Chapter 1: Introduction

1.1 Cancer

Cancer is a genetic disease characterised by uncontrolled cell division and growth. The oldest record of cancer is described by the Egyptians (3000-1500BC) in the "Edwin Smith" and "George Ebers" papyri. Hippocrates is credited with the origin of the word cancer and was the first to recognise the differences in benign (harmless growth unable to spread) and malignant (spread to and able to grow in other parts of the body) (460-370 B.C) which are described in the Hippocratic Corpus, a collection of ancient Greek medical writings. Progress on understanding cancer was slow until the discovery of DNA in 1953 by Francis Crick and James Watson (Watson & Crick, 1953). Since then there has been an explosion in understanding the cell biology and molecular mechanisms of cancer.

Cancer can either be hereditary or sporadic. Hereditary cancers are much rarer than sporadic cancers and arise due to alterations in the germline DNA with contribution from subsequent acquisition of somatic mutations that give rise to neoplasm's in tissues at risk. Sporadic cancers arise solely from the accumulation of somatic changes during the lifetime of an individual. These changes can occur via a number of different mechanisms - mutations, which can be as small as a single nucleotide changes, small or large scale insertions or deletions of DNA, amplification of areas of chromosomes, loss or gain of chromosomes, translocation of separate chromosomal regions resulting in the inappropriate fusion of genes and epigenetic changes such as DNA methylation. More than 300 genes have been causally implicated in cancer via structural rearrangements (Futreal, et al. 2004; http://www.sanger.ac.uk/genetics/CGP/Census/) many of these encode proteins

which are important for regulating cell growth and division or for repairing DNA, alteration of which can lead to an increased rate of somatic mutation. Somatic cancers are usually caused by the accumulation of mutations over time and not just one single mutation, a process known as somatic evolution. Once cancer begins, cells which acquire new mutations undergo natural selection just as animal populations do. If this mutation provides the cells with a growth advantage or enhanced survival they become the dominant cells of the tumour (Nowell, 1976).

Cancer genes can be broadly classified into two categories: oncogenes or tumour suppressor genes. Oncogenes are abnormally activated versions of genes which generally code for proteins important for instructing cells to grow, divide, survive or proliferate. Mutations in these therefore lead to abnormal activation instructing the cells to permanently divide. Tumour suppressors normally regulate processes that regulate cell growth, death and differentiation. Therefore mutations which lead to inactivation or loss of the tumour suppressor protein result in the “brakes” being removed from cell division allowing the cell to continuously divide.

1.1.1 Lung cancer

Cancer Research UK identifies lung cancer as the second most common cancer in the UK with more than 38000 people diagnosed each year. For women in the UK it is the third most common cancer after breast and bowel cancer and for men it is the second most common after prostate cancer. In 2002 1.3 million people worldwide were diagnosed with lung cancer (Ferlay, et al. 2007). Around the world incidences of lung cancer vary widely, for men the highest incidences for lung cancer are in North America and Central and Eastern Europe, whilst for women the highest incidence is in North America and Northern Europe. In the UK there is a North-South divide for lung cancer incidence with higher prevalence in Scotland and

Northern England and lower in Southern England and Wales, estimated by the Office for National Statistics (Quinn, et al. 2005). In the UK 34500 people die each year from lung cancer making it the most common cause of cancer death, accounting for over 20% deaths due to the disease. The link between smoking and lung cancer was identified almost 50 years ago and it has been estimated that smoking is responsible for 90% of lung cancer deaths. Other risk factors for lung cancer can include poor diet, air pollution and exposure to industrial carcinogens (polycyclic aromatic hydrocarbons, asbestos and diesel exhaust fumes). The survival rates have changed very little over the years with only 7% of people diagnosed surviving beyond 5 years (Coleman, et al. 2004).

There are two main histological types of lung cancer small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC accounts for almost 80% of all cases whilst SCLC accounts for the remaining 20% (Marby, et al. 1998). NSCLC can be further divided into the histological subtype's squamous cell carcinoma, adenocarcinoma and large cell carcinoma (Ginsberg, et al. 1997). Tumours are graded by stage with stage I being the least advanced and most localised tumour and stage IV the most advanced with distant metastasis. The survival rates vary hugely with 60-70% of patients with stage IA NSCLC surviving beyond five years, 57% of stage IB but this drops to 1% of patients diagnosed with stage IV. Surgical resection with radiotherapy is the best option in the early stages; patients with later stage NSCLC are often offered chemotherapy but there is only marginal efficacy (Mountain, et al. 1997). Because NSCLC is mainly asymptomatic in the early stages, half of all patients reach stage IV before diagnosis. There is therefore a need for better diagnosis and improved treatment options, including targeted therapies.

