established MCF10A breast acinus system [9,10]. MCF10A are an immortalized, non-transformed human breast epithelial cell line that form a spherical structure, termed an acinus, when cultured on Matrigel. MCF10A acini recapitulate some features of breast node development in vivo in that they have a hollow lumen and show apico-basal polarization [9,11–16]. MCF10A acinar morphogenesis has particular relevance to breast cancer in that genes that have a causal role in breast oncogenesis disrupt in vitro MCF10A morphogenesis. The activation of oncogenic pathways in three-dimensional culture disrupts the specialized cell–cell contacts by enhancing proliferation and increasing cell size, as well the individual cells within the structure can vary in size and shape [9,17,18]. Thus, Matrigel culture of MCF10A cells is an important system for studying breast oncogene function in a three dimensional context [19].

In this report, we find that PI4KIIIβ expression disrupts acinar morphogenesis. The PI4KIIIβ protein specifically accumulates at the basal surface and its ectopic expression induces multi-acinar formation in MCF10A cells. Moreover, an oncogenic activator of PI4KIIIβ, translation elongation factor eEF1A2, stimulates multi-acinar formation in a manner dependent on PI4KIIIβ. Expression of eEF1A2 or PI4KIIIβ disrupts the polarization of PI(4)P and PI(4,5)P_2. Our work shows that dysregulation of PI4KIIIβ, and therefore PI(4)P, leads to the formation of multi-acinar structures. We believe that PI4KIIIβ plays a role in regulating a neoplastic pathway through eEF1A2.

Materials and methods

Cell culture

MCF10A cells were purchased from the American Type Culture Collection (Manassas, VA) and maintained at 37 °C, 5% CO_2 in DMEM/F12 (Invitrogen) supplemented with 5% horse serum (Invitrogen); 20 ng/ml EGF (Peprotech); 0.5 μg/ml hydrocortisone (Sigma); 100 ng/ml cholera toxin (Sigma); 10 μg/ml insulin (Sigma); and 1% antibiotic antimycotic (Invitrogen). PI4KIIIβ-expressing and eEF1A2-expressing stable cell lines were generated using a retrovirus as described by Debnath et al. [10]. The PI4KIIIβ-pLXSN vector is described by Jeganathan et al. [5] and eEF1A2-pLXSN by Amiri et al. [20]. Cells were selected with 300 μg/ml G418 (Bioshop).

Morphogenesis assays

The three-dimensional culture of MCF10A cells is described by Debnath et al. [10]. Briefly, cells were cultured on 100% Matrigel (BD Biosciences) in assay media (DMEM/F12 (Invitrogen)) supplemented with 2% horse serum (Invitrogen); 5 ng/ml EGF; 10 μg/ml insulin (Sigma); 100 ng/ml cholera toxin (Sigma); 1% antibiotic antimycotic (Invitrogen); and 2% Matrigel (BD Biosciences).

siRNA and transfections

PI4KIIIβ siRNA sequence (5′-GGAGGUGUUGGA-GAAAGUCtt-3′) (catalog no. AM51331) and negative control siRNA (catalog no. AM4611) were both obtained from Ambion. siRNA transfections were performed with Lipofectamine RNAiMAX (Invitrogen) according to manufacturer's instructions. Cells were transfected with siRNA in 2D and the following day were washed, trypsinized and re-plated in Matrigel for morphogenesis assays.

Western blotting and immunoprecipitation

Cells were lysed using radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris–Cl, pH 7.4; 1% Triton X-100; 1% sodium deoxycholate; 0.1% sodium dodecyl sulfate (SDS); 1 mM ethylenediaminetetracetic acid (EDTA), pH 7.0; 150 mM NaCl; 1% aprotonin; 1 mg/ml leupeptin; 50 mM NaF; 1 mM Na_3VO_4; 10 mg/ml pepstatin in ethanol; 1 mM phenylmethylsulfonyl fluoride (PMSF) in dimethylsulfoxide (DMSO)). Protein was quantified using Bradford protein assay (Biorad) according to manufacturer's instructions. Approximately 10 μg of protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore). Anti-V5-horseradish peroxide (HRP) (Invitrogen), PI4KIIIβ (BD Biosciences), and β-actin (Sigma) antibodies were used according to manufacturer's instructions.

Immunofluorescence

Immunostaining in 2D was performed on cells plated in six-well plates (Corning) containing coverslips. Immunostaining in three-dimension was performed on cells plated in 8 well plates (BD Biosciences) as described previously by Debnath et al. [10]. Cells in 2D were fixed with 3.7% paraformaldehyde for 20 min at room temperature (RT), permeabilized with 0.1% Triton X-100 for 20 min and blocked with IF buffer (130 nM NaCl, 7 mM Na_2HPO_4, 3.5 mM NaH_2PO_4, 7.7 mM NaN_3, 0.1% bovine serum albumin, 0.2% Triton X-100, 0.05% Tween-20, 10% goat serum) for 1 h. Primary antibodies used in staining: PI4KIIIβ (Millipore), V5 (Sigma) GM130 (BD Biosciences), were allowed to bind overnight at 4 °C. Secondary antibodies used: Alexa fluor 515 goat anti-mouse IgG, Alexa fluor 647 goat anti-rabbit IgG (1:200, 1 h at RT) were all obtained from Invitrogen. Nuclei were stained with Hoescht 33258 at 5 μg/ml (Sigma) for 20 min at RT and then cells were mounted on slides using fluorescence mounting medium (Dako). Slides were imaged with a 100X NA 1.4 oil immersion objective (Olympus) at 1 airy U on a laser-scanning confocal microscope (IX80, Olympus) with Olympus FluoView FV1000 software (Olympus).

Phosphoinositide identification

FAPP1-PH, PLCδ-PH and BTK-PH GFP-tagged reporter constructs (gift from T. Balla) were cloned into pLXSN retrovirus vector at the HpaI site. Retroviruses were created as described previously [21] with the following modifications. Briefly, Phoenix cells were transfected with 7.5 μg of reporter construct DNA. Following 2 days of virus production, the resulting retrovirus was used to infect day 1 Matrigel cultures. Each well was infected with 250 μl of retrovirus supernatant for 1 day. The following day, 250 μl of 2X assay media was added to the cultures, cells were allowed to propagate for an additional 2 days prior to fixation.

Results and discussion

PI4KIIIβ is localized to the basal surface of acinar cultures

We used the mammary epithelial cell line MCF10A to study mammary oncogenesis in vitro. MCF10A cells are an immortalized breast epithelial cell line that form spherical acini when cultured in Matrigel [9]. In three-dimensional culture, MCF10A acini consist of a polarized outer layer of cells surrounding a hollow lumen. It is important to note however, that while an MCF10A acinus physically resembles a breast node, the term acinus has no anatomical connotation and in vitro MCF10A morphogenesis does not completely recapitulate the histological complexity of normal breast [22]. However, many breast cancer oncogenes disrupt MCF10A acinar development. When MCF10A are grown in Matrigel suspension, we observed that the PI4KIIIβ protein has a basal localization on day 5, as well at day 13 (Fig. 1A and Movie 1). These time points represent mid and late stages of acinar development. This localization is visibly different from that of MCF10A growing in monolayer culture where PI4KIIIβ is found diffusely throughout the cytoplasm with little to no staining in the nucleus (Fig. 1A). In many cell lines, PI4KIIIβ has been reported to be localized to the Golgi, but we detect no apparent enrichment there in monolayer culture. In Matrigel culture, the Golgi orient towards the apical side of the acini, and as with monolayer culture, there is no striking Golgi localization of PI4KIIIβ. The PI4KIIIβ protein polarization in three-dimensional Matrigel culture stands in marked contrast to its diffusely cytoplasmic localization in MCF10A and other cell lines grown in monolayer culture [1,23,24]. While this is the first report of PI4KIIIβ to be found localized to the basal surface in three-dimensions, previous monolayer studies in Madin–Darby canine kidney (MDCK) T23 cell lines, which is a polarized epithelial cell line, suggests an effector role for PI4KIIIβ in regulating basolateral traffic [25,26]. The basolateral localization of PI4KIIIβ appears to be mediated via effector molecules that interact with the kinase, rather than the kinase itself [26]. The spatial reorganization of PI4KIIIβ localization during acinar morphogenesis suggests an important role for PI4KIIIβ in acinar development.

Ectopic expression of PI4KIIIβ disrupts acinar morphogenesis

To investigate a functional role for PI4KIIIβ in neoplasia, we generated MCF10A cells that ectopically express PI4KIIIβ (Fig. 2A). MCF10A cells expressing PI4KIIIβ form large multi-acinar structures in Matrigel and are visibly different than the hollow polarized cysts of vector controls (Fig. 2A). Approximately 56% of PI4KIIIβ expressors are multi-acinar, significantly higher than controls (*t*-test, $p<0.0001$). The multi-acinar structures retain a hollow lumen, although each acinus has several distinct lumen. Multi-acinar structures are a hallmark of disruption of normal morphogenic processes and are a common characteristic of breast cancer oncogenes [9,13]. This suggests that PI4KIIIβ might participate in some pathways of breast oncogenesis (see below). In PI4KIIIβ cells, protein localization is not altered and remains at the basal surface while the Golgi remains oriented to the apical surface (Fig. 2B). While the levels of PI4KIIIβ can vary in monolayer culture depending

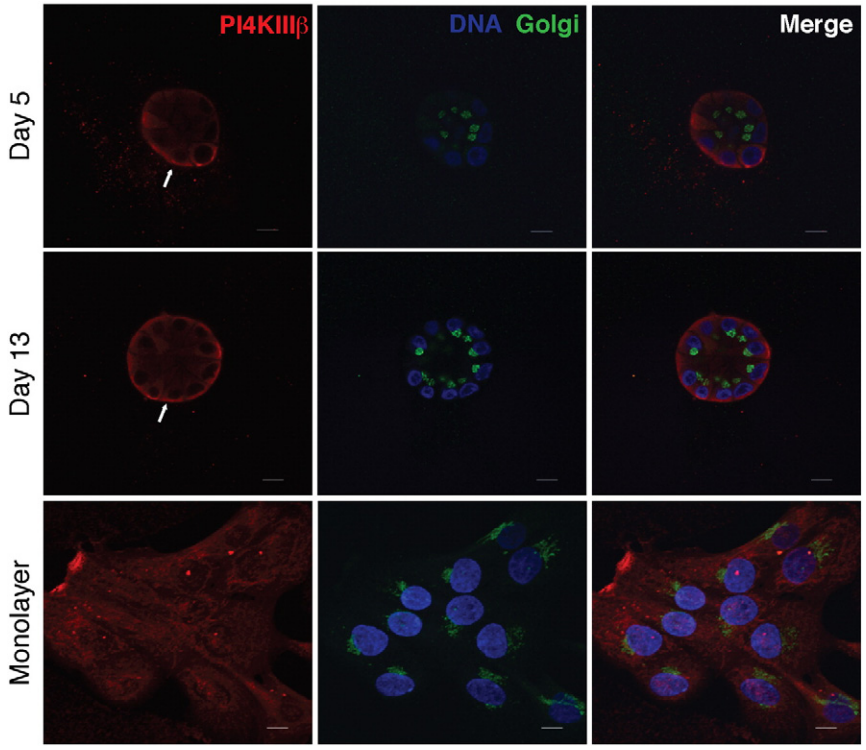

Fig. 1 – The PI4KIIIβ protein localizes to the basolateral surface of breast acini. Confocal microscopy images of MCF10A acini at day 5, 13 in Matrigel and MCF10A cells in monolayer culture. PI4KIIIβ protein is shown in red and arrows indicate localization to basolateral surface of acini. The Golgi (GM130) is shown in green while DNA (Hoechst 33258) is shown in blue. Scale bar indicates 10 μm.

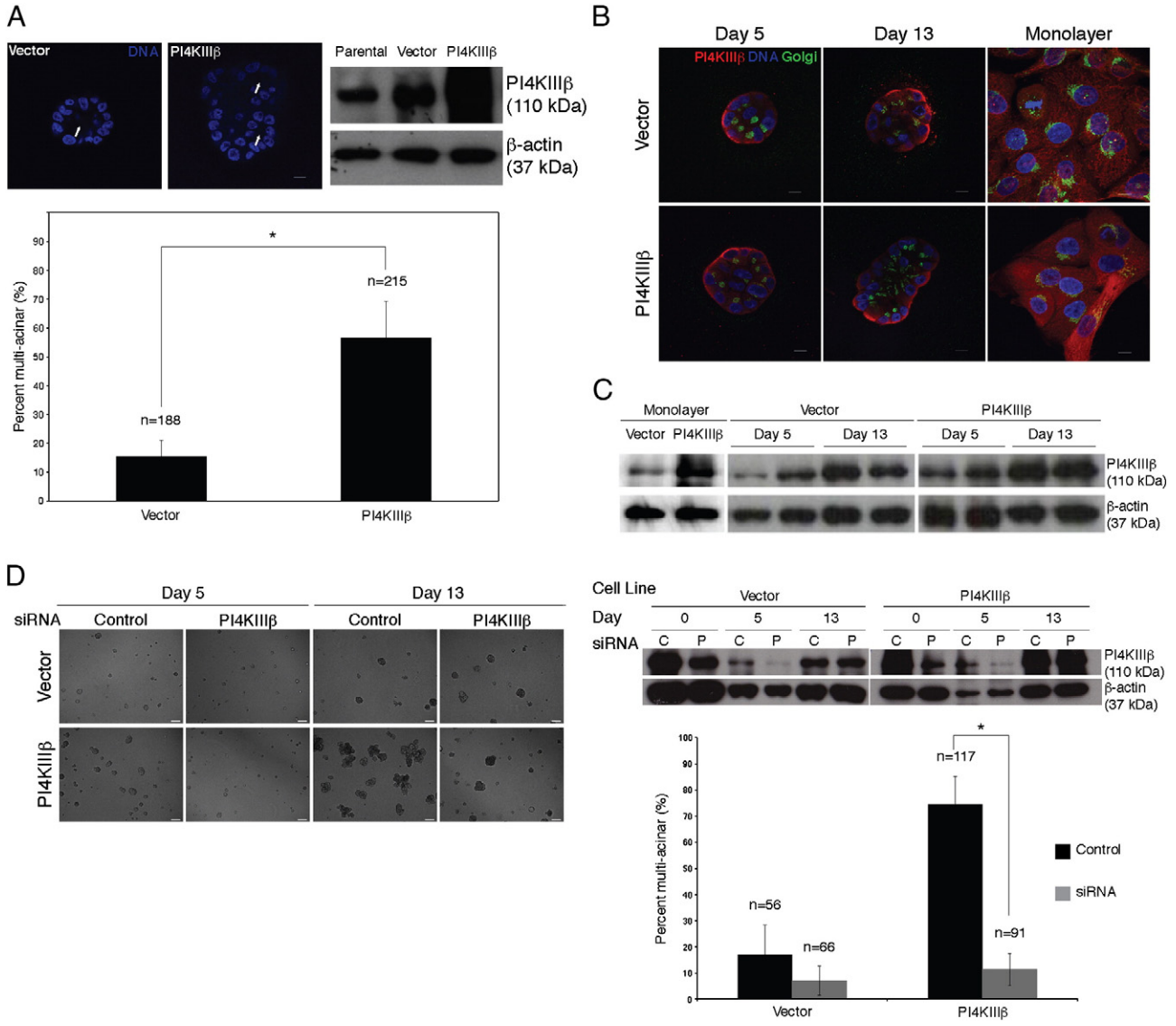

Fig. 2 – Ectopic expression of PI4KIIIβ disrupts acinar morphogenesis. A. Confocal image of MCF10A vector and stable PI4KIIIβ expressors. DNA is shown in blue while arrows indicate lumen. Scale bar indicates 10 μm. Western blot of PI4KIIIβ expression levels in MCF10A cell lines in monolayer culture. β-actin is the loading control. Histogram quantifies the percent of cells forming multi-acinar structures at day 13, with n values indicated. Error bars show standard deviation and (*) indicates statistical significance ($p < 0.0001$). B. Localization of PI4KIIIβ is shown in red at day 5, 13 of Matrigel culture and also in monolayer (2D), with arrows indicating the localization. Golgi is seen in green and DNA is blue. Scale bar indicates 10 μm. C. Western blot analysis showing PI4KIIIβ protein levels in monolayer culture and duplicate reads at day 5 and 13 of Matrigel culture for both the vector and PI4KIIIβ MCF10A cell lines. β-actin is the loading control. D. Phase contrast images of acinar cultures treated with control and PI4KIIIβ targeted siRNA. Scale bar indicates 190 μm. Western blots shows PI4KIIIβ and β-actin protein levels for cells treated with control siRNA (C) and PI4KIIIβ targeted siRNA (P). Multi-acinar phenotype is quantified in the histogram with n values indicated, error bars showing standard deviation and (*) indicates statistical significance ($p < 0.0001$).

on the stage of the cell cycle and cell density, the PI4KIIIβ ectopic expressors have increased levels of PI4KIIIβ when compared to wild-type and vector controls (Figs. 2A and C). Furthermore, PI4KIIIβ levels increase from day 5 to 13 as acini differentiate in both the vector cell and PI4KIIIβ expressing cell lines (Fig. 2C).

To confirm that this multi-acinar phenotype was dependent on PI4KIIIβ, we transiently decreased PI4KIIIβ expression with siRNA and then cultured the cells in Matrigel. As expected, PI4KIIIβ over-expressors reverted to more of a wild-type phenotype following siRNA treatments (Fig. 2D). Of the PI4KIIIβ over-expressors treated with PI4KIIIβ-targeted siRNA 11% were multi-acinar, significantly less than the 75% multi-acinar phenotype observed in the control treated cells (*t*-test, $p < 0.0001$) (Fig. 2D). The decrease in PI4KIIIβ protein levels is lower than controls until at least day 5 and return to levels that are comparable with controls by day 13 (Fig. 2D). Therefore,

increased PI4KIIIβ protein levels have a functional role in disrupting mammary morphogenesis.

