LOSS OF LKB1 TUMOUR SUPPRESSOR FUNCTION PLAYS A ROLE IN MAMMARY GLAND TUMOURIGENESIS AND REGULATION OF CELL METABOLISM.

By

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To my parents

Lamartine and Risomar Andrade

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ABSTRACT

The tumour suppressor kinase LKB1 is involved in many signalling pathways. LKB1 is particularly important for the regulation of cell homeostasis through the phosphorylation of the metabolic regulator, AMPK. Mutations in the catalytic domain of LKB1 cause dysregulation of cell metabolism and enhanced protein synthesis. LKB1-AMPK signalling inhibits mTOR, a protein kinase responsible for cell growth and cell proliferation. Therefore through the inhibition of mTOR, LKB1 impairs cell growth. Because of the role of LKB1 in this signalling cascade, the loss of LKB1 function is relevant in many types of cancers including lung, prostate and breast cancer. I hypothesized that loss of LKB1 plays a role in abnormal cell metabolism and aerobic glycolysis. Therefore the aims of this Thesis were to show that loss of LKB1 leads to HER2/ErbB2+ breast cancer.

The findings detailed in this thesis show that treatment of cells with omega 3 polyunsaturated fatty acids resulted in the activation of LKB1 signalling, leading to AMPK phosphorylation and inhibition of mTOR signalling. In addition, cells expressing LKB1 and treated with DHA show a significant decrease in aerobic glycolysis and the production of ATP. Interestingly, abrogation of LKB1 increased aerobic glycolysis. In the presence of LKB1, DHA-treated mammary epithelial cells showed a diminished migration potential. These findings indicated that activation of LKB1 signalling by DHA plays a role in mammary gland epithelial cell metabolism.

We also investigated a molecular interplay between loss of LKB1 expression and gain of oncogene activity, specifically ErbB2. Tissue microarray analysis confirmed that 31% of HER2+ breast cancer lacked LKB1 expression. Based on this finding we developed a stochastic model of human breast cancer where both loss of LKB1 function and activation of HER2 ($LKB1^{-/-}$NIC mice) in mammary glands were evaluated. The tumours excised from $LKB1^{-/-}$NIC mice were characterized and were demonstrated to have hyperactivation of mTOR signalling and abnormal cell metabolism.

Using the $LKB1^{-/-}$NIC mice we conducted pre-clinical trials to investigate possible therapeutic strategies that could inhibit tumour growth *in vivo*. The mice were treated with mTOR inhibitors alone or in combination with a metabolic inhibitor. The results from this study showed that metronomic daily treatment of mice for 21 days significantly inhibited the growth of tumours and regulated abnormal cell metabolism.

In conclusion, my research provided novel discoveries in the field of LKB1 biology, contributing valuable information about the role of LKB1 in regulating mTOR, cell metabolism and HER2+ breast cancer. The activation of LKB1 promoted by DHA improved the regulatory functions of LKB1 *in vivo*, preventing mTOR hyperactivation and maintaining cell homeostasis. The role of LKB1 in HER2+ breast cancer described in my thesis, places LKB1 expression as a marker for dysregulated cell metabolism and aggressive tumour development. Finally, the use of novel inhibitors that block mTOR and aberrant cell metabolism are valuable therapeutic strategies for the treatment of patients with breast cancer where tumours lack the expression of LKB1.

LIST OF ABBREVIATIONS USED

AA	Arachidonic acid
ACC	Acetyl CoA carboxylase
AICAR	5-aminoimidazole-4-carboxamide ribonucleoside
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate (AMP)–activated protein kinase
ARKs	AMPK-related kinases
ATP	Adenosine triphosphate
BLG	β-lactoglobulin
BRG1	Brahma-related gene 1
BSK	Brain-specific kinases
CaMKKβ	Calcium/calmodulin - dependent kinase β
ChREBP	Carbohydrate response element-binding protein
CPTI	Carnitine palmitoyltransferase I
Cys	Cysteine
DG	Diacylglycerol
DHA	Docosahexaenoic acid
DMEM	Dulbecco`s modified Eagle’s medium
DNA	Deoxyribonucleic acid
ECAR	Extracellular acidification rates
ECL	Chemiluminescence
EtOH	Ethanol
eIFs	Eukaryotic initiation factors

EGFR	Epidermal growth factor receptor
EPA	Eicosapentaenoic acid
ER	Estrogen receptor
FAS	Fatty acid synthase
FBS	Fetal bovine serum
FOXO1	Forkhead box O1 (FOXO1)
GAP	GTPase-activating protein
GEF	Guanine exchange factor
GTP	Guanosine-5'-triphosphate
HER	Human epidermal growth factor receptor
HEX2	Hexokinase 2
HIF	Hypoxia inducible factor
IDH	Isocitrate dehydrogenase
IHC	Immunohistochemistry
IRS1	Insulin receptor substrate 1
KO	Knockout
LDH-A	Lactate dehydrogenase A
LIP	LKB1 interacting protein
LKB1	Liver Kinase B1
MAPK	Mitogen-activated protein kinase
MARK	Microtube-associated protein kinase
MCTs	Monocarboxylate transporters
MEC	Primary mammary epithelial cells

mLST8	Mammalian lethal with sec thirteen 8
MMTV	Murine mammary tumour virus
MO25	Mouse 25
MRI	Magnetic resonance imaging
mtDNA	Mitochondria DNA
mTOR	Mammalian target of Rapamycin
NSCLC	non-small cell lung carcinomas
NRF1/2	Nuclear respiratory factor ½
OCR	Oxygen consumption rates
PAH	Polycyclic aromatic hydrocarbons
PAR	Partition defective proteins
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDH	Pyruvate dehydrogenase
PET	Positron-emission tomography
PFK	6-phosphofructo-2 kinase
PGC-1α	Peroxisome proliferator-activated receptor-γ coactivator 1α
PGE	Prostaglandin E
PI3K	Phosphoinositide-3-kinase
PJS	Peutz-Jeghers syndrome
PKM2	Pyruvate kinase 2
PPAR	Peroxisome proliferator-activated receptor
PR	Progesterone receptor

pS6K	Phosphorylated ribosomal kinase S6
PTEN	Phosphatase and tensin homolog
qPCR	Quantitative PCR
RAPTOR	Regulatory-associated protein of mTOR
RHEB	Ras homolog enriched in brain
RICTOR	Rapamycin insensitive companion of mTOR
ROS	Reactive oxygen species
SCLC	Small cell lung carcinomas
SDH	Succinate dehydrogenase
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Ser	Serine
SGK1	Serum- and glucocorticoid-induced protein kinase 1
SIN1	SAPK-interacting 1
SIRT1-7	Silent Information regulator T1-7
SREBP1	Sterol regulatory element-binding protein-1
STRAD	STE20-related adaptor
STK11	Serine/threonine kinase 11
T2DM	Type 2 diabetes mellitus
TCA	Tricarboxylic acid cycle
TFAM	Transcription factor A mitochondrial
TGFβ	Transforming growth factor beta
Thr	Threonine
TKI	Tyrosine kinase inhibitors

TNBC	Triple negative breast cancer
TSC1/2	Tuberous sclerosis complexes 1/2
TXNIP	Thioredoxin-interacting protein
ULK1	unc-51 like autophagy activating kinase 1
VEGF	Vascular endothelial growth factor
WAP	Whey acidic protein promoter
WT	Wild Type
ω3PUFAs	Omega-3 polyunsaturated fatty acids
ω6PUFAs	Omega-6 polyunsaturated fatty acids
ZMP	AICAR 5'monophosphate
2-DG	2-deoxyglucose
2-HD	2-hydroxyglutarate
3-HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A reductase
4EBP1/2	Eukaryotic translation initiation factor-binding proteins 1/2

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It is good to be important but
It is more important to be good.
(Unknown author)

CHAPTER 1 INTRODUCTION

This chapter includes a bibliography review of the field of the LKB1 tumour suppressor biology that dates back to the first evidence of loss of tumour suppressor-mediated disease in 1920s. LKB1 is a major serine-threonine kinase with multiple functions, herein I described some of LKB1 functions that are highly correlated with cancer and other diseases. The Marignani Laboratory made some important discoveries in LKB1 signalling and contributed to the understanding of LKB1 function and activity. These findings led to my most recent discoveries described in Chapters 4, 5 and 6. Because one of LKB1 main functions is the phosphorylation and activation of AMPK signalling, in this chapter I discussed the importance of AMPK signalling in cancer and other as well as major discoveries in metabolism.

Another important signalling pathway described in this chapter is mTOR signalling. The upregulation of this signalling pathway is highly implicated in cancer and is often altered in many metabolic disorders. In this chapter I discussed LKB1 and AMPK-mediated inhibition of mTOR signalling and the main discoveries that linked mTOR upregulated function to the development of cancer. The final section of this chapter is focused on omega 3 polyunsaturated fatty acids and the main characteristics of this family of fatty acids that are related to maintenance of health. In addition, I discuss how diet influences diseases such as breast cancer and some immunology dysfunctions.

1.0. The tumour suppressor kinase gene LKB1

1.1. Peutz - Jeghers syndrome

In 1921, the Dutch physician Johannes Peutz first described that intestinal polyps with correlated pigmented mucocutaneous lesions were an inherited disorder (Peutz, 1921). Later, in 1949, another scientist, Dr. Harold Jeghers, reported 10 cases of intestinal polyposis that manifested hyper-pigmentation spots on the lips, oral mucosa and digits (Jeghers et al, 1949). All these patients with ages ranging from nine to thirty-nine presented with gastrointestinal polyps. Some were diagnosed with cancer and died of the disease. In cases where family history was available, parents and/or siblings were shown to have manifestation of the disease.

After these two first studies, the disease became known as Peutz-Jeghers Syndrome (PJS) and was associated with the development of early onset hamartomatous polyps throughout the gastrointestinal tract. PJS-hamartomas are most prevalent in the small intestines but cases of hamartomas in the stomach and colon have been reported. Although these polyps were benign, patients had a markedly increase risk for developing cancer at young age when compare to the general population (Mehenni et al, 2006). At the age of 70 years old, the risk reaches 67-85% for these patients. The most frequently observed cancer in PJS is gastrointestinal cancers (38-63%), followed by breast cancer (31%) and gynecological cancers (13-18%) (Hearle et al, 2006; Mehenni et al, 2006).

In 1997, genetic analysis of DNA from 12 PJS patients identified deletion sites on chromosome 19p in the locus site 19p13 without evidence of genetic heterogeneity (Hemminki et al, 1997). One year later, a tumour suppressor kinase was identified with inactivating germ-line mutations in the same locus and the gene was identified as the serine/threonine kinase 11 (*STK11*) gene. Previously, researchers referred to this kinase as LKB1 (Hemminki et al, 1998; Jenne et al, 1998).

Analysis of the LKB1 gene in five non-related PJS patients showed intragenic deletions and premature stop code mutations. All these mutations rendered a truncated protein with loss of kinase activity (Jenne et al, 1998). More recently, approximately 150 mutations have been identified in LKB1 gene, all these mutations have distinct phenotypes and can be associated with more aggressive manifestation of the disease (Mehenni et al, 2006). For example, mutations responsible for loss of kinase activity are highly correlated to earlier manifestation of the disease compared to missense mutations which are less commonly found in PJS (Amos et al, 2004). Because a bi-allelic loss of LKB1 was not necessary for hamartomas polyp development, LKB1 was characterized as a haploinsufficient tumour suppressor gene (Jishage et al, 2002; Miyoshi et al, 2002).

In 2001, a study showed that mice lacking LKB1 expression ($LKB1^{-/-}$) died at embryonic stage E11, highlighting the importance of LKB1 in early development (Ylikorkala et al, 2001). Heterozygous LKB1 mice ($LKB1^{+/-}$) showed similar intestinal polyps found in PJS but had a limited malignant potential

(Bardeesy et al, 2002; Jishage et al, 2002; Miyoshi et al, 2002). However, other studies showed that other oncogenic activating mutations synergize with loss of LKB1 to promote aggressive tumour development in various cancer types (Andrade-Vieira et al, 2013b; Ji et al, 2007; Morton et al, 2010) emphasizing the importance of LKB1 in tumour initiation and progression.

1.2. LKB1 signalling and functions

1.2.1. LKB1 protein kinase

The *LKB1* gene is composed of 10 exons, nine of which encode a 48kDa protein kinase (Fig.1). The human LKB1 protein is composed of 433 amino acids, where the catalytic domain is comprised of residues 44-309. LKB1 is ubiquitously expressed, particularly in liver, pancreas, kidney, heart, lung, ovary, and prostate. The mouse LKB1 homologue is comprised of 436 amino acids with a 88% nucleotide sequence identity to human LKB1 (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The kinase domain contains the vast majority of PJS-associated missense mutations, that result in impaired kinase activity. The N-terminal and C-terminal non-catalytic domains can be auto phosphorylated (Fig.1). A few mutations were found in these domains. Although these mutations do not impair kinase activity, they were detected in sporadic cancer, suggesting a functional relevance. More recently, mutations in the C-terminal were shown to result in impairment of LKB1 downstream signalling (Forcet et al, 2005).

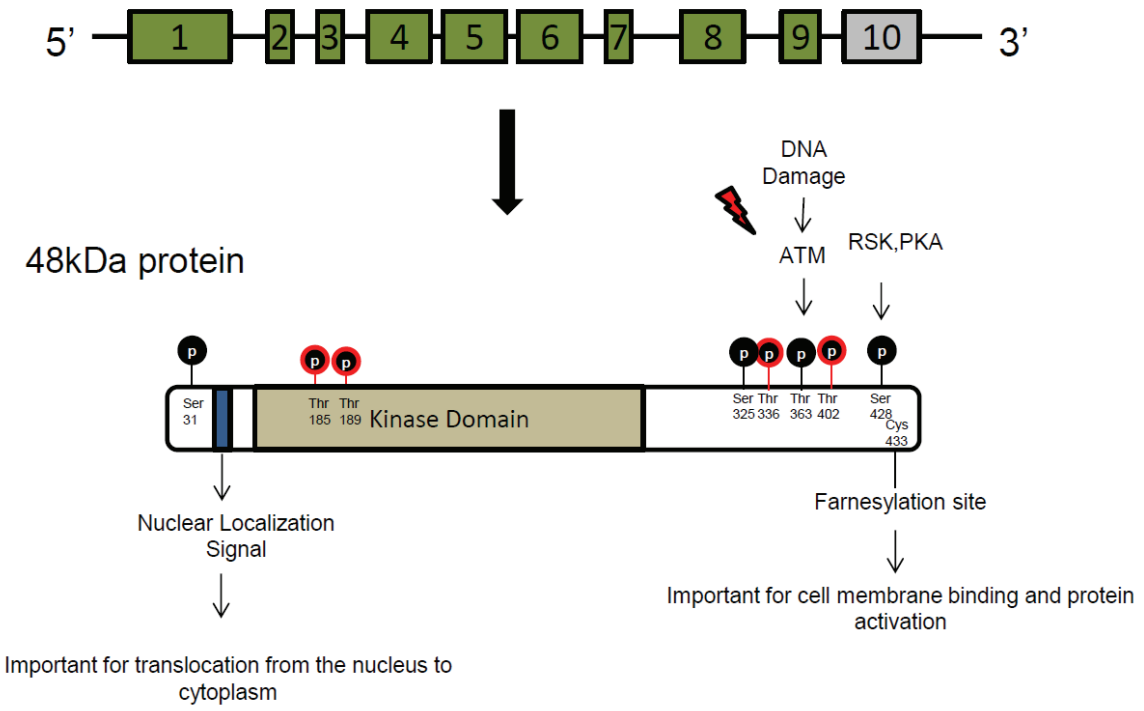

Figure 1 LKB1 gene and protein. Human *LKB1* gene is comprised of ten exons, nine of which (green) are coding and one is not coding (gray). LKB1 kinase protein is a 48kDa protein with three domains, C-terminal, kinase domain and N-terminal domain. Phosphorylation sites are represented by black circles and autophosphorylation sites are represented by red circles.

Post-translational modification sites are common in the C-terminal domain. Four autophosphorylation sites have been described, threonine (Thr) 185, Thr189, Thr336 and Thr402 (Baas et al, 2003; Sapkota et al, 2002a). That correlate with enhanced LKB1 catalytic activity (Baas et al, 2003). LKB1 can also be phosphorylated on serine (Ser) 31, Ser325, Thr363 (mouse Thr366) and Ser428 (mouse Ser431). Mutations of these phosphorylation sites inhibit LKB1-mediated cell growth arrest. Phosphorylation of Ser428 is mediated by the ribosomal S6 kinase (p90RSK) and by cAMP-dependent protein kinase (PKA). Phosphorylation of Thr363 is mediated by ataxia telangiectasia mutated kinase

(ATM) after exposure of cells to ionizing radiation (Sapkota et al, 2002a; Sapkota et al, 2002b; Sapkota et al, 2001). The kinase that phosphorylates Ser31 and Ser325 has not yet been identified (Fig. 1).

The C-terminal domain of LKB1 has a farnesylation site at Cysteine (Cys) 433. This cysteine residue is located at the CAAX motif which is required for prenylation of all proteins (Moores et al, 1991). Mutations of Cys433 to alanine or serine prevent farnesylation and reduce the recruitment of LKB1 to cell membrane (Collins et al, 2000; Houde et al, 2014). In addition, loss of LKB1 farnesylation inhibited LKB1-mediated activation of downstream targets, suggesting that recruitment to cell membranes is important in activating LKB1 and, therefore, phosphorylation of LKB1 targets (Houde et al, 2014).

1.2.2. LKB1 activation

LKB1 is normally found in the nucleus as a result of a nuclear localization signal within the N-terminal domain (Smith et al, 1999). Therefore, to fully perform all the cytoplasmic functions, LKB1 has to be exported from the nucleus by interacting with other proteins since LKB1 lacks a nuclear export domain. Translocation of LKB1 from the nucleus to the cytoplasm is induced upon binding and association with the STE20-related adaptor (STRADα) and the scaffolding mouse 25 (MO25) proteins (Baas et al, 2003; Boudeau et al, 2003).

STRADα serves as an adaptor that promotes the interaction of exportins to LKB1 which facilitates translocation to the cytoplasm. STRADα binding to LKB1 also inhibits importin-α/β binding to LKB1, allowing LKB1 to remain in the

cytoplasm. The kinase domain of LKB1 binds to the pseudokinase domain of STRADα and MO25 acts as a scaffold binding both LKB1 and STRADα (Fig. 2). MO25 forms an interaction with the last three Trp-Glu-Phe amino acids of STRAD serving as a stabilizer of the LKB1-STRAD interaction. The binding complex LKB1: STRAD: MO25 renders LKB1 active (Alessi et al, 2006; Dorfman & Macara, 2008; Zeqiraj et al, 2009). Some LKB1 mutants found in PJS lack the binding sites to LKB1: STRAD:MO25 which greatly inhibits LKB1 function (Boudeau et al, 2004). For instance, the LKB1 mutant, SL26, lacks the binding sites for STRAD which prevents LKB1 export to the cytoplasm and inhibits LKB1-mediated cell cycle arrest (Baas et al, 2003; Hemminki et al, 1998).

Translocation of LKB1 to the cytoplasm enables LKB1-mediated phosphorylation of protein kinases. In the cytoplasm LKB1 phosphorylates and activates AMP-activated protein kinase (AMPK) on Thr172 (Fig. 2). In addition, LKB1 is required to promote the activation of other AMPK-related kinases (ARKs) through phosphorylation of threonine residues equivalent to Thr172 (Fogarty et al, 2010; Hardie). LKB1 phosphorylation of AMPK is one of the most important events that regulate cell energy metabolism and cell proliferation.

1.2.3. LKB1 binding partners

Previously, we found that LKB1 bind to the Brahma-related gene 1 (BRG1) (Marignani et al, 2001) (Fig. 2). BRG1 is a member of the mammalian SWI-SNF chromatin remodelling complex and is required for the chromatin remodelling (Khavari et al, 1993). LKB1 binds to the helicase domain of BRG1 and enhances BRG1 ATPase function. This interaction is required for BRG1-mediated growth

arrest (Marignani et al, 2001). Inactivating mutations of LKB1 and BRG1 are commonly found in lung carcinomas (Matsumoto et al, 2007; Medina et al, 2008; Sanchez-Cespedes et al, 2001), emphasising the importance of LKB1 in cancer.

LKB1 also interacts with a scaffolding leucine-rich protein called LKB1 interacting partner-1 (LIP1) (Smith et al, 2001) (Fig. 2). Recently, LIP1 interaction with LKB1 was implicated in a negative regulation of the transforming growth factor beta (TGFβ) signalling and epithelial-mesenchymal transition (Moren et al, 2011). TGFβ is involved in regulatory cell processes such as cell proliferation, differentiation and survival, dysregulation of TGFβ pathways promotes tumour growth and invasion (Assoian et al, 1984; Boyer et al, 1999).

LKB1 binds to the estrogen receptor alpha (ERα) and increases ERα transactivation (Fig.2). Our group demonstrated that this interaction occurs in the nucleus and is independent of phosphorylation of ERα (Nath-Sain & Marignani, 2009). Because LKB1 mutations in PJS are highly implicated in increasing the risk of breast cancer and ERα signalling is frequently altered in breast cancer, this finding suggested a role for LKB1 in this type of cancer. More recently, another group reported that LKB1 interaction with the methylated form of ERα is related to poor prognosis in breast cancer (Bouchekioua-Bouzaghou et al, 2014). Based on these studies, more research is warranted to understand the role of LKB1 in breast cancer.

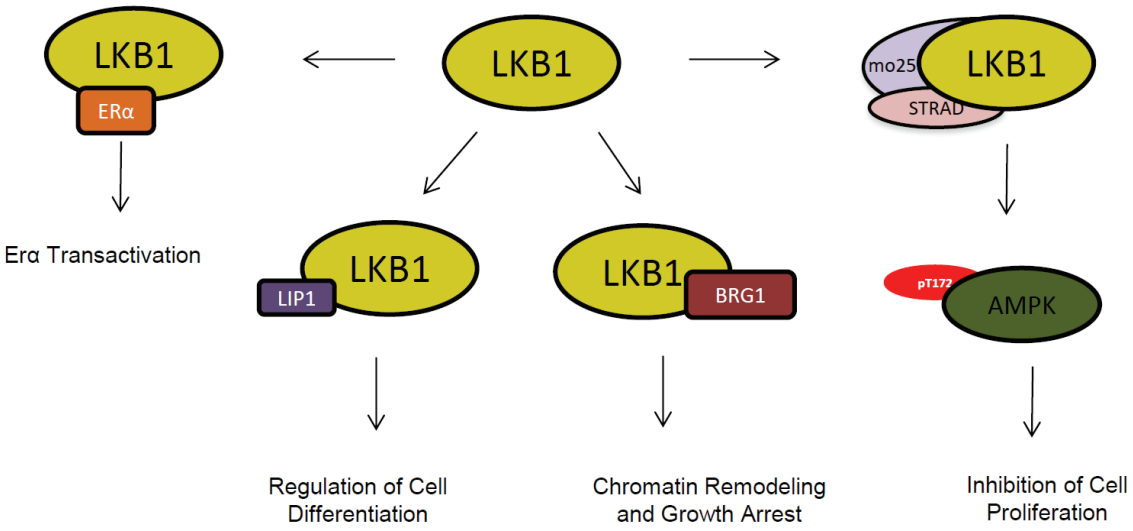

Figure 2 LKB1 binding partners. LKB1 binds to proteins to elicit a variety of functions. LKB1: STRAD: MO binding promotes LKB1 activation, translocation to the cytoplasm and induces phosphorylation of AMPK on Thr172; this phosphorylation event inhibits cell proliferation. LKB1:BRG1 promotes chromatin remodelling and mediates cell growth arrest. LKB1:LIP1 facilitates LKB1 function and is involved in epithelial mesenchymal transition. LKB1: ERα promotes ERα transactivation.

1.2.4. LKB1 in cell polarity

Cell polarity and asymmetry are very important to maintain tissues organization. Epithelial cells, in particular, have a molecular asymmetry that allows proper cell divisions and organization. The apical surface of epithelial cells faces the lumen, and is responsible for absorption, exchange and secretion of molecules (Stevens, 1997). Epithelial cells also interact with adjacent cells through specialized tight junctions, adhesion junctions and desmosomes. The basolateral surface of epithelial cells anchors to a basement membrane composed of laminin polymers, collagen IV, proteoglycans and glycoproteins

(Alberts B., 2007). Loss of this cellular organization is often related to hyperplasia and cancer.

Cellular asymmetry and organization are regulated by a family of proteins sharing the same partitioning phenotype. These proteins are conserved from worms to mammals and are known as partitioning defective proteins (PAR). They are the main proteins responsible for maintaining cellular polarity and asymmetry (Izumi et al, 1998; Kuchinke et al, 1998; Tabuse et al, 1998). In *Caenorhabditis elegans* six PAR proteins were identified and they are all involved in cellular polarization. These proteins have orthologs in many species with the exception of PAR-2 which is a nematode-specific protein.

Mammalian LKB1 orthologs in *Caenorhabditis elegans* and *Drosophila melanogaster,* PAR-4 and dLKB1, respectively, are involved in cellular asymmetry, cell polarization, and formation of epithelial junctions (Martin & St Johnston, 2003; Watts et al, 2000). As expected, mammalian LKB1 is also involved in cell polarity and asymmetric cell division. Recent observations have linked loss of LKB1 to defective morphogenesis in lungs and mammary gland (Lo et al, 2012; Partanen et al, 2012).

Mice model harbouring mutant LKB1 with loss of kinase activity exhibited cell-autonomous branching defects in the lungs (Lo et al, 2012). Interestingly, activation of AMPK rescued cells from defective branching, suggesting that LKB1-AMPK signalling is required for lung morphogenesis. Another study showed that conditional deletion of LKB1 using Cre-recombinase system in the

mammary gland leads to hyper-branching of the mammary ducts. In addition, in this model, coupling LKB1 deletion with oncogenic activation of c-Myc leads to an increase in tumour formation (Partanen et al, 2007).

The mechanism by which LKB1 drives cell polarization is believed to be linked to LKB1 regulation of AMPK and the mammalian microtube-associated protein kinase (MARK) (Lo et al, 2012; Spicer et al, 2003). LKB1 phosphorylation of AMPK and MARKs (in *C. elegans* PAR-1) leads to microtubule cytoskeletal organization and correct partitioning. In addition, in neuronal tissue, polarized migration is regulated by brain-specific kinases BSK1-2 (also known as SAD-A and SAD-B). LKB1 has been known to activating BSK1-2, triggering axon differentiation during neuronal polarization (Barnes et al, 2007; Shelly et al, 2007).

1.3. LKB1 in cancer

Germline mutations in the *LKB1* gene are common event in PJS, this observation led to the discovery of LKB1 as a tumour suppressor kinase. Somatic mutations of LKB1 in sporadic cancers are not a common event with the exception of lung cancer. In contrast, reduced expression and allelic loss of LKB1 are frequently observed in a variety of sporadic cancers. Therefore, LKB1 haplo-insufficiency is often linked to oncogenic events involved in the development of aggressive tumour types.

1.3.1. LKB1 in lung cancer

Lung cancer is the leading cause of cancer-related death in Canada for both males and females (Canadian Cancer Society, 2014). Smoking is mainly responsible for new cases and is associated with an increased risk of lung cancer. Several components of tobacco are implicated in lung mutagenesis, including polycyclic aromatic hydrocarbons (PAH). This substance forms covalent bounds with DNA in the guanines and adenines bases, contributing to DNA transversions mainly G to T in regulatory genes (Greenblatt et al, 1994; Slebos et al, 1991).

Several regulatory genes contribute to lung carcinogenesis when mutated. Mutations in these genes can be activating mutations at the oncogene level or inactivating point mutation in tumour suppressor genes. The main oncogenes found to be activated in lung cancer are BRAF, EGFR, ErbB2, KRAS, PIK3CA and MYC. Inactivation of the tumour suppressor genes LKB1, BRG1, PTEN and P53 are also common (Blanco et al, 2009; Dearden et al, 2013).

Lung cancer is classified in two major groups: non-small cell lung carcinomas (NSCLC) and small cell lung carcinomas (SCLC). NSCLC is also histopathologically divided in squamous cell carcinomas and adenocarcinomas (Beasley et al, 2005; Bhattacharjee et al, 2001). Several genetic mutations are implicated in lung tumourigenesis. In particular, NSCLC have a high frequency of LOH mutations at chromosome 19p (Virmani et al, 1998), where the LKB1 gene is located and inactivated in this type of tumour. In fact, LKB1 mutations are found to be the fourth most common mutation in all types of lung cancer. In NSCLC, LKB1 is the third most common mutated gene following P53 and P16

(Blanco et al, 2009; Carretero et al, 2004; Sanchez-Cespedes, 2007; Sanchez-Cespedes et al, 2002).

Mutations in LKB1 are primarily nonsense, frameshift or intragenic deletions. There is also a large increase on G-T transversion, which explains the fact that LKB1 mutations are frequently found in lungs of smokers. In addition, LKB1 inactivation is found in approximately 34-39% of NSCLC with correlated mutations in KRAS and less frequently with EGFR activating mutations. In Asian patients who have a high frequency of EGFR mutations, LKB1 inactivation is rarely found (Ji et al, 2007; Matsumoto et al, 2007).

In lung tumours carrying KRAS mutations, LKB1 deficiency is an important marker of aggressiveness and short latency. Therefore, therapeutic drugs targeting LKB1 signalling have been explored to treat LKB1-deficient tumours. Recently, Shackelford et al. showed that administration of phenformin and activation of LKB1 signalling prolonged the survival of KRAS-driven tumours in a LKB1-deficient mouse model (Shackelford et al, 2013). Interestingly, another group showed that LKB1 deficiency in KRAS-driven tumours leads to nucleotide biosynthesis deficiency, suggesting that targeting nucleotide metabolism in LKB1-deficient tumours is a strategy to treat this subset of lung cancers (Liu et al, 2013).

Another tumour suppressor gene, *BRG1*, is also found to be mutated in lung cancer. There are a high frequency of nonsense, frameshift mutations and deletions leading to a complete loss of BRG1 function in 35% of NSCLC (Medina

et al, 2004; Wong et al, 2000). Simultaneous mutations in LKB1 and BRG1 are commonly found in lung cancer cell lines and appear to be a result of LOH at chromosome 19p (Medina et al, 2008). Because LKB1 binds to BRG1 and promotes chromatin remodelling (Marignani et al, 2001), this suggests that inactivating mutations of these tumour suppressor genes play a role in early events leading to lung tumourigenesis.

In conclusion, loss of LKB1 function is a major contributor to lung carcinogenesis. The high frequency of LKB1 deficiency in NSCLC and KRAS lung tumours suggests that LKB1 signalling is important to maintain lung tissue integrity. Therefore, activation of LKB1 signalling in lung cancer may affect overall survival of lung cancer patients and provide a better outcome to current therapeutic approaches.

1.3.2. LKB1 in breast cancer

LKB1 loss of function is not commonly found in breast cancer (4.3%). However, loss of LKB1 or reduce expression is found in 25% of invasive breast cancers, predicting a poor prognosis (Fenton et al, 2006). The role of LKB1 in breast cancer is still under investigation. Recently our group and others demonstrated key roles of LKB1 in mammary gland tumourigenesis. As discussed in section 1.2.3., LKB1 plays a role in hormone signalling by binding directly to the ERα and activating downstream signalling (Nath-Sain & Marignani, 2009). Partanen and colleagues also demonstrated that LKB1 plays a role in maintaining mammary epithelial cells polarity, suggesting that LKB1 is involved in the regulation of mammary epithelial organization. Loss of LKB1

markedly compromises the formation of epithelial junctions and leads to hyper branching of mammary ducts (Partanen et al, 2012). More recently, our group showed that 31% of HER2 positive breast cancer lack LKB1 expression. To evaluate the importance of LKB1 in HER2+ breast cancer we generated a mouse model that expressed an activated form of ErbB2 and lacked LKB1 expression (Chapter 5) (Andrade-Vieira et al, 2013b). These studies emphasize the importance of LKB1 in breast cancer development and shows that LKB1 signalling is an important marker for future therapeutic approaches. *For more detailed information on breast cancer refer to section 4.*

1.3.3. LKB1 in other cancers

LKB1 deficiency is found in a variety of other cancers, including pancreatic, prostate and gastrointestinal cancers (Korsse et al, 2013; Pearson et al, 2008). LKB1 activation of AMPK signalling is frequently reduced in these tumours and reflects the importance of maintenance of cellular metabolism. In addition, loss of LKB1 is associated with oncogenic activating mutations, emphasizing the value of LKB1 signalling. More importantly, loss of LKB1 signalling is implicated in aggressive tumour development and, therefore, merits evaluation of LKB1 as a possible marker for a variety of cancers (Co et al, 2014; Morton et al, 2010; Tanwar et al, 2014)

1.4. LKB1 mouse models

Transgenic mouse models have been used for years as a model to study and understand many diseases. The first LKB1 mouse model was developed in

2001, when LKB1 was knocked out in mouse embryos. This first LKB1 knockout (KO) mouse was embryonic lethal at E11. Embryos had severe neural tube deformities and defective vasculature as a result of upregulated VEGF signalling (Ylikorkala et al, 2001). This first study showed the importance of LKB1 in embryonic development and led to the generation of conditional LKB1 KO mice.

The first heterozygous LKB1 ($LKB1^{+/-}$) mice was viable and developed normally until 20 weeks of ages, after which the mice presented with gastric polyps in 93% of the animals. The histopathology of these polyps were similar to those found in PJS (Miyoshi et al, 2002). Another group constructed conditional heterozygous LKB1 mice carrying a null allele ($LKB1^{-}$) and a floxed allele ($LKB1^{fl}$) (Bardeesy et al, 2002). These mice have been widely used to excise LKB1 in various tissues using Cre-recombinase system under different tissues-specific promoters (Andrade-Vieira et al, 2013b; Partanen et al, 2012).

Conditional excision of LKB1 using the Cre-recombinase system provides interesting discoveries of LKB1 function in various organs. In 2005, Sakamoto et al. generated a mouse with LKB1conditional expression, whereby the LKB1 gene was flanked by floxed (fl) allele (Sakamoto et al, 2005). In these mice, LKB1 excision was specifically driven to skeletal and cardiac muscle since the mice had a muscle creatine kinase promoter driving the Cre-recombinase system. The research accomplished using this animal established LKB1 as the major regulator of AMPKα2 (Sakamoto et al, 2005) .

Over the years, many other conditional LKB1 mice were developed. The $LKB1^{fl/fl}$ mice were crossed with mice expressing Cre-recombinase under the control of β-lactoglobulin (BLG) promoter which promoted LKB1 excision in the mammary tissue. These mice developed luminal breast tumours with up-regulation of Cyclin D; in agreement with other LKB1 models, tumours were similar to those found in PJS (McCarthy et al, 2009).

LKB1 has also been excised in the mammary gland using the whey acidic protein promoter (WAP) which is expressed during lactation with modest activity in mid-pregnancy (Wagner et al, 1997). When $LKB1^{fl}$ mice are crossed with WAP-Cre mice, complete excision of LKB1 occurs during lactation (Partanen et al, 2012). Furthermore, the MMTV promoter is also often used to promote excision or activation of proteins directed to the mammary gland (Andrade-Vieira et al, 2013b; Muller et al, 1988; Schade et al, 2009).