Molecular genetic studies have shown that multiple genetic loci contribute to the pathogenesis of sporadic NSCLC. Several inherited cancer syndromes also increase

the risk of lung cancer through rare germline mutations in *EGFR* (T790M mutation associated with acquired drug resistance) (Bell, et al. 2005) and *TP53* (Hwang, et al. 2003). Important events in the development of sporadic NSCLC include mutations in *CDKN2A, TP53, KRAS* and *EGFR* (http://www.sanger.ac.uk/genetics/CGP/cosmic/). Common regions of loss of heterozygosity (LOH) include 3p21.3 (RASSF1A, Ras association domain family member), 9p21 (CDKN2A/p16), 17p13 (TP53) and 3p14.2 (FHIT, fragile histidine triad gene) (Wistuba, et al. 2002). Recently a subset of NSCLC was identified which contained an *EML4-ALK* fusion gene (Soda, et al. 2007). *EGFR* is overexpressed in approximately 60% of NSCLC and its expression has correlated with poor prognosis (Ohsaki, et al. 2000; Nicholson, et al. 2001; Hirsch, et al. 2003). Somatic mutations in *EGFR* occur only in approximately 10% of NSCLC cases of North American or Western European descent (Koaska, 2004; Janne, 2005) but have been shown to be important predictors of response to EGFR-tyrosine kinase inhibitors (TKIs), gefitinib and erlotinib in NSCLC (Paez, et al. 2004; Lynch, et al. 2004). *KRAS* is known to be mutated in 20-30% of cases (Reynolds, et al 1991; Reynolds, et al. 1992; http://www.sanger.ac.uk/genetics/CGP/cosmic/). The presence of KRAS mutations has been is associated with insensitivity to EGFR-TKI (Pao, et al. 2005). *MYC* amplification and overexpression has been identified in 10-20% of NSCLC (Gazzeri, et al. 1994), whilst *P53* mutations have been observed in approximately 50% (Gazzeri, et al. 1994; http://www.sanger.ac.uk/genetics/CGP/CellLines/). *LKB1(STK11)* is a tumour suppressor serine/threonine kinase and is mutated in approximately 30% of NSCLC and rarely in other cancers (Sanchez-Cespedes, et al. 2002; Zhong, et al. 2006; Matsumoto, et al. 2007; Strazisar, et al. 2009; http://www.sanger.ac.uk/genetics/CGP/cosmic/).

1.2 LKB1/STK11

Liver kinase B1 (*LKB1)* was first identified in 1996 by Jun-ichi Nezu of Chugai Pharmaceuticals in a screen designed to identify new kinases. It is highly related to *XEEK1* a substrate for cyclic-Amp dependent protein kinase A (PKA), expressed in the early Xenopus embryo (Su, et al. 1996). In the past decade there has been an explosion in the literature regarding this kinase, in this part of my introduction I will give a brief overview of LKB1 and its roles in cancer.

1.2.1 Role of LKB1 in cancer

1.2.1.1 LKB1 in hereditary cancer

LKB1(STK11) is located on chromosome 19p, this location was first implicated in the hereditary cancer syndrome Peutz-Jeghers Syndrome (PJS) in 1997 (Hemminki, et al. 1997). PJS is characterised by benign hamartomatous polyps especially in the gastrointestinal (GI) tract and marked cutaneous pigmentation of the mucous membranes. The gene responsible for the disease was identified as *LKB1*, after it was found to contain truncating germline mutations in 11 out of 12 PJS families (Hemminki, et al. 1998). Studies using *Lkb1* (+/-) mice revealed that development of hamartomas in the GI tract was not due to LOH of the wild type allele but due to haploinsufficiency (Miyoshi, et al. 2002). A study of PJS patient polyps showed a minority had LOH of *LKB1*, all carcinomas in the study showed LOH of *LKB1* (Entius, et al. 2001). PJS was first described in 1922 by Dr. Johannes Peutz and further described by Dr. Harold Jeghers in 1944 (Peutz 1921; Jeghers 1944; Jeghers, et al. 1949). PJS is a rare autosomal dominant disease and estimates of the incidences range from 1 in 10000 births to 1 in 12000 births (Mallory, et al. 1987; Hemminki, 1999). Patients with PJS have an increased risk of developing malignant tumours in

multiple tissues including; small intestine, colorectal, ovaries, cervical and breast (Tomlinson, et al. 1997; Hemminki 1999; Westerman, et al. 1999; Giardiello, et al. 2000; Mehenni 2006). It is estimated that approximately 93% of PJS patients develop malignant tumours by the age of 43 (Giardiello, et al. 2000) which is thought to be triggered by somatic loss of the second functional allele (Hemminki, et al. 1998). The vast majority of PJS patients are familial however 10- 20% of case arise from *de novo* germline mutations (Boardman, et al. 2000)