Activation of PI4KIIIβ by eEF1A2 induces the formation of multi-acinar structures

To determine whether known biochemical activators of PI4KIIIβ could similarly affect acinar development, we examined the role of eukaryotic elongation factor eEF1A2 in acinar formation. The best characterized function of eEF1A2 is to recruit an amino acylated tRNA to the ribosome during protein translation elongation but it is also a direct PI4KIIIβ activator and can double the enzyme's V_{max} [27–29]. The ability of wild type eEF1A2 to activate PI4K is one of several non-elongation factor functions of the protein. With relevance to this study, we and others have previously found that eEF1A2 is a likely breast and ovarian and lung cancer oncogene based on its transforming capacity, genetic amplification and high expression in these cancers [30–32]. MCF10A cells do not endogenously express eEF1A2 and we generated cell lines that ectopically express it (Fig. 3A). To confirm the interaction of eEF1A2 and PI4KIIIβ in MCF10A cells, a co-immunoprecipitation was performed and found that the two proteins interact in MCF10A (Fig. 3B). As previously reported, only a fraction of eEF1A2 precipitates with PI4KIIIβ. This is likely due to the fact that eEF1A2 is an abundant protein with multiple intracellular functions. When intracellular eEF1A2 protein localization was examined in cells that ectopically express eEF1A2, the protein was found throughout the acini at both day 5 and day 13 in Matrigel. There was visible co-localization of PI4KIIIβ and eEF1A2 at the basal surface (Fig. 3C).

MCF10A-expressing eEF1A2 phenocopy PI4KIIIβ expression and form multi-acinar structures (Fig. 3A). In eEF1A2-expressing cells, PI4KIIIβ is found at the basal surface from day 5 to day 13 and the Golgi remains oriented to the apical surface (Fig. 3D). In order to determine if the multi-acinar phenotype displayed by eEF1A2 was dependent on its interaction with PI4KIIIβ, we transiently decreased PI4KIIIβ expression with siRNA treatment. Upon reduction of PI4KIIIβ, the eEF1A2-expressing cell lines resemble wild-type and control cells. At day 13 the eEF1A2 control siRNA cell lines showed approximately 67% multi-acinar colonies, significantly more than the PI4KIIIβ-targeted siRNA which showed 22% multi-acinar cells (*t*-test, $p<0.0001$) (Fig. 3E). The siRNA experiments indicate that the eEF1A2-dependant morphogenic disruption is mediated by its interaction with PI4KIIIβ.

Since eEF1A2 can increase PI4KIIIβ activity and also leads to the formation of multi-acinar structures, it is likely that increasing PI4KIIIβ lipid kinase activity itself has transformative effects. This suggests that PI4KIIIβ might participate in some pathways of breast oncogenesis and that specific inhibitors of PI4KIIIβ may be useful anti-cancer agents. Some small molecule PI4K inhibitors have been developed, but none, as of yet have shown clinical utility [33,34]. With relevance to breast cancer, the decrease of PI4KIIIβ, through RNAi experiments in the MDA-MB-231 breast cancer cell line, leads to an increase in apoptosis indicating that PI4KIIIβ may play a role in anti-apoptotic signaling and cell survival [35]. However, it is not clear how much excess PI(4)P is required for morphogenic acinar disruption or how much a reduction in PI(4)P is necessary for activating apoptosis. This issue is likely to be complex since PI(4)P and other PIs are found in multiple intracellular locations [1,36]. Given that PI4KIIIβ can supply phosphoinositide substrates for PLC and PI3K/Akt, both pathways implicated in cancer progression, and is associated with increased cell survival, it makes for an interesting and novel target for cancer therapeutics. The PI4KIIIβ gene maps to 1q21, a locus whose amplification has been reported in multiple myeloma, liposarcomas and mucinous adenocarcinoma [37–39]. However, no PI4KIIIβ gene amplification has been reported for breast cancer. Since eEF1A2 is overexpressed in at least 50% of human breast tumors [31], PI4KIIIβ amplification may not be required for oncogenic progression in tumors with elevated eEF1A2 expression as they already have increased PI4KIIIβ activity. In the future, it would be important to investigate whether or not PI4KIIIβ overexpression is sufficient for tumor development in xenograft and transgenic models of cancer.

Ectopic expression of PI4KIIIβ or eEF1A2 alters the distribution of phosphoinositides

We next chose to examine how PI4KIIIβ disrupts acinar morphogenesis. PI4KIIIβ generates PI(4)P from PI, therefore we hypothesized that PI4KIIIβ controls acinar morphogenesis by regulating PI localization. To visualize PI moieties, we generated retroviruses that encode fluorescently tagged reporter constructs that bind to a target lipid. Retroviruses were then used to infect MCF10A cells in Matrigel. To the best of our knowledge, this is the first time that retroviral reporters have been used in Matrigel culture. We utilized the virus due to the toxicity of long-term reporter expression and our inability to transiently transfect MCF10A cells cultured in Matrigel with plasmid-based reporters. The use of reporters is not without its limitations since these reporters only bind to a specific PI in the context of PI-binding proteins.

Initially, we examined the localization of PI(4)P with the FAPP1-PH reporter construct. Upon infection of the Matrigel cultures at day 5, FAPP1-PH was recruited to the basal surface in the parental and vector cell lines. In the PI4KIIIβ and eEF1A2 expressing cell lines we still see a recruitment of FAPP1-PH to the basal surface, however it appears to encroach further into the acini (Fig. 4A). PIs have a well-documented role as spatial and temporal landmarks for compartmentalizing cell components and PI(4,5)P_2 and PI(3,4,5)P_3 have long been thought to be the primary regulators of this process [40]. PI(4)P is the most abundant of the monophosphorylated PIs, and it is often regarded as simply an intermediate along the PI(4,5)P_2 and PI(3,4,5)P_3 synthesis pathways [41,42]. However, PI(4)P is a signaling intermediary in its own right and regulates process such as Golgi tubulation [4,43]. While much work has focused on the role of PI(4)P in the Golgi, closer investigation into its role has revealed a broader subcellular distribution outside of the Golgi [41]. Our report is the first to suggest that PI(4)P might also be important in morphogenesis and we propose that the accumulation of PI(4)P at the basal surface results from the specific localization of PI4KIIIβ protein throughout acinar development.

We then examined the localization of PI(4,5)P_2 with the PLCδ-PH domain. PI4KIIIβ and eEF1A2-expressing cell lines show an enhanced recruitment to the basal surface that appears to delimit the edge of the basal surface, as compared to vector and parental controls (Fig. 4B). Our data is consistent with the idea that increased accumulation of basal PI(4)P, induced by PI4KIIIβ expression, disrupts morphogenesis by causing an increase in PI(4,5)P_2 polarization there. The ability of PI4KIIIβ to affect both PI(4)P and

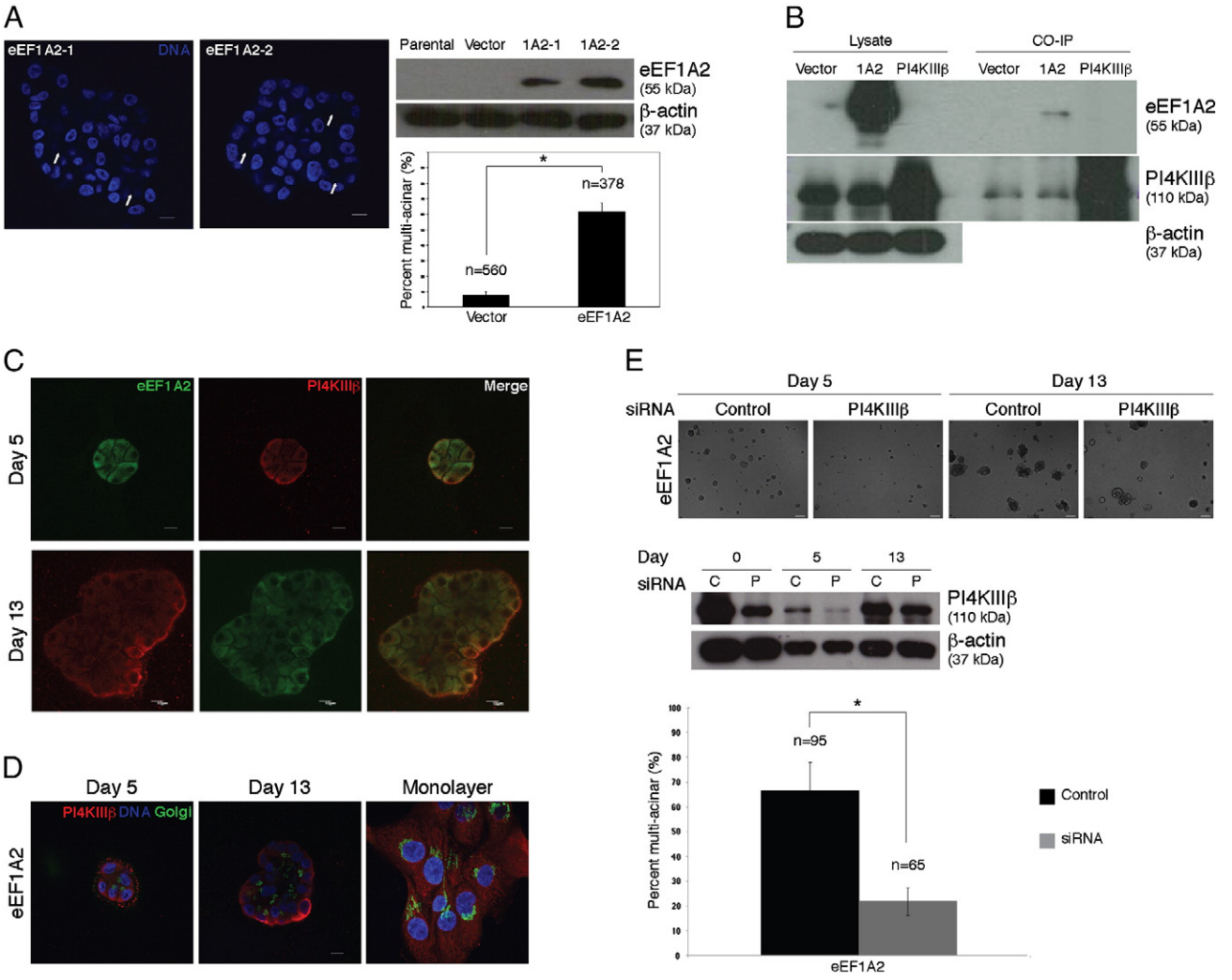

Fig. 3 – Expression of eEF1A2 induces multi-acinar structure formation. A. Confocal images of MCF10A acini expressing eEF1A2 at day 13 with DNA shown in blue. Arrows indicate hollow lumens and scale bar represents 10 μm. eEF1A2 expression in MCF10A cells is shown by Western blot with β-actin as a loading control. Multi-acinar phenotype is quantified in the histogram with n values indicated, error bars represent standard deviation and (*) indicates statistical significance where $p<0.0001$. B. Western blot and co-immunoprecipitation was performed on cell lysates. Lysate lanes show PI4KIIIβ, eEF1A2 and β-actin protein levels in 20 μg of total protein when cultured in monolayer. Co-immunoprecipitation (CO-IP) was performed on membrane-free whole cell lysates of the MCF10A cell lines by using a PI4KIIIβ specific antibody. Interacting proteins were detected by Western blot analysis using PI4KIIIβ and eEF1A2 antibodies. C. Localization of eEF1A2 (green) and PI4KIIIβ (red) are shown at days 5 and 13 of acinar cultures. DNA is shown in blue and scale bars represent 10 μm. D. PI4KIIIβ (red), Golgi (green) and DNA (blue) are shown for day 5 and day 13 in three-dimension culture as well as in monolayer culture (2D). E. Phase contrast images of control and PI4KIIIβ siRNA treated acini. Scale bar represents 190 μm. Western blot analysis shows PI4KIIIβ and β-actin protein levels for the control siRNA (C) and PI4KIIIβ (P) targeted siRNA. Histograms quantify the percent of multi-acinar structures in each condition with n values indicated, error bars represent standard deviation and (*) indicates statistical significance ($p<0.0001$).

$PI(4,5)P_2$ is consistent with our previous work that shows that PI4KIIIβ can control filopodia generation by co-ordinately stimulating the membrane accumulation of these two lipids [5]. $PI(4,5)P_2$ has previously been reported to localize to the apical surface during acinar formation in MDCK, kidney epithelial cells, cultured in three-dimension, so it is likely that MDCK and MCF10A cells use different lipid-dependent strategies to shape acini [6,7,44,45].

We then examined the localization of $PI(3,4,5)P_3$ through the use of the BTK-PH reporter construct. In all of the cell lines, parental, vector, eEF1A2 or PI4KIIIβ expressing, we do not see any specific recruitment or localization of the reporter construct (Fig. 4C). Thus, we propose a model of PI4KIIIβ control of acinar morphogenesis where PI4KIIIβ is required for basal surface identity through its control of PI(4)P and $PI(4,5)P_2$ localization. It is a surprise that $PI(3,4,5)P_3$ is neither substantially polarized during breast morphogenesis nor does its localization change following PI4KIIIβ expression. Previous studies in three-dimension culture using MDCK and HMT-3522 S-1 cells indicated that PI $(3,4,5)P_3$ localizes to the basolateral surface in the canine kidney and breast epithelial cells respectively [8,46,47]. One possible

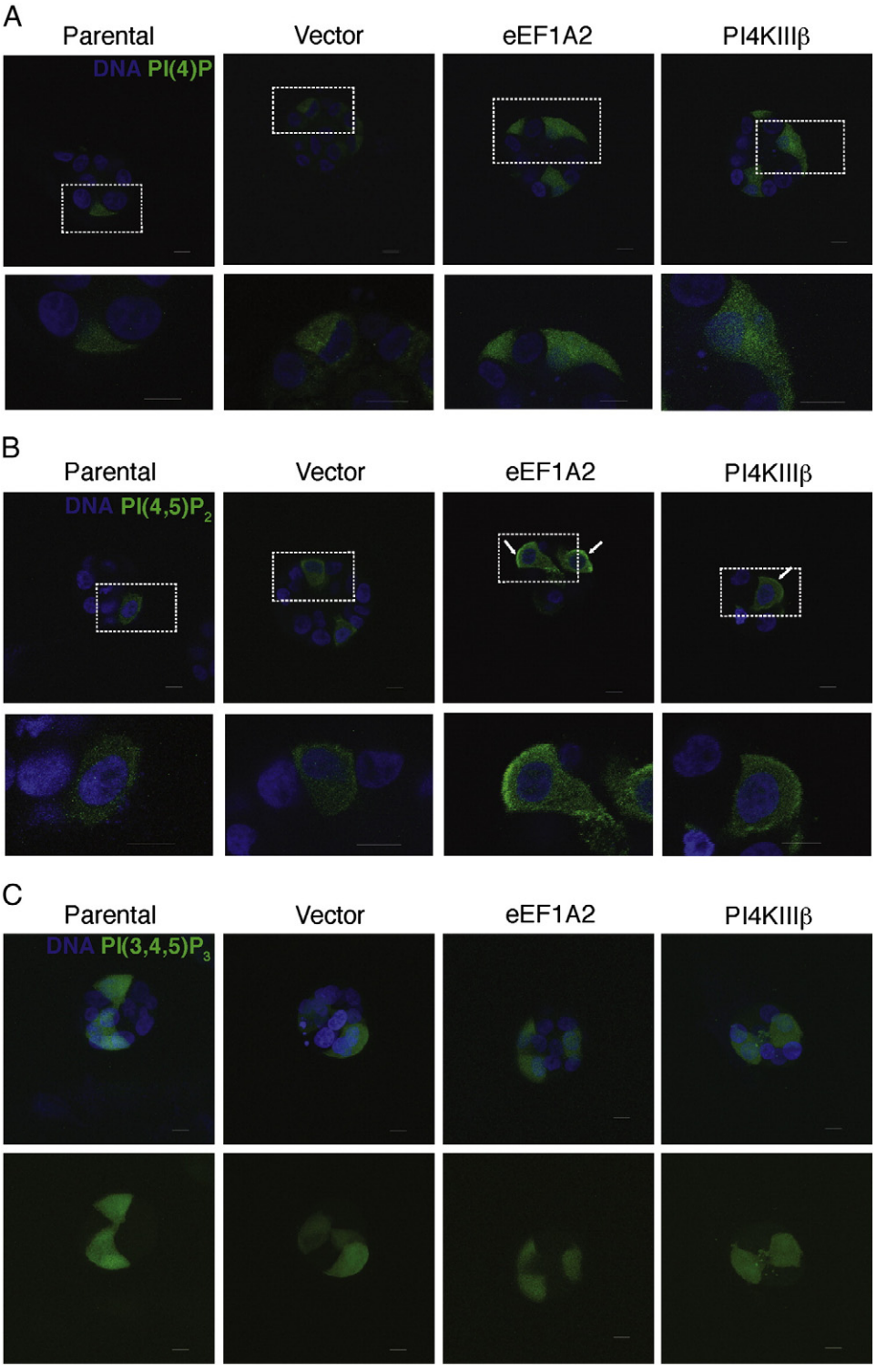

Fig. 4 – Ectopic expression or activation of PI4KIIIβ alters phosphoinositide distribution. A. PI(4)P (green) and DNA (blue) are shown in day 5 three-dimension cultures through the use of the FAPP1-PH-GFP reporter construct. Below, areas enclosed in the boxes are shown at a higher magnification. B. Localization of PI(4,5)P_2 (green) and DNA (blue) is seen in day 5 three-dimension cultures. Increased accumulation of the PLCδ-PH-GFP domain is indicated with arrows. Below, images of enclosed area is shown at a higher magnification. C. Visualization of PI(3,4,5)P_3 (green) is seen with the BTK-PH-GFP reporter. DNA is shown in blue and removed for the lower panels. Scale bar represents 10 μm.

explanation for the discrepancy between our work and the study using MDCK cell lines, is the use of an Akt-PH reporter construct in the MDCK work. It has recently been shown that the Akt-PH domain binds PI(3,4)P_2 as well as PI(3,4,5)P_3. On the other hand, the BTK-PH reporter is believed to bind only to PI(3,4,5)P_3[46,48]. Moreover, the lack of specific recruitment of PI(3,4,5)P_3 during

acinar morphogenesis is further consistent with reports that only cell lines lacking the PTEN phosphatase or having constitutively active PI3K display an increase in PI(3,4,5)P_3[5,27,49]. Overall, our work indicates that PI4KIIIβ and the lipids PI(4)P and PI(4,5)P_2 are key to acinar breast morphogenesis and that PI(3,4,5)P_3 polarization is likely to have a minor role in MCF10A cell three-dimensional morphology.

In conclusion, this study indicates an important role for both PI4KIIIβ and eEF1A2 in the disruption of three-dimensional morphogenesis of MCF10A cells. This alteration in morphology is likely to be mediated by changes in PI(4)P and PI(4,5)P_2 localization, consistent with the idea that both these PIs regulate in vitro mammary development. Since disruption of MCF10A morphogenesis is a hallmark of many breast cancer oncogenes, both PI4KIIIβ and eEF1A2 are likely to be important players in breast cancer and could be targets for anti-cancer therapy.