2.0. AMPK family

2.1. AMP- Activated protein kinase

The crystal structure revealed that AMPK exists as a heterotrimers complex comprised of a catalytic α subunit and regulatory β and γ subunits (Stapleton et al, 1994), that are encoded by the *PRKAA1, PRKAA2, PRKAB1, PRKAB2, PRKAG1, PRKAG2* and *PRKAG3* genes. Each subunit is comprised of multiple isoforms which enable the formation of at least 12αβγ heterotrimer combinations. The α subunit contains the serine/threonine kinase domain which contains the conserved residue (Thr172) phosphorylated by other kinases that promote increased AMPK activity (Hawley et al, 1995). The C-terminal region of

the α subunit forms a globular domain where the β subunit is located linking the α subunit to the N-terminal of the γ subunit. The β subunit acts as a scaffold that bridges the α and γ subunits (Townley & Shapiro, 2007).

Activation of AMPK is dependent on its allosteric activator, 5'- adenosine monophosphate (5'-AMP), which binds the γ subunit (Xiao et al, 2007; Xiao et al, 2011). The γ subunit is arranged in a manner that yields four potential adenine binding clefts. One of these clefts (site 2) appears to be permanently unoccupied and site 4 is always bound to AMP. The other two sites (1 and 3) are responsible for binding AMP, ADP or ATP in a competitive manner. During periods of rest, ATP levels are higher leading to direct binding to sites 1 and 3 (Xiao et al, 2007). When cells undergo metabolic stress the levels of ATP are decreased concomitantly ADP and AMP levels are increased (Xiao et al, 2011). Progressively these two molecules replace ATP in the γ subunit, leading to AMPK phosphorylation and activation (Adams et al, 2004) (Fig. 3).

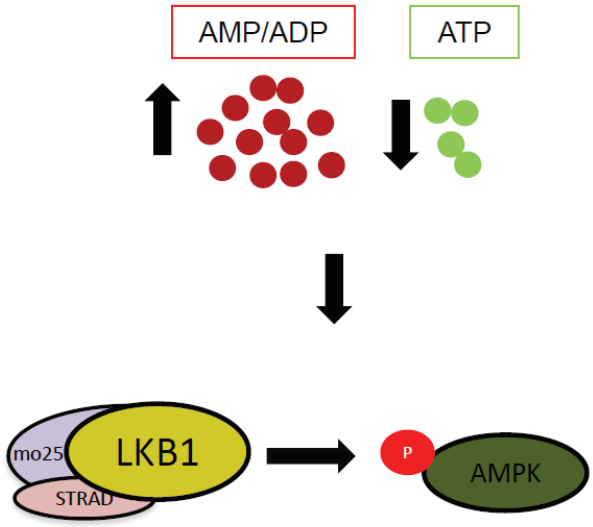

Figure 3 Activation of AMPK signalling. LKB1-mediated AMPK phosphorylation is activated by the increase in AMP and/or ADP levels and decrease in ATP.

Because the regulation of AMPK is dependent on cellular AMP/ATP levels, all the cell metabolic processes that consume ATP or activate catabolic pathways that generates ATP, such as mitochondria oxidative phosphorylation, are highly dependent on AMPK regulation. Therefore, AMPK regulation is one of the most critical events that influences energy homeostasis and cell metabolism. In addition, AMPK regulation is particularly important in biosynthetic events such as lipid and protein synthesis.

2.2. AMPK: master energy kinase

AMPK is known as the master regulator of cellular metabolism (Hardie, 1989). Because of the critical role AMPK plays in cell metabolism, dysregulation of AMPK signalling leads to the development of various metabolic disorders such

as diabetes, cardiovascular disease and obesity. Therefore, AMPK became the subject of various studies aiming to understand the processes that lead to the dysregulation of cell metabolism.

As mentioned in section 2.1. LKB1 activation of AMPK occurs through a series of metabolic switches that takes place in response to energy consumption or anabolism (Adams et al, 2004). Although LKB1 is the main kinase which phosphorylates AMPK, in some cells types, particularly neurons and endothelial cells, another family of upstream kinases, Ca^{++} /calmodulin - dependent kinase β (CaMKKβ) (Hawley et al, 1995), also activate AMPK by phosphorylation.

2.2.1. Activators of AMPK

Pharmacological compounds can trigger AMPK activation (Zhou et al, 2001). One of the most well studied activator of AMPK is metformin. This compound belongs to a class of drugs known as biguanides and it is one of the most commonly used drugs for the treatment of type 2 diabetes (American Diabetes Association, 2013). Metformin increases AMPK activation through inhibition of complex I of the mitochondria, leading to accumulation of AMP and ADP. These metabolites bind to AMPK triggering LKB1-dependent phosphorylation of AMPK (El-Mir et al, 2000; Owen et al, 2000), resulting in increased glucose uptake and inhibition of gluconeogenesis ameliorating diabetic symptoms. Interestingly, patients treated with metformin have statistically less cancer incidence (Decensi et al, 2010). Recently, it was reported that an overall cancer risk reduction of 31% was seen in patients taking metformin in

comparison to other anti-diabetic drugs (Decensi et al, 2010). These findings have raised the possibility of using metformin in cancer therapy (Pollak, 2010).

The action of metformin in cancer involves the reduction of insulin hyper secretion by pancreatic β cells and an increase in insulin sensitivity in tissues. Insulin is a known growth factor; therefore by reducing the plasma levels of insulin, cell growth is also reduced. An increase in insulin sensitivity leads to a more efficient uptake of glucose and reduces hypersecretion of insulin from β cells (Penicaud et al, 1989; Rossetti et al, 1990). These events cause normalization of cell metabolism. This mechanism is supported by a study showing that metformin reverses the increase in plasma insulin and promotes a decrease in tumour growth (Algire et al, 2011).

Metformin as well as other biguanides such as phenformin also increases tumour suppressor-mediated cell signalling. These two compounds are known to reduce tumour growth in mice lacking the phosphatase and tensin homolog tumour suppressor (PTEN) (deleted on chromosome 10) (Huang et al, 2008). Another study showed that the administration of phenformin to mice harbouring LKB1 mutations was efficient to decrease tumour growth (Shackelford et al, 2013).

Phenformin is also a potent inhibitor of mitochondria complex I and has a greater bioavailability crossing efficiently the plasma membrane when compared to metformin (Dykens et al, 2008; Shackelford et al, 2013). Despite this, clinical trials using phenformin failed because of an increase in lactic acidosis that led to death (Bailey & Turner, 1996). However, the use of biguanides as cancer therapy

may still be suitable as combinatory therapy have changed since this initial clinical trial. The effects on tumour growth associated with biguanides administration and the reduction of cancer risk observed in diabetes patients treated with metformin emphasizes its importance in cancer and warrants further investigation. Recently, metformin has entered various cancer clinical trials, including breast cancer (http://www.clinicaltrials.gov).

Another AMPK pharmacological activator, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), is an adenosine analog that mimics AMP on AMPK activation. AICAR is taken up into cells by adenosine transporters and converted to a monophosphorylated form (ZMP). Although, ZMP is a less potent activator than AMP, AICAR have been successfully used for the treatment of metabolic disorders. AICAR activation of AMPK is dependent on LKB1 phosphorylation of Thr172, emphasizing the importance of LKB1-mediated AMPK activation (Hardie; Xiao et al).

In addition to the effects of AMPK, AICAR administration to mice promoted adaptation of extensive exercise and increased muscle endurance after treadmill running experiments (Narkar et al, 2008; Winder et al, 2000). Interestingly, AICAR administration to sedentary mice for 4 weeks led to increase running endurance and upregulation of mitochondria genes. This study demonstrated that AMPK activation enhanced training adaptation and exercise endurance (Narkar et al, 2008). These observations led the World Anti-Doping Agency, the organization responsible to regulate drug abuse in sports, to ban the use of any AMPK activators.

2.2.2. Targets of AMPK

To restore energy levels in the cell, AMPK acts as a master kinase that is able to increase ATP through multiple pathways. One of the main pathways that are switched off upon AMPK activation is protein synthesis and cell growth processes. AMPK inhibits protein synthesis by a series of phosphorylation events initiating by the phosphorylation and activation of the tuberous sclerosis complex 1 (TSC1) and 2 (TSC2). The TSC2 protein contains a GAP (GTPase-activating protein) homology domain, which directly targets the small GTPase Ras homolog enriched in brain (Rheb) (Inoki et al, 2003a; Shaw et al, 2004a; Zhang et al, 2003a). In all eukaryotes, a conserved central modulator, the target of Rapamycin, TOR, regulates protein synthesis. In mammals, mammalian TOR (mTOR) exists in two different complexes, mTORC1 and mTORC2, that are distinguished by downstream targets and Rapamycin sensitivity (Dos et al, 2004) (Fig. 4).

The phosphorylation of TSC2 by AMPK leads to inhibition of mTORC1 signalling (Fig. 4). The phosphorylated form of TSC2 induces conversion of active GTP-bound Rheb to inactive GDP-bound Rheb. Interaction between GTP-bound Rheb and mTORC1 is required for mTORC1 activation, thus TSC2 phosphorylation inhibit mTORC1 activation (Zhang et al, 2003b). This event leads to the inhibition of overall protein synthesis that will be discussed below.

Another important event regulated by AMPK is lipid synthesis and oxidation. The first AMPK substrates described was acetyl CoA carboxylase 1 (ACC1), ACC1 is the key enzyme responsible for fatty acid synthesis. AMPK-

phosphorylation sites are also found in ACC2 isoform and when phosphorylated, diminishes ACC activation (Carling et al, 1989). ACC1 and ACC2 isoforms are found in many tissues such as liver, adipose tissue, skeletal muscle, and heart. The functional distinction is dependent on their subcellular localization. ACC1 is cytoplasmic, while ACC2 is associated with the mitochondria. Despite this, they both promote fatty acid oxidation (discussed in section 2.2.3.) and are identically affected by AMPK phosphorylation (Hardie, 1989).

2.2.3. Lipid metabolism and cancer.

Both isoforms, ACC1 and ACC2, are responsible for malonyl CoA synthesis, the enzyme that regulates fatty acid biosynthesis. In the cytoplasm, ACC1 produces malonyl CoA synthesis that is utilized by fatty acid synthase (FAS) for the synthesis of fatty acids in the cytosol. In contrast, ACC2 produces malonyl CoA synthesis as intermediate of the *de novo* synthesis of fatty acids and as an allosteric inhibitor of carnitine palmitoyltransferase I (CPT I). Inhibition of CPTI blocks the transfer of fatty acyl groups into the mitochondria, thereby diminishing fatty acid oxidation (Ruderman et al, 1999). During energy deprivation ACC2 is inhibited by AMPK-dependent phosphorylation, resulting in enhanced mitochondria oxidation of fatty acids.

Fatty acid synthase is a multifunctional enzyme that catalyzes the synthesis of palmitate through NADPH-dependent condensation of ACC1 and malonyl-CoA. Up regulation of *FAS* gene is observed in various cancers, and is extensively studied in breast cancer (Rossi et al, 2006; Swinnen et al, 2002; Zhang & Powell, 2005). Expression of this enzyme is a hallmark of more

advanced tumours and correlates with poor prognosis as well as high number of metastatic cells (Carvalho et al, 2008; Ross et al, 2008). There is evidence that increased fatty acid biogenesis promoted by FAS is a molecular switch that will drive human epithelial mammary cells progression toward malignancy (Vazquez-Martin et al, 2008).

More recently, a variety of FAS small molecules inhibitors have been shown to inhibit the growth of prostate and lung cancer xenografts (Orita et al, 2007). Studies conducted in xenografts and genetically induced mouse models have shown that FAS inhibitors not only kill cancer cells but also sensitize them to other therapies such as Trastuzumab (Flavin et al, 2011). To date, the use of FAS inhibitors seems very desirable; however the weight loss induced by the increase in mitochondria fatty acid oxidation via stimulation of CPT1 limited the use of these inhibitors. Various therapeutic strategies are being tested to inhibit FAS and avoid damage of normal cells (Agostini et al, 2014; Kant et al, 2013; Lee et al, 2013).

2.2.4. AMPK and glucose metabolism.

The metabolism of glucose is another pathway affected by AMPK. In the skeletal muscle AMPK promotes the translocation of the glucose transporter, GLUT4, to the plasma membrane, increasing glucose flux to the cells (Treebak et al, 2010). The translocation of GLUT1 transporter is indirectly activated by AMPK. Translocation of GLUT1 to plasma membranes is increased through the phosphorylation of Thioredoxin-interacting protein (TXNIP) by AMPK resulting in TXNIP degradation. TXNIP suppresses glucose uptake by direct binding to

GLUT1 inducing the transporter internalization (Wu et al, 2013). Finally, AMPK also promotes glycolysis in the heart through phosphorylation of the glycolytic enzyme, 6-phosphofructo-2 kinase (PFK) which is an important enzyme in the metabolism of glucose (Marsin et al, 2000). The activation of glycolysis by AMPK is not always observed in other tissues, therefore this effect appears to be either tissue specific or metabolic related depending on energy requirement.

2.2.5. AMPK activation leads to transcriptional responses.

AMPK activation also targets long-term responses that lead to epigenetic and transcriptional changes that contribute to the maintenance of energy homeostasis. AMPK activation alters the phosphorylation status of transcription factors, chromatin-remodelling enzymes; including histone deacetylases and modulates gene expression in response to energy deprivation or stress.

One group of these enzymes is the sirtuins, highly conserved NAD^+-dependent histone deacetylases (Imai et al, 2000; Landry et al, 2000), that regulates many epigenetic processes that range from inflammation, cell metabolism, cellular senescence and aging, cellular differentiation, and cell proliferation. In humans, there are seven members of the sirtuin family (SIRT1-7); each has a conserved sequence of 275 amino acids making up the catalytic core domain. SIRT-family members differ through sub-cellular localization. Notably, they are found in the nucleus, cytoplasm or in the mitochondria and are divided into four different classes (I-IV) according to function (Frye, 2000; Michan & Sinclair, 2007; North et al, 2003; Schwer et al, 2002).

In mammals, the best studied of these enzymes is the Silent Information regulator T1 (SIRT1). SIRT1 mediates NAD^+-dependent deacetylation of targeted substrates. SIRT1 is primarily responsible for the deacetylation of lysine residues in a reaction that consumes NAD^+ and releases nicotinamide (Tanny et al, 1999). Because NAD+ and NADH are important coenzymes that mediate catabolic fluxes, SIRT1 acts as a sensor to identify metabolic stresses such as exercise leading to low glucose availability. This regulation leads to post-translation modifications and deacetylation of histones/proteins which promotes the regulation of transcription factors involved in cellular metabolism (Landry et al, 2000).

AMPK activation of SIRT1 is highly dependent on mitochondria generation of NADH. AMPK enhances SIRT1 activity by increasing NADH through an increase in mitochondria β-oxidation (Canto et al, 2009). Knocking down expression of SIRT1 in cells attenuates the effects of AMPK in the expression of genes related to mitochondria biogenesis (Canto et al, 2009). Activation of SIRT1 results in deacetylation and expression of peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) (Canto et al, 2009; Jager et al, 2007) (Rodgers et al, 2005). Increase in PGC-1α expression regulates mitochondria biogenesis (Lagouge et al, 2006). Therefore, AMPK and SIRT1 cooperate to regulate energy metabolism.

2.3. The role of AMPK in disease.

The pathophysiology of diseases such as diabetes, cardiovascular diseases and cancer is a complex process involving the interaction of various

organs and cell signals. A major risk factor related to all these diseases is obesity which results in abnormal lipid and glucose metabolism. An imbalance in these metabolic pathways leads to insulin resistance, glucose intolerance and dyslipidemia.

The number of individuals showing signs of metabolic dysregulation in the developed world has increased to alarming numbers. Common conditions are hyperglycemia, hypertension, dyslipidemia and pro-inflammatory responses. All these factors increase the risk for developing diabetes, cardiovascular diseases and, eventually, cancer. Thus, there is an urgency to find a metabolic target that is associated with these diseases. Because AMPK is the master energy homeostasis protein kinase and controls multiple metabolic pathways, the interest in targeting AMPK signalling for the treatment of these diseases has increased. Regulation of AMPK activation is known to play a role in hepatic fatty acid oxidation and synthesis (Hardie & Pan, 2002), in addition to its role in stimulating skeletal muscle glucose uptake (Hardie, 2004), modulating insulin secretion by pancreatic β-cells (Dufer et al, 2010) and controlling protein synthesis (Shaw et al, 2004b), all these mechanisms are important in the context of treating these diseases.

2.3.1. Diabetes

The key organ associated with lipid and glucose metabolism is the liver. During metabolic stress, AMPK is responsible for regulating hepatic gluconeogenesis and lipid biosynthesis. Impairment of this process contributes to insulin resistance and accumulation of lipids in peripheral tissues, thereby setting

the stage for metabolic dysregulation. In Type 2 diabetes mellitus (T2DM), insulin resistance leads to a dramatic decrease in glucose uptake. AMPK activation by AICAR and metformin was shown to lower plasma glucose by the suppression of gluconeogenesis (Bergeron et al, 2001; Hundal et al, 2000). In addition, conditional knockout of the liver-specific AMPKα2 isoform was shown to promote hyperglycemia. In mice, this increase in overall blood glucose led to glucose intolerance and increased gluconeogenesis (Andreelli et al, 2006). Conversely, a constitutively active form of AMPK led to hypoglycemia (Foretz et al, 2005), reducing hepatic glucose output.

AMPK inhibition of gluconeogenesis also appears to be acting through LKB1. Abrogation of LKB1 inhibits AMPK activation and increases plasma glucose levels (Sakamoto et al, 2004). Bultot and colleagues demonstrated that the suppression of gluconeogenesis by AMPK results from the inactivation of liver glycogen synthase (Bultot et al, 2012). In addition, AMPK phosphorylates a transcription factor named AICAR response element binding protein (AREBP). Phosphorylation of AREBP by AMPK reduced hepatic gluconeogenesis (Shirai et al, 2011).

2.3.2. Cardiovascular disease

AMPK plays an important role in cardiovascular disease as a result of regulation of lipid synthesis and oxidation. The accumulation of fatty acids in the heart is a major promoter of heart attacks and stroke. This accumulation is mainly caused by hyperlipidemia. As discussed in section 2.2.2., AMPK is responsible for inhibiting ACC1 and ACC2, which are necessary for lipid synthesis and

oxidation. Furthermore, AMPK activation reduces lipogenic enzymes and transcription factors involved in lipogenesis.

AMPK reduces lipid accumulation in peripheral tissues (liver and skeletal muscle) by downregulation of the lipogenic transcription factors sterol regulatory element-binding protein-1 (SREBP-1) and carbohydrate response element-binding protein (ChREBP) (Fogarty et al, 2010; Kawaguchi et al, 2002; Li et al, 2011). SREBP-1 is responsible for the expression of various lipogenic enzymes that function in the metabolism of cholesterol and fatty acids. SREBP-1 is involved in up-regulating the expression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase) which is the rate-limiting enzyme coordinating cholesterol biosynthesis. Therefore, enhanced expression of SREBP-1/2 increase triacylglycerol and cholesterol accumulation whereas, AMPK downregulation of SREBP-1/2 lowers lipid synthesis (Li et al, 2011).

Long-term activation of AMPK by AICAR improves cardiac function by attenuating cardiac hypertrophy in rat models (Li et al, 2007). In addition, acute administration of metformin also promotes cardiac protection against myocardial infarction in mice (Calvert et al, 2008). Therefore, activation of AMPK promotes heart protection and contributes to cholesterol and glucose homeostasis. These important observations confirms a role for AMPK in cardiac function; emphasizing the need of further investigation of AMPK activation in cardiovascular disease.

2.3.3. Cancer

The importance of AMPK in cancer was first recognized after the characterization of AMPK as a target of LKB1 signalling (Shaw et al, 2004b).

Since then, the signalling network LKB1-AMPK-mTOR has been the focus of numerous studies (Huang et al, 2008; Zhong et al, 2006). Lack of LKB1 expression decreases AMPK phosphorylation and leads to hyperactivation of mTOR signalling. Activation of mTOR results in cell growth, protein synthesis and major dysregulation of cellular metabolism. Knockout mouse models of LKB1 correlates with aggressive tumour development (Andrade-Vieira et al, 2013b). More recently, mouse models lacking LKB1 expression have been used as a valuable tool to study cancer therapies targeting mTOR signalling (Andrade-Vieira et al, 2013b; Cheng et al, 2014; Garcia-Martinez et al, 2011). Combinatory therapeutic strategies that targets AMPK activation and mTOR inhibition have also been conducted (See Chapter 6) (Andrade-Vieira et al, 2014). *The role of AMPK in cancer will be discussed in details in Chapters 4, 5 and 6.*

3.0. mTOR signalling

3.1. mTOR kinase

Protein synthesis is a highly regulated process in all cells. A number of signalling pathways play distinct roles suppressing or enhancing protein synthesis. One of the most studied pathways involved in the mediation of this process is mTOR, a Ser/Thr protein kinase conserved in all eukaryotes. mTOR exists in two distinct complexes, mTORC1 and mTORC2, that are regulated by different pathways, promoting activation of distinct signalling cascades and with different sensitivities to rapamycin. The main core of mTORC1 is composed of the catalytic mTOR subunit, the regulatory-associated protein of mTOR (RAPTOR) and the mammalian lethal with sec thirteen 8 (mLST8) (Fig. 4).

mTORC2 is composed of the catalytic mTOR subunit, the rapamycin insensitive companion of mTOR (RICTOR), SAPK-interacting 1 (SIN1) and mLST8 (Jacinto et al, 2006; Jacinto et al, 2004; Kim et al, 2002). Exclusively, rapamycin binds to the intracellular FK506-binding protein (FKBP12) which directly interacts and inhibits mTORC1 but not mTORC2 (Brown et al, 1994; Kim et al, 2002). This inhibition leads to a decrease phosphorylation of downstream substrates resulting in diminished cell proliferation. mTORC1 is uniquely regulated by nutrients such as amino acids and is activated by Rheb in response to growth factor signals and nutrient availability (Inoki et al, 2003b; Tee et al, 2003). Therefore, inhibition of mTORC1 suppresses global protein synthesis and decreases ATP consumption towards a growing system.

While mTORC1 is widely characterized, mTORC2 is still under investigation. Functional studies have identified mTORC2 as the kinase responsible for the phosphorylation of AKT on Ser473 (Sarbassov et al, 2005b). Complete activation of AKT is accomplished by the phosphorylation of two residues, Thr308 via PI3K signalling and Ser473 through mTORC2. Phosphorylation of these two residues fully activates AKT, leading to cell survival and proliferation. mTORC2 also phosphorylates the serum- and glucocorticoid-induced protein kinase 1 (SGK1) (Garcia-Martinez & Alessi, 2008). This kinase is important for the regulation of ion transport and cell growth. These findings demonstrated the importance of mTORC2 in regulating cell growth and emphasize the relevance of mTORC2 in dysregulated cell growth.

3.2. mTOR activation

Activation of mTORC1 is promoted by PI3K-AKT signalling in response to growth factors. Activated AKT phosphorylates TSC2 at multiple sites and inhibits the activation of TSC1. AKT also activates mTOR independently of TSC1/TSC2 by phosphorylation and dissociation of Raptor of PRAS40, a mTORC1 inhibitor (Sancak et al, 2007; Thedieck et al, 2007). Pro-inflammatory cytokines such as tumour necrosis factor-α (TNFα) and Wnt signalling are also involved in mTORC1 activation via inhibition of TSC1/TSC2 (Inoki et al, 2006; Lee et al, 2007).

mTORC1 is also activated by amino acids, specifically leucine and arginine (Blommaart et al, 1995; Hara et al, 1998). Amino acids-dependent activation of mTORC1 requires a group of proteins known as Rag GTPases. Mammals have four Rags, namely Rag A, Rag B, Rag C and Rag D. Upon amino acids stimulation, Rag A and Rag B forms a heterotrimer when bound to GTP which enables the interaction of these proteins with Raptor and translocation of mTORC1 to the lysosomal surface (Sancak et al, 2010; Sancak et al, 2008). At the lysosomal surface mTORC1 is activated by Rheb.

3.3. mTOR and regulation of protein synthesis.

After activation, mTORC1 phosphorylates a variety of substrates that promote cap-dependent translation initiation, leading to protein synthesis. The cap-dependent translation is dependent on the assembly of the cap-binding complex. This complex consists of a group of proteins that are recruited to the mRNA 5'-cap to initiate the events that will lead to protein synthesis upon nutrient availability and growth factor signalling via mTORC1 (Fig. 4).

The cap-binding complex is comprised of the eukaryote initiation factors (eIFs). The three main proteins involved in cap-dependent translation are the cap binding protein (eIF4E), a scaffolding (eIF4G) protein and the helicase protein (eIF4A) (Sonenberg, 2008). The expression of these proteins is directly related to enhanced protein synthesis. As a result, eIF4E is proposed to be an oncogene because it is highly expressed in various cancers (Lazaris-Karatzas & Sonenberg, 1992; Ruggero et al, 2004).

Upstream of the cap-binding complex, mTORC1 is responsible for the phosphorylation of two well-characterized downstream proteins, the ribosomal S6 kinases (S6K1/S6K2) and the eukaryotic translation initiation factor-binding proteins (4EBP1/4EBP2). Phosphorylation of S6K is required for its activation and leads to a number of events that will promote translation initiation. S6K phosphorylates the small ribosomal protein, S6 on Ser235 and Ser236, which in turn, promotes the assembly of ribosomes to the mRNA. In addition, S6K have been shown to phosphorylate eIF4B, which is another component of the cap-binding complex. S6K also phosphorylates a negative regulator of eIF4A, programmed cell death protein 4 (PDCD4) mediating its ubiquitin degradation (Fenton & Gout, 2010).

In addition, S6K is involved in a negative feedback loop through insulin receptor substrate 1 (IRS1) attenuating PI3K signalling. Inhibition of mTORC1 by rapamycin in a time-dependent manner leads to up regulation of AKT driven by PI3K signalling and phosphorylation of AKT on Thr308 and Ser473. Thus, inhibition of mTORC1-mediated S6K phosphorylation by rapamycin leads to

blockage of S6K negative feedback loop promoting up regulation of PI3K signalling and cell survival. Due to this feedback loop, the use of rapamycin and analogs, in combination with other therapies, including PI3K inhibitor appears to be a more appropriate therapeutic approach. However side effects may occur as a result of this double blockage, thus further investigation into how to better inhibit mTORC1 is warranted (Fig. 4).

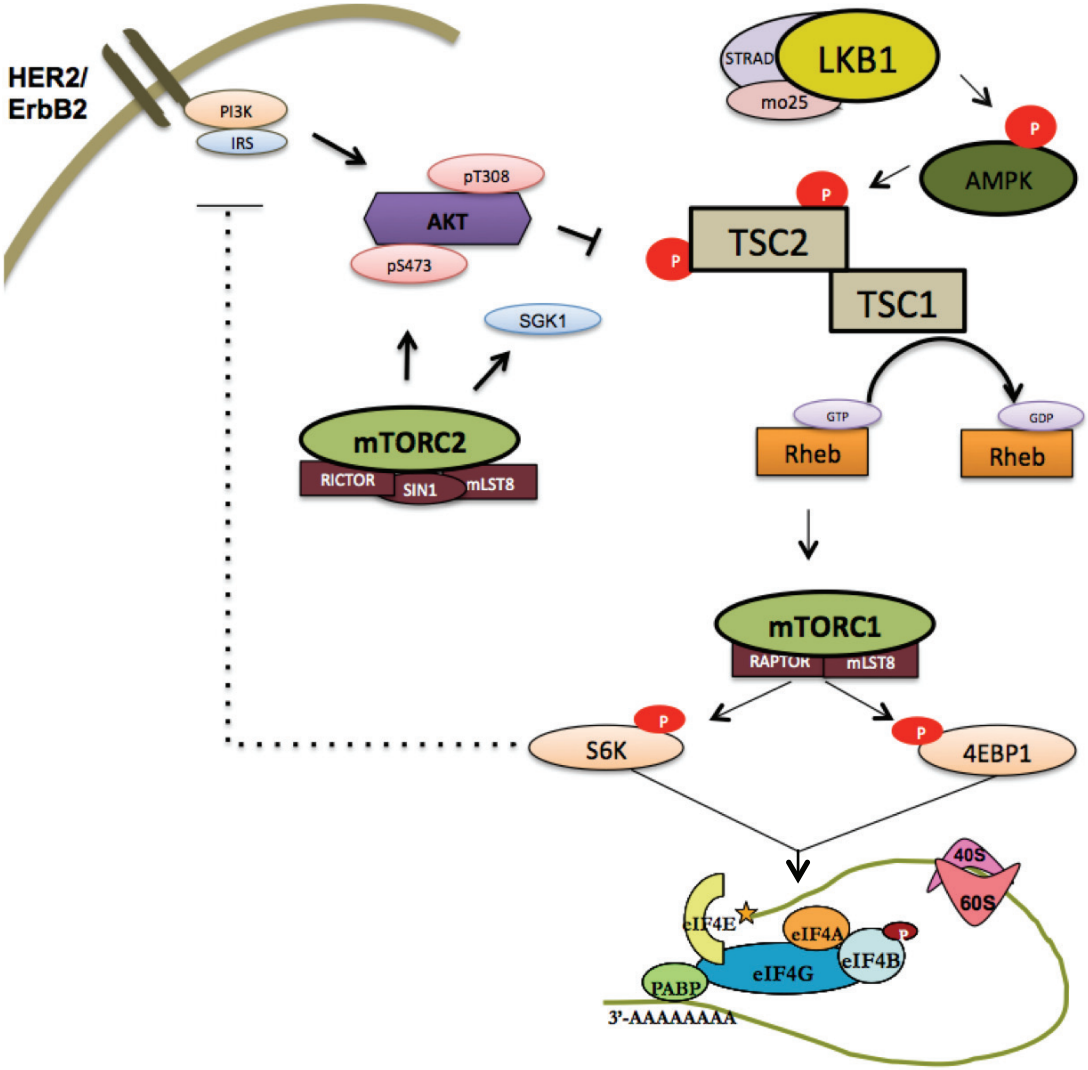

Figure 4 Cell signalling. Tyrosine kinase receptor (HER2/ErbB2) is phosphorylated upon cell growth stimuli. Phosphorylation of HER2 leads to activation of PI3K signalling. Phosphorylation of AKT on T308 and promotes mTORC2-mediated phosphorylation of AKT on S473. Activated AKT inhibits TSC2/TSC1 GEF activity thus maintaining Rheb association with GTP which promotes mTORC1 signalling. Once activated mTORC1 phosphorylated S6K and 4EBP1 leading to cap-dependent translation. Constitutive activation of S6K leads to a negative feedback loop through IRS which blocks further activation of PI3K/AKT. LKB1-AMPK signalling phosphorylates TSC2 activating this protein and promoting GEF. These events lead to the hydrolysis of Rheb-GTP to Rheb-GDP diminishing activation of mTORC1 signalling.

While the phosphorylation of S6K by mTORC1 promotes activation, phosphorylation of 4EBPs by mTORC1 triggers the inhibition. The dephosphorylated form of 4EBPs binds to eIF4E preventing the recruitment of eIF4E to the mRNA 5'-cap. The phosphorylation of 4EBP1 by mTORC1 in multiple sites (Thr37, Thr47, Thr70 and Ser65) is proline directed since phosphorylation sites are identified by an area rich in proline residues (Nave et al, 1999). These phosphorylation events release eIF4E, promoting its association with mRNA 5'-cap,therefore, initiating protein synthesis. The phosphorylation of these sites is sensitive to rapamycin and is commonly used to assess mTORC1 activity (Huang & Manning, 2008).

3.4. mTORC1 signalling regulates lipids synthesis and autophagy.

In addition to protein synthesis, mTORC1 controls, in part, lipid metabolism through the regulation of the SREBP1/2. As mentioned in section 2.3.2., SREBP1/2 is a transcription factor that controls the expression of fatty acids and cholesterol synthesis genes. mTORC1 inhibition leads to downregulation of SREBP1/2 causing the suppression of lipogenesis. mTORC1 activation of SREBP1/2 is through several mechanisms but most importantly, stabilization of SREBP1/2 by its recruitment to the nucleus to activate transcription and lipogenesis of cholesterol and triglycerides (Duvel et al, 2010; Wang et al, 2011).

Autophagy is another process regulated by mTORC1. Cellular adaptation to nutrient starvation leads to recycling process that result in the engulfing of organelles by lysosomes. mTORC1 negatively regulates this process by the

inhibition of the autophagy complex composed by the autophagy-related genes ULK1/Atg13 that are required to initiate autophagy (Ganley et al, 2009; Hosokawa et al, 2009). mTORC1 phosphorylates ULK1 and disrupts the interaction between ULK1 and AMPK, resulting in ULK1 inactivation (Egan et al, 2011).

3.5. mTORC1 signalling regulates the expression of glycolytic enzymes.

Another important mechanism regulated by mTORC1 is glucose metabolism. Recently, mTORC1 was shown to enhance the expression of glycolytic enzymes, resulting in upregulation of glycolysis (Sun et al, 2011; Zha et al, 2011). Glucose catabolism has become the focus of many cancer studies since it was discovered that glycolysis enhances the metabolic changes that leads to the Warburg effect. In 1920's, Otto Warburg observed that highly proliferating cells tend to rely on glycolysis to convert glucose to lactic acid in order to generate ATP (Warburg, 1956). Even though this system is less effective in providing energy compared to mitochondrial oxidative phosphorylation, it yields biosynthetic products that are required by highly proliferating cells. *For more detailed information on Warburg effect refer to section 5.*

There is increasing evidence that mTORC1\2 are involved in up-regulation of glycolysis. Enhanced aerobic glycolysis is frequently found in cells expressing hyperactive mTOR signalling. Recently, it was shown that mTORC1 enhances the expression of the glycolytic enzyme, pyruvate kinase 2 (PKM2) (Sun et al, 2011) by the activation of the proto-oncogene c-Myc. Remarkably, both mTORC1 and mTORC2 synergistically mediate the c-Myc cascade through

the phosphorylation of downstream targets which enhances the expression of many glycolytic enzymes (Masui et al, 2013; Sun et al, 2011). *For detailed information on glycolytic enzymes refer to section 5.*

Regulation of glycolysis is, in part, influenced by LKB1 activity. More recently, our group showed that LKB1 inhibits mTORC1 affecting the expression of glycolytic enzymes. Tumours lacking LKB1 have underlying mechanisms that drive the Warburg effect. These changes are evident from the high levels of metabolites and expression of enzyme characteristic of this effect in tumours lacking LKB1 (Andrade-Vieira et al, 2013b; Shackelford et al, 2009). Notably, the lack of LKB1 enhances cancer cell metabolism specially in lung, kidney, cervical and breast cancers, suggesting the possibility of using LKB1 expression as a biomarker for aberrant metabolism (Andrade-Vieira et al, 2013b; Feng et al, 2012; Rowe et al, 2013; Xiao et al, 2012).

4.0. Breast Cancer

Breast cancer is a complex disease because of the heterogeneous profile of the disease. In Canada, breast cancer is the second most common cancer-related cause of death amongst females. Despite this, research efforts have substantially decreased the number of women dying of breast cancer per year. In 1986, there were 32.0 deaths per 100,000, but in 2014, the rate was 18.4 deaths per 100,000 in Canada (Canadian Cancer Society, 2014).

Clinically, breast cancer is classified based on hormone receptor expression and human epidermal growth factor receptor (HER2) status (Goldhirsch et al, 2009). According to this molecular classification, hormone

positive breast cancer have high levels of estrogen and progesterone receptors (ER and PR, respectively), they have a high responsiveness rates when treated with Tamoxifen® and analogs which target ER receptors. HER2 positive tumours are treated with targeted antibodies therapies such as Herceptin ® (Trastuzumab) (Tebbutt et al, 2013). Tumours that are ER, PR and HER2 negative are known as triple negative breast cancer (TNBC), are highly aggressive, have a low responsiveness to treatments and poor prognosis (Gluz et al, 2009).