1.2.1.2 LKB1 in somatic cancers

Due to the large number of PJS patients who go on to develop malignant tumours, many groups have studied *LKB1* loss in sporadic tumours. The highest incidence of somatic mutations occur in lung cancer where *LKB1* is mutated in approximately 30% of NSCLC (Sanchez-Cespedes, et al. 2002; Zhong, et al. 2006; Matsumoto, et al. 2007; Strazisar, et al. 2009; http://www.sanger.ac.uk/genetics/CGP/cosmic/). *LKB1* mutation has also been found at a lower prevalence in other cancers. Although somatic mutations are absent or extremely rare in breast cancer (Bignell, et al. 1998) immunohistochemistry analysis has revealed loss of LKB1 protein expression in a subset of high grade in situ and breast carcinomas (Fenton, et al. 2006). LKB1 has also been suggested to be a prognostic marker for breast cancer after an association of LKB1 loss with higher histological grade, larger tumour size, worse overall survival, higher relapse rate and presence of lymph node metastases was found (Shen, et al. 2002; Fenton, et al. 2006). Studies of LKB1 protein expression in pancreatic cancer have shown loss of LKB1 expression in between 4-7% of adenocarcinomas (Su, et al. 1999; Sahin, et al. 2003) as well as 27% of intraductal papillary mucinous neoplasms (Sahin, et al. 2003). More recently almost 20% of cervical cancers were found to harbour somatic inactivating mutations in *LKB1* and this has been associated with poor prognosis (Wingo, et al. 2009).

1.2.2 LKB1 structure and function

LKB1 is a serine/threonine kinase with only one human isoform spanning 23kb in the genome comprised of 10 exons, 9 of which code for a 433 amino acid protein. The protein consists of a catalytic domain and non-catalytic N and C-termini. The catalytic domain is distantly related to other protein kinases, whereas the N and C-termini are unrelated to other kinases. LKB1 is expressed in most foetal and adult tissues at varying levels (Hemminki, et al. 1998; Jenne, et al. 1998).

LKB1 is phosphorylated by a number of upstream kinases on; ser-325, thr-366 and ser-431, and can also autophosphorylate, ser-31, thr-185, thr-189, thr-336, ser-404 (Sapkota, et al. 2001 & 2002; Boudeau, et al. 3003). Experiments to mutate these autophosphorylation sites to either glutamine to mimic phosphorylation or alanine to abolish phosphorylation had very little effect apart from residue thr-336. When mutated to glutamate it prevented LKB1 from inhibiting cell growth in G361 melanoma cells (Sapkota, et al. 2002). ATM phosphorylates LKB1 thr-366 in response to DNA damage (Sapkota, et al. 2002; Fernandes, et al. 2005), 90kDa ribosomal S6 kinase (RSK) and protein kinase A (PKA) phosphorylate ser-431 and mutation of this residue also prevents LKB1 from inhibiting the growth of G361 cells (Sapkota, et al. 2001). AMP-activated kinase (AMPK) has been shown to phosphorylate ser-31 (Sapkota, et al. 2002). More recently it has been found that LKB1 is also phosphorylated on ser-307 by protein kinase C-zeta (PKCzeta) which directs nucleocytoplasmic transport of LKB1 with AMPK activation as a consequence, and suppression of apoptosis and angiogenesis (Xie, et al. 2009).

Affinity purification and yeast two-hybrid studies identified two proteins in complex with LKB1, STE-20-related adaptor (STRAD) and mouse protein 25 (MO25) (Baas, et al. 2003; Boudeau, et al. 2003; Brajenovic, et al. 2003). STRAD is a LKB1-specific

adaptor protein and substrate with a STE20-like kinase domain (Baas, et al. 2003). There are two isoforms of STRAD - STRADα and STRADβ which are catalytically inactive due to lacking key residues in the kinase domain. STRADα in particular has been tested for activity and has not shown phosphorylation activity towards any substrates so far (Baas, et al. 2003). MO25 is an armadillo repeat scaffolding-like protein which also exits as two isoforms - MO25α and MO25β. These two proteins are critical for localisation of LKB1 within a cell (Boudeau, et al. 2003). Overexpressed on its own, LKB1 localises mainly to the nucleus due to the nuclear localisation signal (NLS) in its N-terminus, with a small proportion in the cytoplasm. Mutation of the NLS leads to LKB1 redistribution throughout the cell (Sapkota, et al. 2001; Smith, et al. 1999). However cytoplasmically located LKB1 still retains its ability to suppress cell growth indicating the importance of the cytoplasm pool in tumour suppressor activity (Tainan, et al. 2002). When expressed with STRADα and MO25α, LKB1 is activated and localises to the cytoplasm (Baas, et al. 2003; Boudeau, et al. 2003; Boudeau, et al. 2004). A number of *LKB1* point mutations in human cancer affect the ability of the protein to interact with STRADα and MO25α (Boudeau, et al. 2004). This was demonstrated by a *LKB1* mutant which was unable to bind STRADα and failed to induce growth arrest when overexpressed in G361 cells (Baas, et al. 2003). Knockdown of *STRADα* in cells expressing wild type LKB1 also prevented LKB1 from inducing growth arrest (Baas, et al. 2003). Together these data show that STRADα binding to LKB1 is required for LKB1 to induce growth arrest. Recently it has been found that the ability of STRADα to activate LKB1 is dependent on ATP binding as well as MO25α binding (Zeqiraj, et al. 2009). Binding of ATP converts STRADα to a closed conformation with an ordered activation loop and it is this closed "active" conformation that mediates the LKB1 activation rather than kinase activity (Zeqiraj, et al. 2009).