Supplementary materials related to this article can be found online at doi:10.1016/j.yexcr.2011.08.002.

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The prognostic significance of elongation factor eEF1A2 in ovarian cancer ☆

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Abstract

Objective. To determine whether eukaryotic elongation factor 1 alpha 2 (eEF1A2), a transforming gene previously shown to be highly expressed in primary human ovarian tumours, is a prognostic marker.

Methods. We have used an antibody specific for eEF1A2 to measure eEF1A2 protein expression in 500 primary ovarian tumours in a tissue microarray. We have also ectopically expressed eEF1A2 in SK-OV-3 cells, a clear cell carcinoma line that does not normally express eEF1A2.

Results. We have shown that eEF1A2 has high expression levels in ~30% of all primary ovarian tumours. 50% of serous tumours, 30% of endometriod, 19% of mucinous and 8% of clear cell tumours highly express eEF1A2. Ectopic expression of eEF1A2 in the SK-OV-3 clear cell carcinoma line enhances their *in vitro* proliferative capacity and ability to form tumour-like spheroids in hanging drop culture. Expression of eEF1A2 did not alter sensitivity to anoikis, cisplatin, or taxol. In serous cancer, eEF1A2 is an independent prognostic marker for survival and high eEF1A2 protein expression was associated with increased probability of 20-year survival.

Conclusions. eEF1A2 is highly expressed in ovarian carcinomas. Its expression enhances cell growth *in vitro*, and eEF1A2 expression is likely to be a useful ovarian cancer prognostic factor in ovarian cancer patients with serous tumours.

Keywords: eEF 1A2; Protein translation; Oncogene; Spheroid; TMA; Prognostic factor

Introduction

The identification of genetic abnormalities that occur during ovarian cancer development is a necessary part of understanding its root causes. We have previously identified eEF1A2 (eukaryotic elongation factor 1 alpha 2) as a putative ovarian cancer oncogene by virtue of its high expression and gene amplification in primary human ovarian tumours [1,2]. In addition, the eEF1A2 gene is transforming: ectopic eEF1A2 expression in rodent fibroblast cells allows these cells to grow in an anchorage independent fashion and enhances their tumourigenicity when xenografted in nude mice [2].

eEF1A2 is one of two isoforms of the eukaryotic elongation factor 1 alpha: eEF1A1 and eEF1A2 [3,4]. eEF1A proteins bind amino-acylated tRNAs and recruit them to the ribosome during the elongation phase of protein translation [1,5]. In addition to its role in protein elongation, eEF1A2 regulates a multitude of other cellular processes [1,2,6,7]. For example, eEF1A2 is reported to be an inhibitor of caspase 3-dependent apoptosis [8] and deletion of the mouse eEF1A2 homologue, *Eef1a2*, results in immunodeficiency, elevated lymphoid apoptosis and death by 30 days of age [9–11]. eEF1A2 also regulates cell signaling and we have previously reported that eEF1A2 binds and activates the PI4KIIIβ lipid kinase [7]. eEF1A2 also stimulates the Akt serine/threonine protein kinase and activates Akt-dependent cell migration and actin remodeling [6].

The two eEF1A isoforms have markedly different tissue-specific expression patterns: eEF1A1 is expressed ubiquitously while eEF1A2 expression is restricted to the brain, heart and skeletal muscle [1,3,4]. Moreover, eEF1A2 is highly expressed in a subset of ovarian, lung and breast tumours, suggesting an important role for eEF1A2 in oncogenesis [2,12–14]. The role of eEF1A2 in cancer outcome is likely to be complex; high eEF1A2 expression is a marker for good outcome in breast cancer [13] but a marker for poor survival in lung cancer [12]. It is not yet known

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whether eEF1A2 expression has prognostic significance in ovarian cancer.

In this report, we show that ~30% of primary human ovarian tumours have high eEF1A2 protein expression. eEF1A2 is highly expressed in 50% of serous and 30% of endometroid tumours but in a much smaller fraction of clear cell and mucinous carcinomas. Expression of eEF1A2 in ovarian clear cell carcinoma cells enhances *in vitro* proliferation and their ability to form tumour-like spheroid cultures. eEF1A2 expression is also a significant predictor of 20-year survival in ovarian cancer of the serous type.

Materials and methods

Ovarian tumour tissue microarray (TMA)

Tissue arrays were constructed from 500 archival formalin-fixed, paraffin-embedded ovarian tumour samples from Vancouver General Hospital. These included a variety of high and low grade tumours (see Results for detailed information). The tissue microarrays (TMA) were assembled using a tissue-arraying instrument (Beecher Instruments, Silver Springs, MD) as described previously [15]. Briefly, the recipient block had holes created by the instrument with defined array coordinates and a stylet was used to transfer the tissue cores to the recipient block.

Immunohistochemistry

The TMA sections underwent immunostaining on a Ventana Discovery Instrument (Ventana Medical systems, Tucson, Arizona) using a DAB Map Kit (HRP labeled Biotin-Streptavidin System). The staining was done as outlined in [13]. Briefly, the staining steps were: deparaffinization, heat induced antigen retrieval with EDTA pH 8.0 (24 min), hydrogen peroxide quenching 3% H_2O_2 (8 min), eEF1A2 antibody (1:100 dilution) (32 min), biotynilated universal secondary antibody (32 min), streptavidin–biotin peroxidase complex (16 min), DAB (8 min), counterstain with hematoxylin (4 min).

Evaluation of eEF1A2 protein expression

The staining levels of eEF1A2 were evaluated as previously described [16,17]. Briefly, the images of the tissue cores were scanned using a Bacus Laboratories Inc. Slide Scanner (BLISS) (Bacus Laboratories, Inc., Lomabard, IL). WebSlide Browser v.3.98 (Bacus Laboratories, Inc., Lombard, IL) was used to view the images of the arrays and to assess the individual core images. H&E slides were scanned along with immunohistochemical ones and used as a reference to determine the expression of protein to the specific breast carcinoma structures. Images are available through http://www.gpecimage.ubc.ca/tma/webviewer.php. Scoring of the eEF1A2 immunostaining was performed semi-quantitatively, using digital images and 22-in. monitor with hardware color calibration capabilities. Staining was considered to be negative (0) if no staining was seen in the tumour, weak positive (1+), moderate (2+) and strong (3+), depending on the intensity of the staining in the cytoplasm. Scores were entered into a standard Excel worksheet (Microsoft Excel, Microsoft, Redmond, WA) with a sector map matching each TMA section. Scores for duplicate cores were consolidated to a single value per case using an Excel macro developed by DT. If there were discrepant scores for the two, the high value was accepted for the case. Cases were not included if there was no tumour tissues in the core or if the core was cut through. Original scoring tables were deconvoluted together with the core identification file using Deconvoluter 1.10 [18], and the resulting table files imported into SPSS 11.0 for Windows (SPSS Inc., Chicago, IL).

Cell culture and western blotting

SK-OV-3 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and grown according to their instructions. Cells were transfected with 5 ug *EEF1A2* (with C-terminal V5 tag) plasmid and 15 ul Super-Fect (Qiagen) per 60 mm dish and 0.4 mg/ml Zeocin (Invitrogen) was used for selection. Cells were then lysed using radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris–Cl; pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM ethylenediaminetetraacetic acid (EDTA); pH 7.0, 150 mM NaCl) supplemented with 1% aprotonin, 1 mg/ml leupeptin, 50 mM NaF, 1 mM Na_3VO_4, 10 mg/ml pepstatin in ethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF) in dimethylsulfoxide (DMSO). Protein was quantified using Bradford protein assay (Pierce, Rockford, IL, USA) as per the manufacturer's instructions. Approximately 20 ug of protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane. Anti-V5-horseradish peroxide (HRP) (Invitrogen) and actin (Sigma) antibodies were used according to manufacturers' instructions.

Cell proliferation assay and multi-cellular spheroid culture

Cell proliferation assays were performed by culturing cells as mentioned previously. At indicated time points cells were trypsinized and counted by typan blue exclusion. This was performed in triplicate. The multi-cellular spheroids were cultured as outlined by Kelm et al. [19]. Briefly, 15 ul droplets containing 1000 SK-OV-3 cells were placed on the lids of non-adherent, bacterial grade polystyrene Petri dishes (Starstead). Lids were then inverted over Petri dishes filled with 10 ml 1× phosphate buffered saline (PBS).

Anoikis and cell death

To induce anoikis, cells were shaken at 40 rpm in standard tissue culture dishes on an S-500 orbital shaker (VWR) in a 37 °C, 5% CO_2 incubator. Cells were counted using a Z2 Coulter Counter (Beckman Coulter, Fullerton, CA) at set time points. Sensitivity of cells to cisplatin and paclitaxel were performed by adding serial dilutions of either cisplatin or taxol to cells and determining cell number using trypan blue exclusion counting. The IC_{50} was calculated as the drug dose that reduced surviving cell abundance by 50%.

Flow cytometry

In order to analyze the levels of apoptosis by flow cytometry, 500 000 SK-OV-3 cells were shaken at 40 rpm in standard tissue culture dishes on an S-500 orbital shaker (VWR) in a 37 °C, 5% CO_2 incubator. Cells were then removed from the culture dishes by pipetting. Control SK-OV-3 cells were trypsinized from standard tissue culture dishes. Cells were washed with 1× PBS then resuspended in 500 ul 1× Binding Buffer (Sigma). Cells were then stained with 2.5 ul of a 50 ug/ml Annexin V FITC conjugate (Sigma) and analyzed by flow cytometer, FC500 Flow Cytometer Beckman Coutler (Beckman Coulter, Fullerton, CA).

Statistical analysis

Univariable survival analysis was performed using the Kaplan–Meier method and log-rank test. Multivariable survival analysis was performed using the proportional-hazards model. The level of significance for all statistical tests was defined as $p<0.05$. All survival analyses were performed using JMP v6.0.3 (SAS Institute, Cary, NC, USA).

Results

eEF1A2 expression in ovarian cancer

We have previously reported that eEF1A2 mRNA is not detectable in normal ovarian epithelium but is expressed in a fraction of human ovarian tumours [1,2]. To further characterize the expression of eEF1A2 in ovarian cancer, we analyzed eEF1A2 protein expression in 500 ovarian tumours on a tumour microarray. Of the tumours sampled, 42% of those were in stage I, 41% were stage II and 17% were stage III and the entire patient cohort had no macroscopic residual disease after the surgery. The distribution of the subtypes of the tumour samples are as follows: 42% Serous, 26% Clear cell, 25% Endometeroid and 7% Mucinous. The age of

the patients assayed range from 9258 to 32,502 days with a mean age of 21,197±4665 days. The creation and validation of the eEF1A2 antibody used is described elsewhere [13]. Staining was categorized as negative, weak, moderate or high. Representative photographs of the 4 categories are shown in Fig. 1. Overall, 32% of the tumours (159/500) tested have high expression of eEF1A2. As shown in Table 1, the tumour types with the most frequent expression of eEF1A2 were serous and endometriod tumours, with 50% and 30% of these tumours having high levels of eEF1A2 expression respectively. 19% of the mucinous tumours and 8% of clear cell tumours had high expression of eEF1A2.

eEF1A2 increases cell proliferation in vitro

In order to determine the role that eEF1A2 might have in ovarian tumour development, we ectopically expressed eEF1A2 in SK-OV-3 cells. SK-OV-3 cells are derived from a human clear cell ovarian adenocarcinoma and do not endogenously express eEF1A2 (Fig. 2A). eEF1A2 protein expression in three independent SK-OV-3 clones is shown in Fig. 2A. As shown in Fig 2B, SK-OV-3 variants that express eEF1A2 proliferate at a faster rate than their wild type and vector counterparts (Fig. 2B), eEF1A2-negative parental and empty vector controls had a doubling time of between 53 and 61 h. On the other hand, the doubling time for eEF1A2-expressors was substantially less, between 22 and 39 h depending on the cell line. This indicates that expression of eEF1A2 increases the cells *in vitro* proliferative capacity.

Table 1
Evaluation of eEF1A2 expression in ovarian cancer

Histological type	eEF1A2 expression	Percentage of tumours (%)
Serous (n=212)	High	50
	None, low, moderate	50
Endometriod (n=125)	High	30
	None, low, moderate	70
Mucinous (n=31)	High	19
	None, low, moderate	81
Clear cell (n=132)	High	8
	None, low, moderate	92

Expression of eEF1A2 increases the rate of spheroid formation

We next wanted to determine whether eEF1A2 can affect other aspects of *in vitro* cell growth. Multi-cellular spheroids (MCS) have been used as an *in vitro* model system of avascular tumour development [19–24]. When SK-OV-3 cells were grown as MCS by the hanging drop method, they form a three-dimensional sphere approximately 2–3 days after culture initiation. As shown in Fig 3A, SK-OV-3 cells that express eEF1A2 form MCS more rapidly than vector counter parts (Fig. 3A). To quantitate this difference, we estimated the size of each spheroid by counting the

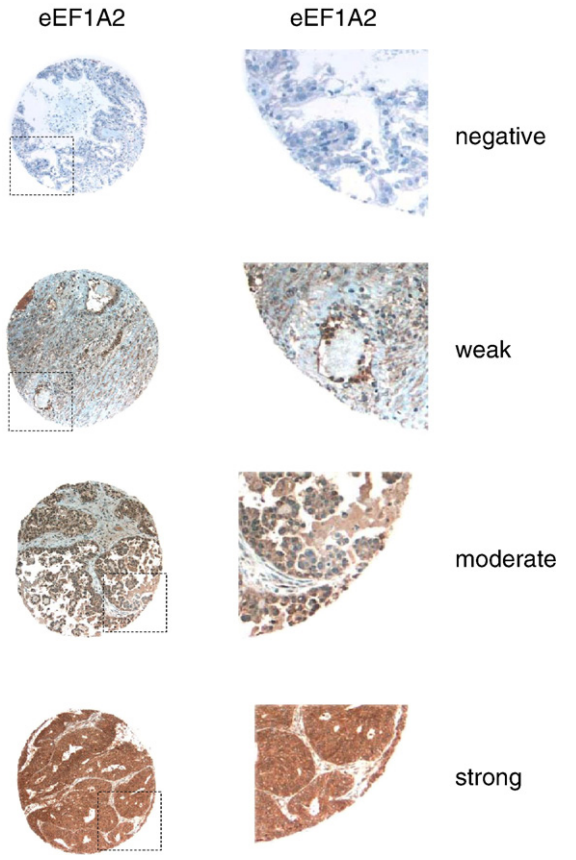

Fig. 1. eEF1A2 expression in primary ovarian tumours in a tissue microarray. Representative examples of eEF1A2 immunostaining in tumours classified as showing negative, weak, moderate and strong expression of eEF1A2. The right column is a higher magnification view of the boxed square of the left column.

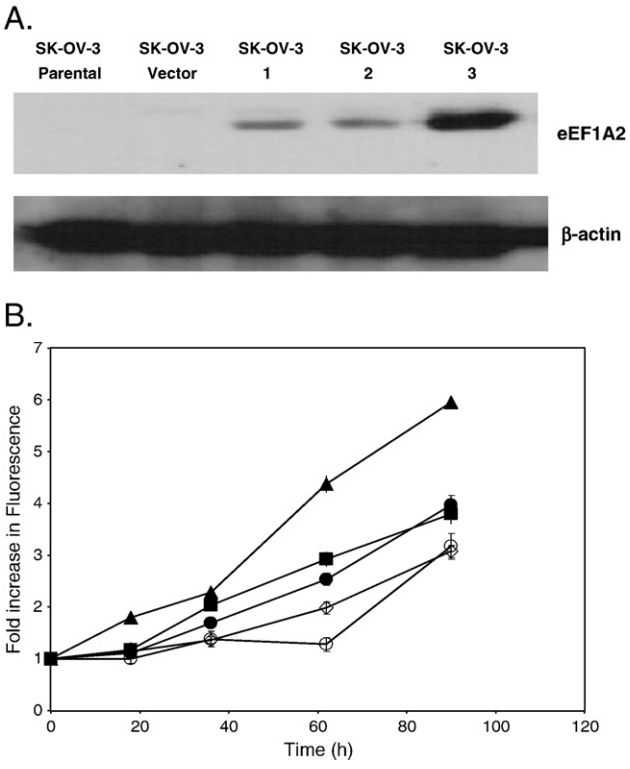

Fig. 2. Expression of eEF1A2 enhances cell proliferation. (A) Western blot of eEF1A2 expression. Parental and Vector controls do not express eEF1A2 while SK-OV-3 cell lines E1, E2 and E3 show detectable eEF1A2 protein expression. (B) eEF1A2-expressing cells (closed symbols) proliferate at a faster rate than the Parental and Vector controls (open symbols).

number of pixels in photographs of each spheroid. As shown in Fig. 3B, the vector controls formed spheroids which were approximately 104,000 pixels in size (Fig. 3B). On the other hand, eEF1A2-expressors have significantly ($p<0.001$, ANOVA) smaller spheroids, 65,000 to 74,000 pixels in size. This indicates that the expression of eEF1A2 in these cell lines is enhancing the adhesive interactions responsible for spheroid formation [23,24].

Expression of eEF1A2 does not affect resistance to anoikis or chemotherapeutics

As eEF1A2 has previously been linked to an increased resistance to apoptosis [8,11], we next determined whether or not expression of eEF1A2 altered cellular susceptibility to anoikis. Anoikis is a specific type of apoptosis that occurs when cells lose cell–matrix adhesion [25–27]. We used shaking culture to induce anoikis and as shown in Fig. 4A, SK-OV-3 cells that express eEF1A2 do not show a substantial difference in overall survival in shaking culture relative to controls. We also measured apoptosis in these cells using Annexin V and find no substantial difference in apoptosis between eEF1A2 expressing and controls SK-OV-3 cells (Fig. 4B). We also investigated whether eEF1A2 might alter the cytotoxicity of cisplatin or taxol. As with anoikis, eEF1A2 expression does not detectably affect the cytotoxicity of either cisplatin or taxol (Table 2).