Breast cancer research has elucidated many signalling pathways linked to tumourigenesis and has developed therapies to treat patient with common breast cancer mutations. Despite this, there are tumours subtypes that are resistant to current therapeutic approaches requiring the development of novel therapies. One of the most common signalling pathways mutated in breast cancer is the HER2/ErbB2 (Tandon et al, 1989). HER2 is a member of ERBB family of receptors, which includes other four members namely EGFR, ErbB3 and ErbB4 (Semba et al, 1985). These receptors are commonly expressed in epithelial cells and are tyrosine kinase receptors. All the members have an analogous structure which stimulates homodimeric and heterodimeric interaction between the family members and stimulates autophosphorylation of tyrosine residues (Schechter et al, 1984). These interactions activate ErbB2 which does not have a known ligand and requires dimerization with other family members for complete activation. The other receptors have ligands that promote their activation (Pinkas-Kramarski et al, 1996; Roskoski, 2014).

The main structure of tyrosine kinase receptors consists of an extracellular domain, a hydrophobic transmembrane region and an intracellular tyrosine kinase domain. The phosphorylation of tyrosine residues activates the receptors and promotes downstream signalling through RAS-MAPK and PI3K-AKT (Soltoff & Cantley, 1996). Aberrant activation of these receptors upregulates survival signalling and promotes tumourigenesis. A number of targeted therapies have been developed over the years to inhibit activation of these receptors (Akiyama et al, 1986; Bos et al, 1997; Cho et al, 2003).

In breast cancer, HER2 signalling is mutated in approximately 25-30% of the tumours (Slamon et al, 1987). These tumours have high resistance rates to monotherapy (Fujita et al, 2006; Nagata et al, 2004). Less than 35% of patients respond to Trastuzumab monotherapy (Vogel et al, 2002). HER2+ breast cancer is commonly treated with HER2- targeted antibodies or small molecule tyrosine kinase inhibitors (TKI). These inhibitors compete with ATP for binding to the kinase domain of the receptors (Furet et al, 1995). Conversely, HER2-targeted antibodies bind to the extracellular domain preventing receptor dimerization and leading to activation of immune response thereby blocking receptor phosphorylation activity (Cooley et al, 1999). Activation of HER2 induces phosphorylation of PI3K-AKT signalling which enhances mTOR activity. Breast cancer patients that acquire resistance to HER2 therapy often carry mutations in other signalling which synergistically promote tumour growth and progression.

Resistance to current therapy has become a challenge, and the development of an alternative treatment is under investigation. One of the

strategies is to combine TKI with antibody therapy. This strategy has been proven to have additive antiproliferative activity in studies of head and neck cancers *in vivo* (Johns et al, 2003). Another alternative treatment is the direct inhibition of PI3K. This strategy is widely accepted since PI3K signalling is hyper-activated in a variety of cancers (Maira et al, 2008), however the efficacy of this therapeutic approach has not proven successful in clinics.

There are a variety of animal models to study activating mutations of HER2 signalling. One of these models is called NIC mouse. The NIC mouse was generated in 2008 and co-expresses activated mutation in ErbB2 and Cre-recombinase in the same mammary cells. This strategy avoids the expression of activated ErbB2 in Cre-recombinase negative cells. Activation of ErbB2 expression is under the control of the murine mammary tumour virus (MMTV) promoter. These mice can be efficiently interbred with floxed mice targeting genes of interest in order to study the implication of combinatorial mutations directed to the mammary gland (Schade et al, 2009; Ursini-Siegel et al, 2008).

Several genes have been shown to play a role in HER2+ breast cancer, one of these gene is the tumour suppressor *PTEN*. PTEN is a dual phosphatase that dephosphorylates phosphatidylinositol phosphates 3 in the cellular membrane. PTEN activation leads to inhibition of PI3K signalling resulting in inhibition of cell proliferation and survival (Franke et al, 1997). Loss of PTEN is highly involved in Trastuzumab resistance in HER2 positive patients and predicts a poor prognosis and diminished overall survival rates (Nagata et al, 2004).

5.0. Regulation of mitochondria.

5.1. Mitochondria.

Mitochondria are responsible for providing energy to sustain all metabolic activity in organisms. The generation of energy involves the transport of electrons between the mitochondrial protein complexes, I-IV. Each complex is comprised of proteins and metabolites responsible for feeding the mitochondria electron transport chain (Brand & Lehninger, 1977). Mitochondria contain two main membranes, the inner membrane and the outer membrane; these membranes are organized to efficiently produce ATP. The inner membrane is the site of oxidative phosphorylation and ATP synthesis (Lehninger & Wadkins, 1962; Schultz & Chan, 2001) .

5.2. Mitochondria biogenesis.

There are several mechanisms regulating mitochondria biogenesis, here I will discuss LKB1-AMPK regulation of mitochondria metabolism. In addition, mitochondria biochemistry will be discussed in section 6.

LKB1 phosphorylation of AMPK increases mitochondria biogenesis in skeletal muscle during metabolic stress and nutrient deprivation (Zong et al, 2002). This mechanism is enhanced by upregulation of transcription factors involved in mitochondria biogenesis such as nuclear respiratory factor 1/2 (NRF1/2). These factors increase the expression of transcription factor A mitochondrial (TFAM), a mitochondria matrix protein, important for the replication of mitochondrial DNA (Canto et al, 2009).

The first evidence connecting AMPK and mitochondria biogenesis was the discovery that constitutively active mutation of AMPKγ3 subunit of AMPK correlated with increased expression of PGC1α (Garcia-Roves et al, 2008). As mentioned in section 2.2.5., an increase in PGC1α enhances mitochondria biogenesis. The activation of PGC1α by AMPK is not well understood, however a proposed mechanism is via SIRT1-mediated deacetylation of PGC1α (Canto et al, 2010). PGC1α expression is also stimulated by metabolic changes such as exercise, starvation and cold temperatures (Wu et al, 1999). The maintenance of mitochondria bioenergetics function is extremely important to maintain energy homeostasis whereby LKB1-AMPK signalling is essential because they increase mitochondria biogenesis.

A plant compound known as resveratrol also enhances mitochondria biogenesis. Resveratrol is a polyphenol with powerful antioxidant properties. Mainly found in grapes, dietary intake of resveratrol activates SIRT1 and AMPK leading to activation of PGC1α signalling. Interestingly, resveratrol also attenuates the oxidative stress typically produced by the high flow of electrons in the mitochondria transport chain (Ungvari et al, 2009). During oxidative stress there is an increase in reactive oxygen species (ROS), which damages the tissues and accelerates the oxidative stress and the aging process. Resveratrol promotes mitochondria biogenesis, downregulates NAPDH oxidases and increases superoxide dismutase activity contributing to diminish levels of ROS, thus reducing cellular damages attributed to aging (Shin et al, 2009).

5.3. Metabolic switch that leads to malignant transformation.

The malignant transformation of normal cells to cancer cells involves a series of steps that are highly dependent on energy metabolism. The early events involved in malignant transformation occur when there is an imbalance of oncogene expression that leads to the generation of metabolically stressed cell populations. These cells often acquire growth advantage over normal cells and, in many types of cancer, mitochondria oxidative phosphorylation (OXPHOS) is attenuated. Rapidly growing cells typically produce necrotic areas due to lack of proper angiogenesis and reduced oxygen supply. The absence of oxygen leads to a metabolic reprogramming required for survival in the extreme micro-environmental conditions (Gatenby & Gillies, 2004).

The lack of oxygen partially converts malignant cells to a glycolytic phenotype in which ATP generation occurs by aerobic glycolysis but is still dependent on OXPHOS (Guppy et al, 1993). The lack of oxygen availability forces the tumour to initiate hypoxic signalling. The hypoxia-induced factor (HIF) mediates another metabolic switch that completely diverts the generation of ATP from OXPHOS and strengthens the glycolytic phenotype (Gottlob et al, 2001). Concomitantly, transcription of glycolytic genes is stimulated by HIF, providing the tumour with tools to sustain a high proliferative rate (Lambert et al, 2010; Wang & Semenza, 1993). The HIF complex is comprised of two oxygen sensitive subunits, HIF1α and HIF2α. Under normal oxygen levels, HIF subunits are hydroxylated and targeted to the proteasome for degradation, however when oxygen levels are decreased, HIF hydroxylation is reduced and the subunits are

stabilized and translocated to the nucleus (Huang et al, 1996; Wang & Semenza, 1995). Once in the nucleus HIF activates the expression of many genes related to energy metabolism. Interestingly, it was reported that cells lacking LKB1 expression have enhanced levels of HIF1α, suggesting that HIF1α is a direct or indirect target of the LKB1 signalling (Shackelford et al, 2009).

Two key enzymes are involved in HIF activation of glycolysis. These enzymes are hexokinase 2 (HEX2) and lactate dehydrogenase (LDH) (Hu et al, 2003; Mathupala et al, 2001). Once glucose enters the cell, HEX2 is the glycolytic rate-limiting enzyme that converts glucose to glucose 6-phosphate incorporating definitely glucose into the cells since this metabolite cannot cross cell membranes. Pharmacological inhibition of HEX2, by a glucose analog 2-deoxyglucose (2-DG) impairs cancer cells growth, emphasising the importance of metabolic drugs for the treatment of cancer (Purohit & Pohlit, 1982; Zhang et al, 2006).

Similarly important for this glycolytic phenotype is LDH, the enzyme that converts the final product of glycolysis, pyruvate to lactate, preventing pyruvate entry into the Krebs cycle. LDH upregulation is considered to be a hypoxia adaptation (Firth et al, 1995). Cancer cells are known to produce high levels of lactate and have enhanced LDH activity which provides high levels of NAD^+ to feed glycolysis. LDH expression is regulated by HIF but also by the oncogene c-MYC, which is highly express in a variety of cancers (Dang et al, 1999; Shim et al, 1997). LDH expression is a hallmark of malignancy and is a critical enzyme in the Warburg effect, attenuating mitochondria OXPHOS.

In cancer cells undergoing hypoxia, impairment of LDH expression reduces ATP concentrations resulting in attenuation of cell growth (Fantin et al, 2006) (Chapter 4 Fig.6). Recently, we showed that tumours lacking LKB1 appeared to have greater levels of lactate and enhanced LDH expression, suggesting a possible role of LKB1 in the maintenance of the glycolytic phenotype (Andrade-Vieira et al, 2013b) (Chapter 5). In addition, we showed that the mutant forms of LKB1 with oncogenic properties (Scott et al, 2007) enhanced the expression of LDH (Andrade-Vieira et al, 2013a) (Chapter 4).

Inevitably the high energy requirements of cancer cells exceed the supply of nutrient. When the supply of glucose is exhausted, tumours cells go through another adaptation event which promotes glutaminolysis. The amino acid glutamine is, catabolized by glutaminase to glutamate, another source of energy. Glutamine feeds the Tricarboxylic acid cycle (TCA) to increase ATP levels and sustains the production of nucleotides that are important in a growing system. The metabolism of glutamine can be accomplished in an anoxia state and promotes the activation of mTOR signalling, enhancing malignancy (Fogal et al, 2015; Jewell et al, 2015). Some cancer cells re-adapt to OXPHOS even with low levels of glucose, notably this is accomplished by the metabolism of glutamine which provides pyruvate, lactate and NADPH required for the TCA cycle (DeBerardinis et al, 2007).

In consequence of the low level of energy, mitochondria biogenesis is activated. Thus, PGC1α mediates the co-activation of several transcription factors to increase mitochondria biogenesis including peroxisome proliferator-

activated receptor alpha (PPARα). Activation of PPARα is also stimulated by AMPK which enhances mitochondria function and stimulates fatty acid oxidation. Simultaneously, AMPK and PPAR activates the expression of many genes encoding nuclear subunits of the respiratory chain and proteins involved in mitochondria DNA (mtDNA) transcription (For reviews see (Ventura-Clapier et al, 2008)). The increase in transcription of mitochondria genes triggers OXPHOS and results in activation of mtDNA replication.

Mitochondria function and the glycolytic pathway are tightly related. Metabolically altered cancer cells promote critical changes to this equilibrium in order to maintain tumour growth. The cumulative modifications in gene and protein expression dictate cell growth and characterize tumour transformation and progression. Understanding cancer metabolism is the key to unveiling the mechanisms involved in uncontrolled cell growth. Thus, targeting tumour metabolism represents a promising strategy for the treatment of cancer.

6.0. The Warburg Effect

In early 1920s, Otto Warburg first published his observations that highly proliferating cells such as cancer cells, rely greatly on glycolysis to produce ATP. These findings also demonstrated that cancer cells favour glycolysis even in the presence of oxygen. Warburg called this process aerobic glycolysis and considered this metabolic switch a growth adaptation. According to Warburg, cancer cells altered glucose metabolism as a result of a diminished mitochondria OXPHOS, leading Warburg to assume that cancer cells had aberrant mitochondria (Warburg, 1956).

Warburg's observations contributed immensely to the study of cancer metabolism and led to important discoveries. In 1950s, Dr. Hans Adolf Krebs demonstrated that OXPHOS renders 34 ATPs while glycolysis renders only 2 ATPs (Krebs et al, 1953), therefore many research groups questioned why aerobic glycolysis conferred cancer cells growth advantages. There are several hypotheses to explain this concept. One explanation is that highly proliferating cells require substrates to sustain nucleic acids, protein and lipids synthesis to maintain rapid growth. In the glycolytic pathway, substrates for the production of these building blocks are produced, facilitating the high metabolic demand. A second explanation is that opting for aerobic glycolysis reduces mitochondria OXPHOS thus leading to diminish production of ROS, which is known to induce apoptosis (Gansauge et al, 1997; Smolková et al, 2011). Therefore, cancer cells continue to proliferate and gain survival advantages over normal cells. A third explanation is that high demand of aerobic glycolysis produces a more acidic extracellular environment due to the production of lactic acid. This phenomenon promotes destruction of the extracellular matrix facilitating the ability of cancer cells to expand, invade and migrate (Gatenby & Gawlinski, 1996; Walenta et al, 2000).

More recently, with the development of the positron-emission tomography (PET) imaging technique, the importance of glucose metabolism in cancer cells became of interest in clinical oncology. The glucose analog tracer used in PET imaging, fluorodeoxyglucose (FdG) (Di Chiro et al, 1982; Weber et al, 1999), is taken up almost immediately by cancer cells emphasizing the increased glucose

uptake by these cells. The levels of FdG uptake often correlate with the aggressiveness of tumours and poor prognosis (Di Chiro et al, 1982; Nieweg et al, 1993), which demonstrated that the glycolytic phenotype is a characteristic of carcinogenesis.

6.1. Glycolytic enzymes and the Warburg Effect.

The increased uptake of glucose leads to upregulation of glycolytic enzymes. As glucose is metabolized to pyruvate key enzymes are expressed to promote glycolysis. Some of these enzymes, such as HEX, pyruvate kinase M1/2 (PKM1/2), pyruvate dehydrogenase (PDH) and LDH are very important to produce substrates required during cell proliferation. They also play an important role in cancer cell metabolism.

Hexokinase is a rate-limiting enzyme required to maintain glucose inside the cell as it phosphorylates glucose to glucose 6-phosphate, a product that is unable to cross cell membranes. Mammals express HEX1-3, however HEX2 is the principal isoform involved in regulation of aerobic glycolysis (Parniak & Kalant, 1985). Inhibition of HEX2 by metabolic competitors depletes glucose metabolism. Currently in pre-clinical trial, 2-DG is a well-studied inhibitor of HEX2. 2-DG is taken up by cells and it is metabolized by hexokinase to phosphorylated-2DG (p-2DG). The accumulation of p-2DG inhibits hexokinase and prevents glucose-6-phosphate dehydrogenase from further metabolizing glucose into the glycolytic pathway (Parniak & Kalant, 1985; Taliaferro-Smith et al, 2009). Therefore, inhibition of HEX2 by 2-DG strongly decreases glycolysis (Aft et al, 2002).

Pyruvate kinase (PK) is comprised of four isoforms; PKL, mainly found in the liver and kidney; PKR found in erythrocytes and PKM1/2 in muscles. PKM1 is found in most adult tissues, however PKM2 is detected in embryonic cells and in rapidly proliferating cells (Bigley et al, 1968). As a result of this, PKM2 expression is linked to malignant transformation and is known to have tumour initiation and progression functions. PKs catalyze the conversion of phosphoenolpyruvate to pyruvate. The active form of PKM2 is a tetramer while the inactive state is a dimer (Ashizawa et al, 1991). The availability of these two forms is determined by cellular energy needs. Activation of PKM2 triggers cell cycle progression through MYC activation and histone modification via EGF-induced expression of cyclin D (Yang et al, 2011). Concomitantly, mTORC1 increases PKM2 expression to promote protein synthesis and cell proliferation (Sun et al, 2011).

At the end of the glycolytic pathway two enzymes play a role in the fate of pyruvate. PDH converts pyruvate to acetyl-coenzyme A, the first substrate to enter the tricarboxylic acid cycle (Nisman & Mager, 1952). The TCA cycle produces ATP but most importantly provides NADH, one of the main products that feed the electron transport chain in the mitochondria. Therefore, PDH expression drives mitochondria energy metabolism and is a key enzyme in oxidative metabolism. As a result of this, PDH upregulation is highly found in tumour stroma supporting the energy needs of growing tumours (Koukourakis et al, 2005).

In cancer cells, a large amount of pyruvate produced by the glycolytic cycle is converted to lactate by LDH. LDH is an enzyme composed by two

different subunits, LDHA and LDHB. These subunits form a tetramer that can be assembled into five different combinations; LDH1 tetramer contains four LDHB; LDH2 contains three LDHB and one LDHA; LDH3 contains two of each LDH, LDH4 contains one LDHB and three LDHA; and finally LDH5 contains four LDHA. LDHA expression is highly correlated with cancer and is predominantly expressed in highly metabolic tissues such as skeletal muscle (Chung et al, 1985; Markert et al, 1975; Takeno & Li, 1989).

High production of lactate triggers an acidification of the cellular microenvironment, which in turn promotes the destruction of the extracellular matrix and cell migration. In addition, lactate efflux initiates a local inflammatory response resulting in the secretion of cytokines and growth factors that will further drive cell proliferation (Shime et al, 2008; Yabu et al, 2011) . Lactate transport is also a key event in malignant cells. The transport of lactate across cellular membranes is regulated by a family of transporters known as monocarboxylate transporters (MCTs).

The MCT family of transporters is comprised of fourteen isoforms, MCT1-14. They are proton-coupled monocarboxylate transporters with distinct roles in a variety of tissues. MCT1 and MCT4 are very similar and play an important role in highly glycolytic tissues such as skeletal muscle and brain (Halestrap & Price, 1999). MCT1 is ubiquitously expressed and transports lactate by sequential binding to H+, followed by lactate binding. The transport across cellular membranes is bidirectional and is dependent on cellular energy.

Energy metabolism is highly dependent on MCT expression and cancer cells are known to have high expression of MCT1 (Fang et al, 2006; Mathupala et al, 2004; Pinheiro et al, 2010). Importantly, therapeutic strategies that target metabolism are also dependent on MCT1 expression to enter the cells. The glycolytic inhibitor, 3-bromopyruvate, is dependent on MCT1 expression to inhibit glycolysis, emphasizing the importance of this transporter in cancer therapy (Birsoy et al, 2013).

Although aerobic glycolysis is observed in numerous cancers, it is not the only metabolic pathway engaged to provide energy to cancer cells. Recently, Lisanti and collaborators (Pavlides et al, 2009) demonstrated that the tumour microenvironment (stromal cells) feeds the tumour cells through the production of pyruvate and lactate. These substrates are taken up by tumour cells to generate energy through the activation of the TCA cycle and mitochondria electron-transport chain. This new concept is known as the Reverse Warburg Effect, and although the cells are still dependent on aerobic glycolysis for the production of pyruvate, they also show increased OXPHOS.

6.2. Cancer mutations associated with cell metabolism.

Enhanced metabolic reprograming is also associated with mutations in metabolic enzymes such as succinate dehydrogenase (SDH) and isocitrate dehydrogenase (IDH) (Grassian et al, 2014), both involved in the progression of the TCA cycle. SDH is composed of four subunits, SDHA, SDHB, SDHC and SDHD. SDH mutations are found in leukemia and paragangliomas. These

mutations are correlated with growth advantages and enhanced activation of regulatory metabolic factors (Baysal, 2007).

Humans have three isoforms of IDH, IDH1-3. IDH1 and IDH2 are involved in the redox regulation of $NADP^+$ and mutation in these enzymes was identified in a variety of tumour types, especially acute myeloid leukemia and gliomas. These mutations cause disruption of OXPHOS and accumulation of (D) 2-hydroxyglutarate (2-HG). This metabolite is found in tumours of patients with IDH1/2 mutations (Grassian et al, 2014; Wu & Zhao, 2013) and is associated with epigenetic changes leading to dysregulated cell growth (Lu et al, 2012; Xu et al, 2011). In addition, mutation in IDH1/2 leads to increase levels of HIF1α which enhances the expression of glycolytic enzymes and, consequently, glycolysis (Sasaki et al, 2012).

7.0. Omega 3 polyunsaturated fatty acids.

Although high rates of lipid synthesis are correlated with increased risk of developing cancer, there is another class of lipids, known as omega-3 polyunsaturated fatty acids (ω3PUFAs) that have protective functions against cardiovascular disease, immunity and cancer (Bang et al, 1971). ω3PUFAs cannot be synthesized in the human body due to the lack of desaturase enzymes that are required for the insertion of double bonds into the ω3PUFAs structure, therefore availability of these fatty acids in humans is dependent on diet (Meyer et al, 2003). Dietary ω3PUFAs is essentially supplied by cold water fishes and walnuts (Weaver & Holob, 1988). Western diets have low levels of ω3PUFAs,

although efforts are being made to increase the levels in a variety of foods (Simopoulos, 2002).

Polyunsaturated fatty acids are derived from phospholipids that undergo hydrolysis by phospholipases. They are mainly found in the cell membrane increasing the flexibility of membranes. PUFAs have multiple carbon-carbon double bonds which are all in the *cis* configuration (Feller et al, 2002). The common nomenclature includes the number of carbon atoms and the number of double bonds in the fatty acids chain. In addition, the ω symbol designates the location of the first double bond in relation to the terminal methyl carbon, for instance, Docosahexaenoic acid (DHA 22:6 ω3) is composed for 22 carbon atoms and has 6 double bonds with the first one on the third carbon atom (Fig. 5).

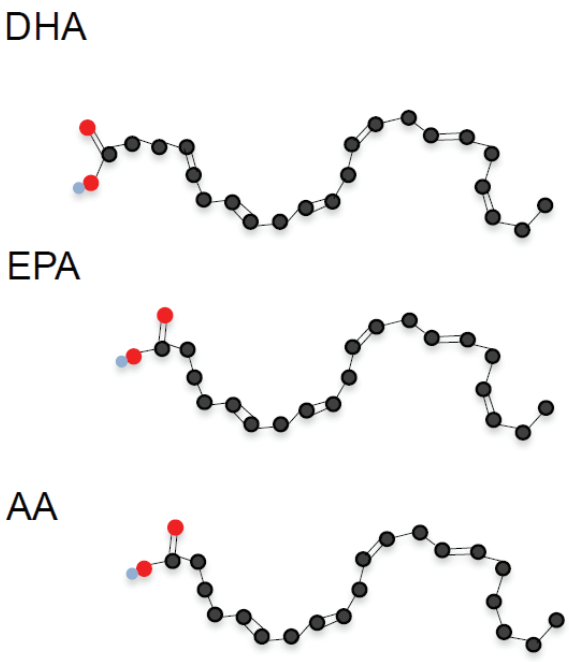

Figure 5. The structure of polyunsaturated fatty acids. The nomenclature of fatty acids is based on the total number of carbon atoms followed by the number of double bonds and the location of the first double bond from the terminal methyl group. Black, red and blue circles are representative of carbon, oxygen and hydrogen groups, respectively. DHA= docosahexaenoic acid, EPA= eicosapentaenoic acid and AA=arachidonic acid.

7.1. History and Discoveries.

Omega 3 polyunsaturated fatty acids were first identified as important to health by an epidemiological study which demonstrated that high levels of ω3PUFAs correlates with lower incidence of metabolic disorders (Bang et al, 1971; Kromann & Green, 1980; Sinclair, 1953). In these epidemiological studies, researchers found that high intake of ω3PUFAs resulted in high levels of ω3PUFAs in their body. The increase in ω3PUFAs levels correlated with a protective effect against chronic inflammation disorders, atherosclerosis and

hypertension. Later, another study found that Japanese with a high intake of ω3PUFAs had low incidence of myocardial infarction and chronic inflammation (Hirai et al, 1980). Other findings also demonstrated that diets rich in ω3PUFAs suppress the growth of cancer cells (Kort et al, 1987).

Cells membranes are significantly altered by different fatty acids composition. Unsaturated fatty acids, for example, are more flexible than saturated fatty acids whereas PUFA-rich cell membranes are more flexible and have a greater fluidity facilitating protein-membrane interactions (Feller & Gawrisch, 2005).Therefore PUFA- rich cell membranes are capable to bend easily without breaking. ω3PUFAs are incorporated to cell membranes partially replacing the fatty acid, arachidonic acid (AA 20:4 ω6), found in cells membranes of practically all tissues (Fig. 5) (Eldho et al, 2003; Rajamoorthi et al, 2005).This incorporation is highly implicated in the downstream effects observed when ω3PUFAs intake is increased (Andrade-Vieira et al, 2013a; Marignani et al, 1996; Marignani & Sebaldt, 1995; Simopoulos, 2002).

7.2. The main Omega 3 polyunsaturated fatty acids.

There are several ω3PUFAs, however the more commonly studied are DHA and eicosapentaenoic acid (EPA 20:5 ω3) (Fig. 5). These two fatty acids are highly implicated in inflammation response and anti-proliferative effects. Several observations demonstrated that dietary supplementation with DHA and EPA reduced tumour growth and decreased the viability of a range of cancer cells such as colon, prostate, breast and hepatocellular carcinoma (Calviello et al, 2004; Friedrichs et al, 2011; Kort et al, 1987). In addition, DHA and EPA are

also implicated in modulating aerobic glycolysis and mitochondrial ROS production emphasising a possible role of these fatty acids in cell metabolism (Andrade-Vieira et al, 2013a; Hsu et al, 2014).

7.3. Importance of Omega 3 polyunsaturated fatty acids in disease.

There are several proposed mechanisms implicating ω3PUFAs in disease. One of the most studied is the role ω3PUFAs play on immune response. Dietary ω3PUFAs supplementation also leads to a decrease in the capacity of monocytes to synthesize interleukins and tumour necrosis factor (TNF), the production of these inflammation mediators is associated with adverse clinical prognosis favouring invasiveness and aggressiveness of a wide range of cancers (Calviello et al, 2004; Mickleborough et al, 2009). Because of this tight relationship between immune system and tumour development, lowering the immune response has a useful effect for the treatment of cancer (Simopoulos, 2002).

Prostaglandins function, in particular, is enhanced in tumour progression, promoting angiogenesis by inducing the production of pro-angiogenic factors including vascular endothelial growth factor (VEGF). In addition, prostaglandins have been implicated in the inhibition of apoptosis and stimulation of cell proliferation. Cellular effects of prostaglandin E (PGE_2) are through G protein-coupled receptors that contain seven transmembrane domains. These receptors are members of a family of E-series prostaglandin (EP) receptors that act in many signalling pathways (Narumiya et al, 1999). Signals through these receptors mediate a variety of distinct effects, some enhancing cellular

malignancy by the activation of PI3K signalling leading to invasion and metastasis (Fulton et al, 2006).

In addition to the role in immune response, ω3PUFAs have shown anticancer effects by inhibiting signalling that drive cell proliferation and/or activating signals that drive tumour suppressor function. AMPK activity is enhanced by ω3PUFA supplementation. The first evidence was published in 2012, demonstrating that ω3PUFAs antagonize the macrophage inflammation response by the activation of AMPK signalling (Xue et al, 2012). Another group showed that LKB1 signalling was enhanced in the brain of animals fed DHA, whereas increasing levels of AA in the brain correlated with diminished activation of LKB1 signalling (Agrawal & Gomez-Pinilla, 2012).

During liver regeneration, ω3PUFAs promoted expression of hepatic tight junctions proteins that support hepatocytes function via activation of LKB1-AMPK signalling (Yan et al, 2011). More recently, our group showed that ω3PUFAs, in particular DHA, activates LKB1 signalling and inhibits cell metabolism in mammary cells. Functionally, DHA treatment in the presence of LKB1 impaired the ability of mammary epithelial cells to migrate (Chapter 3) (Andrade-Vieira et al, 2013a).

CHAPTER 2 OBJECTIVES AND HYPOTHESIS

This chapter summarizes the objectives of my thesis and introduces my hypothesis. For the first objective I described the effects of omega 3 polyunsaturated fatty acids, specifically DHA, in LKB1 signalling. The experiments results showed that DHA enhances LKB1 signalling and inhibits aerobic glycolysis. These findings led to further investigation of the role of LKB1 in cell metabolism. This discovery was published in the Journal of Cancer Biology and Therapy in 2013.

The second objective of my thesis research investigated the role of LKB1 in mammary gland tumourigenesis. Loss of LKB1 expression is involved in a variety of cancers as was discussed in the Introduction Chapter however very little was known about the role of LKB1 in breast cancer, specifically HER2+ breast cancer. The results from this project were published in Plos One in 2013. Based on this discovery I studied possible drug therapies and combinations that targeted tumours lacking LKB1 expression and, simultaneously, tumours that have an upregulation of the mTOR signalling. In this research, I developed a pre-clinical trial studies. This research is part of the third objective of my thesis and was published in Oncotarget in 2014.

2.0 Investigate the role of LKB1 on cell metabolism and mammary gland tumourigenesis.

The main objective of this research was to identify the role of LKB1 in cell metabolism, specifically dysregulation of energy metabolism that leads to abnormal cell growth. This research used both cell culture models and re-engineered mouse models to explore the role of LKB1 in energy metabolism and breast cancer tumourigenesis.

2.1. Specific Objective I

The first specific objective was to determine whether administration of ω3PUFA to transformed cells lines regulated LKB1 signalling. This study was performed using cells lines and primary mammary epithelial cells. In addition, this study determined a role for ω3PUFA in LKB1 signalling, aerobic glycolysis and cell migration (Chapter 4).

2.2. Specific Objective II

The second specific objective was to determine the importance of LKB1 in HER2+ breast cancer. To accomplish this objective, a transgenic mouse model lacking LKB1 and expressing a constitutively active form of ErbB2 receptor was generated. The mouse model was characterized to determine whether mTOR signalling was hyperactive and whether the tumours were metabolically active. Further to this, *in vitro* drug studies were conducted to determine possible therapeutic strategies that inhibited signalling pathways involved in tumour growth and cells proliferation (Chapter 5).

2.3. Specific Objective III

The third objective of this research was to conduct a pre-clinical drug trial using the compounds identified as possible targets in the *in vitro* drug study (Chapter 5). In this study, mice were evaluated for tumours by MRI, followed by 21 days of treatment with selected compounds. In order to determine the compounds efficacy, tumour growth was monitored by MRI and tumour cells were analyzed *ex vivo* (Chapter 6).

CHAPTER 3 MATERIALS AND METHODS

Chapter three contains an extended version of the Materials and Methods section published in manuscripts detailed in Chapters four, five and six. The original version of the manuscripts was modified to include a complete description of how experiments were performed and how results were obtained and analyzed. Some of the methods described in this section were stablished by previous members of the Marignani Laboratory and by collaboration with other researchers. In addition, some of the methods were developed by R. Andrade-Vieira specifically to accomplish the objectives and goals of my thesis.

Methods that were performed by collaborators are specified and methods developed by previous members of the Marignani Laboratory are properly cited. All the materials and methods described herein are published, unless otherwise specified. Additionally, in the appendix A there are two tables of antibodies used with specific concentrations and conditions that were stablished by R. Andrade-Vieira. In the appendix A, I also provided the detailed protocols that I developed for the purpose of this research.

Appendix A: Table of antibodies used and the following protocols: ATP bioluminescent assay, mitotracker red, PCR arrays, seahorse experiment, and adeno-virus infection.

3.1. Cell Culture and Transfection.

MCF7 and HeLaS3 cells (American Type Culture Collection, Manassas,VA) were maintained in DMEM supplemented with 8% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA). Cells were transiently transfected with expression plasmids (indicated in the figures), using PEI (Polysciences, Inc.). Cell cultures were maintained at 37ºC in a humidified atmosphere of 5% CO_2.

3.2. Animals.

All animal husbandry and studies were conducted in strict accordance with the Canadian Council on Animal Care. Protocols #10-009, #12-091 and #13-063 were approved by the Committee on Laboratory Animals (UCLA), Dalhousie University. LKB1$^{fl/fl}$ (129S6-*LKB1*^{tm1Rdp}) (Bardeesy et al, 2002) were from the NCI Mouse Repository while the transgenic mice expressing oncogenic ErbB2 followed by an internal ribosomal entry sequence and a MMTV promoter driving Cre-recombinase (generous gift from Dr. William Muller) (Schade et al, 2009). LKB1$^{fl/fl}$ mice were interbred with NIC mice to generate LKB1$^{-/-}$NIC mice. All mice were palpated every three days to monitor for mammary tumours, grooming abnormalities and weight change. All animals were sacrificed in accordance with the University Committee on Laboratory Animals, Dalhousie University. Primary mammary tumours, primary mammary tumour cells and primary mammary epithelial cells were harvested from LKB1$^{-/-}$NIC, NIC and wild-type control mice. Primary mammary epithelial cells were harvested and immediately used for experimentation.

3.3. Cell lysis and Western blot analysis.

Cells were harvested and lysed as described previously (Marignani et al, 2001). The following cells were used in my Thesis research: MCF7, HelaS3 (American Type Culture Collection) and primary mammary epithelial cells. Primary mammary epithelial cells were isolated from mammary gland of female wild-type mice and from spontaneous mammary tumours in LKB1$^{-/-}$NIC and NIC mice, using a solution of dispase/collagenase (Invitrogen). Epithelial cells were isolated based on surface markers using epithelial cells specific antibodies. For western blot analysis of protein expression, total tumours were lysed and protein concentrations were determined by Bradford analysis. Proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) then transferred to Immobilon-P membrane (Millipore). Membranes were initially blocked in fatty acid free skimmed milk (1%)/TBS or BSA/TBS. The following antibodies were used: anti-eIF4E, anti-eIF4G, anti-4EBP1/2, -phospho-ribosomal protein S6 (S235/236), -ACC, -pACC (S79), -pAMPK (T172), -AMPK, , -AKT (S473), -AKT (T308), -ErbB2, -pERK (T202/Y204), -ERK , -phospho-p90RSK (S380) and –p90RSK (Abcam), glycolytic proteins -LDH-A, -Hex2, -PKM2 and -PDH (all from Cell Signalling), anti-LKB1 (Ley 37D), and actin (Santa Cruz Biotechnologies). Conditions for primary and secondary antibodies were established (Appendix C, Table S1 and S2). Membranes were prepared for chemiluminescence (ECL) and proteins were visualized as previously described (Scott et al, 2007).

3.4. Cap- binding Assay.

Cells lysates (500 μg) were incubated with the m^7-GTP-Sepharose 4B (Invitrogen) and rocked at 4º C for 1.5 h. Following incubation, the complex was washed three times in phosphate-buffered saline and prepared for western blot analysis to determine cap-binding affinity. Recruitment of eIF4E and eIF4G to the cap's affinity resin was determined using anti-eIF4E and -eIF4G antibodies.