1.2.3 LKB1 is a multi-tasking tumour suppressor kinase

LKB1 phosphorylates at least fourteen closely related serine/threonine kinases; AMPKα1, AMPKα2, NUAK1, NUAK2, SIK1, SIK2, QSK, MARK1, MARK2, MARK3, MARK4, BRSK1, BRSK2 and SNRK (Lizcano, et al. 2004; Jaleel, et al. 2005). LKB1 phosphorylates the T-loop activation segment of all these kinases and increases the activity > 50-fold in the presence of STRADα and MO25α (Lizcano, et al. 2004). The first to be discovered and perhaps the best understood is AMPK (Hawley, et al. 2003; Shaw, et al. 2004a; Woods, et al. 2003). This large number of substrates implicates LKB1 in diverse processes such as; energy sensing, metabolism, cell polarity, cell growth and viability, and protein synthesis.

1.2.4 LKB1 and cell polarity

LKB1 regulates epithelial and neuronal cell polarity through phosphorylation of AMPK, MARKs and BRSKs. Initial work was carried out in *C.elegans* with the worm homologue of LKB1 *par4* (Watts, et al. 2000). *Par4* was discovered to be necessary for cell polarity in the first cycle of embryogenesis, loss of function mutants displayed disruption in the asymmetries which are established in the first cycles of embryogenesis (Kemphues, et al. 1988). Further study revealed that mutations in *par4* affect a number of aspects of cell polarity (Morton, et al. 1992). The work in *C.elegans* also led to the discovery of downstream components of PAR4 signalling including PAR1 (MARK) (Kemphues, et al. 1988). In *Drosophila*, mutation of *lkb1* leads defects in anterior-posterior axis formation and epithelial polarity defects (Martin, et al. 2003). More recent work in *Drosophila* using *ampk* mutant and *lkb1* null flies has shown that epithelial cells lose polarity and hyperproliferate under energetic stress (Mirouse, et al. 2007). This work suggested that LKB1 signals through AMPK to regulate epithelial cell polarity and this perhaps connects cellular

energy sensing pathways to cellular structure (Mirouse, et al. 2007). Further evidence linking energy sensing to cell structure via AMPK has come from additional *C.elegans* studies. Acetyl coA carboxylase (ACC) regulates fatty acid synthesis and is phosphorylated by AMPK (Carling, et al. 1987; Carling, et al. 1989; Davies, et al. 1990). Mutation of the *C.elegans* homologue of *ACC pod2*, results in disruption of embryo polarity similar to that resulting from *par4* inactivation, revealing that fatty acid pathways are required for polarity and that LKB1/AMPK signalling are likely key to this process (Rappley, et al. 2003).

The role of LKB1 in mammalian cell polarity was first demonstrated by Baas et al. in 2004. They demonstrated that activation of LKB1 resulted in repolarisation of an unpolarised intestinal epithelial cell via actin cytoskeleton re-organisation, leading to formation of an apical brush border (Bass, et al. 2004). In mice, LKB1 was demonstrated to play a key role in establishing epithelial cell polarity in the pancreatic epithelium. Targeted *LKB1* deletion led to abnormal cystokeletal organisation, defective acinar cell polarity, loss of tight junctions and at the molecular level, inactivation of AMPK/MARK/BRSK (Hezel, et al. 2008). In addition it was shown to be important for pancreatic acinar cell function and viability and suppression of neoplasia (Hezel, et al. 2008). Recent efforts have concentrated on elucidating the pathways acting downstream of LKB1 in cell polarity. In particular, a study in NSCLC cells found that LKB1 is associated with actin and polarises to the leading edge of motile cancer cells where it co-localises with CDC42 and PAK (Zhang, et al. 2008). Paradoxically, mice heterozygous for a *lkb1* null allele spontaneously developed highly invasive endometrial adenocarcinomas which displayed normal cell polarity in spite of alterations in AMPK signalling (Contreras, et al. 2008) suggesting that polarity effects alone are not responsible for LKB1 tumour suppressor activity.