Expression of eEF1A2 indicates good long-term prognosis in serous tumour types

We next investigated whether eEF1A2 expression had prognostic significance for long-term survival in ovarian cancer. Based on the assumption that eEF1A2 is an intensity-based marker, we

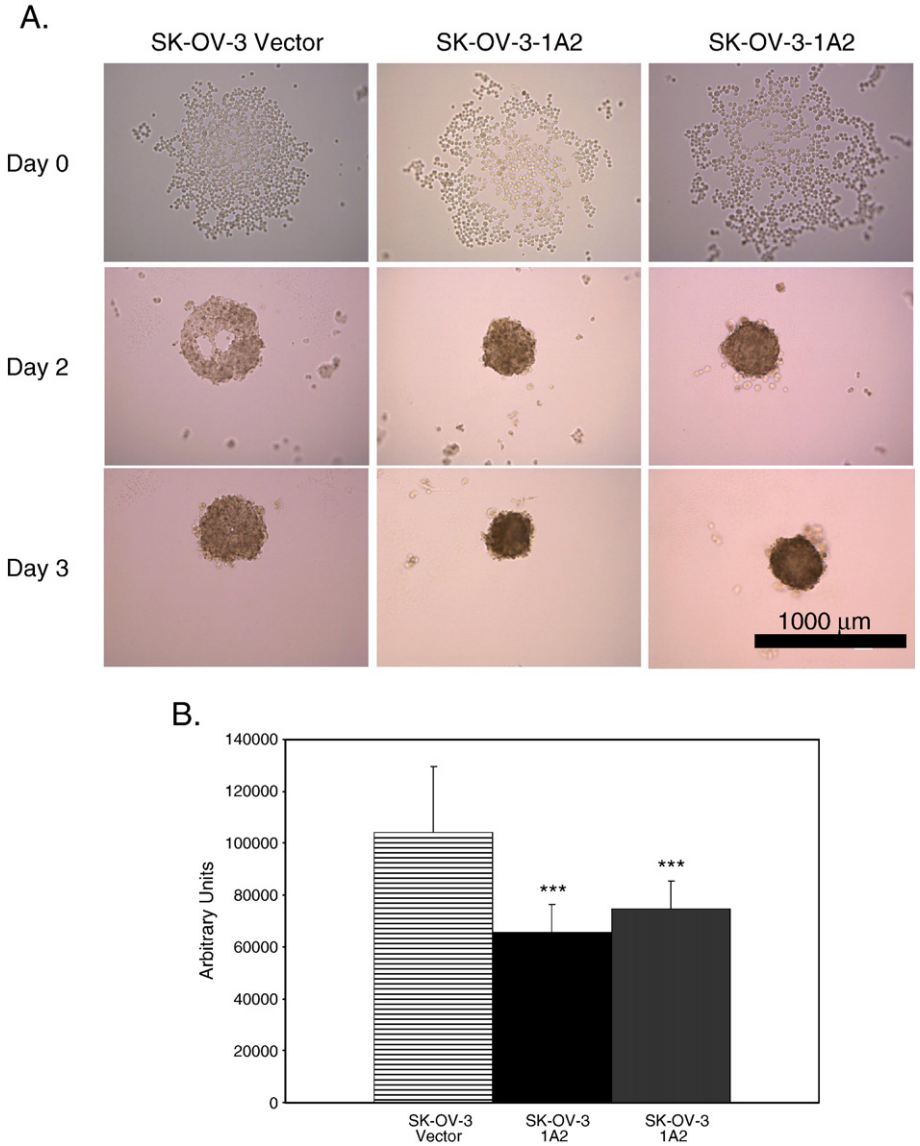

Fig. 3. Expression of eEF1A2 enhances spheroid formation. (A) Representative photographs of SK-OV-3 cells forming tumour-like spheroids in hanging drop culture. Cells (E1 and E3) that express eEF1A2 form spheroids more rapidly than their vector counterparts. (B) Aggregation was quantified after 2 days to quantitate the approximate cross sectional area of the spheroids. Data is the mean and standard deviation of three independent experiments with triplicates measurements. E1 and E3 spheroids are significantly smaller than the vector controls. Significance is indicated by *** ($p<0.001$, ANOVA).

used recursive partitioning, an unsupervised procedure, to divide the tumour cohort into two groups to maximize observable differences in mean disease specific survival time. We portioned the tumours cohort in the high expressors (3) and grouped the negative to moderate staining (0–2) tumours together. When not separated by tumour type, patients whose ovarian tumours had high expression of eEF1A2 had approximately the same 20-year survival outcome as their eEF1A2 low or negative group (Fig. 5A), 50% survival in both groups is approximately 12 years. However, in women with serous tumours, high expression of eEF1A2 was associated with significantly ($p<0.01$, log rank test) increased 20-year survival probability (Fig. 5B). For example, at 10 years after

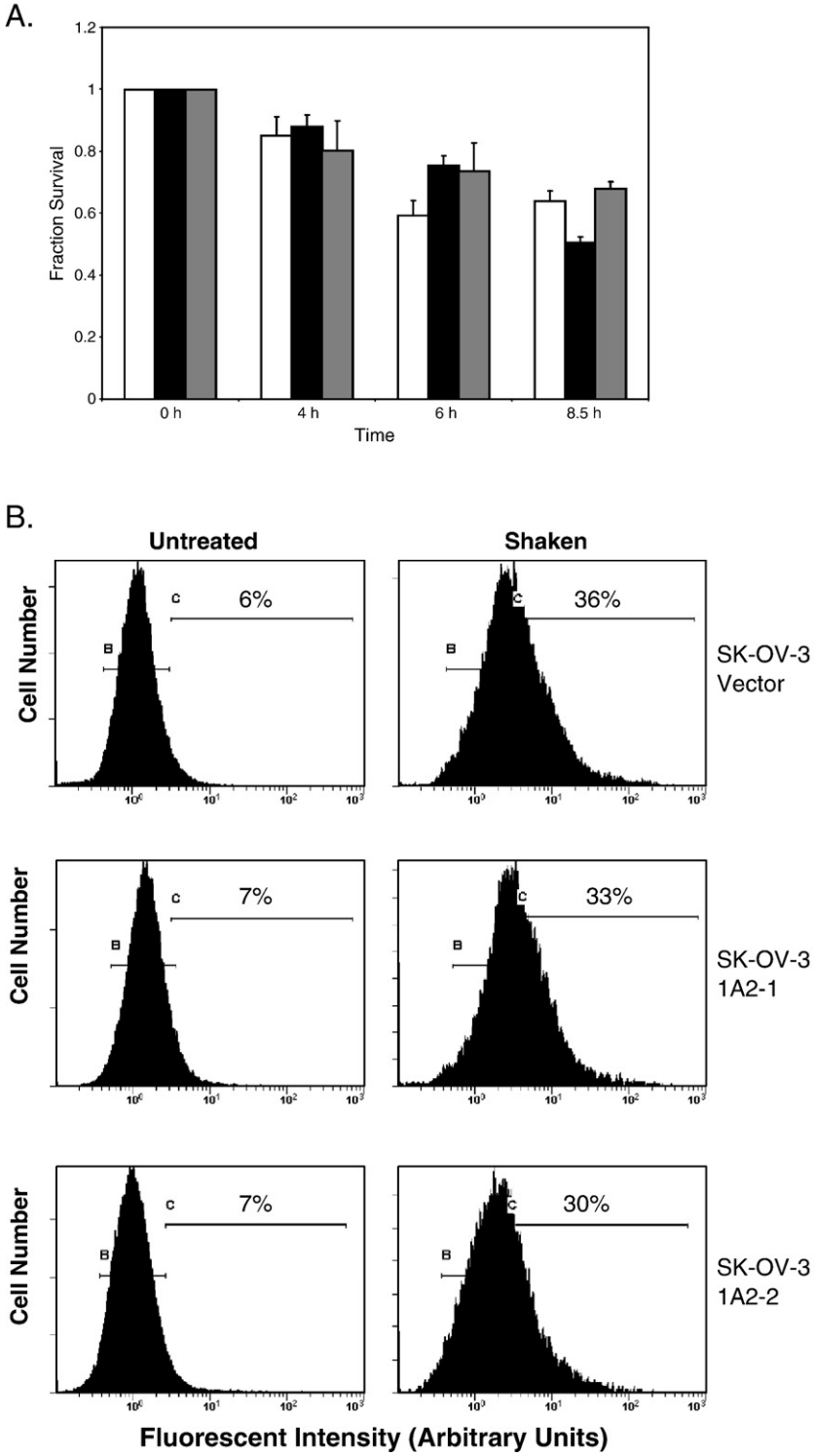

Fig. 4. Expression of eEF1A2 does not detectably affect anoikis. (A) SK-OV-3 Vector only cells (white bar) and E1 (black) and E3 cells (gray) were induced to undergo anoikis and cell number counted at set time points. Data is the mean and standard deviation of triplicate cell counts. (B) Annexin V staining was measured in SK-OV-3 vector and eEF1A2-expressors after 20 h in shaking culture. Figure is representative of three independent experiments.

Table 2
Effect of eEF1A2 on cisplatin and taxol sensitivity

Cell line	Cisplatin IC_{50} (mM)	Taxol IC_{50} (mM)
SK-OV-3-Parental	82.5	35.3
SK-OV-3-Vector	78.8	38.6
SK-OV-3-1A2	75.2	42.8
SK-OV-3-1A2	83.4	35.6
SK-OV-3-1A2	80.5	39.6

diagnosis, the surviving fraction of women with $eEF1A2^{high}$ tumours was ~50%, compared to ~30% in the others. Proportional hazards analysis shows that eEF1A2 is an independent prognostic marker in the serous subtype when age, stage and Silverberg grade are included in the model (Table 3). Other groupings do not lead to statistically significant differences in survival probability (not shown). eEF1A2 was not a significant prognostic marker for endometriod, clear cell or mucinous cancers (not shown).

Table 3
Multivariable analysis of eEF1A2

Term	Levels	Risk ratio	*P* value
eEF1A2			0.0210
	0	1.0 [1.0–1.0]	
	1	0.62 [0.41–0.93]	
Stage			0.0002
	I	0.81 [0.57–1.13]	
	II	0.68 [0.50–0.92]	
	III	1.0 [1.0–1.0]	
Silverberg grade			0.6880
	1	0.92 [0.45–1.59]	
	2	1.15 [0.78–1.78]	
	3	1.0 [1.0–1.0]	
Age	–	NR	0.2414

Discussion

In this report we show that *EEF1A2*, a gene not expressed in normal ovary, is highly expressed in ~30% of human ovarian tumours. 50% and 30% of the serous and endometroid tumours have high expression of eEF1A2, while only 19% and 8% of mucinous and clear cell tumours, respectively, express eEF1A2. This high expression of eEF1A2 in ovarian tumours implies that eEF1A2 expression has some causal role in neoplastic development. We have previously reported that eEF1A2 transforms rodent fibroblasts and enhances their tumourigenicity in nude mice [2]. Consistent with these observations, we find that eEF1A2 expression in SK-OV-3 clear cell carcinoma cells increases their *in vitro* growth rate. eEF1A2 also enhances the ability of SK-OV-3 cells to form *in vitro* spheroids in hanging drop culture. The hanging drop spheroid culture provides a model for studying avascular tumour regions *in vitro* [22]. eEF1A2 expression causes cells to aggregate into spheroids more quickly than their vector control, suggesting that eEF1A2 may be contributing to primary tumour formation *in vivo*.

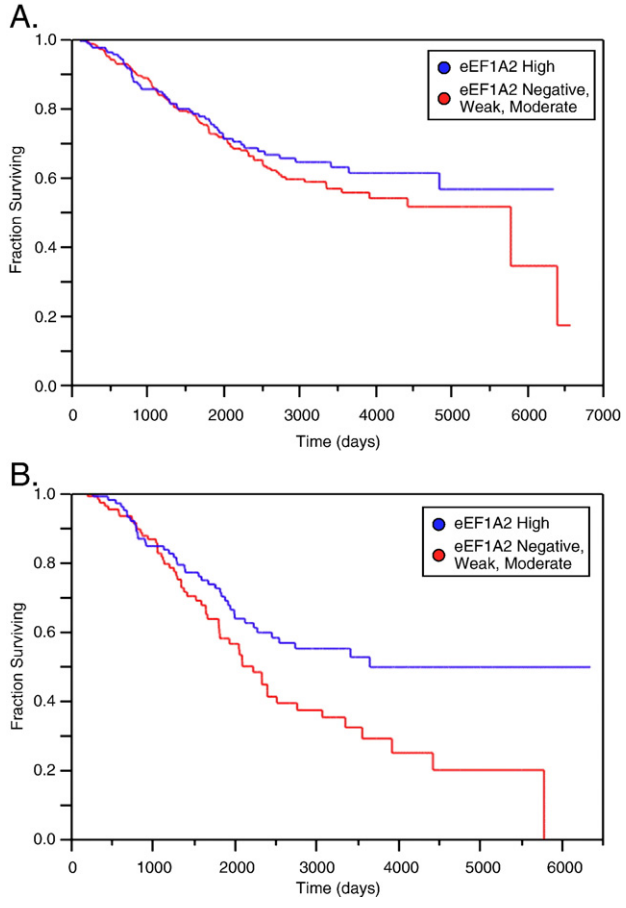

Fig. 5. Prognostic significance of eEF1A2. Fractional survival comparison of patients whose ovarian tumours showed negative, weak and moderate expression of eEF1A2 (red line) and with high expression (blue line) as a function of time. (A) Fractional survival is similar between the two populations when all ovarian tumour types are examined. (B) Long-term fractional survival is increased in serous tumours when they highly express eEF1A2.

Given the ability of eEF1A2 to enhance cell growth *in vitro*, it is therefore somewhat of a paradox that eEF1A2 expression predicts good survival probability in serous cancers. We have previously reported that eEF1A2 expression similarly marks good survival probability in breast cancer [13]. There are several possible explanations for the ability of eEF1A2 to mark good survival. eEF1A2 could affect chemotherapy resistance or alter primary tumour dissemination, both of which have important roles in controlling ultimate clinical outcome in ovarian cancer [28,29]. However, we do not find that eEF1A2 expression has a detectable effect on anoikis or sensitivity to cisplatin or taxol. This was a surprise to us since it has been previously been reported that ectopic expression of S1, the rat eEF1A2 homologue, inhibits caspase 3-dependent apoptosis [8,11]. In addition, homozygous deletion of the mouse eEF1A2 gene, *Eef1a2*, increases lymphoid apoptosis [10]. While these reports indicate that eEF1A2 inhibits apoptosis, we find no effect on apoptosis in SK-OV-3 cells. With respect to tumour dissemination, we have previously reported that eEF1A2 can enhance *in vitro* cell migration and invasion of breast cancer cells [6]. At first glance it would therefore seem probable that eEF1A2 should enhance *in vivo* dissemination of the primary tumour in the peritoneal cavity. However, successful tumour dissemination is a balance between two opposing requirements:

sufficient mobility to leave the primary tumour but sufficient "immobility" to colonize secondary sites [30–32]. We postulate that eEF1A2 might make cells too migratory to successfully colonize secondary sites. Alternatively, eEF1A2 may enhance prognosis by altering other oncogenic process, perhaps tumour self-renewal potential or angiogenic capacity. Further investigation will be necessary to clarify the mechanism by which eEF1A2 leads to increased long-term survival.

eEF1A2 does not predict good survival probability in all cancers. For example, others have reported that eEF1A2 expression is a marker for poor survival in lung cancer [12]. This disparity in prognostic significance of eEF1A2 between ovary, breast and lung cancer implies that eEF1A2 is having markedly different biological effects in the three distinct tissues. An oncogene undertaking different roles and partaking in different signaling pathways often differs as it is expressed in different tumour types [33,34]. The reason why eEF1A2 might be highly expressed in one ovarian tumour sub-type remains unclear. The gene is not detectably expressed in normal mammalian ovary [2] so tumour-specific expression is unlikely to be related to any unique cell type in normal ovary. Similarly, the reason why eEF1A2 predicts long-term survival in only serous tumours is unknown. The origin and developmental history of ovarian tumours is not well characterized, since most tumours are identified only in more advanced stages. Thus, further investigation is necessary to determine the role for eEF1A2 in controlling the development of specific gynecological cancers.

Tomlinson et al. have previously reported that expression of eEF1A2 is associated with the clear cell histology, whereas we have found that high expression of eEF1A2 is only found in 8% of these tumour types as opposed to 75% of those they examined [35]. The discrepancies between the results can be attributed to two possible differences. Firstly, Tomlinson et al. examined eEF1A2 protein expression in a total of was 44 tumours, only 5 of which were of the clear cell type. We have used 500 ovarian tumours, of which 132 were clear cell carcinomas. Secondly, Tomlinson et al. have grouped the tumours examined as either eEF1A2 negative or positive whereas the grouping used here is eEF1A2 negative to moderate expression and high expression of eEF1A2. Therefore, the different sample sizes, as well as the different method of categorizing the expression of eEF1A2 could explain the difference between our two studies.

In summary, we report here that eEF1A2 is highly expressed in ovarian tumours and enhances *in vitro* properties of ovarian cancer cells. Thus, eEF1A2 likely has some causal role in the ovarian neoplastic process. Consistent with this idea, we find that eEF1A2 expression predicts increased survival probability in serous ovarian cancer.