3.5. ATP bioluminescent assay.

MCF7 and HeLaS3 cells were seeded in 12-well plates followed by treatment with PUFA in DMEM/1%FBS. After 48 h cells were harvested and lysed in 100 μL lysis passive buffer (Promega). Primary mammary epithelial cells and tumour cells were also seeded in 12-well plates for 24 hrs prior to conducting the ATP assay that is based on luciferase activity according to ATP concentration. Cells were lysed and protein concentrations were measured using Bradford analysis (BioRad). A total of 2 μg of protein was used to determine cellular ATP level. The ATP Bioluminescent Kit (Sigma) was used according to the manufacturer's protocol to measure cellular ATP concentrations on a Berthold multi-chain luminometer. Standard curves were generated using increasing concentrations of ATP. Final ATP concentrations were calculated as mean ± SEM, $p<0.01$ corrected for total protein concentration of 2 μg.

3.6. Metabolic Assays.

Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured using XF24 analyzer (Seahorse Bioscience). Cells were

plated at a density of $5x10^3$/well in Dulbecco modified Eagle's medium (DMEM) and treated with DHA (25 μM) and EtOH for 1 h in serum free DMEM. Following this, medium was changed to non-buffered DMEM containing glucose (25 mM) and pyruvate (1 mM) and cells were incubated for 1 h in a CO_2-free incubator prior to analysis using the XF24 analyzer. Primary $LKB1^{-/-}$NIC mammary tumour cells were isolated, then plated at a density of $1x10^4$ per well in DMEM. Drug treatments with vehicle, 2DG, NVP-BEZ235, AZD8055 and combination of drugs were performed for 2 hrs, followed by metabolic assays. During the assay glucose (10 mM), oligomycin (0.2 μM) and 2DG (10 mM) were added sequentially. Data analysis was accomplished using Seahorse Bioscience Software.

3.7. Migration Assay.

Primary mammary epithelial cells were either mock-infected with control adenovirus-GFP (AdGFP) or infected with AdGFP-Cre-recombinase (AdGFP-Cre) (Vector Biolabs) for 48h after which cells were trypsinized, then plated into the upper chambers of transwell migration plates (BD BioCoat; 8 μm pore diameter; 6.4 mm polyethylene terephthalate membrane diameter). Cells were allowed to adhere to the surface of the upper chamber insert (24 hrs). DMEM/FBS (8%) was added (1 mL) to the lower chamber to serve as a chemoattractant. Cells were either treated with ethanol control or DHA (25 μM). Plates were returned to the incubator to allow migration for up to 8 h. Non-migrated cells were swabbed off using flat-ended cotton swabs. Migrated cells were fixed and permeabilized with methanol (15 min), stained with crystal violet

solution (15 min), and washed three times with Milli-Q water (2 min per wash). Stained inserts were mounted onto Superfrost Plus glass slides with Zeiss immersion oil (Immersol^{TM}). Images of inserts were acquired using a microscope (Nikon Eclipse TE2000-E) mounted with a camera (RetigaEX 7099) coupled to the SimplePCI imaging program as previously described (Nath-Sain & Marignani, 2009). Cells were visualized and photographed under 2X and 10X objective magnifications. Counts were averaged and reported as Average cells per field. Graph Pad Prism software was used to conduct statistical analysis and to display data graphically. Migration assays were performed by Han, JH. and statistical analysis was performed by Han, JH and Andrade-Vieira, R.

3.8. Fatty acids.

Arachidonic acid (20:4, ω-6) and docosahexaenoic acid (22:6, ω-3) were purchased from Enzo Life Sciences. Stock solutions (100mM) of fatty acids were prepared in ethanol and stored at -80ºC under a blanket of N_2 as previously described (Marignani & Sebaldt, 1995; Marignani & Sebaldt, 1996b) for a period no longer than three months.

3.9. Kinase Inhibitors.

The immunosuppressant and mTOR inhibitor rapamycin was purchased from Calbiochem, mTOR ATP-competitive inhibitor, Torin1 was a generous gift from Drs. Sabatini and Grey (Thoreen et al, 2009). AZD8055, NVP-BEZ235 and BIBW2992 (Afatinib) were purchased from Selleck, Chemicals. Treatments using mTOR inhibitors were based on previous drug studies conducted in the LKB1

conditional mice (Garcia-Martinez et al, 2011; Katajisto et al, 2008; Shaw et al, 2004a). While treatments using the irreversible ErbB1/ErbB2 inhibitor BIBW2992 were based on studies conducted in conditional mouse model of lung cancer (Li et al, 2008). AZD8055 and NVP-BEZ235 treatment protocols were based on previous drug studies conducted in the LKB1 conditional mice (Carretero et al, 2010; Garcia-Martinez et al, 2011; Katajisto et al, 2008; Shaw et al, 2004b). 2DG was purchased from Toronto Research Chemicals. 2DG treatments were based on studies conducted in various conditional mouse models (Goldberg et al, 2012; Maschek et al, 2004; Purohit & Pohlit, 1982).

3.10. Histology.

Whole mounts of mammary gland were stained using carmine (Sigma C1022) and aluminum potassium sulfate (Sigma A7167). Formalin fixed and paraffin embedded tissues were prepared for histology followed by hematoxylin and eosin (H/E) staining. Tissue sections were incubated with primary antibodies: LKB1 (Santa Cruz) 1:30, Ki-67 (Santa Cruz) 1:30, E-cadherin (Cell Signalling) 1:100, pErbB2 1:100 (Santa Cruz), and pS6 (S235/S236) 1:100 (Cell Signalling). Human breast cancer tissue microarrays were purchased from Biomax USA and prepared for immunohistochemistry (IHC) as per manufacturer' recommendations. Blind scoring for all imaging was conducted in triplicate. Images were obtained using Nikon Eclipse TE 2000-E, mounted with a Q-Imaging CCD camera and acquired using the Simple PCI software as previously described (Nath-Sain & Marignani, 2009). Histology experiments were performed by Marignani, PA. and Colp, P.

3.11. Metabolic profile.

Mammary tumours from three separate LKB1$^{-/-}$NIC and NIC mice, and mammary glands from three separate WT mice were excised and analyzed for metabolites by NMR analysis (Chenomx, Edmonton, Alberta, Canada). Samples were prepared and analyzed as previously described (Mercier et al, 2011). Multivariate statistical analysis was performed using SIMCA P+ Version 12.0.1. For NMR data, metabolites were corrected for the weight of the tumour or normal mammary tissue, and reported as mean (μM/mg) ±SEM, $p<0.05$ or $p<0.01$. NMR metabolomics analysis was performed by Chenomx.

3.12. Magnetic Resonance Imaging.

Magnetic resonance imaging (MRI) was conducted using a 3T Agilent MRI system specifically optimized for pre-clinical imaging, using a 21cm Magnex gradient coil and 25 mm quadrature RF coil from Doty Scientific. MRI acquisitions involved 150 micron isotropic resolution 3D balanced-SSFP optimized for fat-tumour contrast. Volumetric tumour estimates were obtained through ellipsoidal model estimates in 3-plane orthogonal views using Agilent's VNMRJ package to measure tumour extent in each of 3 orthogonal directions. 3D rendered tumour volume calculations were performed using the RView software. MRI was performed by Goguen, D under the guidance of Bowen, CV.

3.13. Mitochondria Content.

Primary LKB1$^{-/-}$NIC mammary tumour cells were isolated as described above and plated onto coverslips. Treatments were performed using AZD8055

(100 nM), 2DG (10 mM) and a combination of both. Following this, cells were incubated for 20 min at 37ºC with Mitotracker Red CMX/Ros (Cell Signalling) at a final concentration 100 nM. Cells were then washed 2x with PBS and prepared for analysis by fluorescence microscopy and flow cytometry. Fluorescence microscopy was conducted using a Nikon Eclipse TE 2000-E, mounted with a Q-Imaging CCD camera. Fluorescent images were acquired using Simple PCI software as previously described (Marignani & Sanchez-Cespedes, 2010; Scott et al, 2007). Flow cytometry was conducted using a Becton Dickinson FACS Calibur. Data was acquired using CellQuest software and analyzed using ModFitLT as previously described (Scott et al, 2007).

3.14. Electron microscopy.

Cells were maintained as described above and collected for electron microscopy. Samples were fixed as previously described (Faulkner et al, 2008). Once embedded in 100% epon araldite resin, the samples were placed in a 60°C oven for 48 hrs to cure thoroughly. Thin sections were cut using a LKB Huxley Ultramicrotome with a diamond knife and placed on 300 mesh copper grids. The grids containing the samples were stained, first with 2% x aqueous uranyl acetate for 10 minutes, followed with 2 x 5 minutes distilled water rinses. Lead citrate was then added for 4 minutes, followed with a quick rinse with distilled water. Samples were then allowed to air dry and viewed using a JEOL JEM 1230 Transmission Electron Microscope at 80 kV. Images were captured using a Hamamatsu ORCA-HR digital camera. Electron Microscopy was performed by Bentley, HA.

3.15. Statistics.

Each experiment was conducted using a minimum of three separate samples and reported as mean ± SD or mean ± SEM. The statistical analysis was performed by repeated measures one-way ANOVA, followed by Newman-Keuls or Bonferroni multiple comparison test. Values were statistically significant at $p<0.05$, $p<0.01$, or $p<0.0001$ as indicated. The statistical analysis was performed using Graph Pad Prism software 5. Kaplan-Meier curves, Log ranked tests scores of 3 or higher were considered significant ($p<0.0001$). Kaplan-Meier curve statistical analysis was performed by Marignani, PA.

CHAPTER 4 PUBLISHED MANUSCRIPT

This Chapter refers to the investigation of the effects of omega-3 PUFA on cellular metabolism and LKB1 signalling; the data obtained for this investigation was published in Cancer Biology and Therapy on September 6, 2013. The Material and Methods section was removed and it is part of Chapter 3. The original formatting for this publication was modified in compliance with Dalhousie University Thesis guidelines.

Manuscript Title: Omega-3 polyunsaturated fatty acid promotes the inhibition of glycolytic enzymes and mTOR signalling by regulating the tumour suppressor LKB1.

Authors: Rafaela Andrade-Vieira, Jae H. Han and Paola A. Marignani.

Author's contributions

RA contributed to the design of the experiments, acquisition of data, analysis and interpretation of data and draft of the manuscript. JH contributed to the cell migration assays. PM contributed to the design of the experiments, analysis and interpretation of data, and editing of the manuscript. All authors read and approved the final manuscript.

4.1. Abstract

The omega-3 polyunsaturated fatty acids (ω3PUFAs) are a class of lipids biologically effective for the treatment of inflammatory disorders, cardiovascular disease and cancer. Patients consuming a high dietary intake of ω3PUFAs have shown a low incidence of metabolic disorders, including cancer. Although the effects of ω3PUFAs intake was shown to be involved in the prevention and treatment of these diseases, the underlying molecular mechanisms involved are not well understood. Here, we show that ω3PUFA, docosahexaenoic acid (DHA) enhanced the tumour suppressor function of LKB1. We observed that when LKB1 expressing cells (MCF-7) are treated with DHA, there is an increase in LKB1 activity leading to phosphorylation of AMPK and inhibition of mTOR signalling. Abrogation of LKB1 in MCF-7 by siRNA reversed this phenotype. Furthermore, cellular metabolism was decreased and ATP levels were reduced in response to DHA treatment. The presence of LKB1 further attenuated cell metabolism. More importantly, in mammary epithelial cells expressing LKB1, the rate of glycolysis was decreased as a result of diminished expression of glycolytic enzymes. Functionally, these events lead to a decrease in the migration potential of these cells. Overall, our discovery shows for the first time that LKB1 function is enhanced in response to ω3PUFA treatment, thereby regulating cell metabolism.

4.2. Introduction

Metabolic syndrome is characterized by dysregulation of normal cellular metabolism. Expression of glycolytic enzymes and the energy requirement of cells are altered leading to hypertension, dyslipidemia, insulin resistance and cell growth, culminating in heart diseases, diabetes and cancer. Numerous studies have shown a correlation between metabolic syndrome and aberrant signalling pathways, see reviews (Laplante & Sabatini, 2009; Yecies et al, 2011). In our study we show that altered LKB1 signalling affected energy homeostasis, survival and ultimately cell metabolism.

LKB1 is a tumour suppressor kinase that has many biological functions, and when mutated gives rise to cancers (Marignani & Sanchez-Cespedes, 2010). LKB1 is the primary activator of AMPK, a central regulator of homeostasis, when activated, AMPK leads to the inhibition of mTOR (Lizcano et al, 2004; Shaw et al, 2004a). To date, regulation of LKB1 is poorly understood, however it is known that LKB1 forms a heterotrimeric complex with STRAD, and the scaffolding protein MO25, enhancing LKB1 catalytic activity (Baas et al, 2003; Boudeau et al, 2003). When LKB1 catalytic activity is lost due to mutations, the oncogenic mutants drive tumourigenesis through the activation of oncogenes (Scott et al, 2007).

Regulation of cell metabolism is essential for maintaining human health, while diet is considerably relevant in protecting cellular metabolism and therefore promoting health. Amongst the various dietary products that have been implicated in promoting health are omega-3 and omega-6 polyunsaturated fatty

acids (ω3PUFAs/ ω6PUFAs). ω3PUFAs have been shown to contribute to the prevention of many cancers, including breast, prostate and colon cancers (Friedrichs et al; Menendez et al, 2005) however, the anti-tumourigenic activity of ω3PUFAs is not fully understood.

The ω6PUFAs are primarily obtained from vegetable oils, such as corn oil, while the ω3PUFAs are obtained primarily from cold-water fish (Hubbard et al, 1994; Magrum & Johnston, 1986). Many studies revealed that mice fed a diet rich in ω3PUFAs display reduced risk of developing cancer while mice fed diets rich in ω6PUFAs have the opposite effect (Bartsch et al, 1999; Rose & Connolly, 1990; Rose & Connolly, 1997). These studies concluded that the fatty acid composition of a given diet significantly impacts the risk of developing cancer. However, the molecular under pinning of how dietary fatty acids affect carcinogenesis remains controversial. It is well established that the phospholipid composition of the cell membrane and therefore cell fluidity alters the manner in which cells respond to hormones and growth factors through binding to transmembrane receptors (Galli et al, 1993; Raclot & Groscolas, 1994). While others provide evidence that the ω3PUFA-mediated changes made to eicosanoid molecular species lead to the inhibition of cancer progression (Sebaldt et al, 1990; Singh et al, 1997; Zhu & Conney, 1998). Previously we showed that mice fed various molecular species of ω3PUFA led to alterations in membrane phospholipids, culminating in changes in diradylglycerol (DG) formation (Marignani & Sebaldt, 1996a; Marignani & Sebaldt, 1995; Marignani & Sebaldt,

1996b) and PKC-mediated activity (Marignani et al, 1996). These data strongly support a role for ω3PUFA in regulating cellular signalling events.

Here we report that the ω3PUFA, docosahexaenoic acid (DHA; 20:6 ω-3) enhances LKB1 signalling. We provide evidence that treatment of cells with ω3PUFA decreases cell growth through the inhibition of mTOR in an LKB1-dependent manner. In addition, we show that ω3PUFA treatment diminishes glycolysis by inhibiting the expression of glycolytic enzymes. Interestingly, LKB1 expression enhanced glycolysis inhibition and diminishes migratory behavior upon ω3PUFA treatment. Overall our data suggests that the anti-carcinogenic properties of ω3PUFA are mediated in part through the tumour suppressor function of LKB1, leading to inhibition of mTOR activity and cellular metabolism associated with tumourigenesis.

4.3. Results

4.3.1. DHA increases LKB1 gene expression and stimulates LKB1 activity.

To determine whether ω3PUFA treatment of cells influences LKB1 expression and/or signalling, we performed gene expression analysis and western blotting analysis. HeLaS3 cells were transiently transfected with a LKB1 expression plasmid and empty vector (V) as previously described (Nath-Sain & Marignani, 2009). Following this, cells were treated for 24 hrs with the ω6PUFA arachidonic acid (25 μM), DHA (25 μM) or vehicle (EtOH) control (Marignani & Sebaldt, 1995). After treatment, mRNA was isolated and prepared for quantitative-PCR (q-PCR). In response to DHA, *LKB1* expression was elevated compared to *LKB1* expression in response to AA treatment (Fig. 1A). Protein

expression of LKB1 was elevated in response to DHA treatment compared to AA and vehicle control as observed by western blot analysis (Fig. 1B). Furthermore, the phosphorylation status of AMPK (pAMPK), a direct target of activated LKB1, was enhanced compared to pAMPK in response to AA and vehicle treatments. Moreover, phosphorylation of ACC, a target of AMPK, was also enhanced (Fig. 1B).

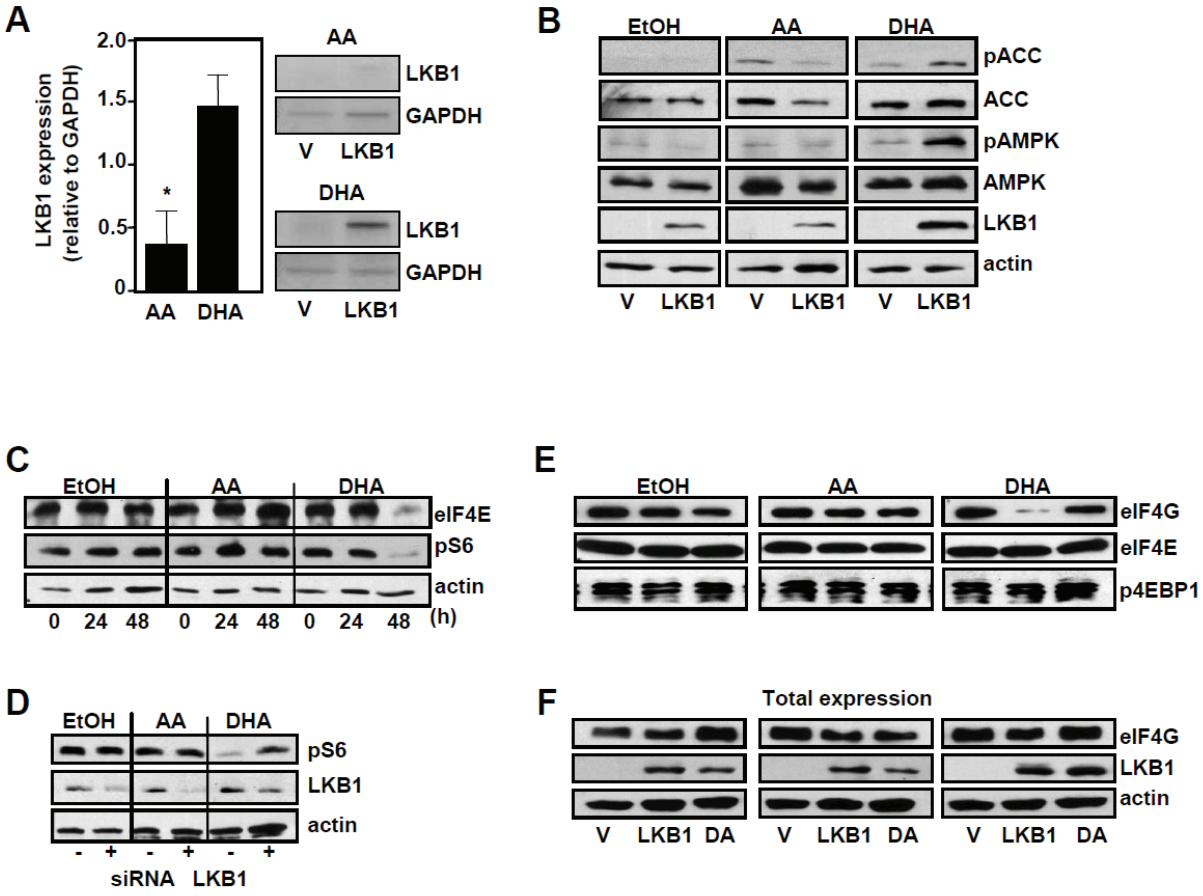

Figure 1 Dietary PUFA enhances LKB1 signalling. (A) q-PCR analysis of LKB1 gene from HeLaS3 cells expressing ectopic LKB1 followed by treatment with vehicle, AA and DHA at final concentration of 25 μM. Results are the mean ± SEM; n=4. * $p<0.03$. (B) Cells were treated with AA or DHA (25 μM each) followed by western blot analysis for expression of proteins using anti-LKB1 (Ley), -pAMPK, -AMPK, -pACC and -ACC antibodies. (C) MCF7 cells were treated for 48h with PUFAs and vehicle followed by western blot analysis of pS6 and eIF4E. (D) MCF7 cells were transfected with siRNA-LKB1, incubated then treated with PUFAs, followed by western blot analysis for expression of proteins using anti-LKB1 and -pS6 antibodies. (E) HeLaS3 cells were transfected with LKB1, D194A and expression plasmids. Cells were treated with PUFAs (25 μM) for 48 h followed by cap binding assay. Western blot analysis for cap binding proteins was determined using anti- eIF4E, -eIF4G and -4EBP1 antibodies. (F) Lysates for total protein expression of LKB1 and eIF4G are also shown. Actin was used as loading control. Data is representative of three separate experiments. Vector (V).

4.3.2. Inhibition of mTOR by LKB1 in response to PUFAs.

Using MCF7 cells, we evaluated the effect of PUFA on mTOR activity. MCF7 cells were treated with AA, DHA and vehicle. Western blot analysis showed that the phosphorylation status of S6 (pS6), downstream target of mTOR, was decreased in response to DHA-treatment compared to AA and vehicle control treated cells (Fig. 1C). Interesting, expression of eIF4E was also decreased (Fig. 1C). To confirm whether DHA played a role in LKB1-mediated inhibition of mTOR signalling, we selectively knocked down the expression of LKB1 in MCF7 using LKB1 siRNA as previously described (Nath-Sain & Marignani, 2009). After transfection, cells were treated with AA, DHA and vehicle. The results showed that in the presence of endogenous LKB1, DHA treatment leads to inhibition of S6 phosphorylation, however when LKB1 expression was attenuated, pS6 expression was comparable to that of AA and vehicle control (Fig. 1D).

It is known that the activation of mTOR leads to the phosphorylation of downstream targets, culminating in translation initiation events (Fingar et al, 2004). The recruitment of protein to the mRNA cap and the assembling of the cap-binding complex is the first step in this mechanism. Thus, we analyzed cap recruitment in the presence of LKB1 and D194A, a mutant that lacks catalytic activity (Scott et al, 2007). In response to DHA treatment, recruitment of eIF4G to the cap was considerably reduced in the presence of LKB1, compared to recruitment in the presence of D194A and vector control (Fig. 1E-F). Amongst

transfectants, we observed no difference in the recruitment of eIF4G, eIF4E and 4EBP1 in response to AA or vehicle control (EtOH).

4.3.3. ω3PUFA-mediated alterations in cellular ATP levels are enhanced by LKB1.

The Warburg effect provides an explanation as to how cancer cells are able to maintain high metabolic rates thereby generating ATP that are necessary to provide energy requirements of a growing cancer (Warburg, 1956).

In recent years, ω3PUFAs have been observed to inhibit glycolytic genes, having an impact on the metabolic requirements of cancer cells (Dentin et al, 2005; Jump et al, 1994). Therefore we asked whether DHA alters cell metabolism in the presence of LKB1. To address this question we used two cell lines, MCF-7 and HeLaS3. Briefly, MCF-7 cells were transiently transfected with control (scrambled) and siRNA-LKB1 as previously described (Nath-Sain & Marignani, 2009) and HeLaS3 cells were transiently transfected with a LKB1 expression plasmid. Following transfections, cells were treated with PUFA, and ATP assays were performed. In the presence of endogenous LKB1, ATP levels in MCF-7 cells were reduced compared to MCF-7 cells where LKB1 expression was attenuated by siRNA (Fig. 2A). In HeLaS3 cells, ectopic expression of LKB1 resulted in a reduction in ATP levels compared to HeLaS3 cells alone (LKB1 ^{null}) (Fig. 2B).

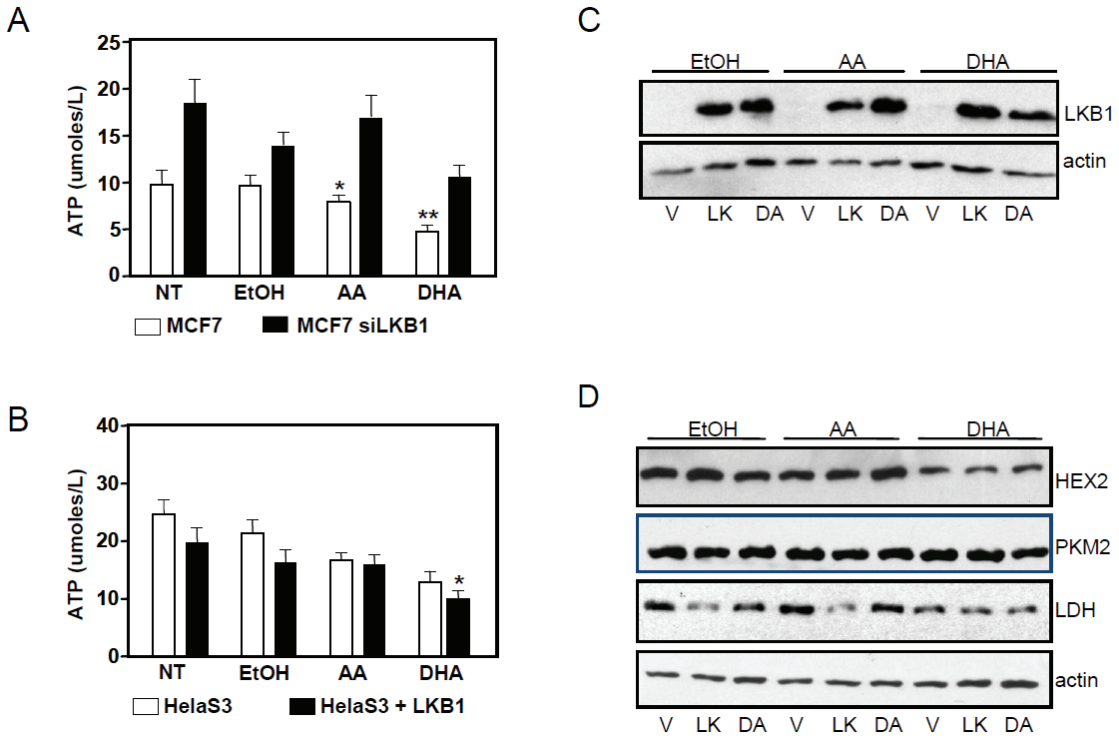

Figure 2 DHA treatment contributes to LKB1-mediated depletion of cellular ATP. (A) ATP levels were determined in MCF7 and MCF-7 siRNA-LKB1 cells in response to PUFA treatment. Results are representative of three experiments in triplicate, mean ± SEM, *p<0.001 and **p<0.05 compare to MCF-7 siRNA LKB1 cells. (B) ATP levels were determined in HeLaS3 cells alone and HeLaS3 cells transfected with a LKB1 expression plasmid. Results are representative of three experiments in triplicate, mean ± SEM,*p<0.001 compare to AA treatments. (C) HeLaS3 cells were transfected with LKB1 and D194A expression plasmids followed by treatment with AA, DHA and vehicle for 24h. (D) Expression of HEX2, PKM2, and LDH-A were determined by western blot analysis in HeLaS3 cells. Actin was used as loading control.

4.3.4. ω3PUFA treatments lead to decrease in glycolytic enzymes.

Aerobic glycolysis consists of a chain of reactions which converts glucose into pyruvate yielding two molecules of ATP. In this process there are a number of enzymes involved in each step, some of which are strongly associated with the metabolic switch that lead to the Warburg effect. The gene expression of these enzymes was shown to be inhibited by ω3PUFA (Dentin et al, 2005). Here we investigated whether LKB1 played a role inhibiting glycolysis and therefore enzymes expression upon ω3PUFA treatments. HeLaS3 cells were transiently transfected with LKB1 and D194A expression plasmids (Fig. 2C), followed by treatment with vehicle, AA (25 μM) and DHA (25 μM) for 24 hours. Cells were harvested and protein expression of hexokinase 2 (HEX2), pyruvate kinase (PKM2) and lactate dehydrogenase A (LDH-A) was determined by western blot analysis. Expression of HEX2 and LDH were decreased in the presence of DHA. The expression of LKB1 lead to a decrease in LDH protein levels under all the treatments. We did not observe any effect in PKM2 expression. Treatment with AA did not alter the expression of these proteins (Fig. 2D).

We investigated whether LKB1 catalytic mutants, shown to have oncogenic properties (Scott et al, 2007), would reverse DHA-mediated inhibition of these glycolytic enzymes. HeLaS3 were transiently transfected with LKB1, kinase dead K78I (KI) and oncogenic mutants of LKB1 found in PJS and breast cancer, namely D194A (DA) and R304W (RW) expression plasmids (Nath-Sain & Marignani, 2009; Scott et al, 2007), followed by treatment with DHA. In the presence of LKB1 catalytic mutants, there was a distinct effect in the expression

of these enzymes compare to the expression observed in cells harboring wild type LKB1 in response of DHA treatment. The expression of HEX2 (Fig. 3A) was significantly enhanced by D194A and K78I mutants ($p<0.01$), similarly the expression of LDH was significantly increased by K78I mutant, compare to LKB1 expression ($p<0.007$) (Fig. 3C). PKM2 expression was not significantly affected by any of the LKB1 mutants (Fig. 3B).

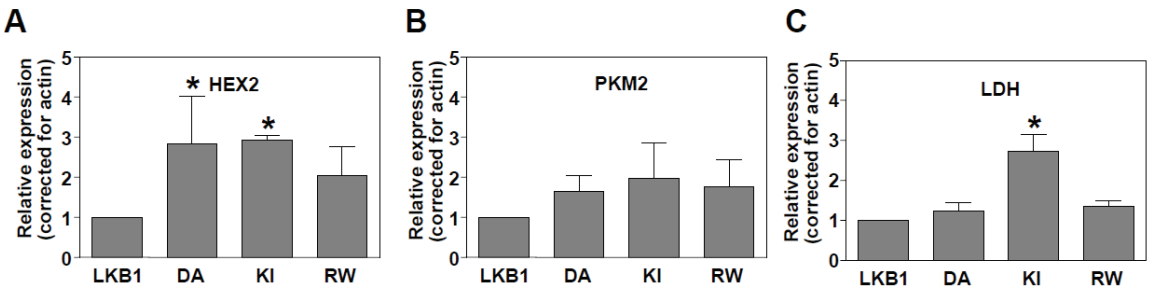

Figure 3. LKB1 activity in response to ω3PUFA treatment alters expression of glycolytic enzymes. (A) HeLaS3 cells were transfected with vector (V), LKB1 (LK), oncogenic mutants D194A (DA) , R304W (RW) and catalytic deficient mutant K78I (KI) as previously described (Marignani et al, 2007) (Scott et al, 2007), followed by treatment with DHA. Western blot analysis shows the expression of HEX2 (A), PKM2 (B) and LDH-A (C); actin was used as loading control. Data is representative of three experiments and expressed as relative expression corrected for actin and compared to expression of LKB1, mean ± SD; HEX2 *$p<0.01$ and LDH-A, *$p<0.0006$.

4.3.5. ω3PUFA treatment decreases glucose metabolism and cell migration.

We investigated the effects of DHA treatment in the metabolism of mammary epithelial cells. To gain insights into the effects of DHA in mammary energy metabolism, we use the Seahorse XF Analyzer to measure the cells

extracellular acidification rates (ECAR) that correlate with lactate production through glycolysis, thus reflecting glycolysis rates (Wu & Huang, 2007) and oxygen consumption rates (OCR). Mammary epithelial cells harboring flox-*lkb1* alleles were infected with Cre-recombinase adenovirus to excise the *lkb1* gene (Fig. 4A). After successfully excision, the cells were treated for 1 h with DHA or control, following ECAR and OCR analysis.

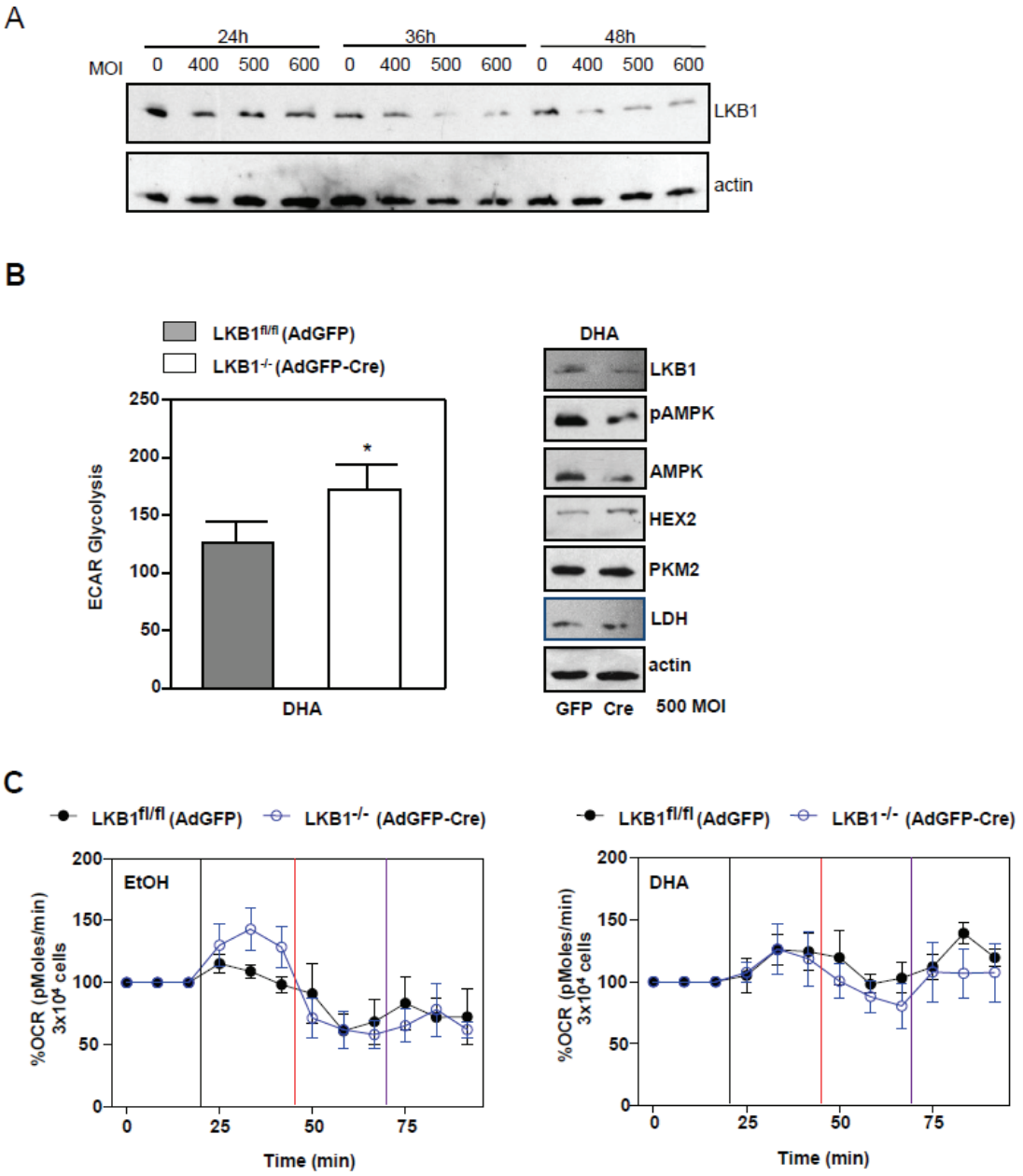

Figure 4. LKB1 signalling promotes inhibition of glycolysis in primary cells. (A) To determine the MOI of adenovirus, primary epithelial cells were infected with AdGFP-Cre for 24h, 36 and 48h to identify appropriate multiplicity of infection (MOI). (B) Extracellular acidification rate (ECAR) using an MOI of 500 was used to infect cells with AdGFP and AdGFP-Cre. Values are representative of four experiments in duplicate, mean ± SEM, *p<0.03. Western blot analysis of -LKB1, pAMPK, AMPK, HEX2, PKM2 and LDH-A expression in cells treated with DHA and infected with AdGFP and AdGFP-Cre. (C) Oxygen consumption rate (OCR) in cells treated with DHA and control. The data is representative of three independent experiments.