1.2.5 The role of LKB1 in energy sensing and metabolism through AMPK regulation

LKB1 is the major AMPK regulator (Hawley, et al. 2003; Shaw, et al. 2004a; Woods, et al. 2003). AMPK is a heterotrimeric protein, consisting of a catalytic α-subunit which contains the key phosphorylation site for LKB1 threonine-172 (thr-172) required for activation. The β and γ subunits are regulatory subunits (Figure 1-1). At the cellular level under conditions of energetic stress, when ATP levels drop and AMP levels rise, AMPK becomes activated by the binding of AMP to the γ-subunit, preventing dephosphorylation of thr-172 (Hardie, et al. 1999; Kahn, et al. 2005; Sanders et al. 2007). AMPK activation acts as a metabolic checkpoint when cells are under stresses such as hypoxia or nutrient deprivation. AMPK then activates ATP-generating processes and suppresses cell growth and ATP-consuming processes such as biosynthesis (Figure 1-1) (Shaw, et al. 2004b). AMPK acts not only at the cellular level but due to its roles in glucose and lipid metabolism in specialised tissue such as muscle and liver it plays an important role in the bioenergetics of the whole organism (Kahn, et al. 2005). LKB1 is therefore implicated in various metabolic disorders including obesity, diabetes and metabolic syndrome. All of these are associated with an increased risk of cancer through mechanisms which are not yet well understood (Lou, et al. 2005).

AMPK influences many aspects of metabolism through direct and indirect regulation of enzymes involved in protein synthesis (discussed later), fatty acid metabolism, glucose homeostasis and mitochondrial biogenesis. With LKB1 as the master regulator of AMPK (Hawley, et al. 2003) it therefore follows that LKB1 influences these processes. It should be noted however that *LKB1*-deficient cells still have some basal activity of AMPK and low level of phosphorylation at thr-172 (Hawley, et al. 2003; Woods, et al. 2003; Carretero, et al. 2007). There are two isoforms of AMPK α1 and α2 and recent data suggests that the different isoforms of AMPK have

distinct functions and are regulated differently *in vivo*, with the AMPKα2 isoform playing the main role in metabolic adaptation (McGee, et al. 2008). During ischemia LKB1 has been shown to preferentially phosphorylate the α2 isoform of AMPK (Sakamoto, et al. 2006). There are other kinases found to phosphorylate and activate AMPK. One such kinase is Ca^{2+}/CaM-dependent protein kinase kinase (CaMKK) after significant basal activity and phosphorylation of AMPK in LKB1-deficient cells stimulated by Ca^{2+} ionophores was found implicating CAM kinases (Hawley, et al. 2005). It has also been found that transforming growth factor-beta-activated kinase (TAK1), a member of the MAPK3 family also phosphorylates AMPK on thr-172 (Momcilovic, et al. 2006) and that this phosphorylation plays an important role in energy sensing and cellular metabolism in mice (Xie, et al. 2006).

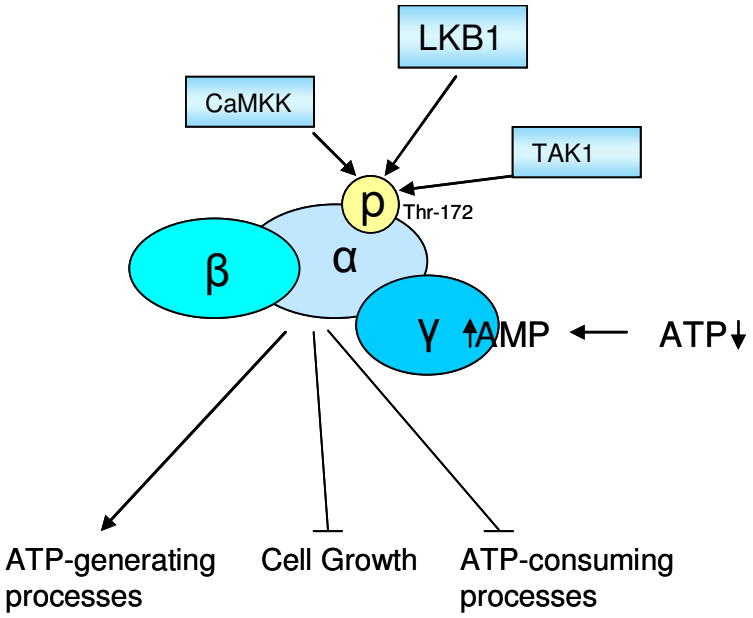

Figure 1-1. AMPK structure and regulation. AMPK is a heterotrimeric protein which consists of a catalytic α-subunit, and regulatory β- and γ-subunits. AMP levels rise under conditions of stress and bind to the γ-subunit of AMPK. This prevents the catalytic subunit from being dephosphorylated. LKB1 is the major regulator of phosphorylation of thr-172 on the AMPK α-subunit, other activators are thought to be Ca^{2+}/CaM-dependent protein kinase kinase (CaMKK) and transforming growth factor-beta-activated kinase (TAK1). Active AMPK switches off ATP-consuming processes and inhibits cell growth whilst promoting ATP-generating processes such as fatty acid oxidation.