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ORIGINAL ARTICLE

eEF1A2 activates Akt and stimulates Akt-dependent actin remodeling, invasion and migration

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eEF1A2 (eukaryotic protein elongation factor 1 alpha 2) is a protein translation factor that is likely a human oncogene by virtue of its capacity to transform mammalian cells and its high expression in tumors of the ovary, breast and lung. Here, we show that expression of eEF1A2 is sufficient to stimulate the formation of filopodia in BT549 human breast cancer cells and non-transformed Rat2 cells. Filopodia formation in eEF1A2-expressing cells is dependent on the activity of phosphatidylinositol-3 kinase (PI3K), and the ROCK and Akt kinases. Furthermore, eEF1A2 expression is sufficient to activate Akt in a PI3K-dependent fashion and inactivation of eEF1A2 by short interfering RNA reduces Akt activity. Using breast cancer cell line BT 549, we show that eEF1A2 expression stimulates cell migration and invasion in a largely PI3K- and Akt-dependent manner. These results suggest that eEF1A2 regulates oncogenesis through Akt and PI3K-dependent cytoskeletal remodeling.
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Keywords: eEF1A2; Akt; filopodia; protein translation; actin; cell motility

Introduction

eEF1A2 (eukaryotic protein elongation factor 1 alpha 2) is one of the two isoforms of protein elongation factor eEF1A (eEF1A1 and eEF1A2). eEF1A proteins are GTP-binding proteins that interact with amino-acylated tRNA and recruit them to the ribosome during the elongation phase of protein translation. The two human isoforms share >90% identity and have essentially the same function during protein translation (Knudsen *et al*., 1993). eEF1A1 is expressed ubiquitously whereas mammalian eEF1A2 expression is limited to the heart, brain and skeletal muscle (Knudsen *et al*., 1993; Lee *et al*., 1993; Kahns *et al*., 1998).

eEF1A2 is likely to be an important human oncogene (Lee, 2003). *EEF1A2*, the gene encoding eEF1A2, is amplified and its mRNA overexpressed in ~30% of primary human ovarian cancers (Anand *et al*., 2002). Expression of wild-type eEF1A2 transforms rodent fibroblasts and increases their tumorigenicity in nude mice (Anand *et al*., 2002). Amplification of *EEF1A2* and increased eEF1A2 mRNA and protein overexpression has also been reported in lung and breast tumors (Wang *et al*., 2004; Tomlinson *et al*., 2005; Kulkarni *et al*., 2006). In lung cancer, high expression of eEF1A2 correlates with increased Ki-67 expression and is associated with poor prognosis (Wang *et al*., 2004). Furthermore, eEF1A2 may also have a role in metastatic development and it is overexpressed in metastatic rat mammary adenocarcinoma cell lines relative to non-metastatic controls (Pencil *et al*., 1993; Edmonds *et al*., 1996). Although these observations implicate eEF1A2 in oncogenesis, little is known about the molecular mechanisms by which eEF1A2 could enhance tumor development.

eEF1A proteins have cellular functions in addition to their canonical role in translation elongation. eEF1A from several species and genera binds to actin filaments and microtubules both *in vivo* and *in vitro* (Condeelis, 1995). Binding of *Dictyostelium* eEF1A to F-actin enhances actin bundling (Yang *et al*., 1990), suggestive of a role for eEF1A in actin cytoskeleton remodeling. Two C-terminal domains in the *Dictyostelium* eEF1A protein directly bind actin (Condeelis, 1995). These domains are distinct from sequences that bind GTP, tRNA or are responsible for GTP hydrolysis. *Saccharomyces cereviseae* has two eEF1A genes (*TEF1* and *TEF2*), both of which more closely resemble human eEF1A1 than eEF1A2 gene. *TEF1* proteins that are deficient in actin bundling are competent in translation elongation, indicating that actin interaction and peptide elongation are independent functions of the protein (Gross and Kinzy, 2005). Although ectopic *TEF1* or *TEF2* expression in *S. cereviseae* leads to a general disorganization of the actin cytoskeleton (Munshi *et al*., 2001), the effect that mammalian eEF1A2 expression has on the eukaryotic actin cytoskeleton has yet to be fully investigated.

eEF1A proteins have also been implicated in phosphatidylinositol (PI) signaling. PIs are negatively

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charged, low-abundant, membrane-bound phospholipids that serve as regulators of multiple signaling pathways (Carpenter and Cantley, 1990; Fruman *et al*., 1998; Overduin *et al*., 2001; Meijer and Munnik, 2003). An eEF1A-related protein, PIK-A49, has been purified from carrot cells based on an ability to increase the *in vitro* lipid kinase activity of phosphatidylinositol-4 kinase (PI4K) (Yang *et al*., 1993). PI4Ks catalyse the phosphorylation of the D4 carbon of the inositol ring PI (Heilmeyer *et al*., 2003). Phosphatidylinositol-4 phosphate is an obligate precursor of phosphatidylinositol-(4,5) biphosphate and phosphatidylinositol-(3,4,5) triphosphate (PIP_3). PIP_3 is a lipid second messenger that activates diverse signaling pathways important in oncogenesis, among them activation of the Akt serine/threonine kinase (Vivanco and Sawyers, 2002). Akt directly and indirectly controls the activity of many oncogenic pathways, including proliferation, growth, apoptosis and actin filament remodeling (Vivanco and Sawyers, 2002; Qian *et al*., 2004). Recently, eEF1A1 has been determined by mass spectroscopy to be a possible binding partner for Akt2 and ectopic eEF1A2 expression has been shown to increase Akt abundance (Lau *et al*., 2006). However, functional aspects of eEF1A/Akt interaction remain unexplored.

In this report, we find that eEF1A2 is a novel activator of Akt. Akt activation by eEF1A2 is dependent on PI3K. Furthermore, eEF1A2 induces filopodia production in rodent and human cell lines and enhances cell invasion and migration in an Akt- and PI3K-dependent manner. This indicates an important role for eEF1A2 in controlling phosphatidylinositol signaling, actin remodeling and cell motility.

Results

eEF1A2 expression induces rearrangement of the actin cytoskeleton

To examine a role for eEF1A2 in actin remodeling, we ectopically expressed eEF1A2 in cell lines that normally do not express eEF1A2. As shown in Figure 1a, eEF1A2 protein is expressed in the monocyte chemoattractant factor-7 (MCF-7) human breast adenocarcinoma line, but is not detectable in the BT549 human breast ductal carcinoma cancer cell line nor the rat Rat2 non-transformed fibroblast line. eEF1A2 protein is also detectable in the rat ovarian epithelial ROSE 199 cell line (Figure 1a, right panel) (Adams and Auersperg, 1985). The eEF1A2 antibody used for this analysis recognizes an eEF1A2 epitope (KVERKEGNASGVSLLEALDT) that is identical between the rat and the human eEF1A2 isoforms (Kulkarni *et al*., 2006). High expression level of eEF1A2 protein was detected in selected cell lines (Figure 1b). Cells infected with empty pLXSN or transfected with an empty pCDNA3.1 vector were used as controls.

To examine a role for eEF1A2 in controlling the actin cytoskeleton, we stained eEF1A2-expressing cells and controls with phalloidin. Control BT549 cells showed prominent actin stress fibers, a thin but recognizable

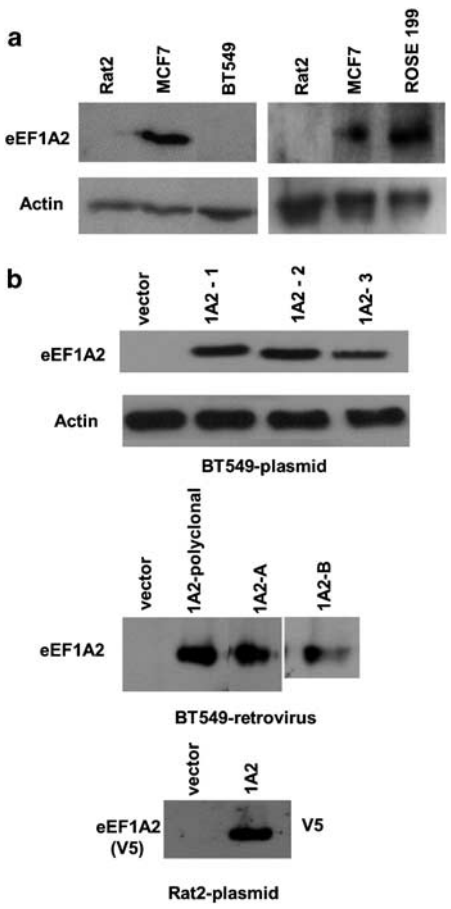


Figure 1 eEF1A2 expression in cell lines. (**a**) Western Blot (WB) of eEF1A2 expression. Human BT549 and rat Rat2 cells do not naturally express eEF1A2 whereas human MCF7 breast cancer and rat ROSE 199 cells do express eEF1A2 protein. Expression was measured using an eEF1A2-specific antibody. (**b**) Stable expression of eEF1A2 protein in BT549 and Rat2 cell lines. 1A2-1, 1A2-2, 1A2-3, 1A2-A and 1A2-B refer to clonal cell lines. 1A2-polyclonal and Rat2-1A2 are cell lines derived from pooling several (>10) drug-resistant colonies after selection. The expression of eEF1A2 was analysed by immunoblotting using anti-eEF1A2 or anti-V5 antibodies. Actin was used as a loading control. eEF1A2-expressing BT549 cells were generated using a plasmid vector or a retrovirus.

lamellipodia, but very few filopodia-like structures (Figure 2a). Filopodia are pencil-like bundles of parallel actin fibers that emerge from the cell lamellipod. Filopodia exist in many cell types and have a role in activating and sustaining cell migration (Carragher and Frame, 2004; Chodniewicz and Klemke, 2004). eEF1A2-expressing BT549 cells showed a dramatic increase in the number and length of filopodia-like structures emerging from the lamellipod (Figure 2a). These filopodia are $>10\,\mu$M in length. Filopodia in control BT549 cells, when present, were $<1\,\mu$M. In many eEF1A2-expressing cells, the filopodia are not only greater in number but also exhibit branching as they project outward. Branched filopodia were not observed in any control BT549 cells. Filopodia structures in the eEF1A2-expressing cells are somewhat polarized, meaning that they are often longer and more branched at one

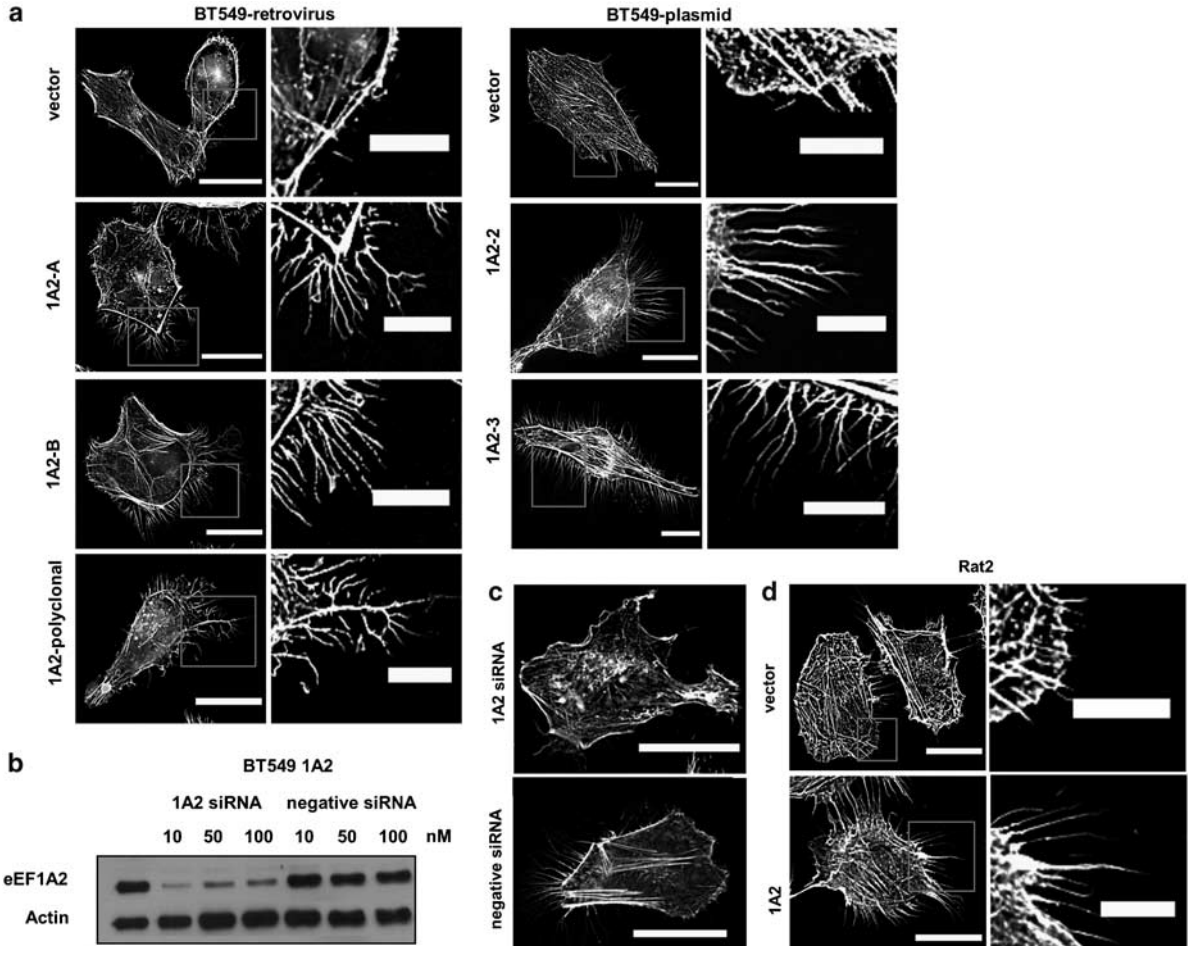


Figure 2 eEF1A2 expression induces actin cytoskeletal rearrangement. (**a**) Fluorescence deconvolution micrographs of eEF1A2-expressing and control BT549 cells with actin visualized by phalloidin (Alexa Fluor 546). Right panels are magnified versions of the boxed area of the left panel. The scale bars in each of the left and right panels are 30 and 12 μM in length, respectively. (**b**) WB of eEF1A2-expressing BT549 cells with eEF1A2 siRNA or negative siRNA control. (**c**) Fluorescence micrographs of eEF1A2-expressing cells treated with either eEF1A2 or control siRNA for 24 h and stained with phalloidin to examine actin filaments. (**d**) Fluorescence micrographs of eEF1A2-expressing and control Rat2 cells stained with phalloidin. Right panels are magnified versions of the boxed area of the left panel. The scale bars in each of the left and right panels are 30 and 12 μM in length, respectively.

side of the cell (Figure 2a). Increased filopodia number and length was observed in all eEF1A2-expressing BT549 clones, independent of whether they were made by retroviral infection or plasmid transfection. This indicates that observed eEF1A2-dependent actin remodeling is unlikely to be owing to chance clonal variation. To further confirm that the increase in filopodia formation was dependent on eEF1A2, we inhibited eEF1A2 by adding eEF1A2 short interfering RNA (siRNA) to eEF1A2-expressing BT549 cells. Using this siRNA, we are generally able to reduce the eEF1A2 level by $\sim$90% relative to control siRNA (Figure 2b). Addition of eEF1A2 siRNA resulted in a reduction in filopodia formation relative to control siRNA (Figure 2c).

To determine whether eEF1A2 could induce filopodia formation in other cell types, we next expressed eEF1A2 in non-transformed rodent Rat2 cells. Rat2 does not express detectable eEF1A2 protein (Figure 1a). Like BT549 cells, eEF1A2 expression activated the appearance of filopodia structures in Rat2 cells (Figure 2d). Filopodia were not observed in control Rat2 cells. No discernable alteration in lamellipodia or stress fibers was observed upon eEF1A2 expression. The ability of eEF1A2 to induce filopodia formation in both transformed (BT549) and non-transformed (Rat2) cell lines indicate that eEF1A2's effect on actin remodeling is unlikely to be cell-line specific.

To further characterize eEF1A2-induced filopodia, we observed their formation by video time-lapse microscopy. As shown in Figure 3a, filopodia were visible by light microscopy only in eEF1A2 expressing cells. These filopodia protruded outward from the eEF1A2-expressing cell in the general direction of cell movement, indicating that they do not derive from cytoplasmic retraction (Figure 3a). To further study the filopodia structures, we stained eEF1A2-expressing BT549 cells with a VASP antibody and phalloidin (Figure 3b). VASP is a member of the Ena (*Drosophila* enabled)/VASP (vasodilator-stimulated phosphoprotein)

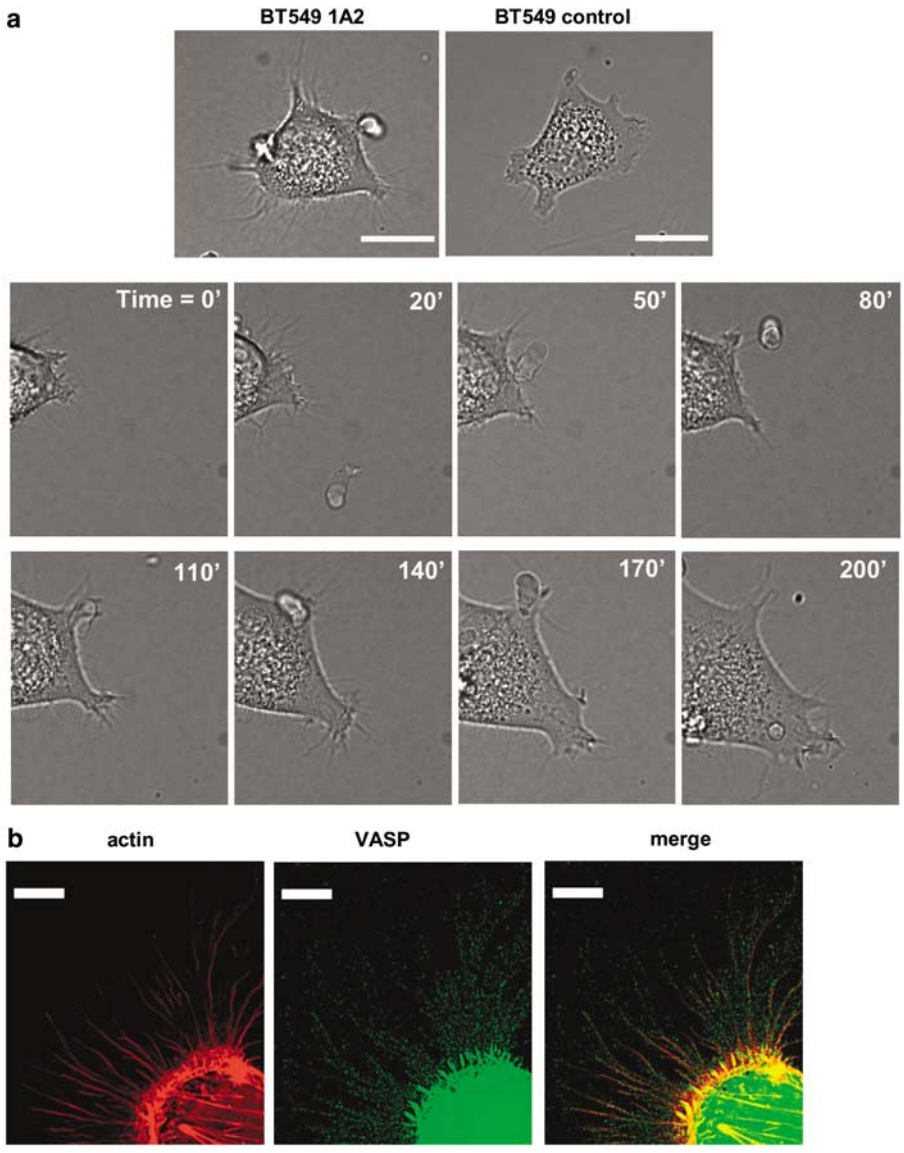


Figure 3 Structures seen in eEF1A2-expressing cells have the criteria of filopodia. Upper panels: phase contrast micrographs of control and eEF1A2-expressing BT549 cells. Lower panels time-lapse phase contrast microscopy of filopodia in an eEF1A2-expressing BT549 cell. Elapsed time is indicated in each panel. Images were captured every 5 s for 4 h. A full video of this sequence is found in the Supplementary Figures (S1). (**c**) Fluorescence micrographs of eEF1A2-expressing BT549 cell stained for actin (phalloidin) and VASP.

proteins, which are localized to focal adhesions, along stress fibers and at the tips of lamellipodia and many filopodia (Reinhard *et al*., 1992; Svitkina *et al*., 2003). VASP is a key player in the formation of filopodia. In eEF1A2-expressing cells, VASP was mostly detectable at the base of the filopodia and between and within the actin fibers.

eEF1A2-dependent actin reorganization requires P13K, Akt and ROCK activity

PI3K is involved in many cellular processes, including growth, survival and actin filament remodeling (Vivanco and Sawyers, 2002). To test the hypothesis that eEF1A2 may regulate filopodia through PI3K-dependent signaling, we added LY294002, a specific inhibitor of PI3K to eEF1A2-expressing cells and observed filopodia formation. As shown in Figure 4a and b, addition of LY294002 reduced the size and number of filopodia structures in both Rat2 and BT549 cells. This reduction was dose-dependent: filopodia shorten to $<5\,\mu$M in $10\,\mu$M LY294002 and branching is reduced in both Rat2 and BT549 cells. LY294002 ($20\,\mu$M) reduced filopodia number to wild-type levels in both cells. LY294002 had no visible effect on lamellipodia or stress fibers. These observations indicate that PI3K activity is required for actin filament rearrangement induced by eEF1A2.