Overall, we observed that in primary cells expressing LKB1$^{fl/fl}$ (AdGFP) there was a decrease in ECAR compare to cells lacking LKB1$^{-/-}$ (AdGFP-Cre) expression (Fig. 4B) in response to DHA. Because this decrease in glycolysis could be related to changes in the expression of glycolytic enzymes, we analyzed the protein levels of HEX2, LDH-A and PKM2. In cells expressing LKB1$^{fl/fl}$ (AdGFP) the HEX2 and LDH-A expression was decreased compared to cells lacking LKB1$^{-/-}$ (AdGFP-Cre) expression (Fig. 4B). We did not observe any changes in PKM2 expression. Similarly, in HeLaS3 expressing ectopic LKB1, we observed low expression of HEX2 and LDH-A in response to DHA treatment. As in primary cells, we did not observe any changes in PKM2 expression. Therefore the expression of LKB1, in both transfected and primary cells results in an impairment of aerobic glycolysis upon ω3PUFA treatment. In addition, we observed that treatment with DHA led to a modest increase in OCR compared to vehicle (EtOH) treated cells. Further to this, we did not observe differences in OCR between LKB1$^{fl/fl}$ (AdGFP) and LKB1$^{-/-}$ (AdGFP-Cre) cells (Fig. 4C). In conclusion, cells lacking LKB1 expression are more inclined to rely on glycolysis as the main source of energy, a mechanism commonly observed in malignant cells transformation.

The acidic environment caused by lactate production and co-secretion of protons leads to destruction of extracellular matrix thereby promoting cell migration and therefore metastasis (Langbein et al, 2006). Because of the low glycolytic rate in cells expressing LKB1, we investigated whether LKB1 expression would affect the ability of cells to migrate in response to DHA. Here,

primary mammary epithelial cells harvested from LKB1$^{fl/fl}$ mice were infected with AdGFP and AdGFP-Cre, following transwell migration assays. Immunofluorescence was performed simultaneously to confirm expression of GFP (Fig. 5A). We observed that cells expressing LKB1$^{fl/fl}$ (AdGFP) have reduced migration compared to cells lacking LKB1$^{-/-}$ (AdGFP-Cre) expression in response to DHA treatment (Fig 5B). Based on these results we proposed a model of how the activation of LKB1 signalling in response to DHA treatment lead to inhibition of glycolysis and consequently to a decrease in cell migration (Fig. 6).

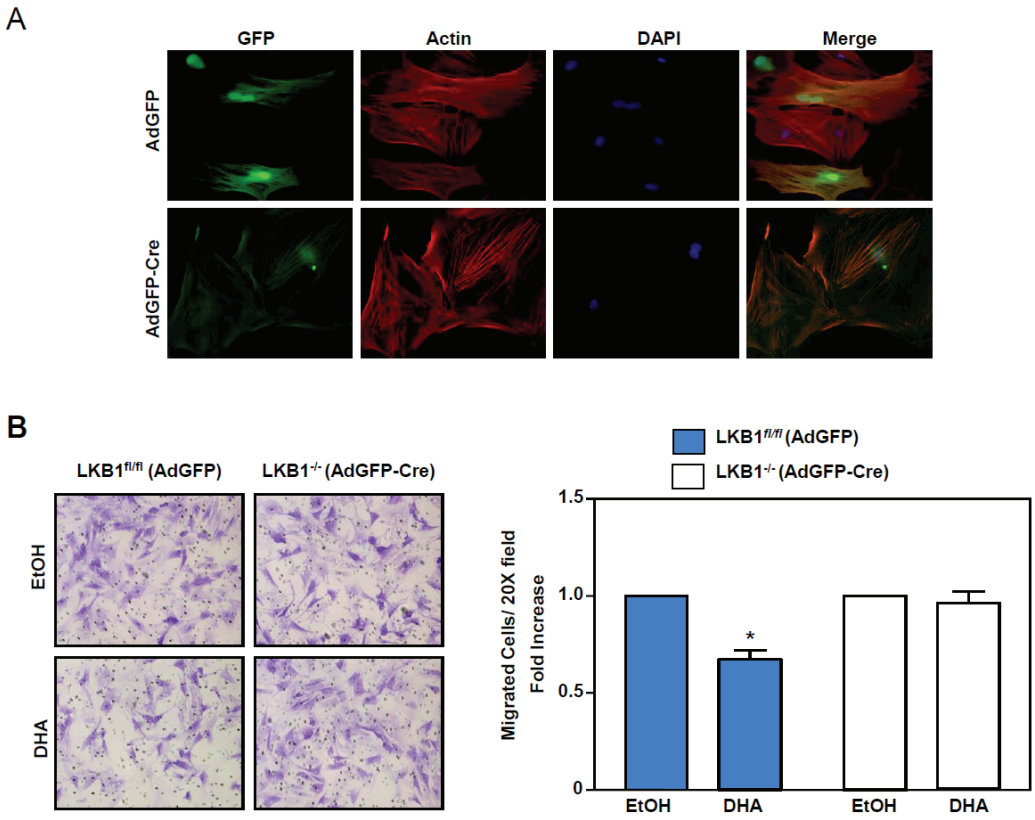

Figure 5. LKB1 modulate cell migration in response to ω3PUFA treatment. (A) Mammary epithelial cells infected with AdGFP and AdGFP-Cre, followed by fluorescence staining for actin using Rhodamine phalloidin (red) and for nuclei using 4′, 6-diamidino-2-phenylindole (DAPI) (blue). (B) Transwell assays of cell treated with DHA or control. Data shows fold increase of cells migration. Data are representative of three experiments in triplicate, mean ± SEM,*p<0.02.

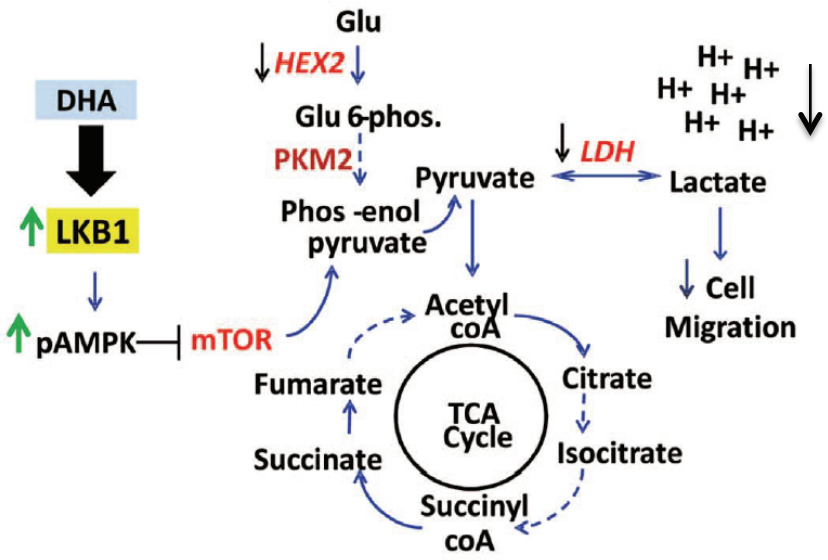

Figure 6. Proposed model for LKB1 modulation of glycolysis and cell migration. In response to ω3PUFA treatment, LKB1 signalling is activated, leading to the phosphorylation and activation of AMPK. pAMPK, in turn, inhibits mTOR signalling leading to the attenuation of aerobic glycolysis. Activation of LKB1 significantly decreases the expression of HEX2 and LDH-A whereby the conversion of pyruvate to lactate is diminished, thus lowering the release of protons (H^+) and restoring pyruvate to the tricarboxylic acid cycle (TCA) cycle. These events reduce the production of ATP through aerobic glycolysis and decrease cell migration. Conversely, the attenuation of LKB1 expression promotes an increase in glycolysis and hyper expression of HEX2 and LDH-A. These enzymes favor the production of lactate, which, in turn, increases acidification of the environment, and leads to the destruction of extracellular matrix, promoting cell migration.

4.4. Discussion

While the benefits of a ω3PUFA diet on cancer are recognized, the mechanism by which these fatty acids are able to suppress tumour growth is poorly understood. Here, we show that DHA ameliorates the tumour suppressor function of LKB1. LKB1 protein and gene expression levels were enhanced by DHA treatment, thereby furthering LKB1-mediated suppression of mTOR signalling. We show that DHA activates LKB1 signalling to promote AMPK

phosphorylation, whereas the more common ω6PUFA, AA does not alter LKB1 activity. The activation of AMPK is implicated in reducing cell proliferation and regulation of cell metabolism (Carretero et al, 2007; Faubert et al, 2013), also AMPK reduces lipid accumulation in peripheral tissues (liver and skeletal muscle) by inhibition of fatty acid synthesis and stimulation of fatty acid oxidation (Zhang et al, 2009). Therefore, a high ω3PUFA diet may regulate the activation of AMPK and play a role in cancer metabolism as well as in other metabolic diseases such as diabetes and cardiovascular disease. Recently, others have shown that ω3PUFA enhances activation of the LKB1-AMPK pathway in rats during partial hepatectomy. This study showed a significant increase in the phosphorylation of AMPK upon ω3PUFA administration (Liu, 2011). Interestingly, another study showed that LKB1 activity was enhanced by DHA and decreased by AA in the brain of rats (Agrawal & Gomez-Pinilla, 2012).

Unlike most normal cells, tumour cells derive a significant amount of energy from glycolysis rather than through oxidative phosphorylation (known as the Warburg effect) (Warburg, 1956). Therefore the inhibition of glycolysis can greatly affect tumour growth, since the majority of energy produced by the cancer cells is through glycolysis. We showed that LKB1 tumour suppressor activity is enhanced by ω3PUFA thereby mediating the inhibition of glycolysis and glycolytic enzymes. Recently, the p53 tumour suppressor was shown to suppress glycolysis by controlling the expression of p53-inducible glycolysis and apoptosis regulator (TIGAR), in part through the activation of AMPK (Bensaad et al, 2006).

The similarity between the two tumour suppressors; LKB1 and p53, is the activation of AMPK in the suppression of glycolysis.

Although aerobic glycolysis is recognized as an inefficient energy process, the Warburg effect is a mechanism by which cancer cells acquire growth advantage over normal cells (see review (Gatenby & Gillies, 2004)). Since mTOR is found to enhance transcription of glycolytic enzymes, thereby increasing the capacity of cells to execute glycolysis (Cairns et al, 2011; Laplante & Sabatini, 2009; Sun et al, 2011), the inhibition of mTOR signalling leads to the suppression of glycolysis. Here we show that in the presence of LKB1, DHA treatment results in a decrease in ATP production and aerobic glycolysis as observed by the inhibition of glycolytic enzymes expression (HEX2 and LDH-A). In addition, we show that the reduction in the expression of rate-limiting glycolytic enzymes was dependent on LKB1 catalytic activity since the expression of HEX2 and LDH-A was enhanced in the presence of LKB1 oncogenic mutants. Interestingly, others have shown that activation of AMPK suppresses the Warburg effect and inhibits tumour growth *in vivo.* This study also showed that cells lacking AMPK had elevated ATP levels (Faubert et al, 2013), supporting our discovery that LKB1 activation of AMPK signalling plays a vital role in cell metabolism, driving the Warburg effect.

We found that mammary epithelial cells expressing LKB1 displayed decreased glycolysis as measured by ECAR. Expression of LKB1 in these cells also contributed to reduced protein expression of HEX2 and LDH-A. These effects were influenced by DHA treatment, implying that dietary intake of

ω3PUFA could attenuate excessive aerobic glycolysis. Functionally, cell migration is highly influenced by glucose metabolism. In the past decade, many studies have shown that increased in glucose metabolism promotes cell migration by increasing lactate levels (Langbein et al, 2006; Masur et al, 2011; Walenta et al, 2000). Consequently, elevated lactate leads to cellular acidification which then promotes the destruction of the extra cellular matrix and predispose cells to migration (Langbein et al, 2006; Masur et al, 2011; Walenta et al, 2000). In our study we show that cells expressing LKB1 migrate significantly less than cells that do not express LKB1 when treated with DHA.

Recently, we showed that primary mammary epithelial cells that lack LKB1 expression in combination with gain of ErbB2 expression showed an aggressive tumour development with abnormal metabolism caused by the Warburg effect (Andrade-Vieira et al, 2013b). In our current study, we showed for the first time that LKB1 tumour suppressor function is enhanced in response to DHA treatment, resulting in the inhibition of mTOR signalling. In the presence of LKB1, the suppression of cellular ATP levels and reduced expression of glycolytic enzymes was enhanced in response to DHA treatment. These findings confirm the importance of diet in the regulation of cellular metabolism. This discovery is an example of how ω3PUFA can affect signalling pathways to control elevated glycolysis in cancer cells and decrease glucose metabolic rates. Hence, the effects of DHA on LKB1 signalling would benefit patients with metabolically active tumours. Recent efforts to develop anti-tumourigenic strategies that target the glycolysis process have yet to prove successful. Therefore the enhanced tumour

suppressor activity of LKB1 associated with DHA treatment may represent an effective strategy in conjunction with traditional therapeutics to suppress tumour growth.

Competing interest

The authors declare no competing interests.

Acknowledgements

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CHAPTER 5 PUBLISHED MANUSCRIPT

This Chapter refers to the development and characterization of a novel mouse model of breast cancer, here referred as STK11$^{-/-}$/NIC. STK11$^{-/-}$/NIC mice are referred as LKB1$^{-/-}$/NIC in all other chapters. The data obtained from this study was published in the Plos One on February 22, 2013. The material and methods section was removed and it is part of Chapter 3. The original formatting for this publication was modified in compliance with Dalhousie University Thesis guidelines.

Manuscript Title: Loss of *lkb1* expression reduces the latency of ErbB2-mediated mammary gland tumourigenesis, promoting changes in metabolic pathways.

Authors: Rafaela Andrade-Vieira, Zhaolin Xu, Patricia Colp and Paola A. Marignani

Author's contributions

RA contributed to the design of the experiments, acquisition of data unless otherwise specified, analysis and interpretation of data and draft the manuscript. PC contributed to immunohistochemistry experiments. ZX contributed to pathological analysis of tissues. PM contributed to the design of the experiments, animal husbandry, genotyping of mice, analysis and interpretation of data, immunohistochemistry experiments, scoring of tissue microarrays and draft the manuscript. All authors read and approved the final manuscript.

5.1. Abstract

The tumour suppressor kinase LKB1 is mutated in a broad range of cancers however; the role of LKB1 mammary gland tumourigenesis is not fully understood. Evaluation of human breast cancer tissue microarrays indicated that 31% of HER2 positive samples lacked LKB1 expression. To expand on these observations, we crossed $STK11^{fl/fl}$ mice with mice genetically engineered to express activated Neu/HER2-MMTV-Cre (NIC) under the endogenous *Erbb2* promoter, to generate $STK11^{-/-}$/NIC mice. In these mice, the loss of *lkb1* expression reduced the latency of ErbB2-mediated tumourigenesis compared to the latency of tumourigenesis in NIC mice. Analysis of $STK11^{-/-}$/NIC mammary tumours revealed hyperactivation of mammalian target of rapamycin (mTOR) through both mTORC1 and mTORC2 pathways as determined by the phosphorylation status of ribosomal protein S6 and AKT. Furthermore, $STK11^{-/-}$ /NIC mammary tumours had elevated ATP levels along with changes in metabolic enzymes and metabolites. The treatment of primary mammary tumour cells with specific mTOR inhibitors AZD8055 and Torin1, that target both mTOR complexes, attenuated mTOR activity and decreased expression of glycolytic enzymes. Our findings underscore the existence of a molecular interplay between LKB1-AMPK-mTORC1 and ErbB2-AKT-mTORC2 pathways, suggestive that loss of LKB1 expression may contribute to a marker for hyperactivated mTOR in HER2 positive breast cancer and warranting further investigation into therapeutics that target LKB1-AMPK-mTOR and glycolytic pathways.

5.2. Introduction

Loss of function mutations in the tumour suppressor serine-threonine kinase LKB1, also referred to as STK11, is responsible for Peutz-Jeghers Syndrome (PJS), an autosomal-dominant disorder characterized by mucocutaneous hyper-pigmentation and benign gastrointestinal hamartomatous polyps (Avizienyte et al, 1999). In PJS, the risk of breast cancer is the second highest after gastrointestinal cancers (Lim et al, 2003), while in non-PJS population, a correlation between loss of LKB1 expression in breast cancer, as determine by tissue microarray (TMA) and poor prognosis has been identified (Fenton et al, 2006). We previously discovered that LKB1 is a coactivator of estrogen receptor alpha (ERα) through direct binding with the hormone receptor (Nath-Sain & Marignani, 2009). In this study, we demonstrate for the first time a functional link between LKB1 and ERα, broadening the scientific scope of LKB1 and laying the groundwork for further investigations into the role of LKB1 in breast biology. For more detail about LKB1 signalling in disease please refer to the following review (Marignani & Sanchez-Cespedes, 2010).

When in complex with pseudokinase STRAD and adaptor protein MO25, LKB1 catalytic activity is enhanced (Baas et al, 2003) allowing for the phosphorylation and activation of AMPK on threonine 172 (Lizcano et al, 2004; Shaw et al, 2004b). A link between the LKB1-AMPK pathway and disease pathways is tuberin, the product of the tuberous sclerosis complex 2 gene (*TSC2*) that represses mammalian target of rapamycin (mTOR) activity when phosphorylated by AMPK on S1387 (Corradetti et al, 2004). mTOR is a

serine/threonine kinase that is the catalytic component of two distinct signalling pathways, mTOR complex 1 (mTORC1) and mTORC2 (Sarbassov et al, 2005a). Both complexes are activated in response to growth factor signals mediated by phosphoinositide 3-kinase (PI3K)/AKT signalling and the availability of nutrients to the cell (Sarbassov et al, 2005a). Broad spectrums of cancers express aberrant mTOR signalling, whereby several pivotal proteins such as AMPK, AKT and S6 kinase (S6K) are functionally altered.

Previously, conditional hypomorphic LKB1$^{fl/fl}$ mice (Sakamoto et al, 2005) were crossed with ovine beta-lactoglobulin gene (BLG)-Cre mice to excise *lkb1* from mammary glands of multiparous mice. In this model, loss of LKB1 expression gave rise to tumours by 16 months on average (McCarthy et al, 2009). More recently Klefström and colleagues (Partanen et al, 2012) analyzed the role of LKB1 and c-Myc in mammary gland development and tumourigenesis with a specific emphasis on the maintenance of epithelial integrity. Interestingly the outcome of the Klefström study is in agreement with our earlier work (Scott et al, 2007) that describes how LKB1 catalytic deficient mutants gain oncogenic properties, driving the expression of oncogenes. How the loss of LKB1 expression leads to changes in downstream signalling pathways and how these pathways may be involved in mammary gland tumourigenesis require further investigation.

The ErbB family is implicated in mediating oncogenesis of epithelial-derived cancers and is reported to be overexpressed in approximately 20-30% of invasive breast cancers, more specifically in high grade ductal carcinoma *in situ*

(DCIS) along with other oncogenes, cyclin D1 at 40-50% (Lodén et al, 2002), and c-Myc at 15-25% (Varley et al, 1987). While 30-60% of breast cancer express mutations in the tumour suppressor p53 and approximately 10% express mutations in the polyposis syndrome tumour suppressor phosphatase and tensin homolog (PTEN), mutations in Neu/HER2 (ErbB2) are often found in conjunction with loss of function mutations in tumour suppressor proteins (Li et al, 1997). Interestingly, inactivating mutations in PTEN are associated with Herceptin resistance (Chandarlapaty et al, 2012; Nagata et al, 2004)

To explore the interplay between Neu/HER2 and PTEN, conditional PTEN mice were interbred with constitutively active Neu/HER2 mice (MMTV-NIC) (Schade et al, 2009) (referred to herein as NIC mice), resulting in a dramatic increase in the rate of oncogenesis with corresponding amplification of the PI3K/AKT pathway but not mTOR signalling pathway (Schade et al, 2009).

5.3. Results

5.3.1. Loss of LKB1 expression in high-grade breast cancer.

We evaluated commercially prepared human breast cancer tissue microarrays (TMAs) (Table 1). Duplicate core samples (102) of high-grade tumours with corresponding HER2 expression status were scored for LKB1 expression by immunohistochemistry (IHC) (Table 1, Fig. 1). We found that 31% (32/102) tumours lacked LKB1 expression compared to tumours that expressed LKB1, $p<0.05$ (Table 1). We also observed that LKB1 expression was localized to luminal epithelium of normal breast tissues (Fig. 1A, C). LKB1 expression was scored as LKB1-ve (null) for no detectable LKB1 staining (Fig. 1B), LKB1+ (low),

LKB1++ (medium) and LKB1+++ (high), compared to antibody controls (Supplemental Fig.1, Appendix C).

Table 1 LKB1 expression in high grade breast cancer. IDC: Invasive ductal carcinoma, DCIS: Ductal carcinoma *in situ*.

Table 1. LKB1 Expression in high grade breast cancer					
	HER2+ve			HER2 -ve	
TMA Cores	LKB1-	LKB1+	LKB1+++	LKB1-ve	LKB1+ve
IDC 94	31 ± 1.4 (33%)*	24 ± 2.0 (26%)	11 ± 1.5 (12%)	19 ± 0.8 (20%)**	9 ± 0.5 (9%)
DCIS 8	1 ± 0.3 (12.5%)	2 ± 0.3 (25%)	1 ± 0.3 (12.5%)	3 ± 1.1 (37.5%)**	1 ± 0.3 (12.5%)

*p<0.05 compared to LKB1+ and LKB1+++ expression **p<0.05 compared to LKB1+ve LKB1 expression

Invasive ductal carcinoma (IDC)
Ductal carcinoma *in situ* (DCIS)

Table 1 LKB1 expression in high grade breast cancer.

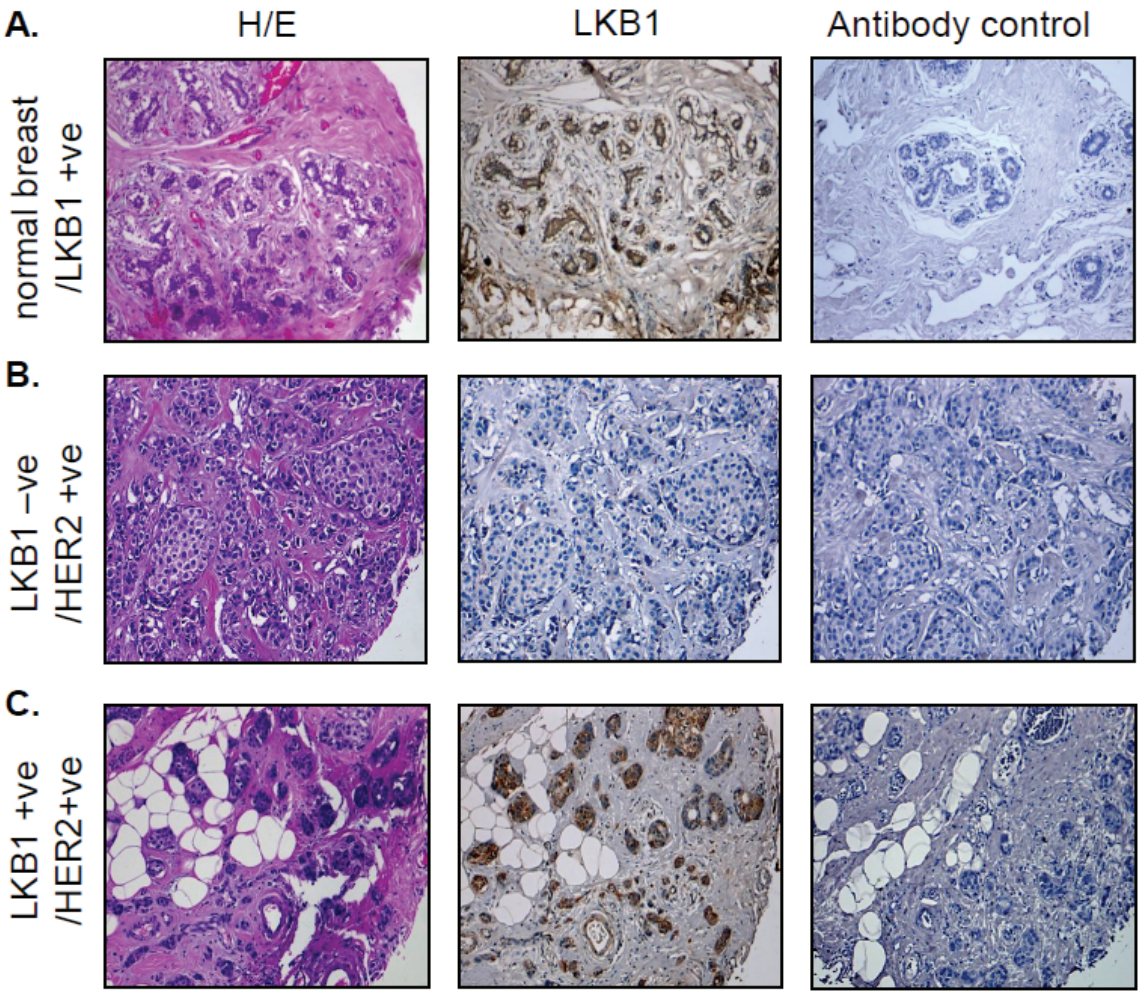

Figure 1. Loss of LKB1 expression in high grade tumours. A, Human breast cancer tissue microarrays containing 120 duplicate core samples were analysed by IHC for LKB1 expression. H/E staining (left column), LKB1 expression (middle column), and antibody control (right column). **A,** LKB1 expression in normal mammary gland, **B,** loss of LKB1 expression/HER2 positive and **C,** LKB1 positive/HER2 positive.

5.3.2. Activated Neu/HER2 enhances onset of tumourigenesis.

Given our results from human TMA analysis that indicate 31% of HER2 positive breast cancers lack LKB1 expression (Table 1, Fig. 1), we investigated whether the loss of LKB1 expression would alter the latency of ErbB2-mediated tumourigenesis. To explore the possibility, we developed a stochastic model of

human breast cancer by breeding STK11$^{fl/f}$ mice (FVB)(Bardeesy et al, 2002) with mice genetically engineered to express activated Neu/HER2-MMTV-Cre (FVB) under the control of endogenous *Erbb2* promoter, referred to as NIC mice (Schade et al, 2009), to generate STK11$^{-/-}$/NIC mice. Kaplan-Meir curves show a significant reduction in the latency of tumour onset in nulliparous STK11$^{-/-}$/NIC mice (Fig. 2A) compared to NIC mice ($p<0.0001$). By 147 days, 50% of STK11$^{-/-}$/NIC mice presented with tumours, compared to NIC mice at 197 days and nulliparous WT (FVB) mice that did not developed tumours (Fig. 2A).

Western blot analysis confirmed a significant reduction in LKB1 expression in STK11$^{-/-}$/NIC tumours compared to NIC tumours and WT mammary tissue, and elevated expression of ErbB2 in NIC and STK11$^{-/-}$/NIC tumours (Fig. 2B) as determined by densitometry. Characterization of STK11$^{-/-}$/NIC (top panel) and NIC (middle panel) by IHC confirmed DCIS (Fig. 2C) with loss of LKB1 expression, elevated expression of ErbB2, and pS6, compared to wild-type (bottom panel) mammary glands. Analysis of pS6 expression using imaging software confirmed a significant difference between pS6 expression in STK11$^{-/-}$/NIC tumours compared to NIC tumours (Fig. 2D, $p<0.0001$), suggesting hyperactive mTOR activity in STK11$^{-/-}$/NIC tumours. We did not observe any difference in tumour histopathology between STK11$^{-/-}$/NIC and NIC tumours. Nor were we able to observe significant metastasis to organs such as lung. Whole-mount analysis of mammary tissue adjacent to primary tumours from STK11$^{-/-}$/NIC (top panels) and NIC (bottom panels) (Fig. 2E) mice displayed abnormal luminal growth that likely represent early tumours.

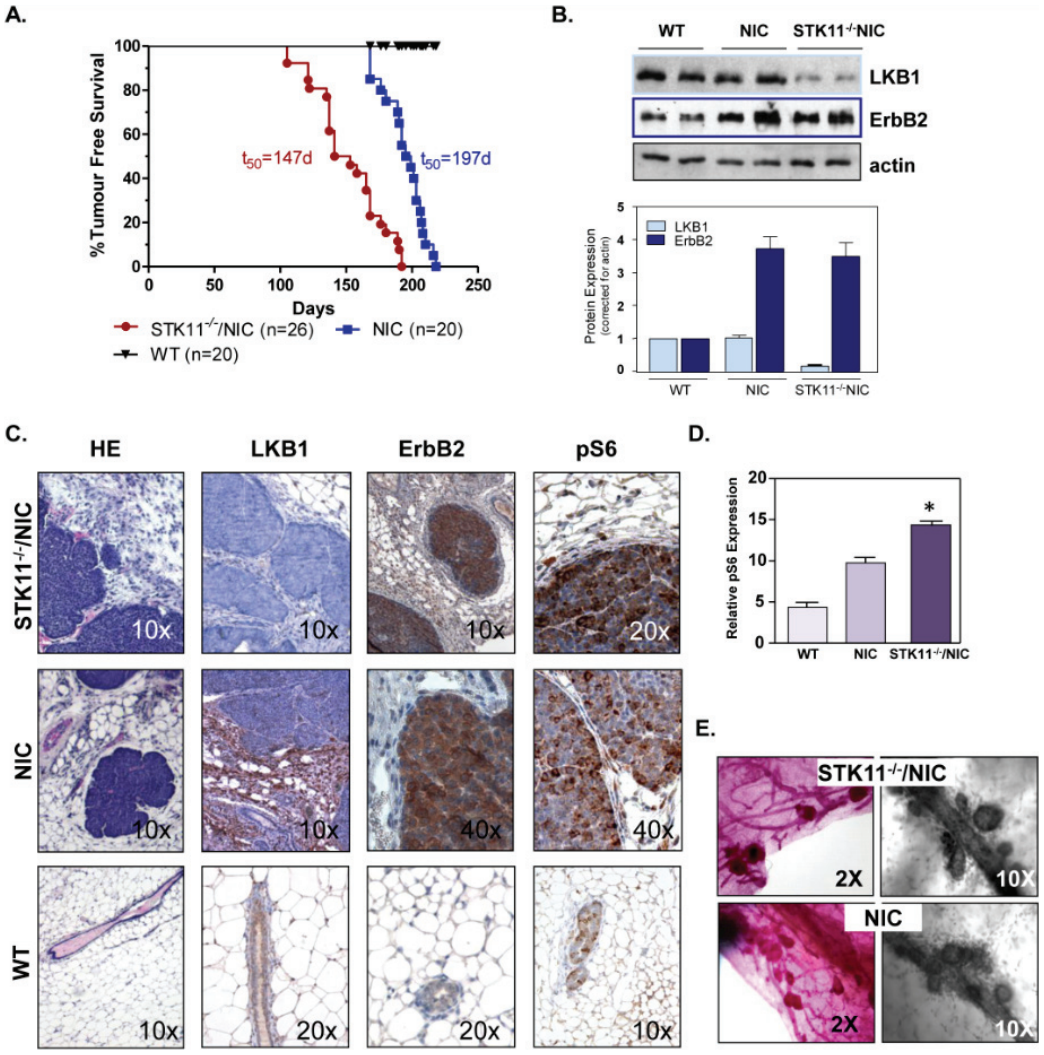

Figure 2. Loss of LKB1 expression reduces latency of ErbB2-mediated carcinogenesis. A, Kaplain-Meir percent tumour free survival curves for WT, STK11$^{-/-}$/NIC, and NIC mice. Log Ranked Test, $p<0.0001$. T_{50} represents time in days where 50% of the females presented with tumours and n is the number of female mice analysed. **B,** Western blot analysis confirming expression of LKB1, ErbB2, and actin (loading control) using antibodies detailed in the Methods section. Graphic representation of protein expression corrected for actin ± SD. **C,** Tumours were evaluated for LKB1, ErbB2, and pS6 expression in STK$^{-/-}$/NIC, NIC and WT mice. **D,** pS6 expression was quantified using three randomly selected IHC samples from STK$^{-/-}$/NIC, NIC and WT mice. Data is represented as relative pS6 expression mean ± SEM, $p<0.0001$. **E**, Carmine alum staining of mammary gland wholemounts from STK11$^{-/-}$NIC (top panel, 2x and 10x magnification) and NIC (bottom panel, 2x and 10x magnification) mice. Data are

representative of three separate experiments, using three separate mice from each genotype.

5.3.3. mTOR and ErbB2 signalling.

Based on the results described above, we evaluated the expression of downstream targets of mTOR in primary mammary epithelial cells (MECs) harvested from STK11$^{-/-}$/NIC, and NIC mice, in the absence or presence of mTORC1/mTORC2 and ErbB1/ErbB2 inhibitors (Fig 3A). In MECs from STK11$^{-/-}$/NIC mice, phosphorylation of S6 and AKT (S473 and T308) were elevated compared to levels in NIC mice in response to treatment with mTOR selective inhibitor rapamycin (Rapa). Here, Rapa attenuated the phosphorylation of S6 (Fig. 3Ba), while the phosphorylation status of AKT at residues S473 and T308 were unchanged (Fig. 3Ba). Since Rapa is selective for mTORC1, we tested whether inhibition of mTORC1 and mTORC2 by the ATP-competitive inhibitor Torin1 (Thoreen et al, 2009) altered mTOR activity. In MECs from STK11$^{-/-}$/NIC mice, we observed a significant reduction in the phosphorylation status of pS6 in response to Torin1 in STK11$^{-/-}$/NIC, and NIC mice (Fig.3Bb). Furthermore, we observe inhibition of the phosphorylation status of both pAKT-S473 and pAKT-T308 in response to Torin1. Next we tested AZD8055, a novel ATP-competitive inhibitor of mTOR that inhibits both mTORC1 and mTORC2 complexes thereby preventing feedback loops through AKT (Chresta et al, 2009). As with Torin1, we observe a reduction in the phosphorylation status of S6 along with inhibition of AKT-pS473 and inhibition of AKT-pT308 (Fig. 3Bc).