1.2.5.1 LKB1/AMPK signalling in liver and gluconeogenesis

Gluconeogenesis is the process by which hepatic glucose production maintains glucose supply under low nutrient conditions to the brain and red blood cells. It is activated by the production of glucagon from α-pancreatic cells which stimulates transducer of regulated CREB activity 2 (TORC2) to translocate to the nucleus of hepatic cells (Koo, et al. 2005). Binding of TORC2 to CREB activates CREB and induces peroxisome proliferator-activated receptor-{gamma} coactivator 1α (PGC1α) which leads to transcription of genes involved in the energetically demanding process of gluconeogenesis (Yoon, et al. 2001; Herzig, et al. 2001). If ATP levels drop sufficiently or the cells are under stress, AMPK is activated and can override the glucagon/fasting signals and suppress gluconeogenesis (Long, et al. 2006) by sequestering TORC2 in the cytoplasm (Koo, et al. 2005). Deletion of *LKB1* in the livers of mice resulted in decreased AMPK activation, indicated by lack of phosphorylation and hyperglycemia (Shaw, et al. 2005). It also resulted in increased expression of a number of gluconeogenic and lipogenic genes, in particular PGC1α. In the livers of the *LKB1* knockout mice TORC2 was located mainly in the nucleus and its phosphorylation levels were lower. AMPK and another LKB1 regulated kinase salt-induced kinase (SIK) are responsible for phosphorylation of TORC2 which results in its retention in the cytoplasm, loss of *LKB1* in the liver results in decreased AMPK activity, TORC2 relocalisation to the nucleus and increased expression of gluconeogenic genes via CREB and PGC1α (Shaw, et al. 2005)

1.2.5.2 LKB1/AMPK signalling in fatty acid metabolism and cholesterol biosynthesis

One of the best characterised downstream targets of AMPK in fatty acid metabolism is acetyl coA carboxylase (ACC). Phosphorylated ACC is often used as a marker for AMPK activity (Trumble, et al. 1995; Winder, et al. 1996; Winder, et al. 1997). Phosphorylation of ACC by AMP-bound activated AMPK inhibits ACC, preventing the production of malonyl-coA the allosteric inhibitor of the rate-limiting enzyme of fatty acid oxidation carnitine palmitoyltransferase 1(CPT1) (Ruderman. et al. 1999). AMPK restores the energy balance through inhibition of ACC, reducing the levels of malonyl-coA, thus allowing CPT1 to transport the long-chain acyl-coA into the mitochondria for oxidation (Figure 1-2) (Merrill, et al 1997; Merrill, et al. 1998). Comparing mice with skeletal/cardiac muscle specific knockout of *Lkb1* to wild type mice, loss of *Lkb1* has been shown to directly affect the malonyl-coA levels and fatty acid oxidation, an effect not observed in the wild type mice (Thomson, et al. 2007). In addition to phosphorylation of direct targets AMPK can also regulate the expression of a number of genes associated with metabolism. Fatty acid synthase is a key enzyme associated with lipogenesis and AMPK can suppress the glucose induced expression of fatty acid synthase through suppression of the expression of the lipogenic transcription factor SREBP-1 (Zhou, et al. 2001). In addition it also regulates expression of pyruvate kinase and ACC (Foretz, et al. 1998; Woods, et al. 2000). Studies in *C.elegans* have highlighted the importance of LKB1/AMPK signalling in diapause (physiological state of dormancy). When *C.elegans* larvae enter dauer (a form of stasis which allows them to survive harsh conditions), they arrest feeding yet remain active and motile and are stress resistant with an extended lifespan. Mutants of *LKB1* or *AMPK* enter dauer normally but show premature death after rapidly consuming stores of triglycerides (Narbonne, et al. 2009). The slow-release of triglycerides is essential for survival through dauer and LKB1/AMPK signalling acts to ration the reserves of triglycerides in adipose tissues. AMPK also

inhibits cholesterol synthesis pathways by inhibiting the rate-limiting enzyme HMG-coA reductase (Hardie, et al. 1989; Sato, et al. 1993; Henin, et al. 1995).