As filopodia formation is dependent on PI3K, we next determined eEF1A2 could directly bind PI3K or was an activator of PI3K activity. To this end, we transiently expressed eEF1A2 in BT549 cells using an eEF1A2-adenovirus. As shown in Figure 4c, ectopically expressed eEF1A2 did not co-immunoprecipitate with

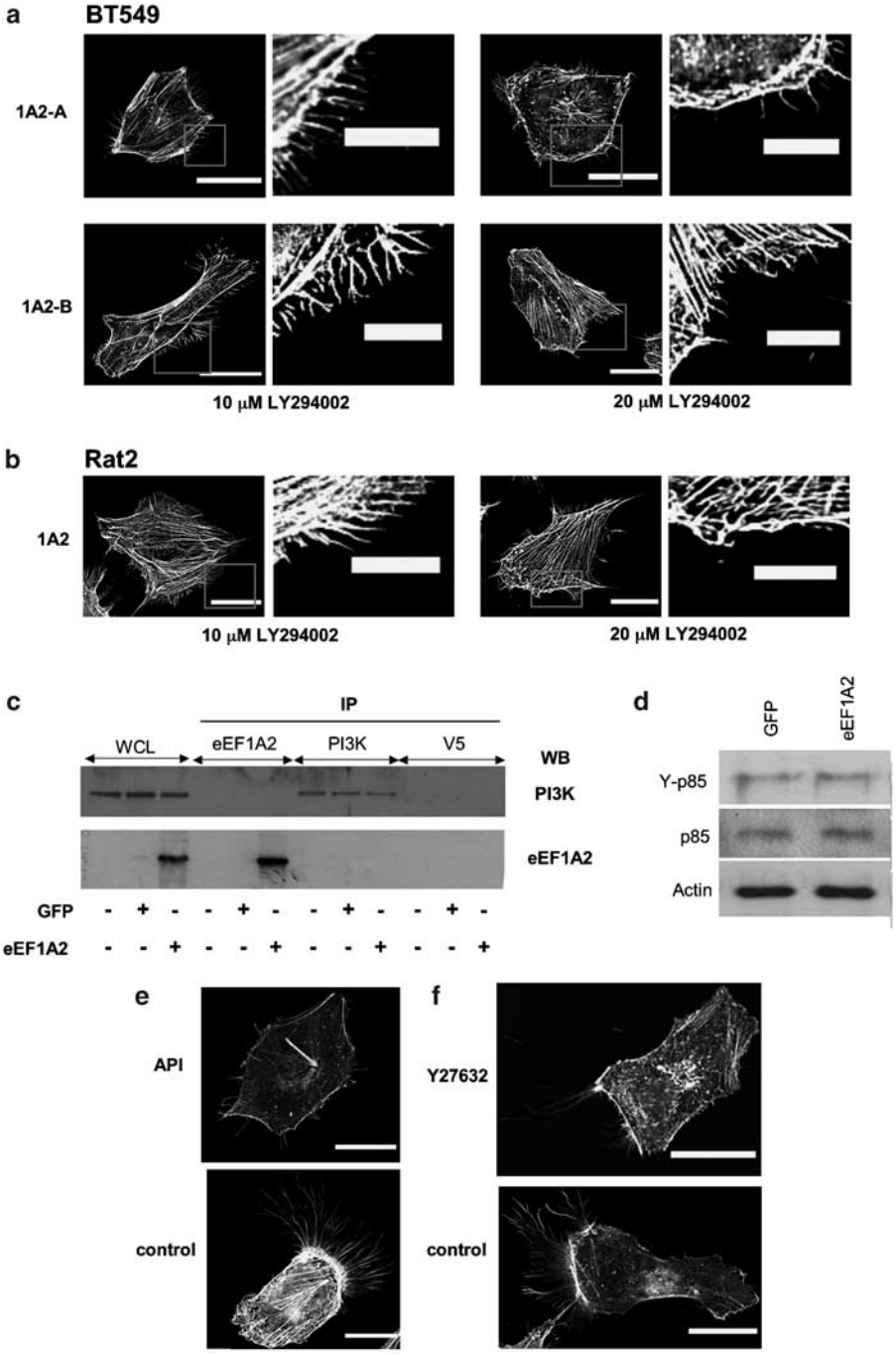


Figure 4 PI3K is required for eEF1A2-induced filopodia formation. (**a** and **b**) BT549 and Rat2 cells expressing eEF1A2 were cultured in the presence of the indicated concentrations of LY294002, a PI3K-specific inhibitor, overnight. Right panels are magnified versions of the boxed area of the left panel. The scale bar in the left and right pictures is 30 and 12 μM in length, respectively. (**c**) eEF1A2 does not physically interact with PI3K. BT549 cells were infected with an adenovirus for GFP or eEF1A2 or mock infected. Antibodies for either eEF1A2 or PI3K were used for immunoprecipitation (IP) from whole cell lysates. An anti-V5 antibody is used as an IP control. Co-immunoprecipitating proteins were detected by WB using antibodies specific for eEF1A2 and PI3K. The whole-cell lysate lane (WCL) contains 100 μg of total cellular protein and each IP was performed using 100 μg of protein lysate. (**d**) eEF1A2 does not activate PI3K. BT549 cells were infected with an Adenovirus for GFP or eEF1A2 and the levels of the indicated proteins assayed by WB. PI3K activation is measured by phosphorylation of tyrosine 508 (**e** and **f**) cells were treated with 1 μM API-2, an Akt inhibitor (**e**) or 10 μM Y27632, a ROCK kinase inhibitor overnight and stained with phalloidin. Vehicles applied to control cells.

endogenous PI3K. Furthermore, eEF1A2 expression neither altered the overall abundance of PI3K (Figure 4c) nor the activation status of PI3K (Figure 4d). PI3K activity was measured by phosphorylation of tyrosine 506 (Y508), the p85 subunit of PI3K, a marker for P13K activity (Chen *et al*., 2004).

A key target of PI3K is Akt. Akt is involved in many biological processes, among them are proliferation,

apoptosis and growth (Vivanco and Sawyers, 2002). It has also been shown that PI3K remodels actin filaments through the activation of Akt (Qian *et al*., 2004). To test whether Akt is also involved in filopodia formation by eEF1A2, we used Acute Panic Inventory-2 (API-2), a specific inhibitor of Akt (Yang *et al*., 2004) to eEF1A2-expressing cells. As shown in Figure 4e, addition of API-2 greatly reduced the formation of filopodia in eEF1A2-expressing BT549 cells, indicating that Akt is required for eEF1A2-dependent filopodia formation.

Recently, it has been shown that PI3K regulates filopodia dynamics through Akt and ROCK (Tornieri *et al*., 2006). ROCK kinase is a downstream effector of the RhoA GTPase (Riento and Ridley, 2003). To investigate whether Rho signaling has any role in the formation of filopodia by eEF1A2, we used ROCK inhibitor Y27632. As shown in Figure 4f, addition of Y27632 decreased the number and length of filopodia to the wild-type level in eEF1A2-expressing BT549 cells, suggesting the importance of ROCK kinase in the formation of filopodia by eEF1A2.

Expression of eEF1A2 is sufficient to activate Akt in a PI3K-dependent manner

As filopodia production by eEF1A2 was, at least in part, dependent on Akt activity, we determined whether eEF1A2 might be involved in Akt activation. Akt is activated by its membrane translocation and phosphorylation at Thr 308 and Ser 473. Thus, phosphorylations of Thr 308 and Ser 473 serve as surrogate markers of Akt activation. To determine whether the expression of eEF1A2 has any effect on Akt activation, we transiently expressed eEF1A2 in BT549 cells using an eEF1A2-adenovirus and then used Western blotting to determine the phosphorylation status of Thr 308 and Ser 473. As shown in Figure 5a, infection of eEF1A2 increased phosphorylation of both Thr 308 and Ser 473 relative to GFP-infected cells. Moreover, BT549 cells that stably express eEF1A2 also showed increased levels of phosphorylation at both Thr 308 and Ser 473 sites relative to control cells (Figure 5b). We next used siRNA to inactivate eEF1A2 in MCF7 breast cancer cells. Endogenous eEF1A2 is readily detectable in wild-type MCF7 cells (Figure 1a). In these cells, eEF1A2 siRNA reduced Akt phosphorylation somewhat (1.8 when normalized to actin). Inhibition of Akt activity was not complete, suggesting that as eEF1A2 has a physiological role in controlling Akt activity, there are likely to be eEF1A2-independent pathways of Akt activation. To determine whether Akt activation by eEF1A2 was dependent on PI3K activity, we treated eEF1A2-overexpressing BT549 cells with LY294002. As shown in Figure 5d, LY294002 inhibited the activation of Akt in a dose-dependent manner. However, addition of rapamycin, an inhibitor of mTOR/Raptor complex,

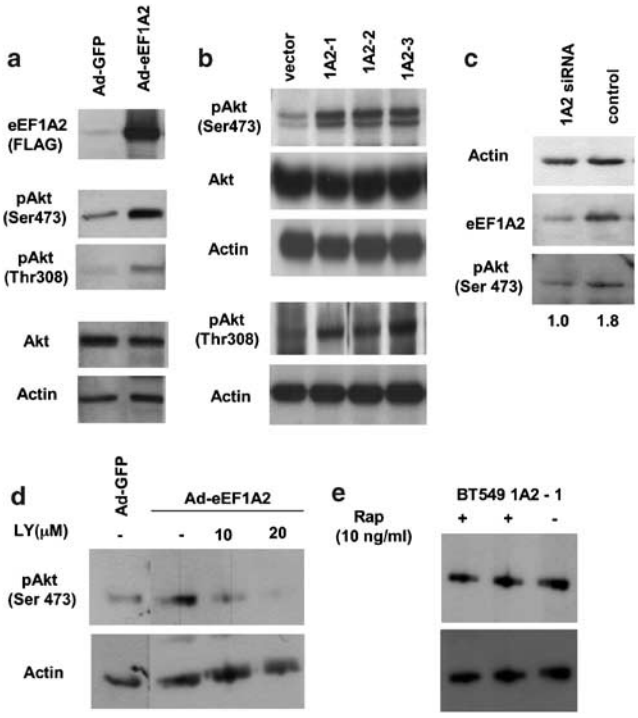


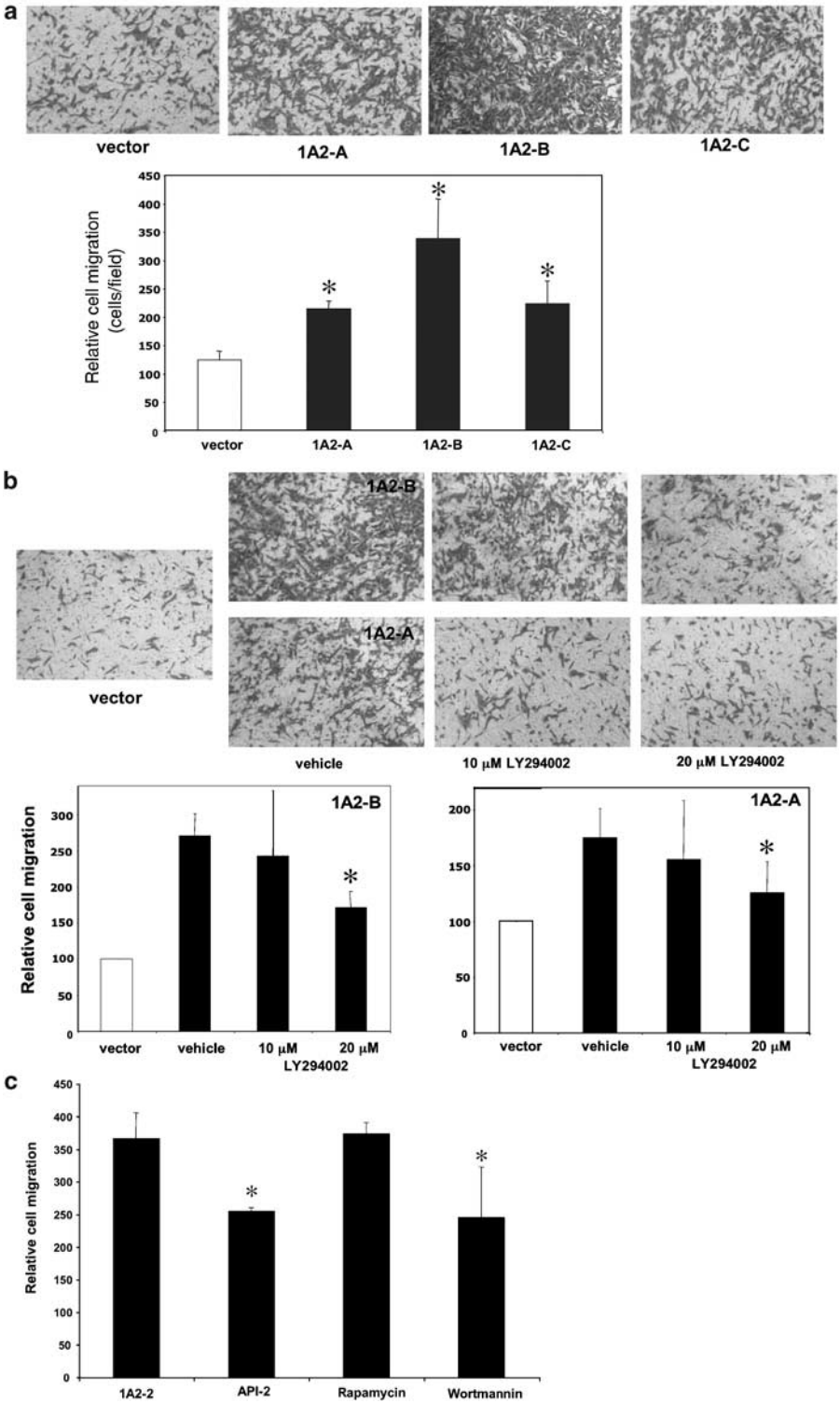
Figure 5 eEF1A2 activates Akt in a PI3K-dependent manner. Total protein extracts from eEF1A2-expressing cells were analysed by WB using Akt, phospho-Akt (Ser 473), or phospho-Akt (Thr 308) antibodies. The protein extracts from cells expressing GFP or the empty vector were used as controls and actin used as a loading control. (**a**) Akt-phosphorylation 24 h after infection with an eEF1A2 of GFP-adenovirus. (**b**) Akt-phosphorylation in stable eEF1A2-expressing BT549 cells. (**c**) Akt phosphorylation in wild-type MCF7 cells that have been treated with an eEF1A2 siRNA. Numbers reflect pAkt band intensity normalized to the actin loading control and is representative of three independent experiments. (**d**) Akt-phosphorylation, 24 h after infection with an eEF1A2 adenovirus and a 2-h treatment with indicated concentrations of LY294002. (**e**) Akt-phosphorylation of eEF1A2-expressing BT549 cells that were treated with rapamycin for 2 h.

Figure 6 eEF1A2 expression increases cell migration. (**a**) The indicated clones of eEF1A2-expressing BT549 cells were serum-starved overnight and placed in a transwell migration chamber. The photographs show a representative field from each of the cell lines that have migrated into the chamber. In the lower panel, migration is expressed as a percentage of vector only controls and is the mean and s.d. of triplicate independent experiments. Enhanced migration is statistically significant ($P<0.05$, Students's *t*-test) and marked by an asterisk. (**b**) eEF1A2-induced migration is inhibitable by PI3K inhibition. Indicated eEF1A2-expressing BT549 cells were serum-starved overnight, and incubated with LY294002 for 2 h at the indicated concentrations before being placed in the transwell chamber. The micrographs show a representative field of cells that have migrated into the chamber. In the lower panel, migration is expressed as a percentage of vector only controls and is the mean and s.d. of triplicate independent experiments each with triplicate counts. (**c**) eEF1A2-expressing BT549 cells were treated with 1 μM API-2 (overnight), 10 ng/ml rapamycin (2 h) or 10 nM wortmannin (30 min) and subjected to cell migration as above. As control cells were treated with vehicles only, migration is expressed as a percentage of vector only controls and is the mean and s.d. of triplicate independent experiments, each with triplicate counts. Migration inhibition is statistically significant ($P<0.05$, Students's *t*-test) and marked by an asterisk.

had no affect on Akt activation by eEF1A2 (Figure 5e). Taken together, our results indicate that eEF1A2 is a functional regulator of Akt activity.

Expression of eEF1A2 increases cell migration

Filopodia structures are critical for cell migration and invasion (Chodniewicz and Klemke, 2004) and activation of Akt has been reported previously to increase cell migration and invasion (Arboleda *et al*., 2003). Enhanced formation of filopodia and Akt activation in eEF1A2-overexpressing cells suggests that eEF1A2 might be involved in both migration and invasion. To assess whether overexpression of eEF1A2 is sufficient to increase cell migration, we measured the migration of BT549 cells using transwell migration assays. As shown in Figure 6a, eEF1A2 expression significantly enhanced



cell migration relative to control cells (Student's *t*-test, $P<0.05$). To determine whether PI3K activity is required for this increase in cell migration, we measured migration in the presence of LY294002. As shown in Figure 6b, PI3K inhibition reduced the extent of migration significantly in eEF1A2-expressing cells (Student's *t*-test, $P<0.05$). However, the magnitude of this migration in the presence of 20 μM LY294002 was still higher than the control, and increasing LY294002 dosage had no further inhibitory effect on migration but did cause cytotoxicity (data not shown). To investigate whether Akt has any effect on the cell migration by eEF1A2, we added Akt inhibitor, API-2, to the BT549 cells overexpressing eEF1A2. As in the case of LY294002 and wortmannin, addition of API-2 significantly reduced cell migration (Student's *t*-test, $P<0.05$), but it did not completely abolish it to the same level as control cells (Figure 6c). Similarly, addition of wortmannin, another PI3K inhibitor, at the concentration of 10 nM significantly (Student's *t*-test, $P<0.05$) decreased the magnitude of cell migration in eEF1A2-expressing cells (Figure 6c). These results suggest that activation of PI3K and Akt is important in eEF1A2-induced cell migration.