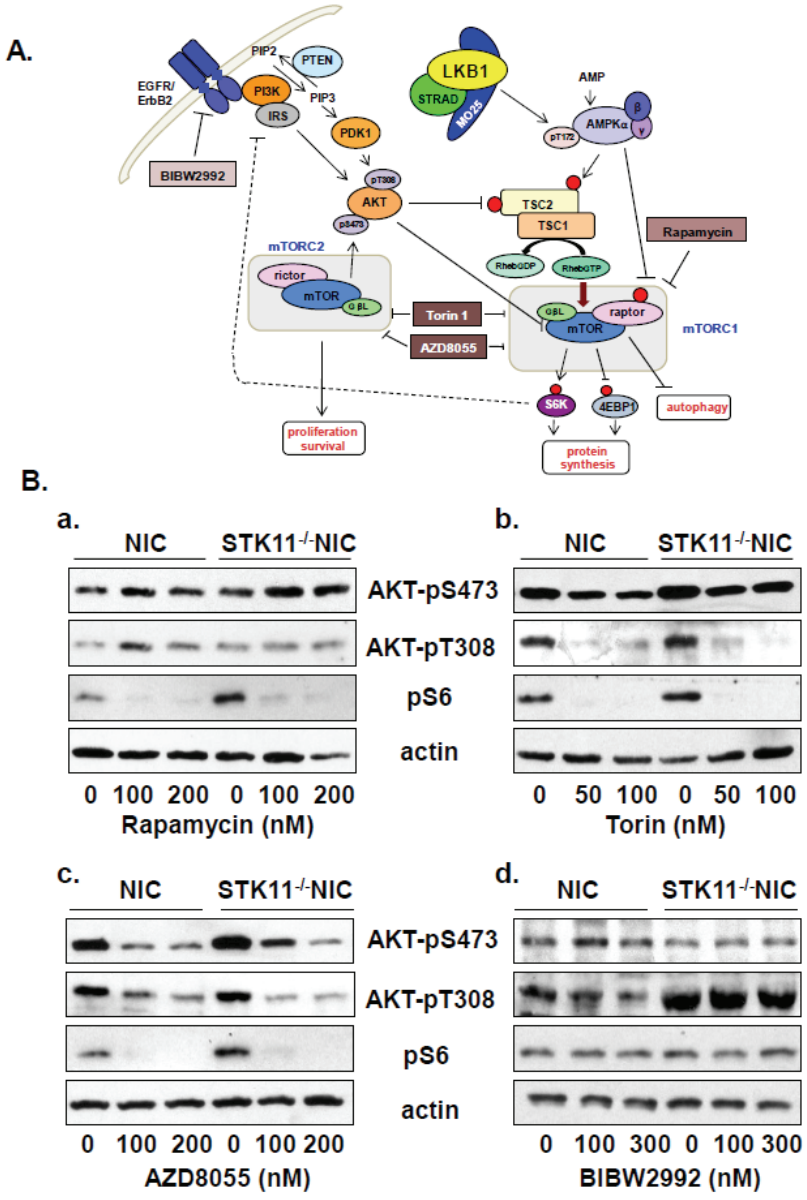

Figure 3. Inhibition of mTOR signalling. A, LKB1 in complex with STRAD and MO25 phosphorylates AMPKα on T172. AMPK phosphorylates tuberous TSC2 on T1227 and S1345 in response to metabolic stress. Loss of LKB1 expression leads to impaired regulation of AMPK, leading to impaired regulation of mTORC1. Rapamycin is an allosteric inhibitor of mTORC1 that prevents pS6 by S6K1. Rapamycin also blocks the negative feedback loop onto PI3K via S6K1, eliciting pro-survival. Torin1 and AZD8055 are selective ATP-competitive mTOR inhibitors of both mTORC1 and mTORC2, thereby blocking both protein synthesis and pro-survival pathways mediated by mTOR. With Torin1 and AZD8055, inhibition of mTOR prevents the phosphorylation of S6 by S6K and the

phosphorylation of AKT on S473 by mTOR. BIBW2992 irreversibly inhibits the ATP binding pocket within the kinase domain EGFR and ErbB2, whereby phosphorylation of AKT on the conserved T-loop T308 is inhibited. **B,** MECs isolated from tumours harvested from NIC and STK11$^{-/-}$/NIC mice were treated at concentrations as indicated with the following **a,** Rapamycin for 30 min. **b,** Torin1 for 2h. **c,** AZD8055 for 2h and **d,** BIBW2992 for 2 h. Expression of AKT-pS473, AKT-pT308 and pS6 was determined in response to treatments by western blot analysis using antibodies detailed in the Material and Methods Chapter. Data are representative of three separate experiments, using three separate mice from each genotype.

To test whether 5- aminoimidazole-4-carboxamide ribonucleoside (AICAR) could restore AMPK activity in the absence of LKB1 and therefore inhibit mTOR activity, we treated MECs from STK11$^{-/-}$NIC with AICAR (up to 2 mM for 1h) followed by western blot analysis for protein expression. As previously shown by others (Carretero et al, 2007; Sakamoto et al, 2005; Shaw et al, 2004a) AICAR did not activate AMPK in the absence of LKB1, as we did not observe any changes in the phosphorylation status of ACC or S6, the substrate of S6K (data not shown).

Since mammary tumours from STK11$^{-/-}$/NIC mice are positive for elevated ErbB2 expression (Fig. 2B), and 31% of HER2 positive breast cancers show loss of LKB1 expression (Table 1, Fig. 1), we investigated the effect of a next generation small molecule that targets the EGFR (ErbB1) and HER2, BIBW2992 (BIBW), on mTOR signalling. BIBW is an anilino-quinazoline that irreversibly binds to critical residues in the ATP binding pocket within the kinase domain; Cys 773 of EGFR and Cys 805 of HER2, thereby rendering the tyrosine kinases catalytically dead (Eskens et al, 2007). Compared to tumours from NIC mice,

AKTp308 expression was elevated in STK11$^{-/-}$/NIC mice (Fig, 3Bd). Treatment of mammary tumours cells from STK11$^{-/-}$/NIC and NIC mice with BIBW did not lead to changes in AKT phosphorylation, nor did we observe changes in the phosphorylation status of S6 (Fig. 3Bd).

5.3.4. NMR analysis of glycolysis metabolites.

Tumours require changes in metabolism in order to support growth, proliferation and survival as first described by Warburg in the 1920s (Warburg, 1956). The Warburg effect, when cancer cells metabolically remodel by shifting from oxidative phosphorylation to aerobic glycolysis to generate ATP despite the presence of oxygen and being less efficient then TCA (Duvel et al, 2010; Zaugg et al, 2011). Recent reports in the literature highlight the importance of AMPK/mTOR in glycolysis (Shaw et al, 2004a; Shaw et al, 2005). Since LKB1 is a regulator of AMPK function (Shaw et al, 2004b; Shaw et al, 2005), we tested whether cell metabolism contributed to mammary gland tumourigenesis in our model. First we found that the expression of LDH and PDH in STK11$^{-/-}$/NIC whole mammary tumours were elevated compared to wild-type mammary glands (Fig. 4A). Next we determined whether ATP levels were different between normal mammary tissues and mammary tumours by bioluminescence assay. Here we found that ATP levels in primary cells prepared from STK11$^{-/-}$/NIC tumours was significantly increased compared to levels in primary cells from WT mammary glands (23.5 ± 2.0μM and 15.9± 0.9μM, $p<0.01$ respectively) (Fig. 4B).

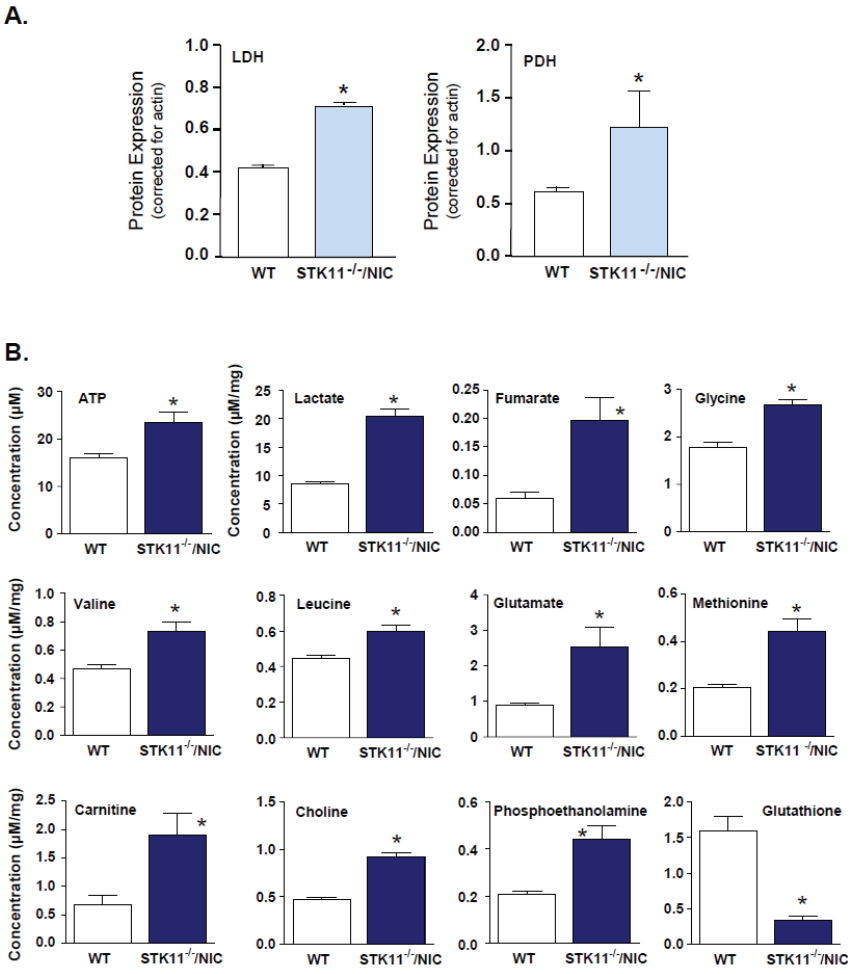

Figure 4. Analysis of metabolites from mammary tumours. Mammary glands from WT mice and tumours from STK11$^{-/-}$/NIC mice were excised and analyzed for **A,** expression of LDH, and PDH glycolytic enzymes by western blot analysis. Data are representative of three separate experiments, using three separate mice from each genotype and corrected for actin expression, mean ± SD, $p<0.05$. **B,** Mammary glands from WT mice and tumours from STK11$^{-/-}$/NIC mice were excised and analyzed by NMR. ATP concentrations are represented as mean of values from 2 μg of total protein from MEC lysates ± SEM, $p<0.01$. NMR data represents the mean of tumours from STK11$^{-/-}$/NIC mice and mammary glands form WT mice, corrected for the weight of the individual tumours/tissues and reported as μM/mg ±SEM, $p<0.05$. Tumours from three separate STK11$^{-/-}$/NIC mice and mammary tissue from three separate WT mice were analyzed.

Next we measured metabolites in tumours by NMR and found that lactate was significantly elevated in STK11$^{-/-}$/NIC tumours compared to WT mammary glands (20.4 ± 2.2 μM/mg and 8.5 ± 0.5 μM/mg, respectively; $p<0.05$). Since elevated lactate production is a hallmark of cancer metabolism (Vander Heiden et al, 2009), our results suggest that lactate from tumours could then be used by adjacent epithelial cells to generate ATP through TCA cycle as would fumarate, which is significantly elevated in STK11$^{-/-}$/NIC tumours compared to levels in WT mammary glands (0.19 ± 0.03 and 0.06 ± 0.01 μM/mg, respectively; $p<0.05$). In addition, we observed elevated levels of glycine in STK11$^{-/-}$/NIC tumours compared to WT mammary glands (1.8 ± 0.09 and 2.7 ± 0.1 μM/mg, respectively; $p<0.05$), supporting the biosynthetic requirements of the growing tumour.

Compared to WT mammary glands, branched-chain amino acids (BCAA) were significantly elevated in STK11$^{-/-}$/NIC mammary tumours, specifically valine and leucine. BCAAs are used for translation and are preferentially metabolized by cancer cells to serve as nitrogen sources for the production of the non-essential amino acid glutamate, a nutrient required by cancer cells in addition to glucose. Glutamate can then be converted to the oxidative substrate α-ketoglutarate and/or converted to pyruvate, both of which are then used in TCA. Compared to WT mammary tissue, glutamate was significantly elevated in STK11$^{-/-}$/NIC tumours ($p<0.05$).

During translation, the essential amino acid methionine is incorporated into the N-terminal position of all proteins as well as serving as an intermediate for biosynthetic products including membrane phospholipids. In STK11$^{-/-}$/NIC

tumours, we observe an increase in methionine, compared to WT tissues (0.44 ± 0.15 and 0.21 ± 0.01 μM/mg, respectively; $p<0.05$). Carnitine was significantly elevated in STK11$^{-/-}$/NIC tumours, compared to WT tissues (1.9 ± 0.0.7 and 0.68 ± 0.3 μM/mg, respectively; $p<0.05$), thereby serving as a precursor for mitochondrial ATP production through β-oxidation of fatty acids, satiating the energy requirement of growing tumours (Duvel et al, 2010; Zaugg et al, 2011). The dysregulation of lipid metabolism in tumours is reflected by significantly elevated levels of choline (0.92 ± 0.04 μM/mg), and phosphoethanolamine (0.44 ± 0.05 μM/mg), compared to levels in WT mammary tissues (0.47 ± 0.02 and 0.20 ± 0.01 μM/mg, respectively; $p<0.05$). In addition, we observed a reduction in redox metabolism as reflected by the antioxidant enzyme glutathione in STK11$^{-/-}$/NIC tumours, compared to WT tissues (1.6 ± 0.2 μM/mg and 0.33 ± 0.06 μM/mg, respectively; $p<0.05$), thereby providing a micro-environment that is beneficial to the development of the tumours.

5.3.5. Inhibition of mTOR induces alteration in glycolytic enzymes.

In our model, we observed that in the absence of LKB1 expression, mTOR is hyperactivated thereby enhancing glycolytic enzymes (Fig. 4). Since STK11$^{-/-}$/NIC mammary tumours exhibit a high concentration of lactate secretion (Fig. 4B), a characteristic of the Warburg effect, we investigated whether inhibition of mTORC1 and mTORC2 would translate into changes in lactate dehydrogenase (LDH) expression, as well as pyruvate dehydrogenase (PDH).

We observed that rapamycin treatment led to modest reduction in LDH expression in STK11$^{-/-}$/NIC cells compared to NIC; however, we did not observe

changes in PDH expression (Fig. 5a). Previous work by others show that rapamycin inhibits lactate secretion in mTORC1 hyperactive cells (Duvel et al, 2010). Next we investigated whether treatment of primary tumour cells with Torin1 and AZD would alter the expression of these enzymes. PDH expression was not altered in response to Torin1 treatment in $STK11^{-/-}$/NIC compared to NIC MECs (Fig. 5c) however PDH expression was modestly reduced in response to AZD (Fig. 5d). In response to both Torin 1 and AZD treatments (Fig. 5c, d) we observed a reduction in LDH expression in $STK11^{-/-}$/NIC compared to NIC MECs. We did not observe any differences in the expression of these enzymes while treating cells with BIBW (Fig. 5b). These data suggest that the hyperactivation of mTOR through loss of LKB1 expression in mammary tumours contributes to LDH expression.

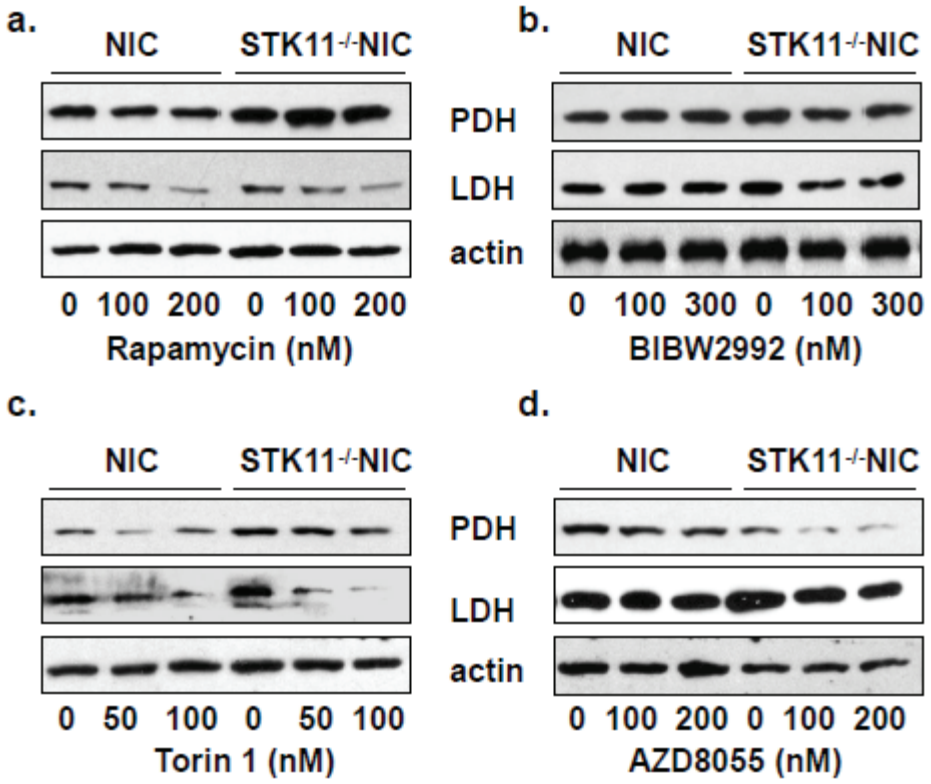

Figure 5. Expression of glycolytic enzymes in mammary tumours. MECs were isolated from STK11$^{-/-}$/NIC tumours and NIC mammary glands, followed by treatment with **a,** Rapamycin for 30 min. **b,** BIBW2992 for 2 h, **c,** Torin 1 for 2 h and **d,** AZD8055 for 2 h, followed by western blot analysis for LDH, and PDH. Data are representative of three separate experiments, using three separate mice from each genotype.

5.4. Discussion

Breast cancer is a complex disease due to the interplay between numerous proteins and genes at the molecular level. Because of the stochastic nature of this disease, characterization of critical signalling events that lead to the development and progression of breast cancer require further exploration. Since little is understood about the complex interplay between LKB1 and oncogenes in breast cancer, we evaluated the expression of LKB1 in human breast cancer

tissue microarrays and found that 31% of HER2 positive highly invasive cancers were null for LKB1 expression. These data along with studies conducted by others that report reduced expression of LKB1 is correlated with poor prognosis (Fenton et al, 2006), strongly support a role for LKB1 in breast cancer and suggest molecular interplay between LKB1 and HER2 signalling pathways in breast cancer.

In an effort to understand the molecular interplay between LKB1 and HER2, we developed a mouse model where *lkb1* was conditionally inactivated in combination with conditional expression of activated ErbB2 and Cre recombinase in the same mammary epithelial cell (Schade et al, 2009). We observed a significant reduction in the latency of mammary gland tumour onset in $STK11^{-/-}$ /NIC mice compared to NIC mice. We attribute the reduced latency to hyperactivation of mTOR signalling network in response to the loss of LKB1 expression (Fig. 2 and 3). Since LKB1 is a negative regulator of mTOR activity through the phosphorylation of the energy sensor AMPK. In the absence of LKB1, mTOR is active and phosphorylates S6K1, that in turn phosphorylates pS6, ultimately leading to protein translation (Shaw et al, 2004b). Interestingly, previous work by others show that $PTEN^{-/-}$/NIC mice rapidly developed mammary gland tumours with corresponding amplification of the PI3K/AKT pathway. However, mTOR signalling was determined not to be a contributing factor in tumourigenesis (Schade et al, 2009). More recently, others have shown that breast cancer cell lines with PTEN loss of function, were not sensitive to second generation mTOR inhibitors, specifically PP242 (Weigelt et al, 2011). Overall, our

data suggest that hyperactivation of mTOR signalling in response to loss of LKB1 function and gain of ErbB2 oncogenic activity leading to tumourigenesis may be mechanistically different from PTEN/ErbB2-mediated mammary gland tumourigenesis.

We investigated specific inhibitors of mTOR and ErbB2 signalling pathways (Fig. 3A), Rapa treatment was effective at inhibiting mTOR, however Rapa did not inhibit the feedback loop through S6 kinase as we did not observe inhibition of pAKT S473. These data are consistent with the finding from the literature that highlights the Rapamycin-resistant function of mTORC1 (Thoreen et al, 2009). We did not observe inhibition of pS6 or pAKT in response to BIBW2992 treatment, suggestive that inhibition of ErbB2 was not sufficient to inhibit mTOR signalling. However, the ATP-competitive inhibitors of mTORC1-mTORC2, Torin 1 and AZD8055, inhibited pS6 and AKT (pS473 and pT308), proving to be more efficacious at inhibiting mTOR in our model.

Previous work by others have explored the effects of inhibiting mTOR and MAPK signalling and found that Torin 1 (Thoreen et al, 2009) and AZD8055 (Chresta et al, 2009; Garcia-Martinez et al, 2011) treatment of primary cells, cell lines and xenografts inhibit mTORC1/mTORC2 (mTOR), as well as expression of cyclin D1, and cyclin D3, while increasing expression of the cell cycle inhibitor p27Kip1(Chresta et al, 2009; Garcia-Martinez et al, 2011; Thoreen et al, 2009). In PTEN/LKB1 deficient mice that develop B-cell follicular lymphoma, AZD8055 treatment resulted in the inhibition of the phosphorylation of AKT substrates PRAS40 and FOXO1/3a, as well as the N-myc downstream regulated gene1

(NDRG1) (Garcia-Martinez et al, 2011). Others have shown similar results in several cells lines including the more common MCF7, HEK293, and A549 cells (Chresta et al, 2009). More recently, AZD8055 treatment of Calu-6 xenografts were found to inhibit mTOR signalling (Holt et al, 2012) similar to findings from our own study. Furthermore, the authors showed that AZD8055 treatment increased cleaved PARP as well as led to a modest increase in BIM-extra long (BIM-EL), the pro-apoptotic BH3 Bcl-2 family member(Holt et al, 2012). In this study, combination treatment with AZD8055 and the MEK inhibitor, AZD6244 (selumetinib), proved to be more efficacious at cleaving PARP and increasing BIM-EL, then AZD8055 alone. Given the recent work by others, pathways identified in their studies may be of interest in our model.

Metabolites are the final product of the genome and are therefore reflective of mutations, deletions, epigenetic or transcriptional modification and are defined as the total quantitative collection of small molecular weight compounds. We observe a difference in the ATP levels between $STK11^{-/-}$/NIC mammary tumours and WT mammary tissue, reflective of the loss of LKB1 expression and therefore regulation of AMPK activity, leading to hyperactivation of mTOR through both mTORC1 and mTORC2 pathways. Previous work by others have demonstrated that activation of mTORC1 is sufficient to stimulate specific metabolic pathways that include glycolysis, the oxidative arm of the pentose phosphate pathway, and de novo lipid biosynthesis (Duvel et al, 2010). Metabolic adaptation of $STK11^{-/-}$/NIC tumours is consistent with defects in fatty acid oxidation, protein synthesis and redox potential (Fig. 4). Therapies that

target multiple metabolic products in combination with aberrant signalling pathways, such LKB1-AMPK-mTORC1 and ErbB2-AKT-mTORC2 may be a consideration for future targeted cancer treatments, particularly for HER2 resistant trastuzumab-refractory breast cancer (Chandarlapaty et al, 2012). Currently there are significant efforts to reverse the Warburg effect through AMPK agonists that would re-establish the conduit between energy and growth signalling to shut down tumour growth. The most common of these agonists are the anti-diabetic drugs metformin and phenformin. It remains to be seen, whether these drugs will be useful in the treatment of cancers with enhanced mTOR activity resulting from dysregulation of multiple signalling pathways that include LKB1.

Recent studies have shown that metabolism and increased glycolysis are two important processes responsible for inducing and sustaining the malignant transformation of normal cells (Duvel et al, 2010; Vander Heiden et al, 2009). Previous work showed inhibition of mTOR by treatment with rapamycin decreased the expression of LDH, PDH and hexokinase II (Duvel et al, 2010). In our model, we observe enhanced expression of LDH and PDH (Fig. 4A) and corresponding elevated lactate production (Fig. 4B), allowing cancer cells to maintain glycolytic metabolism and contribute to the increased acidification of the microenvironment (Gatenby, 1996). In agreement with our findings, others have shown that hyperactivated mTOR leads to elevated expression of LDH (Zha et al, 2011). As such, targeted inhibition of LDH and PDH metabolic enzymes may serve as therapeutic targets to block cancer cell metabolism. Torin1 and

AZD8055 both reduced the expression of glycolytic enzymes LDH and to a lesser extent PDH from primary tumour cells (Fig. 5c and d). In our model these data suggest that loss of the LKB1/AMPK regulation of mTOR is a strong driver of aerobic glycolysis.

Overall, our discovery confirms that loss of LKB1 expression leads to the development of breast cancer in conjunction with accelerated ErbB2-mediated oncogenesis. The outcome of our study suggests that the loss of LKB1 expression in HER2 positive breast cancer may contribute as a marker for hyperactivation of mTOR, warranting further investigation into combinatorial therapeutics that target LKB1-AMPK-mTOR and the glycolytic pathway.

Competing interest

The authors declare no competing interests.

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CHAPTER 6 PUBLISHED MANUSCRIPT

This chapter refers to the results obtained in the pre-clinical drug studies using LKB1$^{-/-}$/NIC mice. The data obtained from this study was published in the Oncotarget on November 25, 2014. The Material and Methods section was removed and is part of Chapter 3. The original formatting for this publication was modified in compliance with Dalhousie University Thesis guidelines.

Manuscript Title: Pre-clinical study of drug combinations that reduce breast cancer burden due to aberrant mTOR and metabolism promoted by LKB1 loss.

Authors: Rafaela Andrade-Vieira, Donna Goguen, Heidi A. Bentley, Chris V. Bowen, and Paola A. Marignani.

Author's contributions

RA contributed to the design of the experiments, acquisition of data unless otherwise specified, analysis and interpretation of data and drafted the manuscript. DG contributed to the treatment, MRI experiments and MRI data analysis. HB conducted the transmission electron microscopy. CB contributed to MRI experiments. PM contributed to the design of the experiments, animal husbandry, genotyping of the mice, analysis and interpretation of data, and drafted the manuscript. All authors read and approved the final manuscript.

6.1. Abstract

Cancer therapies that simultaneously target activated mammalian target of rapamycin (mTOR) and cell metabolism are urgently needed. The goal of our study was to identify therapies that effectively inhibited both mTOR activity and cancer cell metabolism in primary tumours *in vivo*. Using our mouse model of spontaneous breast cancer promoted by loss of LKB1 expression in an ErbB2 activated model (referred to as LKB1$^{-/-}$NIC mice), we evaluated the effect of novel therapies *in vivo* on primary tumours. Treatment of LKB1$^{-/-}$NIC mice with AZD8055 and 2-DG mono-therapies significantly reduced mammary gland tumourigenesis by inhibiting mTOR pathways and glycolytic metabolism; however, simultaneous inhibition of these pathways with AZD8055/2-DG combination was significantly more effective at reducing tumour volume and burden. At the molecular level, combination treatment inhibited mTORC1/mTORC2 activity, selectively inhibited mitochondria function and blocked MAPK pro-survival signalling responsible for the ERK-p90RSK feedback loop. Our findings suggest that loss of LKB1 expression be considered a marker for metabolic dysfunction given its role in regulating AMPK and mTOR function. Finally, the outcome of our pre-clinical study confirms therapies that simultaneously target mTORC1/mTORC2 and glycolytic metabolism in cancer produce the best therapeutic outcome for patients harboring metabolically active HER2 positive breast cancers.

6.2. Introduction

The metabolic branch of mammalian target of rapamycin (mTOR) signalling is primarily dependent on the energy sensing 5' AMP-activated protein kinase (AMPK) and is under-utilized as a strategy to target aberrant mTOR signalling. The main activator of AMPK is the serine-threonine tumour suppressor kinase LKB1, which is responsible for phosphorylating AMPK on Thr172, leading to the activation of the energy sensor (Lizcano et al, 2004; Shaw et al, 2004b). Both somatic and sporadic mutations identified in LKB1 are responsible for numerous malignancies (Marignani & Sanchez-Cespedes, 2010). Arguably, targeting activating mutations in the phosphoinoside-3-kinase (PI3K)/AKT pathway have proven to be a viable strategy for inhibiting mTOR, however, in cancers that are mutant for LKB1 (Marignani & Sanchez-Cespedes, 2010), AMPK-mediated negative regulation of mTOR will be compromised. As such, the metabolic branch of mTOR signalling, mTORC1, will be hyperactive, particularly if there are associated activating mutations in oncogenes. Because hyperactive mTOR is often found in cancers associated with activating mutations in the PI3K/AKT signalling pathway, significant effort has been made to develop therapeutic strategies that target PI3K/AKT signalling. Current treatment strategies are at various stages of clinical trial, specifically NVP-BEZ235, PF-04691502 and BKM120 (Maira et al, 2008; Yuan et al, 2011). These new compounds are promising, however there may be limitations as these drugs are highly dependent on tumour sub-type, are specific to particular genetic alterations, and may lead to the activation of negative feedback loops that

acerbate resistance or recurrence. By exclusively targeting activating mutations in the PI3K/AKT branch of mTOR signalling, it stands to reason that if these same cancers express mutation in LKB1 or express isoforms of the pseudokinase STRADα that render LKB1 catalytically deficient (Marignani et al, 2007), the tumours may initially regress in response to treatment. However because AMPK activity is DYSREGULATED and the mTORC1-MAPK feedback loop is activated (Carracedo et al, 2008), the cancer will invariably return and/or be resistant to future treatments.

Recently we discovered that 31% of HER2 positive breast cancer lacked expression of LKB1 (Andrade-Vieira et al, 2013b). Based on this discovery we developed a mouse model of breast cancer where an activating mutation in the ErbB2 oncogene was combined with loss of LKB1 expression ($LKB1^{-/-}$NIC mice) (Andrade-Vieira et al, 2013b). In this model, we observed that loss of LKB1 activity promoted tumour growth by significantly reducing the latency of ErbB2-mediated tumourigenesis. Furthermore, tumourigenesis was strongly associated with hyperactivation of mTOR and dysregulation of cell metabolism, giving rise to metabolically active tumours. We found that inhibition of mTOR with AZD8055, a novel ATP-competitive inhibitor of mTOR that inhibits both mTORC1 and mTORC2 (Chresta et al, 2009), inhibited mTOR signalling and expression of glycolytic enzymes, lactate dehydrogenase (LDH) and pyruvate dehydrogenase (PDH), in primary breast cancer cells isolated from $LKB1^{-/-}$NIC mice (Andrade-Vieira et al, 2013b). Further to this, we and others observed a reduction in LDH

expression and therefore lactate, in response to inhibition of mTOR by rapamycin (Leontieva & Blagosklonny, 2014; Leontieva et al, 2011).

It is clear that alternative treatment strategies are necessary to overcome hyperactivated mTOR and dysregulation of cell metabolism attributed to the loss of LKB1 regulation of AMPK signalling pathways. Given that aerobic glycolysis plays a significant role in tumourigenesis, targeted regulation of ATP production may present a viable option for the treatment of cancer. 2-deoxyglucose (2-DG) inhibits a rate-limiting step in glycolysis after it is taken up by the cell and metabolized by hexokinase to phospho-2-DG (p-2-DG), a competitive inhibitor of hexokinase (Parniak & Kalant, 1985; Zhong et al, 2009). As a mono-therapy, 2-DG-mediated growth suppression appears to be offset by a concomitant AKT activation through phosphorylation of Thr308 and Ser473 (Tsurutani et al, 2006). We conducted pre-clinical trials to investigate novel mono-therapies and combinatorial therapies that targeted mTOR and metabolism in mammary gland tumourigenesis. In our study, we specifically evaluated inhibition of both mTORC1 and mTORC2 with AZD8055 in combination with 2-DG, in LKB1$^{-/-}$NIC mice with aggressive primary breast cancer (Andrade-Vieira et al, 2013b). Using magnetic resonance imaging (MRI), we monitored changes in tumour growth in response to treatments and elucidated the role metabolism plays in our model. Herein we confirm that targeted combinatorial therapy that simultaneously inhibits mTOR signalling and glycolysis is a viable strategy for the treatment of aggressive primary breast cancer.

6.3. Results

6.3.1. Loss of LKB1 expression enhances cell metabolism.

We previously observed that ATP levels in LKB1$^{-/-}$NIC primary mammary tumour cells were elevated compared to wild-type mammary epithelial cells, as were other important metabolites quantified from whole tumours (Andrade-Vieira et al, 2013b). To determine whether loss of LKB1 expression is responsible for enhanced breast cancer cell metabolism, we analyzed the presence of metabolites in whole LKB1$^{-/-}$NIC mammary tumours compared to tumours harvested from NIC control mice and from wild-type (WT) mammary glands by NMR analysis (Chenomx Inc.) (Fig.1). Normally, when cellular AMP levels are elevated, the binding of AMP to AMPK enhances the substrate readiness of AMPK for phosphorylation/activation by the LKB1 complex, resulting in inhibition of mTOR signalling and fat synthesis (Hawley et al, 2003; Scott, 2007). Thus, AMPK serves as a metabolic switch that senses the energy requirements of the cell. Metabolic analysis of AMP concentrations in LKB1$^{-/-}$NIC tumours (0.001 ± 0.00 μM/mg), NIC tumours (0.13 ± 0.05 μM/mg) and WT (0.59 ± 0.14 μM/mg) mammary tissues indicate that AMP concentrations are significantly reduced in mammary tumours compared to WT tissue ($P<0.05$). Under these circumstances, AMPK activity in LKB1$^{-/-}$NIC tumours would be compromised on two counts; loss of LKB1 activity and reduced AMP concentrations, whereas in NIC mice, AMPK activity would be less impacted since NIC tumours express LKB1 comparable to WT mammary tissues (Andrade-Vieira et al, 2013b). As a result, regulation of

mTOR in LKB1$^{-/-}$NIC mice is increased, as is the regulation of metabolic pathways.

Of the biosynthetic metabolites that are necessary for the maintenance of cell metabolism and mitochondria function, acetate is used in cells as part of acetyl-CoA group and is the first molecule to enter the Krebs cycle. Acetate concentrations in LKB1$^{-/-}$NIC tumours (0.73 ± 0.03 μM/mg), was significantly elevated compared to NIC tumours or WT mammary tissue (0.22 ± 0.04 and 0.22 ± 0.04 μM/mg, respectively; $P<0.05$). The amino acids isoleucine and alanine are direct sources of ions for cell metabolism, as they are found in mitochondria and are precursors for the synthesis of key elements of the Krebs cycle and metabolic pathways. In LKB1$^{-/-}$NIC tumours, isoleucine (0.44 ± 0.02 μM/mg) was significantly elevated compared to NIC and WT gland (0.31 ± 0.03 and 0.21 ± 0.02 μM/mg, respectively; $P<0.05$). Alanine levels were modestly elevated in LKB1$^{-/-}$NIC (3.84 ± 0.41 μM/mg) tumours compared to NIC (2.71 ± 0.23 μM/mg) and WT (3.13 ± 0.42 μM/mg), however the differences were not statistically significant. Taurine plays a role in antioxidant and anti-inflammatory pathways, with low levels of taurine implicated in a variety of metabolic diseases (Schaffer et al, 2009; Suzuki et al, 2001). In LKB1$^{-/-}$NIC tumours, a significant reduction in taurine (2.66 ± 0.31 μM/mg) concentration was observed compared to NIC and WT gland (4.68 ± 0.13 and 7.23 ± 0.38 μM/mg, respectively; $P<0.1$). Niacinamide is incorporated into NAD coenzyme and is involved in a variety of mitochondria enzymatic reactions. In our model, niacinamide was significantly elevated in LKB1$^{-/-}$NIC tumours (0.57 ± 0.007 μM/mg) compared to NIC and WT tissues

(0.38 ± 0.05 μM/mg and 0.41 ± 0.03 μM/mg, respectively; $P<0.05$) (Fig. 1). These results suggest that the loss of LKB1 promotes ErbB2-mediated mammary tumourigenesis, in part, through metabolic processes since analysis of NIC tumours displayed less metabolic activity than tumours from LKB1$^{-/-}$NIC.