1.2.5.3 AMPK signalling in skeletal muscle and mitochondrial biogenesis

AMPK signalling plays a vital role in skeletal muscles during exercise. It is activated by muscle contraction and can mediate glucose uptake in an insulin-independent manner as demonstrated in patients with Type 2 diabetes (Koistinen, et al. 2003). It also increases fatty acid oxidation which leads to a decrease in intramyocyte lipid accumulation and increases the sensitivity of muscles to insulin (Ruderman, et al. 2003). Chronic activation of AMPK either by the agonist 5-amino-imidazole carboxamide riboside (AICAR) or by exercise training results in transcriptional activation of mitochondrial β-oxidation enzymes via PGC1-α (Suwa, et al. 2003), the expression of the enzyme hexokinase and glucose transporter GLUT4 and increased levels of glycogen (Holmes 1999). In rodent skeletal muscle AMPK targets the transcription factors nuclear respiratory factor 1 (NRF-1) and phosphorylates PGC1α which together regulate the expression of mitochondrial genes (Bergeron, et al. 2001; Zong, et al. 2002; Puigserver, et al. 2003; Jager, et al. 2007). AMPK activation in muscle also leads to the increase in NAD^+ levels and the deacetylation and activation of PGC1-α and FOXO1 by SIRT1 (Canto, et al. 2009). AMPK also increases the density of mitochondria in response to chronic energy deprivation (Bergeron, et al. 2001; Zong, et al. 2002).

1.2.5.4 AMPK as a therapeutic target

Due to its role in various metabolic pathways including glucose and lipid homeostasis, AMPK has become a therapeutic target for type 2 diabetes, various metabolic disorders and more recently cancer. Metformin and AICAR are AMPK agonists and widely used in experimental settings to study AMPK signalling. Metformin is also used in the clinical setting to treat type 2 diabetes. On average patients with type 2 diabetes display three times the normal level of gluconeogenesis, and metformin reduces this by a third (Hundal, et al. 2000). A Phase I study of metformin in combination with temsirolimus (an mTOR inhibitor) is being trialed in advanced solid tumours including breast, endometrial, kidney and lung. In 2001 the mode of action of metformin was found to be through activating AMPK (Zhou, et al. 2001) and in liver its action has been shown to require LKB1 (Shaw, et al. 2005). Thiazolidinediones (TZDs) are activators of peroxisome proliferator activated receptor γ (PPARγ) but more recently have been shown to activate AMPK in a mode independent of PPARγ transcription (LaBrasseur, et al. 2006). There are a number of other natural compounds and hormones which activate AMPK including leptin, interleukin-6, cannabinoids and α-lipoic acid.