Expression of eEF1A2 is sufficient to increase cell invasion

To investigate a role for eEF1A2 in invasion, we coated transwells with Matrigel to simulate the basal lamina. As shown in Figure 7, significantly (Student's *t*-test, $P<0.05$) more eEF1A2-expressing cells invaded through the matrix than control cells, suggesting that eEF1A2 is an enhancer of cell invasion. Furthermore, this enhanced invasion in eEF1A2-expressing cells was significantly (Student's *t*-test, $P<0.05$) inhibited by LY294002 and API-2, indicating a dependence of eEF1A2-induced invasion on PI3K and Akt activities (Figure 7a and b). However, like the case with eEF1A2-induced migration by LY294002/wortmannin or API-2 never completely reduced invasion to wild-type levels,

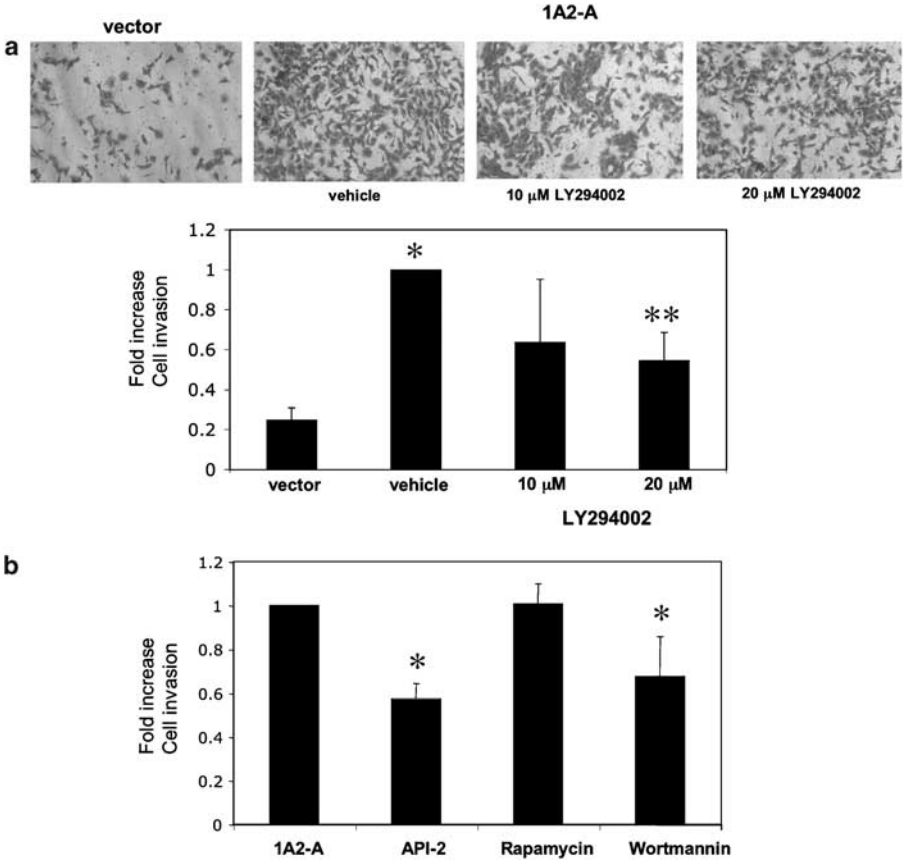


Figure 7 eEF1A2 expression increases cell invasion. eEF1A2-overexpressing BT549 were serum-starved overnight. After pre-incubation with LY294002 or DMSO (vehicle) for 2 h, cells were subjected to the invasion assay using Matrigel-coated transwells for approximately 48 h. The photographs show a representative field from each of the cell lines that have invaded through the Matrigel. In the lower panel, invasion is expressed as a percentage of vector only controls and is the mean and s.d. of triplicate independent experiments with triplicate counts. Enhanced migration of 1A2 relative to the vector only control is statistically significant ($P<0.05$, Students's *t*-test) and marked by a single asterisk. Similarly, the ability of LY294002 to attenuate invasion inhibition is statistically significant ($P<0.05$, Students's *t*-test) and marked by a double asterisk. (**b**) eEF1A2-expressing BT549 cells were treated with 1 μM API-2 (overnight), 10 ng/ml rapamycin (2 h) or 10 nM wortmannin (30 min) and subjected to cell invasion as above. As control cells were treated with vehicles only, invasion is expressed as a percentage of vector only controls and is the mean and s.d. of triplicate independent experiments, each with triplicate counts. Invasion inhibition is statistically significant ($P<0.05$, Students's *t*-test) and marked by a single asterisk.

suggesting the existence of PI3K-independent pathways through which eEF1A2 stimulates invasion and migration. Addition of mTOR/Raptor inhibitor, rapamycin, had no effect on either cell migration or invasion by eEF1A2, indicating that mTOR/Raptor pathway is not involved in these processes (Figures 6c and 7b).

Expression of eFF1A2 has no effect on spreading and adhesion

Cell adhesion and spreading are cellular processes that occur during cell migration. To determine whether eEF1A2 had any effect on cell spreading, we used video microscopy to observe the behavior of BT549 cells on fibronectin-coated plates. eEF1A2-expressing cells and controls were trypsinized, plated and observed for lamellipodia formation. As shown in Figure 8a, by 10 min, control cells begin to form a visible lamellipod (arrow) and by 28 min have developed large lamellipodia. eEF1A2-expressing cells formed a lamellipod with similar kinetics, indicating that eEF1A2 expression is not affecting cell spreading, as measured by lamellipod formation. This result is consistent with our observation (Figure 2) that eEF1A2 does not affect lamellipodia appearance. To determine whether eEF1A2 affected cell adhesion, we trypsinized BT549 cells and added them to fibronectin-coated coverslips. At various times after cell addition, coverslips were washed and fixed to remove the unstably attached or detached cells and to observe cells that had strongly adhered to their growth substrate. The number of adhering cells therefore serves as an indirect measure of cell adhesion. As shown in Figure 8b, control and eEF1A2-expressing cells had similar adhesive parameters. Thus, eEF1A2 detectably affects neither cell spreading nor the kinetics of the adhesion process.

Discussion

eEF1A2, protein elongation factor eEF1A2, is likely to be an important oncogene (Thornton *et al*., 2003). eEF1A2 is highly expressed in 30–60% of tumors of the ovary, breast and lung (Lee, 2003; Li *et al*., 2005; Tomlinson *et al*., 2005; Kulkarni *et al*., 2006). Wild-type eEF1A2 has transforming properties: it enhances focus formation, allows growth in soft agar and increases the tumorigenicity of mouse and human cells in nude mice (Anand *et al*., 2002). However, the mechanism by which eEF1A2 induces oncogenesis is unclear. Here, we show that eEF1A2 activates the Akt serine/threonine kinase and stimulates actin remodeling that is dependent on PI3K, Akt and ROCK. eEF1A2 expression also makes cells more motile and invasive *in vitro*. We propose that eEF1A2 promotes tumor development through PI3K-dependent activation of Akt and an Akt-dependent increase in filopodia formation and motility.

eEF1A2 is one of two isoforms of eEF1A, eEF1A1 and eEF1A2. eEF1A proteins are relatively well conserved during evolution, and eEF1A homologes have been identified in yeast and *Caenorhabditis elegans*, among others. Beyond its role in translation, eEF1A has additional cellular functions. Interaction of eEF1A with

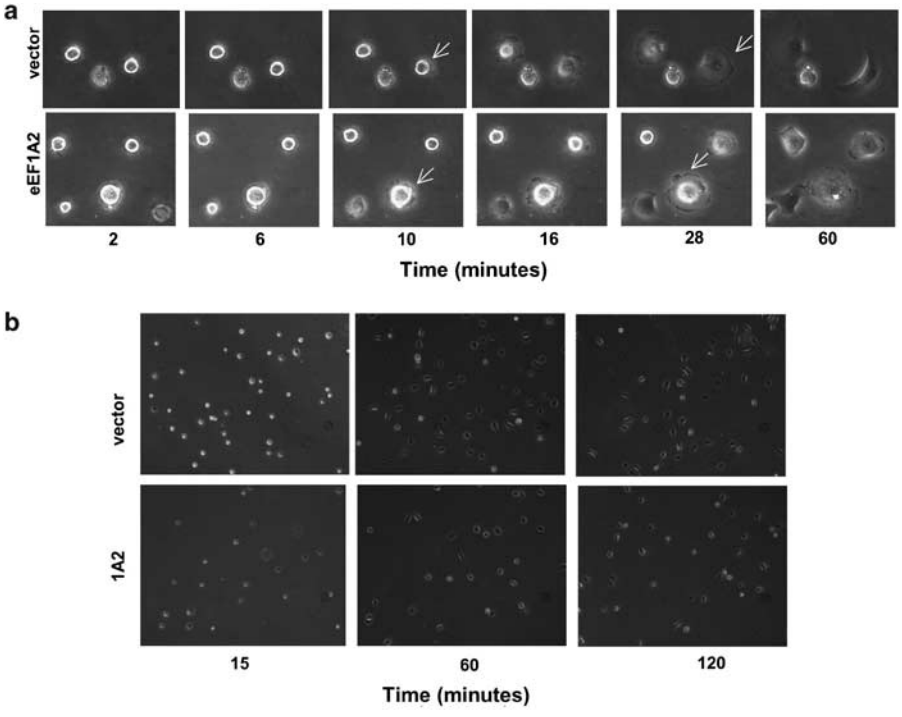


Figure 8 eEF1A2 expression has no effect on cell spreading and adhesion. (**a**) eEF1A2 expressing cells were trypsinized, and placed on a growth substrate containing fibronectin and observed as a function of time using time-lapse video microscopy on a heated stage. White arrows mark the first appearance of lamellipodia in the plated cells. (**b**) Cells were trypsinized and plated onto fibronectin-coated coverslips. At indicated time points, cells were washed with PBS, fixed using 3.7% formaldehyde and adherent cells were counted.

the actin cytoskeleton was first demonstrated in *Dictyostelium* (Yang *et al*., 1990) and eEF1A proteins of several animal genera have been reported to bind F-actin and bundle them *in vitro* (Yang *et al*., 1990; Edmonds *et al*., 1996; Munshi *et al*., 2001). There are two *S. cerevisiae* eEF1A homologues (*TEF1*, *TEF2*); both are more related to eEF1A1 than eEF1A2. In the Tef1 protein, actin bundling and translation elongation are separable enzymatic activities because Tef1 point mutations that inhibit actin binding and bundling have no substantial effect on the translation (Gross and Kinzy, 2005). The two functions are also physically separate on the eEF1A protein: two domains in the C-terminus of eEF1A bind actin whereas the GTP binding, hydrolysis and tRNA interacting domains are found in the eEF1A N-terminus (Hershey, 1991; Condeelis, 1995). Ectopic expression of Tef1 or Tef2 proteins in *S. cereviseae* reduces the accumulation of F-actin structures at the bud (Munshi *et al*., 2001) but the effect of eEF1A on the actin cytoskeleton of mammalian cells has not been reported previously. We find that eEF1A2 expression increases the formation of filopodia structures in rodent and human cells. Filopodia are bundles of parallel actin that protrude outward from the cell membrane. Protrusive actin structures have an important role in driving cell migration and invasion, particularly in metastatic tumor cells (Yamaguchi *et al*., 2005). Consistent with an involvement of filopodia in cell movement, we found that eEF1A2 causes cells to be more migratory and invasive *in vitro*.

The increased invasiveness of eEF1A2-expressing cells suggests that eEF1A2 might have a role in tumor metastasis. In fact, eEF1A has been reported previously to be overexpressed in metastatic rat mammary adenocarcinoma cell lines compared to non-metastatic ones (Pencil *et al*., 1993; Edmonds *et al*., 1996). As metastatic development is the ultimate cause of death in cancer, the ability of eEF1A2 to increase migration predicts that eEF1A2-expression would be associated with poor prognosis. Consistent with this idea, lung cancer patients whose tumor have high levels of eEF1A2 have a reduced probability of survival compared to their non-eEF1A2-expressing counterparts (Li *et al*., 2005). In addition, high eEF1A2 expression is associated with severe tumor grades and metastasis in several cancer cell lines (Pencil *et al*., 1993; Edmonds *et al*., 1996; Li *et al*., 2005). We have recently determined the prognostic significance of eEF1A2 in breast cancer (Kulkarni *et al*., 2006). To our astonishment, we find that high eEF1A2 protein expression correlates with an increased probability of 20-year survival (Kulkarni *et al*., 2006). eEF1A2 expression is independent of other breast cancer prognostic factors. It is a surprise to us that expression of an Akt activator and an enhancer of cell migration correlates with enhanced survival probability. For example, expression of Snail, an inducer of cell migration, correlates with poor survival in breast cancer (Moody *et al*., 2005). Because of its transforming capacity and its high tumor-specific expression, eEF1A2 is likely to be promoting tumor growth in breast cells. However, we speculate that though eEF1A2 may activate several oncogenic processes (i.e. Akt activation and enhancing migration), it may be a less potent breast cancer oncogene than others. Alternatively, eEF1A2 may alter some malignant process that inhibits patient mortality. For example, a capacity for eEF1A2 to promote cell proliferation may make the tumor more susceptible to chemotherapy or eEF1A2 expression may drive tumor cells into a more differentiated and therefore less malignant state. Possibly, the enhancement of motility by eEF1A2 may preclude the successful colonization of metastatic sites because migratory cells that leave the primary tumor may be too motile to stably colonize secondary sites. The observation that eEF1A2 expression is a marker of good prognosis in the breast (Kulkarni *et al*., 2006) and a marker of poor prognosis in the lung (Li *et al*., 2005) may reflect the different microenvironments and physiology of these two malignancies.

The PI3K and Akt pathway regulates many cellular processes, including cell adhesion, proliferation, survival and cytoskeletal rearrangement (Vivanco and Sawyers, 2002). Our observation that eEF1A2 activates Akt suggests a plausible explanation for the capacity of eEF1A2 to transform cells *in vitro* and suggests that eEF1A2 promotes tumor development through Akt. Furthermore, we find that the capacity of eEF1A2 to stimulate cell migration is Akt dependent. Overexpression of Akt2 has been shown to increase invasion and metastasis in human breast and ovarian cancer cells in a PI3K-dependent manner (Arboleda *et al*., 2003). The mechanism by which eEF1A2 could activate Akt is unknown, although we speculate that phosphatidylinositol signaling is involved (see below). Akt2 has recently been identified as an interacting partner of eEF1A1 (Lau *et al*., 2006). It is therefore possible that binding of eEF1A1 or eEF1A2 to Akt may directly activate the kinase, although the biological significance of eEF1A1/Akt interaction has yet to be determined. It is also unknown whether eEF1A2 directly binds any Akt isoform. Chang and Wang (2006) found that overexpression eEF1A2 in mouse NIH 3T3 fibroblast cells resulted in increase of the Akt level in these cells. However, we did not see any increase in the steady state of Akt levels in BT 549 breast cancer cells or Rat2 cell. Although a role for eEF1A2 in controlling Akt abundance is possible, eEF1A2 does not alter steady-state Akt levels in our hands.

We have found that induction of filopodia by eEF1A2 is dependent on PI3K, Akt and ROCK signaling. The ability of eEF1A2 to activate migration and invasion in breast cancer cells *in vitro* is largely, but not completely, PI3K-dependent. Therefore, eEF1A2 is likely to be involved in both PI3K-dependent and independent pathways that control filopodia formation and cell migration and invasion. Overexpression of PI3K has been shown to cause an increase of both lamellipodia and filopodia formation in chicken embryo fibroblast cells as well as a decrease of actin stress fibers (Qian *et al*., 2004). Akt has been postulated to modulate actin remodeling through two substrates: Girdin

(Enomoto *et al*., 2005) and Pak1 (Zhou *et al*., 2003). Girdin is an actin bundling protein (Enomoto *et al*., 2005) and Pak1 is a member of the PAK (p21-activated kinase) family serine/threonine kinases that phosphorylate several proteins that directly or indirectly stimulate actin remodeling (Kumar *et al*., 2006). In the future, it will be interesting to determine whether Girdin or Pak1 is involved in eEF1A2-dependent filopodia formation.

Our study shows that eEF1A2 overexpression only affects filopodia but not lamellipodia or stress fiber formation in a PI3K/Akt-dependent manner. The ability of eEF1A2 to activate filopodia only indicates that eEF1A2 does not regulate all types of actin structures in mammalian cells. Although eEF1A proteins from yeast and *Dictyostelium* have been reported to have actin bundling activity, it is not clear whether eEF1A2 regulates filopodia by directly bundling actin. It has been suggested, however, that eEF1A cross-links actin filaments in a way that exclude other proteins to cross-link F-actin (Owen *et al*., 1992), suggesting a direct interaction between eEF1A2 and actin filaments. Because eEF1A2 induction of filopodia is dependent on PI3K, eEF1A2 may regulate actin rearrangement not through direct actin bundling but through phosphoinositide-dependent signaling. The dependence of eEF1A2 induction of filopodia on ROCK as well as PI3K is consistent with this idea. Because ROCK is a Rho effector and Rho activity is dependent on PI3K, we hypothesize that eEF1A2 activates actin rearrangement by stimulating PI(3,4,5)P generation and activating ROCK through Rho A. Because we find that the eEF1A2-dependent activation of Akt is dependent on PI3K activity, we also propose that Akt activation by eEF1A2 is via PIP_3 generation.