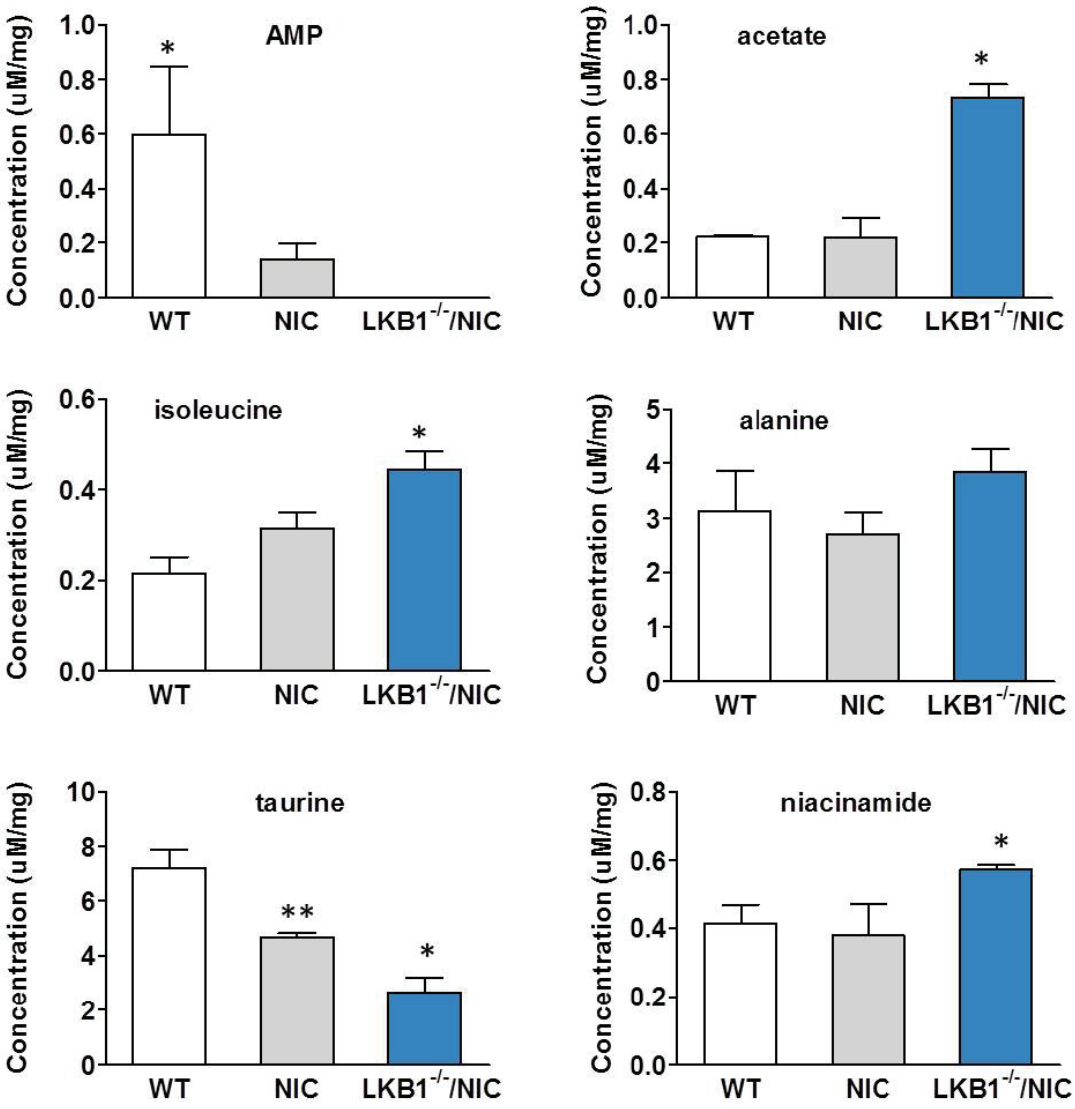

Figure 1. Enhanced metabolism in primary tumour cells lacking LKB1 expression. NMR analysis of metabolites in LKB1$^{-/-}$NIC mammary tumours, NIC tumours and wild-type (WT) mammary gland. For each group, data is reported as three separate samples (μM/mg) ±SEM, $P<0.05$. One-way ANOVA followed by Bonferroni post-hoc test for multiple comparisons and *P* values were calculated. *LKB1$^{-/-}$NIC significantly different from NIC and WT, **NIC significantly different from WT.

6.3.2. Inhibition of mTOR and PI3K impairs tumour growth.

Having confirmed that the loss of LKB1 in our model is responsible for enhanced metabolic activity, we were interested in whether treatment of $LKB1^{-/-}$ NIC mice *in vivo* with compounds that target the PI3K pathway and mTOR would be effective at inhibiting tumour growth. $LKB1^{-/-}$NIC mice at 20 weeks (Andrade-Vieira et al, 2013b) received daily intraperitoneal (i.p.) administration for 21 days and tumour volume was determined weekly using caliper measurements. We observed that mice treated with NVP-BEZ235 (10mg kg^{-1}) resulted in a significant reduction in tumour growth (22.58 ± 10.65, n=3 mean ± SD, $P<0.01$) by day 21 of treatment, compared with vehicle treated mice (40.19 ± 6.97, n=3 mean ± SD) (Fig. 2A, B). We treated mice with the mTOR inhibitor AZD8055 (20mg kg^{-1}) and found that inhibition of mTORC1 and mTORC2 significantly inhibited tumour growth (4.72 ± 1.19, n=3 mean ± SD, $P<0.001$) compared with vehicle treated mice (Fig. 2A, B). Further to this, tumour volume in response to AZD8055 treatment was significantly reduced compared with tumour volume in response to NVP-BEZ235 treatment ($P<0.01$) (Fig. 2A, B). Tumour volume in response to treatments was similar up to day 14, after which there was a significant impairment in tumour growth in response to AZD8055 treatment compared with vehicle treatment (2.5 ±0.9 and 19.29 ±12.8, n=3 mean ± SD, $P<0.01$ respectively) (Fig. 2A).

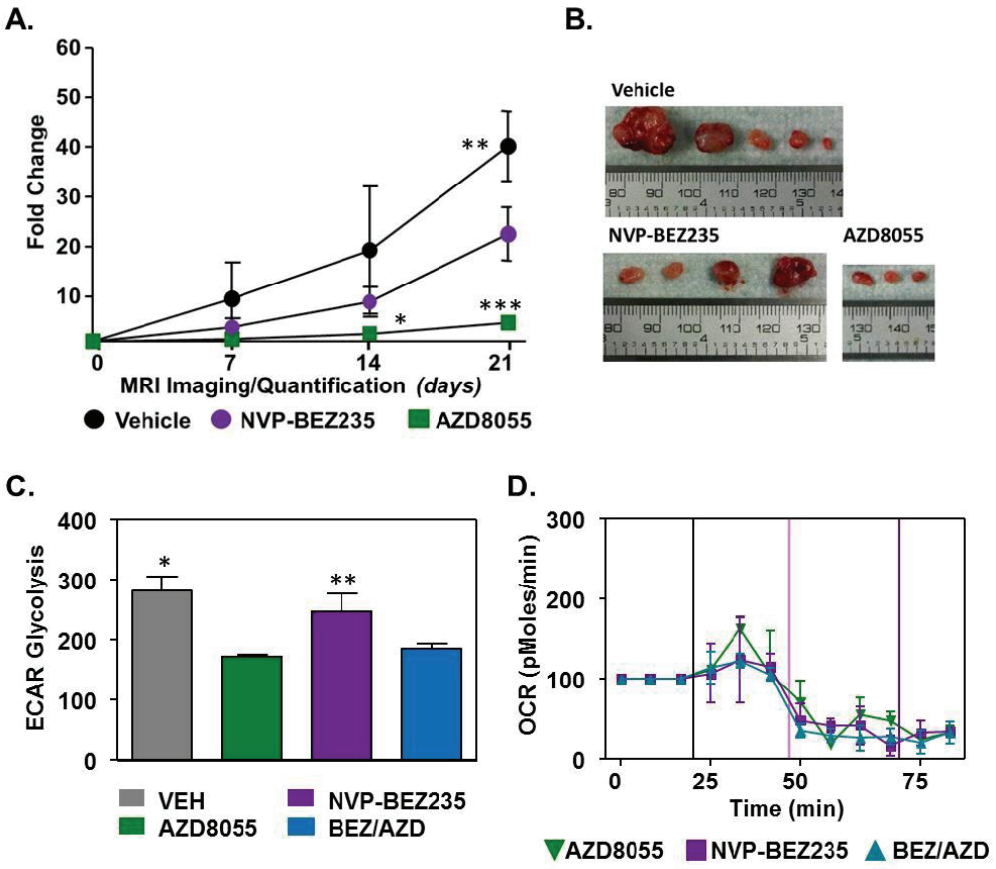

Figure 2. Effects of PI3K and mTOR inhibition on primary tumour development. (A) 20 week old mice were treated with vehicle, NVP-BEZ235 (10 mg/kg) and AZD8055 (20 mg/kg) daily for 21 days. Tumour volume was evaluated weekly by caliper measurements. Data represents mean of three independent mice ± SD, P<0.05. One-way ANOVA followed by Bonferroni post-hoc test for multiple comparisons and *P* values were calculated. *AZD8055 compared with Vehicle. **Vehicle compared with NVP-BEZ235, ***AZD8055 compared with NVP-BEZ235 and Vehicle. (B) Representative tumours excised from LKB1$^{-/-}$NIC after 21 days of treatment with indicated drugs. (C) ECAR and (D) OCR measurements of primary mammary tumour cells isolated from LKB1$^{-/-}$ NIC treated with AZD8055, NVP-BEZ235 and BEZ/AZD. Data is representative of three separate mice per treatment group; mean ± SD, P<0.05, One-way ANOVA, followed by Bonferroni post-hoc test for multiple comparisons and *P* values were calculated. *Vehicle compared with AZD8055 and BEZ/AZD combination, **NVP-BEZ235 compared with AZD8055 and BEZ/AZD combination.

6.3.3. The effects of drug therapy on mitochondria function.

Previously, we showed that treatment of primary breast cancer cells isolated from LKB1$^{-/-}$NIC mice with AZD8055 significantly inhibited mTORC1/mTORC2, as well as inhibition of glycolytic enzymes identified as drivers of the Warburg effect (Andrade-Vieira et al, 2013b). To determine whether mitochondria function is altered in our model, we treated LKB1$^{-/-}$NIC primary breast cancer cells with AZD8055 (100 nM), NVP-BEZ235 (100 nM) alone and combination AZD8055/NVP-BEZ235 (100 nM/100 nM) followed by analysis of aerobic glycolysis (Fig. 2C) and oxygen consumption rates (Fig. 2D). Using the Seahorse XF24 analyzer, we observed that extracellular acidification level (ECAR), a marker of aerobic glycolysis, was significantly decreased in response to both AZD8055 treatment (172 ± 5.2 mpH/min) and NVP-BEZ235 + AZD8055 combination treatment (184.3 ± 14.8 mpH/min) compared with NVP-BEZ235 treatment alone (246.7 ± 51.2 mpH/min; **P<0.05) and vehicle (281.3 ± 24.0 mpH/min; *P<0.05).

Aerobic glycolysis in NVP-BEZ235-treated cells was not different from aerobic glycolysis in vehicle-treated cells (Fig. 2C). In the same experiments, oxygen consumption levels were decreased in response to mono- and combination therapies, indicative of decreased metabolic function (Fig. 2D). Collectively, this data suggests that both AZD8055 and NVP-BEZ235 mono-therapy decreased tumour growth in LKB1$^{-/-}$NIC mice, however the inhibition of mTOR by AZD8055 was significantly more effective at preventing tumour growth

compared with NVP-BEZ235 treatment alone. Given that NVP-BEZ235 is a poor inhibitor of AKT and PDK1 (Baumann et al, 2009; Maira et al, 2008), and inhibition of mTOR by AZD8055 prevents the activation of both AKT-T308 and AKT-S473 (Andrade-Vieira et al, 2013b), AZD8055 is a better treatment for breast cancer in our model.

6.3.4. Inhibition of tumour growth, in response to 2-DG and AZD8055 treatments.

Having shown that treatment of LKB1$^{-/-}$NIC primary mammary tumour cells with AZD8055 inhibited key glycolytic enzymes, namely PDH and LDH, we wanted to explore beyond our previous *ex vivo* findings (Andrade-Vieira et al, 2013b). Because mTOR is a regulator of aerobic glycolysis by promoting activation of glycolytic enzymes (Masui et al, 2013), we evaluated whether it was feasible to simultaneously inhibit glycolysis and mTOR activity in LKB1$^{-/-}$NIC mammary tumours by treating mice daily for 21 days with low dose 2-DG (25 mgkg^{-1}) alone, AZD8055 (20 mgkg^{-1}) alone and 2-DG plus AZD8055 (25 mgkg^{-1} plus 20 mgkg^{-1}). For these longitudinal studies, mice were pre-screened by magnetic resonance imaging (MRI) at 19 weeks of age to identify early tumour bearing mice, after which treatments were initiated at week 20 with daily injections (i.p) for 21 days. Treatment duration was determined by ethical endpoint tumour burden of 10% body weight. Treatments were well tolerated by the mice for the duration of the study and no variation in weight gain was observed. Tumour volume was measured by MRI every seven days with

representative mice shown at Day 0 and Day 21 for each treatment (Fig. 3A). We did not observe any differences in tumour volume after treatment for seven days (Fig. 3B); however by 14 days of treatment, tumour volumes were significantly reduced in response to AZD8055 and in combination treatment, compared with treatment with 2-DG or vehicle. Compared with Day 0, vehicle treatment for 21 days resulted in increased in tumour volume by 42.3 ±10.4 fold, whereas both AZD8055 and 2-DG significantly inhibited tumour growth by Day 21, compared with Day 0 (3.7 ± 1.6 and 14.1 ± 2.4 fold, respectively) (Fig 3A-B). Compared with start of treatment, the combination of AZD8055 + 2-DG significantly prevented tumour growth (0.85 ± 0.4 fold) by Day 21 compared with vehicle (42.3 ±10.4 fold), 2-DG (14.1 ± 2.4 fold) and AZD8055 (3.7 ± 1.6 fold) mono-therapies (Fig 3. A-B). Tumours were harvested at the end of 21 days and in agreement with MRI volumetric analysis, tumours were consistently smaller from combination treated mice compared to mono-therapies and Vehicle treatments (Fig. 3C). Furthermore, tumour burden in response to treatment was significantly different between 2-DG treated mice and those treated with AZD8055 alone or combination treatment (Fig. 3D).

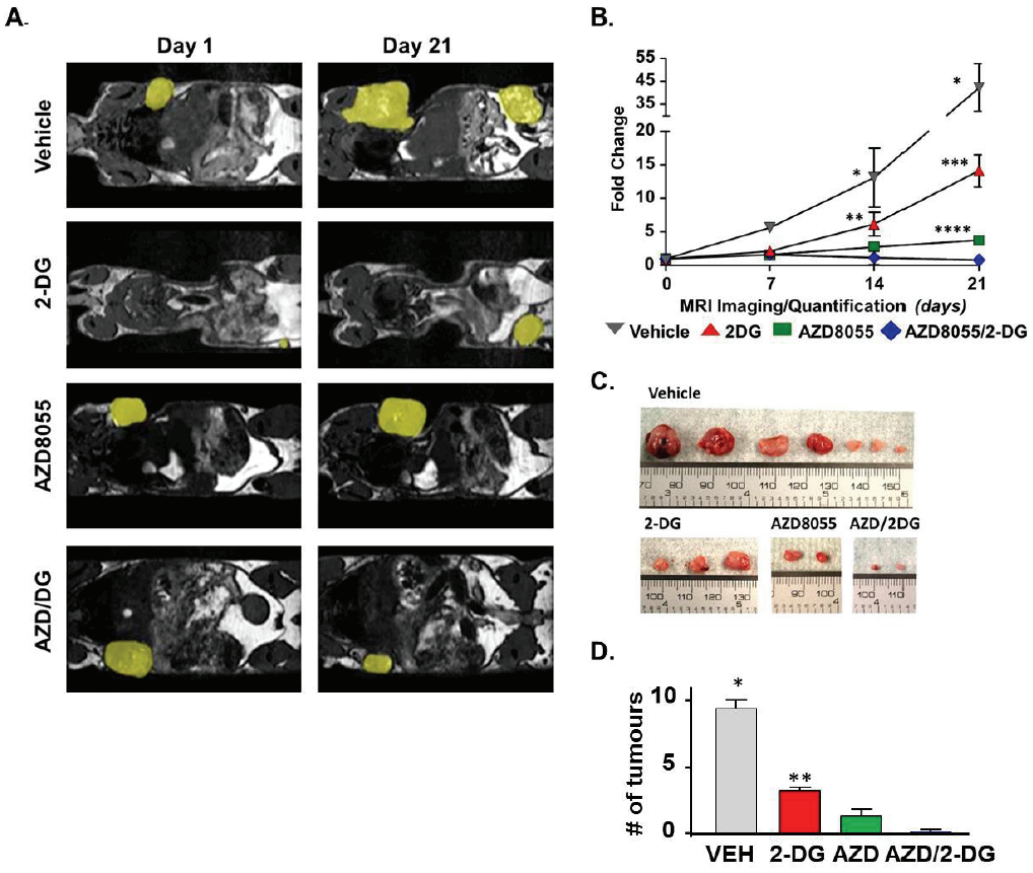

Figure 3. Pre-clinical evaluation of AZD8055 plus 2-DG combination treatment. (A) At 20 weeks of age, mice were treated with vehicle, AZD8055 (20 $mgkg^{-1}$) alone, 2DG (25 $mgkg^{-1}$) alone or a combination of AZD plus 2DG daily (i.p.) for 21 days. Primary tumours were visualized by MRI every 7 days and tumour volume was quantified. Yellow patches highlight mammary tumours. (B) Changes in tumour volume are represented as fold change from Day 0, start of treatment. Data is representative of five - eight mice per treatment group ± SD, *P<0.0001 Vehicle compared to all treatments, **P<0.01 2-DG compared to Vehicle. ***P<0.0001 2-DG compared to AZD and combination. ****P<0.01 AZD compared to combination. Two-way ANOVA followed by Bonferroni post-hoc test for multiple comparisons and *P* values were calculated. (C) Representative tumours excised from $LKB1^{-/-}$NIC mice after 21 days of treatment with indicated drugs. (D) The average number of mammary tumours per $LKB1^{-/-}$NIC mouse after 21 days of treatment. Data is representative of three separate mice per treatment group ± SD, **P<0.01. One-way ANOVA followed by Bonferroni post-hoc test for multiple comparisons and *P* values were calculated.

6.3.5. Mitochondria content shifts in response to AZD8055 and 2-DG.

To determine whether there was a difference in mitochondria function between LKB1$^{-/-}$NIC primary mammary tumour cells and WT mammary epithelial cells, we measured mitochondria content in LKB1$^{-/-}$NIC and WT cells using mitotracker red CMX/ROS (Fig. 4A). We observed that LKB1$^{-/-}$NIC primary mammary tumour cells had greater mitochondria content than WT mammary epithelial cells. In addition, we analyzed mitochondria morphology by electronic and fluorescence microscopy (Fig. 4B). Morphologically, mitochondria in LKB1$^{-/-}$ NIC cells were enlarged with increased cristae density compared to mitochondria in WT mammary epithelial cells, suggestive of increased ATP-production capacity.

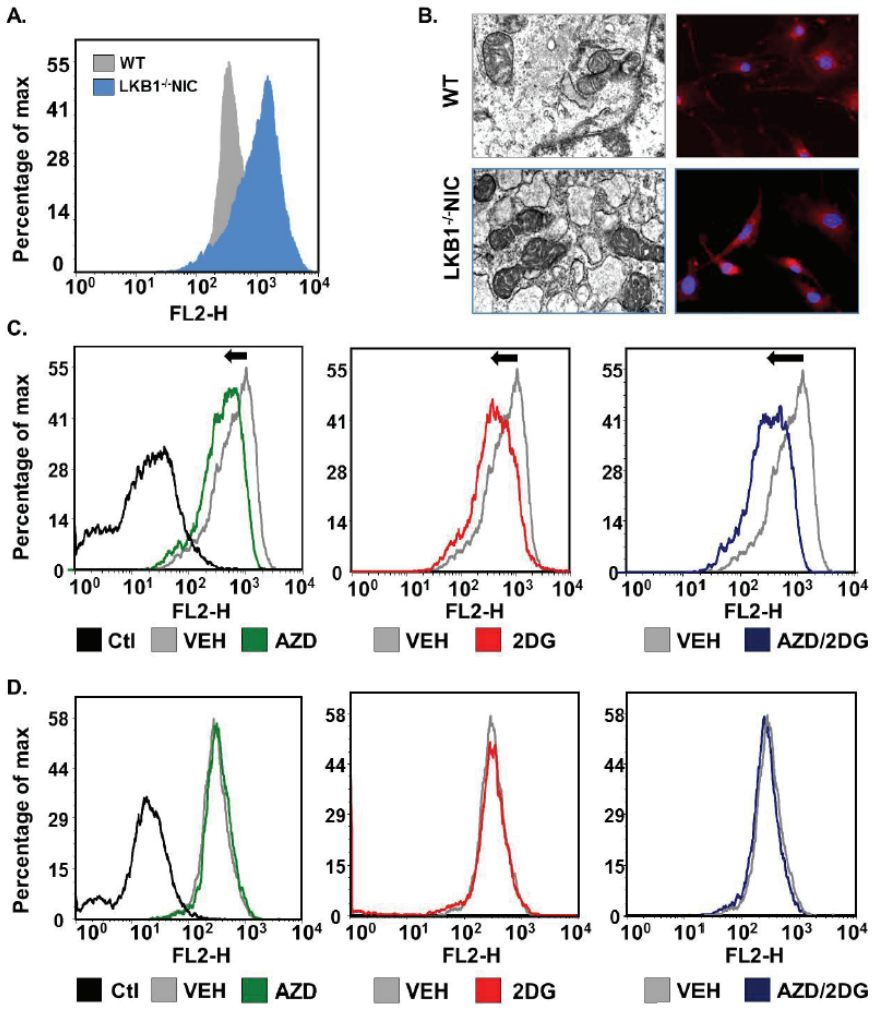

Figure 4. Primary mammary tumour cells undergo metabolic shifts in response to treatments. Mitochondria content was assessed using mitotracker Red. (A) Primary mammary epithelial tumour cells were isolated from LKB1$^{-/-}$NIC mice and compared to mammary epithelial cells isolated from wild-type mice. (B) Transmission electron microscopy showing mitochondria (left image) and Fluorescence Red CMX-ROS (right image) were used to visualize mitochondria. DAPI was used to visualize nuclei. (C) Mammary tumour epithelial cells isolated from LKB1$^{-/-}$NIC mice and (D) Mammary epithelial cells isolated from wild-type mice were treated for 2h with indicated drugs and incubated with mitotracker for 20 min. Representative histograms of mitochondria content are shown for LKB1$^{-/-}$ NIC (C) and WT (D). Non- stained cells are displayed as negative control (Ctl). Data is representative of three separate mice for each treatment group.

To determine the effect of inhibiting mTOR and/or glycolysis on mitochondria content, we treated cells with AZD8055, 2-DG and AZD8055 + 2-DG for 2 hours, followed by metabolic activity analysis of mitochondria. In LKB1$^{-/-}$ NIC cells, both mono- and combination therapies resulted in a shift in mitochondria content to the left; indicative of reduced mitochondria content, compared with vehicle-treated LKB1$^{-/-}$NIC cells (Fig. 4C). In response to combination treatment, we consistently observed a greater reduction in mitochondria content compared with mono-therapies. For WT mammary epithelial cells, we did not observe any difference in mitochondria content between mono-therapies, combination therapy, or vehicle treatment (Fig. 4D). These results suggest that both AZD8055 and 2-DG as mono-therapies or in combination are well tolerated by mitochondria, as not altering mitochondria biogenesis of normal cells. Furthermore, these results suggest that loss of LKB1 signalling in breast cancer reduces the ability of cells to overcome metabolic stress; however, treatments that target aberrant glycolysis and mTOR signalling drive the cells to overcome metabolic stress, ultimately resulting in decreased tumourigenesis, as observed in our pre-clinical study (Fig. 3).

Given that we observed a decrease in mitochondria content in response to AZD8055 alone, 2-DG alone and combinatorial therapy, we investigated whether there were corresponding changes in mitochondria function that would challenge the energy requirements for rapidly growing tumours. We investigated the capacity of LKB1$^{-/-}$NIC primary mammary tumour cells for aerobic glycolysis (ECAR), and oxygen consumption rate (OCR) in response to treatments. Using

the Seahorse XF analyzer, we treated LKB1$^{-/-}$NIC primary mammary tumour cells using 2-DG, AZD8055 and combination of both. We observed that treatment of cells with 2-DG (160.0 ± 1.5 mpH/min), AZD8055 (169 ± 17.9 mpH/min) and combination treatment (200.7 ± 26 mpH/min) significantly inhibited ECAR levels compared to Vehicle treatment (281.3 ± 13.8 mpH/min; $P<0.05$) (Fig. 5A). Treatment of cells with AZD8055 alone or combinatorial treatment significantly reduced OCR compared with 2-DG treatment alone (Fig. 5B). This data strongly supports that combination treatment of AZD8055/2-DG reduces mitochondria function and aerobic glycolysis in LKB1$^{-/-}$NIC mammary tumour cells.

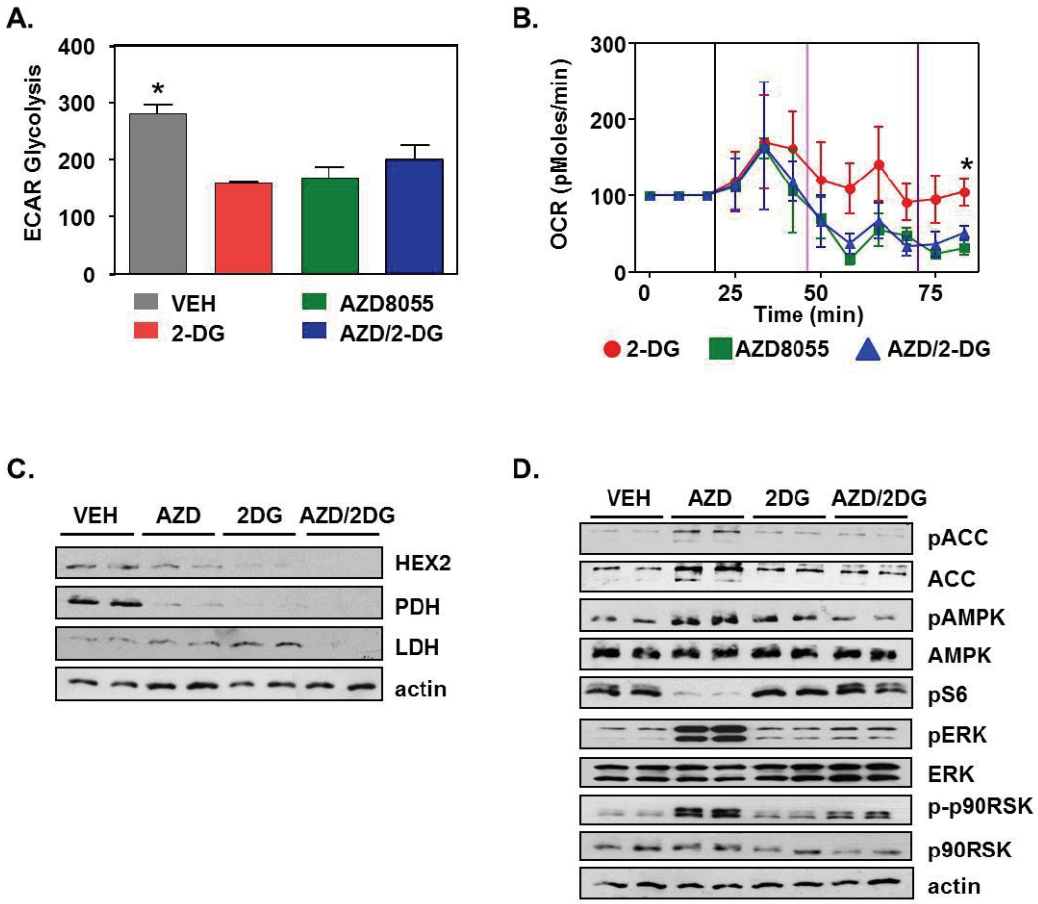

Figure 5. Pro-survival pathways inhibited in response to treatment. Primary mammary tumour cells were isolated from LKB1$^{-/-}$NIC mice, treated with VEH, AZD, 2-DG and AZD/2-DG combination, followed by analysis of mitochondria function. (A) ECAR was determined using cells isolated from three separate mice per treatment group, in duplicate; mean ± SEM, P<0.05, One-way ANOVA, followed by Bonferroni post-hoc test for multiple comparisons and *P* values were calculated. *VEH compared with AZD, 2-DG and AZD/2-DG combination. (B) OCR was determined using cells isolated from three separate mice per treatment group, in duplicate; mean ± SEM, P<0.05, *2-DG treatment compared with AZD, and AZD/2-DG combination. (C) LKB1$^{-/-}$NIC mice were treated for 21 days with VEH, AZD, 2DG, and combination of AZD/2-DG. Mammary tumours were harvested and proteins were prepared for western blot analysis. Duplicate protein samples were loaded and analyzed. Data is representative of three separate mice per treatment. (D) LKB1$^{-/-}$NIC mice were treated for 21 days with VEH, AZD, 2DG, and combination AZD/2-DG. Tumours were harvested and proteins were prepared for western blot analysis. Duplicate samples were loaded and analyzed. Data is representative of three separate mice per treatment.

As tumours develop, faulty metabolic switches allow for growth advantages over normal cells. mTOR enhances transcription of glycolytic enzymes, thereby increasing the glycolytic metabolism (Cairns et al, 2011; Laplante & Sabatini, 2009; Sun et al, 2011). Thus, the inhibition of mTOR would be a strategy for controlling glycolytic metabolic switches. Further to this, the inhibition of the rate-limiting enzyme HEX2 could augment the effects of mTOR inhibition. To explore whether mono- or combination therapies altered the expression of glycolytic enzymes in LKB1$^{-/-}$NIC mammary tumours, we analyzed expression of HEX2, LDH and PDH by western blot analysis of whole tumours harvested from mice that had been treated with AZD8055, 2-DG, combination treatment and Vehicle for 21 days (Fig. 5C). AZD8055 treatment of LKB1$^{-/-}$NIC mice modestly reduced expression of HEX2 compared with 2-DG treatment *in vivo* however; combination therapy exhibited the strongest inhibitory effect on HEX2 expression. LDH expression was reduced only in response to combination therapy, compared with mono-therapies, while all treatments inhibited expression of PDH compared with Vehicle treatment (Fig. 5C). These results suggest that treatment of LKB1$^{-/-}$NIC mice with AZD8055 in combination with 2-DG, leads to molecular changes in metabolic switches that contribute to inhibition of tumourigenesis (Fig. 3).

Previous work by others has shown that prolonged inhibition of either PI3K or mTOR by drugs or genetic means, leads to activation of a negative feedback loop that activates MAPK signalling (Carracedo et al, 2008; Garcia-Martinez et al, 2011; Serra et al, 2013). As such, we examined the effect of mono-therapies and

combination therapy on the expression of both AMPK and MAPK signalling pathways in whole mammary tumours from LKB1$^{-/-}$NIC mice by western blot analysis (Fig. 5D). In response to AZD8055 treatment for 21 days, tumour volume and burden were significantly reduced (Fig. 3), while the expression of pACC, the direct target of AMPK, and pAMPK were elevated, and expression of pS6, the target of S6 kinase, was markedly reduced, compared with other treatments. As expected (Carracedo et al, 2008; Garcia-Martinez et al, 2011; Serra et al, 2013), the phosphorylation status of p90RSK and ERK were elevated in response to prolonged inhibition of mTOR by AZD8055, compared with other treatments (Fig. 5D). Given that 2-DG is a known activator of pro-survival pathways via PI3K and insulin-like growth factor receptor 1 (Maschek et al, 2004; Zhong et al, 2009), in our model both tumour volume and burden were significantly reduced, compared with vehicle treatment (Fig. 3), however the phosphorylation status of S6, ACC and AMPK were unchanged from vehicle-treated mice. The phosphorylation status of p90RSK and ERK was also unchanged compared to vehicle-treated (Fig. 5D). Finally, treatment of LKB1$^{-/-}$ NIC mice for 21 days with combination therapy showed the greatest impact on tumour volume and burden (Fig 3) with no change in the phosphorylation status of S6 compared with 2-DG treatment alone and Vehicle-treated mice (Fig. 5D). Interestingly, the phosphorylation status of ERK and RSK were unchanged in AZD/2DG-treated mice despite the increase observed in response to AZD8055 mono-therapy. These results suggest that the simultaneous inhibition of mTORC1/mTORC2 and glycolytic pathways prevents the mTORC1 negative

feedback loop, and likely enhances AZD8055- and 2-DG-mediated growth inhibition since activation of the MAPK pathway is prevented.

6.4. Discussion

In the present study we evaluated the effect of mono-therapies and combination therapy on spontaneous primary mammary tumours from LKB1$^{-/-}$NIC mice that we previously characterized as hyperactive for mTOR and enhanced metabolic activities promoted by the loss of LKB1 expression and gain of ErbB2 function (Andrade-Vieira et al, 2013b). The LKB1$^{-/-}$NIC mouse model is representative of spontaneous primary human breast cancers that are HER2 positive with dysregulated metabolic activity. Initial characterization *ex vivo* of primary mammary tumour cells confirmed that inhibition of mTOR by AZD8055 significantly reduced AKT, mTORC1/mTORC2 and glycolytic activities (Andrade-Vieira et al, 2013b). Pre-clinical longitudinal studies that targeted the PI3K and p70S6K pathways with competitive NVP-BEZ235 inhibitor was not as effective at reducing tumour volume and burden as targeting mTOR with AZD8055 or glycolysis with 2-DG mono-therapies. A plausible explanation for this difference is that NVP-BEZ235 is a poor inhibitor of AKT and PDK1 (Baumann et al, 2009; Maira et al, 2008), whereas AZD8055 inhibition of mTOR prevents the activation of both AKT-T308 and AKT-S473 (Andrade-Vieira et al., 2013a).

Recently, LKB1 inactivation has been shown to cooperate with activating oncogene mutations to drive tumour progression in various models of cancer (Andrade-Vieira et al, 2013b; Garcia-Martinez et al, 2011; Liu et al, 2013; Morton

et al, 2010). The primary substrate of LKB1 is the central regulator of energy homeostasis and metabolic checkpoint, AMPK. At the nexus between growth factor receptor signalling and cellular energy metabolism, activated AMPK regulates protein and lipid synthesis, inhibits mTORC1 through activation of tuberous sclerosis complex 2 (TSC2) and phosphorylation of raptor (Corradetti et al, 2004; Dos et al, 2004; Lizcano et al, 2004; Shaw et al, 2004a). When LKB1-AMPK signalling is functional, regulation of the metabolic branch of mTOR signalling is intact regardless of whether PI3K/AKT or receptor tyrosine kinase signalling is aberrant. In our LKB1$^{-/-}$NIC mouse model of primary breast cancer, LKB1-AMPK signalling is significantly compromised, thus mono-therapy with the dual ATP-competitive PI3K/mTOR inhibitor NVP-BEZ235 was insufficient to block tumourigenesis whereas the combination of NVP-BEZ235 and AZD8055 resulted in reduced mitochondria function comparable to AZD8055 mono-therapy alone. Unlike NVP-BEZ235, AZD8055 inhibits phosphorylation of mTORC1 and mTORC2 substrates, namely p70S6K and 4E-BP1, leading to significant inhibition of cap-dependent translation, and AKT at residues S473/T308, respectively (Andrade-Vieira et al, 2013b; Chresta et al, 2009). Our pre-clinical data strongly suggest that treatment with AZD8055 as a mono-therapy is sufficient to inhibit primary mammary gland tumourigenesis in LKB1$^{-/-}$NIC mice, however the negative feedback loop that leads to the activation of MAPK signalling (Carracedo et al, 2008; Garcia-Martinez et al, 2011; Serra et al, 2013) could lead to relapse. Thus, a novel combinatorial approach that targets metabolic processes is warranted.

Therapies that activate the AMPK signalling pathway such as the biguanides metformin and phenformin have been used for the treatment of diabetes. Given the role these compounds play in regulated glycolytic metabolism as well as mitochondria function (Dykens et al, 2008; Shackelford et al, 2013), clinical trials are underway for the treatment of cancer, highlighting the importance of targeting cancer cell metabolism in metabolically active diseases. Since aerobic glycolysis is a major source of energy and provides biosynthetic products for protein and lipid synthesis, targeted inhibition of glycolysis would ultimately impact tumour growth. Inhibition of the rate-limiting step in glycolysis with 2-DG leads to depletion of ATP (Parniak & Kalant, 1985; Zhong et al, 2009), potentially shifting the Warburg Effect (Warburg, 1956). Results from Phase II clinical trials using 2-DG for the treatment of osteosarcomas and lung cancer, suggest that mono-therapy with 2-DG may not be as promising as combinatorial therapy. However in combination with other targeted treatments such as paclitaxel, inhibition of glycolysis with 2-DG sensitized tumours to the chemotherapeutic agents (Maschek et al, 2004). In another study, treatment of LNCaP prostate cancer cells with 2-DG in combination with the AMPK activator metformin, activated pro-death pathways *in vitro* inducing p53-dependent apoptosis via the energy sensor pathway AMPK (Ben Sahra et al, 2010).

In our study, treatment of LKB1$^{-/-}$NIC mice that are hyperactive for mTOR and metabolically active (Fig. 6A) with 2-DG mono-therapy (Fig. 3 and 6B) inhibited tumourigenesis. Both tumour volume and burden were reduced in response to reduced aerobic glycolysis and mitochondria function, however

AZD8055 mono-therapy (Fig. 3 and 6C) was significantly better at inhibiting tumour volume, burden and mitochondrial function. A possible explanation as to why 2-DG underperforms may be due to concomitant induction of AKT thereby activating pro-survival pathways (Cheng et al, 2012; Zhong et al, 2008; Zhong et al, 2009). With this in mind, prolonged treatment of $LKB1^{-/-}$NIC mice with AZD8055 or 2-DG have challenges, in that both mono-therapies lead to enhanced activity of pro-survival pathways. However combining both AZD8055 and 2-DG treatment for 21 days synergized treatment affects, overriding pro-survival pathways, reducing tumour volume, burden and mitochondria function. Thus, targeted treatment of hyperactive mTOR and aberrant glycolysis with combination therapy, AZD8055/2-DG inhibited the activation of ERK-mediated survival pathways associated with prolonged inhibition of mTOR (Carracedo et al, 2008; Serra et al, 2008), as well as activation of AKT associated with 2-DG treatment (Cheng et al, 2012; Zhong et al, 2008; Zhong et al, 2009) (Fig.3 and 6D).

Interestingly, we observed phosphorylation of S6 in response to 2-DG monotherapy and combination therapy (Fig. 5C), despite inhibition of pro-survival pathways. A possible explanation for this could be that loss of LKB1 expression and therefore catalytic function, may contribute to the phosphorylation of S6 under an energy depletion state. Our findings are in agreement with work by others that evaluated 2-DG treatment of non-small cell lung cancer cells (NSCLC) that lacked expression of LKB1 compared to NSCLC cells that express LKB1 (Dong et al, 2013). In this study, treatment of cells that lacked the

expression of LKB1, with 2-DG, did not reduce the phosphorylation status of S6 compared to the phosphorylation status of S6 in cells that express LKB1 (Dong et al, 2013). Phosphorylation of TSC2 by AMPK is required for translation regulation and S6 phosphorylation events (Inoki et al, 2003b) and more recently, activated AMPK is necessary for 2-DG mediated inhibition of pS6 (Ben-Sahra et al, 2013). Therefore the loss of LKB1 expression leads to reduced AMPK activation and impaired activation of TSC2 in response to energy deprivation caused by 2-DG treatment, emphasizing the importance of LKB1 in the regulation of energy homeostasis.

Mitochondria content was significantly reduced in response to combination treatment of LKB1$^{-/-}$NIC primary mammary tumour cells compared with wild-type primary mammary epithelial cells that express a functional LKB1-AMPK signalling pathway (Fig. 4C). Since LKB1 and AMPK are necessary for biogenesis, cells lacking the LKB1-AMPK pathway would be more sensitive to treatment with these compounds as mono-therapies and/or in combination, while mitochondria content remains intact in WT mammary epithelial cells. These results suggest that AZD8055 and/or 2-DG compounds would not be detrimental to mitochondria function in normal cells when administered systemically.

In conclusion, we conducted pre-clinical trials using LKB1$^{-/-}$NIC mice to test mono-therapies and combinatorial therapies that targeted mTORC1/mTORC2 and metabolism in primary tumours. We discovered that treatment of mice with AZD8055 or 2-DG as mono-therapies was more effective at inhibiting mammary gland tumourigenesis than targeting PI3K pathways with

competitive NVP-BEZ235 inhibitor. We also demonstrate that metabolically active cancer cells are more susceptible to the effects of metabolic drugs then normal mammary epithelial cells. We discovered that simultaneous inhibition of mTOR and metabolism with AZD8055 plus 2-DG combination was significantly more effective at inhibiting tumourigenesis and preventing sustained tumour growth that would occur through the activation of MAPK survival pathways. The outcome of our pre-clinical study emphasizes that mono-therapies directed towards ErbB2/HER2, PI3K or hyperactive mTOR alone, may not be sufficient to cause complete regression of primary tumours and/or prevent resistant phenotypes from developing. Furthermore, our discovery strongly supports the practice of evaluation of LKB1 expression in tumours as a marker for aberrant mTOR and metabolic signalling. Moreover, our model of primary breast cancer can be used as a tool to study the effectiveness of novel mono-therapies and combination therapies that are directed towards cancer that are hyperactive for mTOR and aberrant cancer metabolism. Future studies could take into consideration the role hyperactive mTOR and metabolism play in metastatic disease, and how best to treat this more lethal form of breast cancer and whether cell autonomous mechanisms are involved. As such, combination therapies that simultaneously target these pathways will provide the best clinical outcome for the treatment of metabolically active breast cancer.

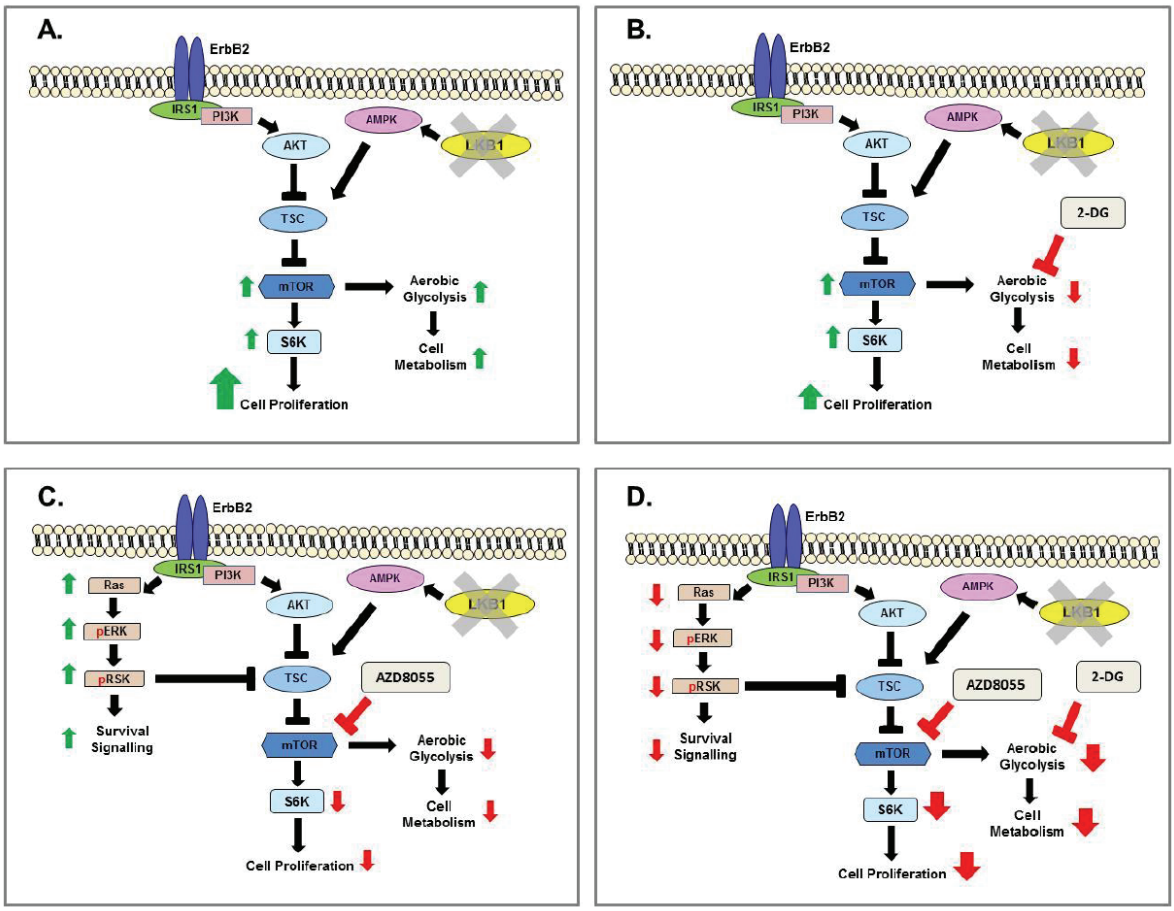

Figure 6. Schematic models describing signalling pathways described in the study. (A) Loss of LKB1 expression in ErbB2/HER2 mouse model of primary breast cancer leads to hyperactivation of mTOR and enhanced cancer cell metabolism. (B) Schematic shows inhibition of aerobic glycolysis leading to decreased cell metabolism. 2-DG treatment does not affect mTOR activity, therefore in absence of LKB1 expression and presence of ErbB2/HER2, mTOR remains hyperactive. (C) Schematic shows inhibition of mTOR signalling thereby preventing phosphorylation of S6K, inhibition of cell proliferation and a decrease in aerobic glycolysis due to low expression of glycolytic enzymes. Prolonged inhibition of mTOR leads to activation of pERK-p90RSK, activating pro-survival pathways. (D) Schematic shows simultaneous inhibition of mTOR and aerobic glycolysis. In this model, glucose enters the cell but is not completely metabolized in response to 2-DG treatment. As such, pro- survival pathways that would be activated in response to prolonged inhibition of mTOR by AZD treatment are suppressed via IRS1 signalling. The simultaneous inhibition of aberrant mTOR signalling and cell metabolism by AZD and 2-DG leads to a significant reduction in tumour growth, burden and aberrant signalling pathways.

Conflicting interest

The authors have no conflicting financial interests.

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CHAPTER 7 DISCUSSION

This chapter includes an overall discussion of the thesis and takes into consideration all the experiments results as part of the main hypothesis for this research. All three manuscripts (Chapters 4, 5 and 6) had specific discussions that focus on the discoveries made in each particular publication. Herein, I discussed how all the findings contributed to LKB1 field, metabolism and HER2+ breast cancer. In addition, I discuss more recent discoveries on drug therapeutics and how these therapies can be effective for the treatment of patient bearing aberrant mTOR signalling and cellular metabolism.

I also discuss the benefits of omega 3 polyunsaturated fatty acids in breast cancer development, how dietary intake of omega 3 polyunsaturated fatty acids can influence cellular signalling pathways and future perspectives for treatment of breast cancer. Lastly, I discuss pre-clinical trials and the lessons learned from my studies and findings. The future plans for this research are also discussed and take into consideration therapeutic strategies that will possibly influence how HER2+ breast cancer patients will be assessed and how a therapeutic plan can be selected based on aberrant cell signalling.

7.1. LKB1 signalling pathway.

LKB1 is a tumour suppressor protein kinase with multiple functions. This thesis mainly discussed the role of LKB1 in cancer and cellular metabolism, with an emphasis on the ability of LKB1 to phosphorylate AMPK and the downstream events promoted by the activation of AMPK. LKB1-AMPK signalling promotes the inhibition of mTOR signalling and ACC1 activity therefore reducing protein and lipid synthesis, respectively. The inhibition of protein and lipid synthesis decreases proliferation rate inhibits cell growth and cell metabolism. The findings detailed in this research described novel mechanisms to activate LKB1 signalling, characterized the role of LKB1 signalling in HER2+ breast cancer and conducted a pre-clinical trial to evaluate the use of specific therapeutic approaches to treat tumours that are hyperactive for mTOR and abnormal for cell metabolism. In this discussion, I will give insights into the importance of my discoveries in the LKB1, mTOR and metabolic fields. Additionally I will discuss the new perspectives for the treatment of HER2+ breast cancer taking into consideration resistance to current therapy.

7.2. The effects of ω3PUFA in LKB1 signalling.

The novel findings detailed in this research showed that ω3PUFA is an activator of LKB1 signalling. Unlike AMPK, LKB1 does not have any known activators; therefore this discovery showed that LKB1 signalling is activated by dietary supplementation with ω3PUFA in mammary cells. Importantly, this discovery has a significance to breast cancer and may affect general population dietary supplementation with ω3PUFA. The role of ω3PUFA is well studied in

cardiovascular disease (Fan et al, 2003; Oda et al, 2005; Sun et al, 2008). The findings from these previous studies suggest that ω3PUFA supplementation exerts anti-inflammatory effects ameliorating the symptoms that lead to the development of cardiovascular disease. Conversely, the role of ω3PUFAs in cancer is controversial as a result of studies in prostate cancer. There are recent studies showing a negative, positive or null effect of ω3PUFAs on prostate cancer (Brasky et al, 2013; Chavarro et al, 2007; Park et al, 2007). Based on our discovery, we showed that DHA activation of LKB1 signalling promoted downregulation of mTOR signalling, inhibition of protein synthesis and reduced aerobic glycolysis. Interestingly, abrogation of LKB1 expression reduced the beneficial effects of DHA in breast cancer cells. According to these findings, ω3PUFA may elicit anti-breast cancer effects that are dependent on LKB1 signalling. In addition, we showed that DHA is involved in cell metabolism through the inhibition of aerobic glycolysis and lactate-mediated cell migration.

Aerobic glycolysis provides the biosynthetic metabolites necessary to sustain high proliferative cells. Mitochondria oxidative phosphorylation is reduced and the production of lactate as the final product of glycolysis is enhanced. Lactate production increases the acidification of microenvironments promoting malignant transformation (Walenta et al, 2000). Therefore we hypothesized that the effects of DHA on cell migration was through the inhibition of LDH leading to the depletion of lactate. All together the results described in this research, strengthens the importance of ω3PUFA in regulating cellular signalling and aerobic glycolysis in breast cancer cells. Moreover, the results of this research

warrant further investigation into the effects of DHA in mammary gland tumourigenesis.

7.3. The role of LKB1 in HER2+ breast cancer.

As a tumour suppressor, lack of LKB1 correlates with tumour development, specifically in lung cancer due to the high number of LKB1 mutations found in NSCLC (Carretero et al, 2004; Matsumoto et al, 2007; Sanchez-Cespedes et al, 2002). Previous findings linked the loss of LKB1 expression to breast cancer since LKB1 plays a role in the co-activation of ERα (Nath-Sain & Marignani, 2009) and in maintaining mammary epithelial cell polarity (Partanen et al, 2012). However, little evidence showed the importance of LKB1 in HER2+ breast cancer.

In our mouse model of breast cancer, loss of LKB1 expression caused hyperactivation of the mTOR signalling which markedly enhanced tumour aggressiveness, reducing tumour latency and deregulating cell metabolism. The upregulation of mTOR signalling in LKB1$^{-/-}$/NIC mammary tumours was promoted by two events. The first event was loss of LKB1 expression which impaired the ability of AMPK to inhibit mTOR. The second event was constitutive activation of PI3K signalling which increased mTOR functions. In addition to these two events, mTOR is activated by amino acids and we showed that metabolic profiling of tumours isolated from this model had a marked accumulation of amino acids contributing to mTOR activation. Upregulation of mTOR in combination with defective AMPK activation also leads to inactivation of autophagy promoted by ULK1. Cells bearing these mutations are found to have large number of

mitochondria with abnormal morphology and altered metabolism (Egan et al, 2011). We showed that it is feasible to reduce mitochondria content in LKB1$^{-/-}$ /NIC cells without affecting mitochondria of WT cells. Inasmuch as the administration of these compounds did not alter mitochondria content in WT cells, the use of this therapeutic approach may be considered in future clinical trials.

The loss of LKB1 expression in 31% of HER2+ breast cancer showed the importance of evaluating LKB1 signalling pathways in this type of tumour. Lack of LKB1-mediating inhibition of mTOR activity is critical in the selection of therapeutic strategies considering that the current HER2+ breast cancer therapies do not use specific inhibitors of mTOR signalling or suppress aberrant cell metabolism. In this research, we considered the activating mutation of ErbB2 in addition to loss of LKB1 to evaluate the best therapeutic approach to inhibit tumour growth. Notably, combinatorial inhibition of mTOR signalling and glycolysis significantly decreased tumour growth. These results demonstrated the importance of considering multiple signalling pathways to obtain the best outcome to treat patients with HER2+ breast cancer.

In summary, this finding emphasizes the importance of evaluating LKB1 expression in breast cancer as a biomarker for aberrant metabolism. Furthermore, evaluation of a novel therapeutic approach that target dual inhibition of mTOR and cell metabolism conferred a promising strategy to treat breast cancer. Clinical trials are required to examine the efficacy of these compounds for the treatment of HER2+ breast cancer.

7.4. HER2+ Breast cancer: perspective on treatments.

HER2+ breast cancer is highly resistant to monotherapy, there are several potential mechanisms involved in resistance. As discussed in Chapter 1, HER2+ resistant breast cancer is treated with combinatorial antibody therapy and TKI therapy. However, there is evidence implicating activation of mTOR signalling in HER2-associated resistance. Dysregulation of mTOR signalling greatly increases survival response compromising the efficacy of HER2+ breast cancer treatment. Abnormal activation of mTOR was observed in tumours lacking PTEN which correlated with poor response to Trastuzumab (Lu et al, 2007; Nagata et al, 2004; O'Brien et al, 2010). Other study, showed that PI3K-independent activation of mTOR promotes resistance to TKI (Brady et al, 2015). Therefore, in our model, where lack of LKB1 promotes mTOR activation there is a likelihood of resistance.

Specific inhibition of PI3K signalling leads to activation of a feedback loop that limits the efficacy of these inhibitors. Similarly, we and others showed that long term inhibition of mTOR leads to activation of MAPK signalling, increasing survival pathways (Andrade-Vieira et al, 2014; Serra et al, 2013). A combinatorial inhibition of PI3K-mTOR signalling using NVP-BEZ235 possibly would give the best outcome. However, we showed that NVP-BEZ235 does not inhibit tumour growth as efficiently as the mTOR inhibitor AZD8055 (Andrade-Vieira et al, 2014). A possible explanation is that treatment with NVP-BEZ235 over time activates one of the feedback loops that lead to an increase in AKT Thr308 phosphorylation (O'Brien et al, 2014).

A recent phase three clinical trial, showed evidence that inhibition of mTOR signalling in combination with Trastuzumab therapy prolonged survival of patients resistant to Trastuzumab (Andre et al, 2014). The authors used a rapamycin analog namely, Everolimus, which inhibits mTORC1. This study lacked the characterization of pro-survival, which is important to assess breast cancer recurrence. Based on our discoveries, the inhibition of mTORC1 and 2 produces the best outcome. The challenge faced while using this strategy is the possibility of feedback loop leading to MAPK activation. However, we showed that inhibition of cell metabolism prevents MAPK activation, highlighting the potential benefit of combinatorial therapy.

The combination of AZD8055 and Trastuzumab also needs to be addressed. Because Trastuzumab is a strong inhibitor of HER2+ and has been shown to inhibit phosphorylation of ERK (Serra et al, 2011), there is a chance that combination of Trastuzumab and AZD8055 may promote the strongest inhibition of tumour growth. There is also an alternative option to antagonize resistance by inhibiting metabolism. We showed that 2-DG has significant antitumour activity; however side effect in humans may occur as a result of prolonged inhibition of glycolysis. This is important to access for eventual use of 2-DG in clinic. In our study, we did not observe any side effects in WT mice related to 2-DG leading to hypoglycemia such as loss of weight or mitochondria dysfunction. In the future, the use of metabolic inhibitors will probably be more attractive due to the high specificity of these inhibitors and efficacy in targeting abnormal signalling.

In conclusion, there are several mechanisms to overcome resistance in HER2+ breast cancer. Many of these mechanisms rely on the characterization of specific mutations in genes linked to resistance. Once these mutations are identified and characterized, more efforts can be made towards finding the appropriated therapeutic strategy. In my studies, I showed how targeting specific mutations can achieve the best tumour growth inhibition response. Therefore, based on my observations, I believe that loss of LKB1 expression will be considered a central determinant of resistance to HER2+-targeted therapy, and treatment strategies should take into consideration LKB1 signalling in the future.

7.5. Future work.

A future direction for my work could be to investigate the effects of ω3PUFA on tumour growth of NIC mice. The effects of ω3PUFA on the current treatment of breast cancer are unknown and there is a need for further investigation. Investigating the combination of current therapy with dietary increase of ω3PUFA will provide beneficial information for breast cancer patients undergoing treatments. Finally, this study warrants further investigation of the efficacy of AZD8055/2-DG novel inhibitory strategy to treat HER2+ breast cancer.

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APPENDIX A PROTOCOLS

The protocols listed in this appendix were developed and/or modified by Andrade-Vieira, R. during the course of this research.

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ATP bioluminescent assay

1. Count cells using cell counter (Trypsinize cells, add media, centrifuge, add 1ml of PBS and count, see protocol). Resuspend cell to a final concentration of $1x10^5$ cells in DMEM media + 8%FBS. Place cells in a 12 or 6 well plate**.** Next day change the media to DMEM + 1%FBS, treat cells in the afternoon.
2. Treat cells with drugs. After treatment lysis the cell in 100μL of passive lysis buffer (Promega). Use 2μL of the lysate for Bradford analysis. Use 2μg of protein per sample for analysis.

Luminometer

1. Turn on Luminometer and computer. Open the Berthold program. Go to maintenance and wash the injectors. Prime injectors.
2. Use the M injector (right injector). Fill it up to 5mL with ATP mix (Sigma). Protect ATP mix from light.
3. Open new file and workload. Load the tubes in the luminometer and press start. Follow instructions in the computer screen. Open the ATP standard curve_1 assay, add 5mL in the M injector and press "OK".
4. After use, remove the tubes and replace the water in the injector. Always wash two times before turn it off.

Attention: Run the ATP standard curve before run the sample. Use the dilution 1:40 of the ATP mix, 20 seconds delay and volume injected of 100μL. To change the settings go to setup- measurements- edit measurements. Choose the ATP standard Curve and make the changes. The Tube luminometer sensitivity is 10^{-15} mol or 1 amol.

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Mitotracker Red CMX-Ros

1- The vials come in a desiccated state. Dissolve the lyophilized MitoTracker in sterile DMSO to a final concentration of 1mM (add 94uL of DMSO to vials).
Note: The reduced rosamine MitoTracker probes (M7511, M7513) are quite sensitive to oxidation, especially in solution, and must be stored under argon or nitrogen, at –20°C and protected from light. Use the solution immediately after preparation.

2- Count cells ($5x10^4$ cells in p60 plates) and plate 24h before the experiment.

3- Before incubation wash the cells in PBS and trypsinize cells. Place cells in flow cytometry tubes and centrifuge cells for 5min 900rpm.

4- Dilute probes to working concentration (100nM) in DMEM serum free. Add media (225uL) plus probes (24uL) to the cells and incubate for 20min at 37ºC in the incubator. Keep one tube without probe to set up flow cytometry machine.

5- Centrifuge cells after incubation and add PBS to wash. Wash 2X in PBS.

6- Add 300uL of PBS to live cells and perform flow cytometry.

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RT^2 profiler PCR arrays

A. PCR Template

Combine the cDNA template (102µL) with the RT^2 SYBR Green master mix (1350µL) and add 1248µL of H_2O. Add equal aliquots of this mixture (25µL for 96-well plate) to each well of the same PCR array plate containing the predispensed gene-specific primer set and perform PCR.

B. Instrument settings

1. Open the Mx3000P with dissociation curve. The program is already saved under the file "RT profiler PCR array protocol template".
2. On the plate setup page click the square button with the word "all" on the top left corner.
3. On the right panel select well-type: Unknown. Choose FAM and ROX for collect fluorescence data. Select Reference dye: ROX
4. Click next to go to thermal profile setup page. The program is saved. Make sure the endpoint data collection maker is place above the step 2 of segment 2 and the all points data collection maker is present between the step 2 and step 3 of segment 3.

Program:

Segment 1: 95ºC – 10min
Segment 2: 95ºC – 15sec
60ºC – 1min } 40 Cycles
Segment 3: 95ºC – 1min
55ºC – 30sec
95ºC – 30sec

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Seahorse Experiment

1- Split cells and plate $1x10^4$ cells/well (primary mammary cells). Add calibrant media in the seahorse experimental plate 24h prior experiments and leave it in the incubator without CO_2.
2- Next day prepare assay media. Use seahorse media and add 25mM GLU (glucose) and 1mM NaPyr (sodium pyruvate).

For 50mL: Add 1.25mL of GLU and 50uL of NaPyr. Stock solutions are 1M.

3- Add 600uL of assay media and place the cells in the incubator without CO_2 for 1h.
4- Prepare injections (may change dependent on experiments):

GLU (Port A) – 10mM final concentration
Add: 6.9uL GLU (1M) 193.2 uL
+ 23.1 uL media X 28 = 646.8 uL

Oligomycin (Port B) – 0.2uM final concentration
Add: 1.38uL OLI (O.1mM) 38.64uL
+ 28.62 uL media X 28 = 801.4uL

2-DG (Port C) – 10mM final concentration
Add: 6.9uL 2-DG (1M) 193.2 uL
+ 23.1 uL media X 28 = 646.8 uL

5- Add 30uL of each drug in the right port. The seahorse plate has 4 ports. Each drug goes in their individual port.
6- Start seahorse program, set the normalization with the right number of cells. Place the experimental plate for calibration, after calibration remove plate and place the cells plate. Use cycle Mix 3min Wait 2min and Measure 3min.

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Adeno-Cre virus infection

The multiplicity of infection (MOI) of virus is calculated based on plaque-forming unit (PFU) provided by the virus company and number of cells desired. For primary mammary cells start off at $5x10^4$ cells per 6-well plate. Cells cannot be confluent. Test at least five MOI concentrations (e.g 0, 100, 200, 400, 500).

For example:

MOI of 100 is calculated as follow:

$$\text{Virus volume } (\mu L) = \frac{100MOI \times \text{number of cells} \times 1000 \text{ (for volume in microliter)}}{PFU}$$

1. Split cells and plate them in a 6-well at $5x10^4$ cells.
2. Next day change media to serum free DMEM for the addition of virus. Add Virus.
3. After 24h change media to 8%FBS DMEM.
4. Harvest cells at 24h and/or 48h after infection.
5. Run gel and western blot.

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2/24/2015

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APPENDIX C SUPPLEMENTAL MATERIAL

This appendix includes supplemental figures as supportive information to this research.

Supplemental Figure 1 – This figure was published as part of the supporting information of (Andrade-Vieira et al, 2013b).

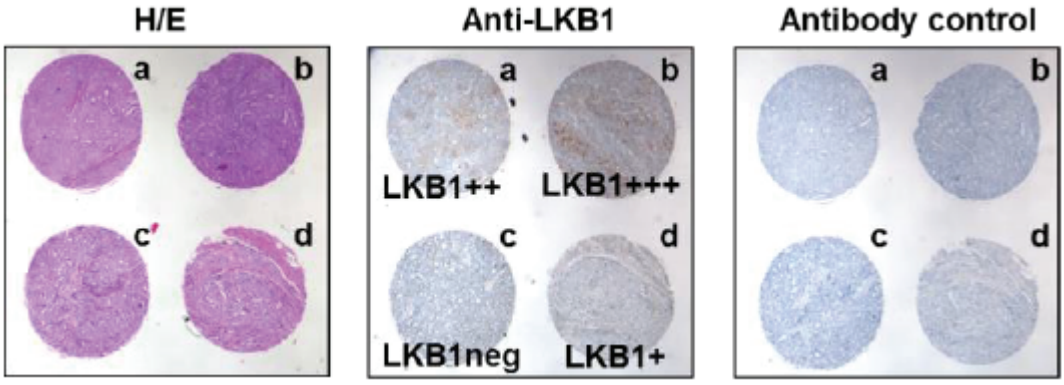

Figure S1 LKB1 antibody conditions for immunohistochemistry. Human breast cancer tissue microarrays of invasive ductal carcinoma (Biomax USA) were used to establish the concentration of anti-LKB1 antibody for staining by IHC. Left panel represents H/E staining, central panel represents anti-LKB1 staining; **a,** LKB1++ (medium expression), **b,** LKB1+++ (high expression), **c,** LKB1neg (null expression) and **d,** LKB1+ (modest expression), and right panel represents antibody control.

Supplemental Figure 2

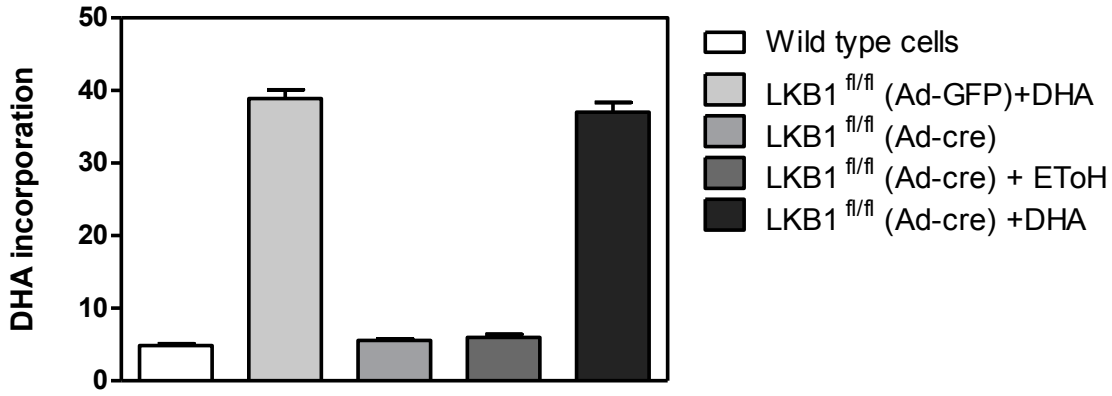

Figure S2 DHA incorporation. Mammary epithelial cells were infected with Ad-GFP and Ad-Cre following treatment with DHA and control (EtOH) for 24hrs. DHA incorporation was determined by lipidomics analysis performed in collaboration with Dr. David Ma.

Supplemental Table 1

Supplemental T1 Antibodies conditions. Antibodies conditions for each of the proteins used in this research. RT: room temperature, TBS: Tris Buffered Saline, TBST: Tris Buffered Saline Tween, BSA: Bovine Serum Albumin.

	Blocking	Primary	Wash	Secondary	Wash
Ley	1hr. at RT in TBS +1%milk	o/n at 4C, 1:5000 TBST + 1% BSA	3 x 10 min.	2 hr RT. 1:2000 TBST + 2% milk	3 x 10 min.
ACTIN	1hr. at RT in TBS +1%milk	o/n at 4C, 1:7000 TBST + 1% BSA	3 x 10 min.	2 hr RT. 1:2000 TBST + 2% milk	3 x 10 min.
AMPK	1hr. at RT in TBS +1%milk	o/n at 4C, 1:1000 TBST + 1% BSA	3 x 10 min.	2 hr RT. 1:2000 TBST + 2% milk	3 x 10 min.
pAMPK	1hr. at RT in TBS +1%BSA	o/n at 4C, 1:1000 TBST + 1% BSA	3 x 10 min.	2 hr RT. 1:2000 TBST +1%BSA	3 x 10 min.
AKT473	1hr. at RT in TBS +1%BSA	o/n at 4C, 1:1000 TBST + 1% BSA	3 x 10 min.	2 hr RT. 1:2000 TBST +1%BSA	3 x 10 min.
AKT308	1hr. at RT in TBS +1%BSA	o/n at 4C, 1:1000 TBST + 1% BSA	3 x 10 min.	2 hr RT. 1:2000 TBST +1%BSA	3 x 10 min.
ACC	1hr. at RT in TBS +1%milk	o/n at 4C, 1:2000 TBST + 1% BSA	3 x 10 min.	2 hr RT. 1:2000 TBST + 2% milk	3 x 10 min.
pACC	1hr. at RT in TBS +1%BSA	o/n at 4C, 1:1000 TBST + 1% BSA	3 x 10 min.	2 hr RT. 1:2000 TBST +1%BSA	3 x 10 min.
4EBP1/2	1hr. at RT in TBS +1%BSA	o/n at 4C, 1:1000 TBST + 1% BSA	3 x 10 min.	2 hr RT. 1:2000 TBST +1%BSA	3 x 10 min.
eIF4E	1hr. at RT in TBS +1%milk	o/n at 4C, 1:5000 TBST+ 1% BSA	3 x 10 min.	2 hr RT. 1:2000 TBST + 2% milk	3 x 10 min.
eIF4G	1hr. at RT in TBS +1%milk	o/n at 4C, 1:2000 TBST + 1% BSA	3 x 10 min.	2 hr RT. 1:2000 TBST + 2% milk	3 x 10 min.

Supplemental Table 2

Supplemental T2. Antibody conditions. Antibody conditions for each of the proteins analyzed in this research. RT: room temperature, TBS: Tris Buffered Saline, TBST: Tris Buffered Saline Tween, BSA: Bovine Serum Albumin.

	Blocking	Primary	Wash	Secondary	Wash
ERK	1hr. at RT in TBS +1%BSA	o/n at 4C, 1:1000 TBST + 1% BSA	3 x 5 min.	2 hr RT. 1:3000 TBST +1%BSA	3 x 5 min.
pERK	1hr. at RT in TBS +1%BSA	o/n at 4C, 1:1000 TBST + 1% BSA	3 x 5 min.	2 hr RT. 1:3000 TBST +1%BSA	3 x 5 min.
P90RSK	1hr. at RT in TBS +1%milk	o/n at 4C, 1:2000 TBST+ 1% BSA	3 x 5 min.	2 hr RT. 1:2000 TBST + 2% milk	3 x 5 min.
pP90RSK	1hr. at RT in TBS +1%BSA	o/n at 4C, 1:1000 TBST + 1% BSA	3 x 5 min.	2 hr RT. 1:2000 TBST +1%BSA	3 x 5 min.
HEX2	1hr. at RT in TBS +1%milk	o/n at 4C, 1:2000 TBST + 1% BSA	3 x 5 min.	2 hr RT. 1:2000 TBST + 2% milk	3 x 5 min.
LDH	1hr. at RT in TBS +1%milk	o/n at 4C, 1:5000 TBST + 1% BSA	3 x 5 min.	2 hr RT. 1:2000 TBST + 2% milk	3 x 5 min.
PDH	1hr. at RT in TBS +1%milk	o/n at 4C, 1:1000 TBST + 1% BSA	3 x 5 min.	2 hr RT. 1:2000 TBST + 2% milk	3 x 5 min.
PKM2	1hr. at RT in TBS +1%milk	o/n at 4C, 1:10000 TBST + 1% BSA	3 x 5 min.	2 hr RT. 1:2000 TBST + 2% milk	3 x 5 min.