A role for eEF1A proteins in phospho-inositol signaling has been suggested previously by the identification of the carrot eEF1A homologe PIK-A49 as direct activator of PI4K (Yang *et al*., 1993). Because PIK-A49 has not been cloned as a full-length complementary DNA (cDNA), it is unclear whether PIK-A49 is a bona fide eEF1A gene, but protein sequences of PIK1A9 have a high homology to eEF1A proteins from multiple species. Because PI4K activity has the potential to regulate the downstream abundance of important lipid second messengers PI(4,5)P and PI(3,4,5)P, eEF1A2 may also activate filopodia formation through activation of PI(3,4,5)P abundance by increasing PI4K activity. A recent report by Pendaries *et al*. (2006) indicate that PI5P created by the *Shigella* parasite can activate Akt in a PI3K-dependent fashion, suggesting that phospholipids abundance may activate PI3K-dependent signaling . It is possible that eEF1A2 may enhance PI (3,4,5)P generation by activating PI4K. Recent identification of Akt2 as a potential interacting partner of eEF1A1 and Fascin (an actin bundling protein) and mRif (mouse Rho in filopodia) as eEF1A2-binding proteins (Chang and Wang, 2006; Lau *et al*., 2006) suggest that eEF1A2 controls filopodia formation by bringing a complex of actin and PI4K/Akt/Rho signaling proteins together.

The two human eEF1A isoforms (eEF1A2 and eEF1A2) are very similar proteins (92% amino-acid identity). The two isoforms appear to have the same activity in protein elongation (Kahns *et al*., 1998). However, it has yet to be determined whether both isoforms have equivalent oncogenic capacity or have the same ability to alter cell motility. We have been unable to directly test the *in vitro* effect of high eEF1A1 expression because we have been unsuccessful at generating cell lines ectopically expressing high levels of eEF1A1 (D Purcell and JM Lee, unpublished observations). We speculate that high levels of eEF1A1 may be toxic to a cell or that some cellular feedback mechanism exists to prevent steady-state eEF1A1 levels from increasing beyond some threshold level. If eEF1A1, like eEF1A2, were oncogenic, we would expect that, like eEF1A2 (Lee, 2003), the gene for eEF1A1 would be commonly amplified during oncogenesis and that some tumors would have high eEF1A1 mRNA expression. The eEF1A1gene, *EEF1A1*, maps to 6q14.1. This region is not frequently amplified in any malignancy and is deleted in squamous cell carcinoma, osteosarcoma and prostate cancer (Nathrath *et al*., 2002; Verhagen *et al*., 2002; Fitzsimmons *et al*., 2003). This suggests that 6q14.1 contains a tumor suppressor. Similarly, we have not detected high expression of eEF1A1 in ovarian or breast tumors (Anand *et al*., 2002 and unpublished data). Although the issue of eEF1A1's oncogenicity will require further investigation, based on the lack of tumor-specific *EEF1A1* amplification or gene overexpression, we speculate that eEF1A1 is unlikely to be an oncogene, an idea consistent with the high level of eEF1A1 expression in normal tissue.

The ability of eEF1A2 to enhance migration and Akt activation suggests that eEF1A2 can be a target for anticancer therapy. The drug Aplidin, a derivative of didemnin B, an eEF1A-binding drug that inhibits GTP hydrolysis and translation elongation (Crews *et al*., 1994), is currently undergoing clinical trials as an anticancer agent (Jimeno *et al*., 2004). Because eEF1A2 is associated with poor prognosis in lung cancer (Li *et al*., 2005), these patients would be predicted to benefit from eEF1A2 inactivation.

In summary, we report several novel functions of the eEF1A2 oncogene: eEF1A2 expression activates Akt and stimulates cell migration, invasion and filopodia formation in an Akt and PI3K-dependent pathway. These observations are consistent with the idea that eEF1A2 may promote tumor development through phosphatidyl-inositol signaling and actin remodeling.

Materials and methods

Cell lines

Rat2, Phoenix and BT549 cell lines were purchased from the American Tissue Culture Collection (ATCC, Manassas, VA, USA) and were grown in ATCC recommended media in 5% CO_2 at 37°C in 1005 humidity. ROSE 199 cells were a gift of Dr Nelly Auersperg (UBC).

Plasmid constructs, siRNA and cell line preparation
To generate an eEF1A2 retrovirus, full-length eEF1A2 with the C-terminal V5 tag was subcloned into pLXSN (Clontech, Mountain View, CA, USA) in *Eco*RI and *Xho*I sites. Retrovirus was generated in Phoenix Ampho cells. Fifty percent confluent BT549 were infected with 1 ml of viral supernatant in the presence of 4 μg polybrene (Sigma, Oakville, ON, Canada) and selected using G418 for 2 weeks. For eEF1A2 adenovirus generation, eEF1A2 was subcloned into pShuttle-IRES-hrGFP-1 in *Eco*RV and *Xho*I sites and the virus was manufactured by the central facility of the University of Ottawa. eEF1A2 and GFP control adenovirus were used at an multiplicity of infection (MOI) of 200. To produce eEF1A2-pCDNA 3.1, full-length eEF1A2 with the C-terminal V5 tag was subcloned into pcDNA 3.1/GS (Invitrogen, Burlington, ON, Canada) in *Eco*RI and *Xho*I sites. Lipofectamine 2000 (Invitrogen) was used for transfection as recommended by the manufacturer. Zeocin was used to select stable cell lines. The sequence of eEF1A2-siRNA is 5′-UCGAACUUCUCAAUG GUCCTT-3′. Transient transfection of siRNA was carried out using 4 μl of Lipofectamine 2000 and 5 μl of siRNA (100 nM).

Western blotting and immunoprecipitation
Cells were lysed using radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-Cl; pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM ethylenediaminetetraacetic acid (EDTA); pH 7.0, 150 mM NaCl) supplemented with 1% aprotonin, 1 mg/ml leupeptin, 50 mM NaF, 1 mM Na_3VO_4, 10 μg/ml pepstatin in ethanol, 1 mM phenylmethylsulfonyl flouride (PMSF) in dimethylsulfoxide (DMSO). Protein concentrations were determined by Bradford protein assay (Pierce, Rockford, IL, USA). Approximately 30 μg of protein extracts were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane. Anti-V5-horseradish peroxide (HRP) (Invitrogen), anti-FLAG (Sigma), β-actin (Sigma), Akt, phospho-Akt (Cell Signaling Technology, Danvers, MA, USA), goat anti-mouse IgG, HRP-conjugate (Upstate Cell Signaling Solutions) and anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technology) were used according to the manufacturer's recommendations. Anti-eEF1A2 was manufactured as described (Kulkarni *et al*., 2006) and was used at 1:2000 dilution. Protein bands were visualized by chemiluminescence reagent enhanced chemiluminescence (ECL) (Amersham, Piscataway, NJ, USA). For PI3K/eEF1A2 interaction, BT549 cells were grown to 50% confluence and transduced with either GFP or eEF1A2 adenovirus (200 MOI) or left untransduced. After a 24-h infection period, cells were lysed in RIPA buffer. Out of the total protein, 100 μg from each case were pre-cleared with protein A agarose (Upstate Cell Signaling Solutions) or protein G sepharose (Amersham Biosciences, Piscataway, NJ, USA) for 1 h at 4°C. 2–4 μg of anti-PI3K p85 (Upstate Cell Signaling Solutions, Charlottesville, VA, USA) or anti-V5 (Sigma) antibody were added to lysates and incubated overnight at 4°C. The antibodies used for the Western blot are: PI3Kp85 (1:2000 in TBST), Y-pPI3Kp85 (Santa Cruz Biotechnology Inc.; 1:1000 in TBST, Santa Cruz, CA, USA), Flag M2 (Sigma; 1:2000 in TBST), and V5 (1:2000 in TBST), anti-rabbit IgG-HRP conjugate (Cell Signaling Technology; 1:5000 in TBST), and anti-mouse IgG (Upstate Cell Signaling Solutions; 1:5000 in TBST).

Immunofluorescence and video microscopy
Cells were grown on coverslips coated with 50 μg/ml poly-D-lysin (Sigma) before fixation or video microscopy. For immunofluorescence staining, cells were fixed with 3.7% formaldehyde for 1 h and permeabilized by 0.1% Triton X-100 for 5 min. Following blocking with 2% goat serum and 1% bovine serum albumin (BSA), cells were labeled with Alexa Fluor 546 phalloidin (Molecular Probes, Burlington, ON, Canada) at 1:40 dilution for 1 h VASP monoclonal antibody (Transduction Laboratories, San Jose, CA, USA) was used at 1:50 dilution followed by secondary antibody Alexa Fluor 488-goat antibody at 1:450 dilution. After washing three times with PBS, cells were mounted on slides using fluorescent mounting medium (Dako Cytomation, Golstrup, Denmark). Slides were analysed by Leica DM IRE2 using 635 nm filter. Images were acquired by Retiga 12 bit camera (Leica, Richmond Hill, ON, Canada) and deconvoluted using Volocity 3.1 software (Improvision, Lexington, MA, USA). Images for time lapse microscopy were captured using the Axiovert 200 ZEISS microscope and ZEISS camera, and analysed by Axioversion Rel4.5 software.

Cell migration and invasion assays
Cells were serum-starved overnight. The top chambers of 6.5-mm Corning Costar transwells (Corning, NY, New York, USA) were loaded with 0.2 ml of cells (5×10^5 cells/ml) in serum-free media. Complete media 0.6 ml was added to the bottom wells and cells were incubated at 37°C overnight. Cells on the top layer were removed and the images of the cells at the bottom of the membrane were captured using a Canon camera and a Zeiss Axio Vert microscope. The mean values were obtained from three individual experiments using Excel Microsoft software and subjected to Student's *t*-tests. For cell invasion assay, cells were serum-starved overnight. The 24-well cell culture inserts (8 μm pore size, BD Biosciences, San Jose, CA, USA) were loaded with 0.5 ml of cells (5×10^5 cells/ml) in serum-free media. Complete media (0.5 ml) was added to the bottom wells and cells were incubated at 37°C for about 2 days. Cells were fixed, stained and analysed as above.

Adhesion and spreading assays
Adhesion and spreading assays were performed as described in Rodrigues *et al*. (2005). Images were acquired by the Retiga 12 bit camera (Leica). Images for time-lapse microscopy for adhesion assay were captured using the Leica DM IRE2 microscope.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).

APPENDIX C

CURRICULUM VITAE

Dixie E. Pinke, Ph.D.

Summary of Qualifications

- Completed a Ph.D. in Biochemistry at the University of Ottawa
- Author of 3 peer review journal articles
- Has been invited to speak at two international research conferences
- Obtained two competitive Doctoral Research Fellowships
- Over six years experience in a research environment
- Experience collecting, analyzing and reporting on quantitative and qualitative data
- Experience in the use and maintenance of statistical data sets
- Experience creating databases and analyzing data using MS Excel
- Advanced knowledge of Microsoft Word, Excel, PowerPoint, Outlook and Adobe Creative Suites Software
- Strong interpersonal skills and the ability to work as part of a team or independently. Have level 1 Coaching Certification.
- Excellent communication skills both orally and written
- Excellent organizational, planning, time management and multitasking skills
- Ability to work under pressure of deadlines and changing priorities with little or no supervision
- Good analytical, problem solving and judgment skills
- Self-motivated and results oriented
- Speaks English (complete fluency) and French (Ontario Bilingual Certificate)
- Government of Canada French SLE results: CBB

Education

Ph. D. Candidate - Department of Biochemistry **2007 – 2012**
University of Ottawa

M. Sc. Candidate – Department of Biochemistry **2006 –2007**
University of Ottawa

B.Sc. (Honours) – Department of Biochemistry **2001 – 2005**
University of Ottawa; Graduated Cum Laude

Publications

1. **Pinke, D.E.** and J.M. Lee. 2011. Control of acinar morphogenesis by the lipid kinase PI4KIIIβ. *Experimental Cell Research*. 317 (17): 2503-2511.
2. **Pinke, D.E.,** Kallogher, S., Francetic, T., Huntsman, D., and J. M. Lee. 2008. The prognostic significance of elongation factor eEF1A2 in ovarian cancer. *Gynecologic Oncology*. 108(3):561-568.
3. Amiri, A., Noei, F., Jeganathan, S., Kulkarni, G, **Pinke, D.E.,** & J.M. Lee. 2007. eEF1A2 activates Akt and induces Akt-dependent actin remodeling and enhances cell invasion and migration. *Oncogene*. 26: 3027-3040.

Invited Speaking Engagements

1. **Pinke, D.** Control of three-dimensional breast morphogenesis by cooperation between elongation factor eEF1A2 and the PI4KIIIβ inositol kinase (October 2009) European Molecular Biology Organization (EMBO) Presentation (Heidelberg, Germany)
2. **Pinke, D.** Control of three-dimensional breast morphogenesis through protein elongation factor eEF1A2 (June 2008) Gordon Research Conference Presentation (Biddeford, Maine, USA)

Awards, Distinctions and Fellowships

Doctoral Research Award **2009 – 2012**
Canadian Breast Cancer Foundation

Doctoral Research Award **2008 – 2009**
Canadian Institute of Health Research

Excellence Scholarship - University of Ottawa **2008 – 2012**

Research Travel Grants - FGPS - University of Ottawa **2008, 2009**

Admission Scholarship - University of Ottawa **2007 – 2008**

Regional, Provincial and National Lifesaving Competition **1996 – 2002**
Obtained medals at all levels of competition, including a silver medal at the Canadian Lifesaving Championship.

Employment History

Teacher's Assistant: University of Ottawa, Ontario **2005 – 2012**
- Trained students in laboratory techniques and developed report writing skills
- Explained concepts and techniques
- Lectured on class material that required clarification
- Communicated feedback on student's progress
- Adapted to different learning styles
- Evaluated reports and exams
- Motivated students with a professional and dynamic attitude

Let's Talk Science (Volunteer) – Mentor **2006 – 2009**
- Participated as a mentor in the scientific outreach program
- Prepared activities to spark youth in the community's interest in science
- Judged science fairs for children ranging from elementary school to high school
- Gave presentations to high school students
- Answered questions to prospective university science students
- Used vocabulary appropriate to the situation

Aquatic Shift Supervisor: City of Ottawa **2002 – 2008**
Splash Wave Pool, Ontario

- Supervised shifts of upwards of 15 staff while responsible for ensuring all emergency situations were handled properly
- Responsible for dealing with clientele, specifically focused on client satisfaction
- Responsible for planning and supervision of staff's weekly and monthly training
- Helped staff improve lifesaving, instructional, teamwork and leadership skills while providing new staff with mentoring and guidance

Camp Coordinator: City of Ottawa **2002**

- Responsible for staffing and scheduling, ensured that equipment was available for the planned activities
- Supervised up to 5 counselors and 30 children
- Responsible for planning and coordinating daily activities and weekly field trips
- Provided employer with end of season summary and initiatives to improve for the following year

Experience

- Seven years experience in a research laboratory: conducting research and analyzing and reporting experimental data
- Experience in preparing major research proposals
- Proven experience in grant writing and editing
- Experience with data entry, compiling data and research findings into spreadsheets
- Experience creating databases and analyzing data using MS Excel
- Experience collecting, analyzing and reporting on quantitative and qualitative data
- Experience in data management, including the manipulation of large relational databases, verification and validation of data quality
- Experience in data management (including manipulation of large relational databases, verification and validation of data quality) and evaluation of database and surveillance systems
- Experience in planning and developing methods and protocols to obtain, validate, manipulate and analyze data
- Experience working with health-related data
- Experience in evaluating databases
- Experience planning and developing methods and protocols to obtain, validate, manipulate and analyze data
- Experience synthesizing surveillance information for public use
- Experience conducting research
- Experience in preparing tables, charts, diagrams and in writing reports
- Experience in the use and verification of data for the purpose of integration into publications and databases
- Significant experience with data analysis methods and tools
- Experience creating and running experimental designs
- Experience with data gathering and analysis techniques

- Experience writing peer-reviewed publications
- Experience in working as part of a research team addressing complex problems
- Experience in delivering course lectures
- Experience explaining concepts and techniques
- Experience handling multiple projects concurrently
- Experience in project management, including: establishing goals, priorities, deadlines and managing towards expected outcomes
- Significant experience in training lab personnel
- Significant experience in nucleic acid and protein extraction and analysis
- Significant experience in immunological methods: Western Blot, immunohistochemistry, immunocytochemistry, ELISA, etc.
- Significant experience in mammalian cell culture: including primary cell lines and transformed cell lines. In both traditional monolayer culture as well as three-dimensional culture
- Experience in PCR
- Experience in microarray experiments
- Experience in extracting RNA and DNA from tissues and cells and analysis
- Experience in handling and conducting research with small laboratory animals
- Experience in gene and protein engineering
- Experience in fluormetric and luminescence
- Experience in cloning procedures
- Experience with flow cytometry

Knowledge

- Advanced knowledge of Microsoft Word, Excel, PowerPoint and Outlook
- Knowledge of Adobe Creative Suites Software
- Knowledge of standard laboratory operating procedures and laboratory safety practices
- Knowledge of practices and standards related to laboratory quality systems
- Knowledge of statistical methods used in data analysis
- Knowledge of laboratory techniques used to the analysis of cells and biological macromolecules including proteins and nucleic acids
- Knowledge of search methods on literature relevant to specific projects
- Knowledge of graphic creation basics
- Knowledge of data dissemination trends
- Knowledge of research methods
- Knowledge of scientific principles of internal and external validity and rigor
- Knowledge of quality control and assurance programs as they apply to a research laboratory
- Knowledge of laboratory techniques used to the analysis of cells and biological macromolecules including proteins and nucleic acids
- Knowledge of search methods on literature relevant to specific projects

Abilities

- Ability to communicate effectively both orally and in writing
- Ability to communicate results effectively to non-research audiences

- Ability to interact easily with clinical and research staff
- Ability to work under pressure of deadlines and changing priorities with little or no supervision
- Ability to deal with deadlines, to set priorities and to work independently
- Ability to plan, coordinate multiple projects and duties
- Ability to synthesize information from a variety of sources
- Ability to work in a team environment as well as independently
- Ability to work with various personalities
- Ability to investigate practical laboratory problems and provide solutions
- Ability to statistically validate results and identify data trends
- Ability to collect, analyze and interpret data
- Ability to maintain accurate and timely data records and documentation
- Ability to operate and maintain modern laboratory equipment
- Ability to conduct literature and data searches
- Ability to manage: Ability to react to situations related to a number of subjects such as: client satisfaction, employee requests, budgetary questions, activity reports, etc.

Personal Suitability

- Demonstrates poise, tact and diplomacy under pressure
- Able to work in a fast-paced environment and able to multi-task
- Has strong leadership capabilities
- Respectful of diversity
- Effective interpersonal relations
- Dependable
- Displays good judgment
- Takes initiative and is reliable and adaptable
- Thorough and detail oriented
- Works well within a team
- Superb time management skills
- Self-motivated and results oriented
- Planning skills, both long term and short term

Languages

- English (complete fluency) and French (Ontario Bilingual Certificate)
- Government of Canada's French SLE results: CBB

Thank you for your time and consideration