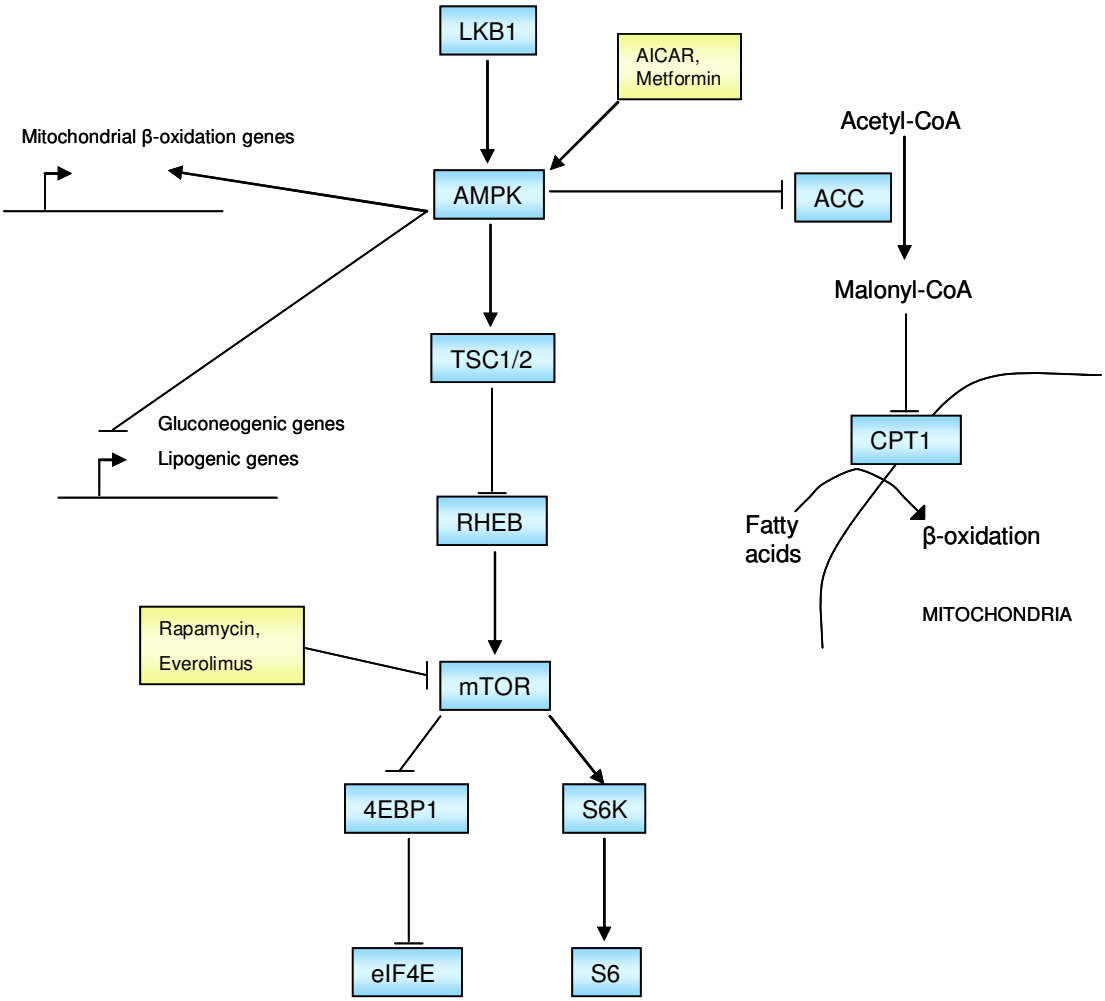

Figure 1-2. A simplified overview of the known components of the LKB1/AMPK/mTOR pathways. LKB1 signals to activate AMPK under conditions of stress. AMPK inhibits translation and protein synthesis through inhibition of mTOR signalling. AMPK also enhances β-oxidation of fatty acids through inhibition of acetyl-coA carboxylase (ACC), promotes transcription of β-oxidation genes and represses transcription of gluconeogenic and lipogenic genes. In yellow boxes are targeted compounds. For references see main text.

1.2.6 LKB1/AMPK signal to control protein synthesis and translation through mTOR

Perhaps the best understood role of AMPK is negative regulation of mTOR (mammalian target of rapamycin). mTOR is an important regulator cell growth. It is a central downstream component of both nutrient and growth factor signalling pathways (Figure 1-2). Upstream of mTOR are a number of tumour suppressors including LKB1, PTEN, TSC1/2, and NF1 which are mutated in various hamartoma syndromes. AMPK phosphorylates and activates tuberin (TSC2) which forms part of the tuberous sclerosis complex, consisting of the tumour suppressors TSC2 and its binding partner hamartin (TSC1). Downstream of TSC1-2 is RAS homolog enriched in brain (RHEB), TSC2 inactivates RHEB through its GTPase-activating protein domain (Tee, et al. 2003a), and RHEB has been shown to activate mTOR (Garami, et al. 2003). AMPK exerts its effects on mTOR through TSC2 activation leading to inhibition of RHEB and decreased activity of mTOR. The TSC1-2 complex is central hub for inputs from a number of different signalling pathways besides AMPK, which include PI3-kinase (PI3K) and MAPK. Inactivation of *PTEN* in Cowden disease leads to constitutive activation of PI3K signalling and inhibition of TSC2 by AKT phosphorylation (Inoki, et al. 2003; Tee, et al. 2003b). ERK through direct and indirect mechanisms also inactivates TSC2 on sites distinct to those by AMPK and AKT (Roux, et al 2004; Ma, et al. 2005). Mutations in *NF1* are responsible for Neurofibromatous Type 1 (Xu, et al. 1990), loss of NF1 leads to constitutive activation of RAS signalling and as a consequence PI3K signalling, both of which feed into the mTOR pathway. Loss of LKB1, TSC1/2, PTEN or NF1 all lead to hyperactivation of RHEB and constitutively active mTOR in various hamartoma syndromes (Rosner, et al. 2008).

1.2.6.1 mTOR

Rapamycin was first identified from a soil sample from Easter Island in the 1970's. The bacterial strain *Streptomyces hygroscopicus* in the soil produced an anti-fungal metabolite, later identified and named rapamycin. As well as immunosuppressive properties rapamycin was found to inhibit the proliferation of mammalian cells. Utilising yeast mutagenesis screens the cellular target of rapamycin was identified as TOR (Heitman, et al. 1991). mTOR is a serine/threonine kinase which regulates a number of cellular processes including survival, proliferation, protein synthesis, transcription and motility (reviewed in Dunlop, et al. 2009) mTOR exists as two complexes mTORC1 and mTORC2 which differ in their binding partners and sensitivity to rapamycin (Loewith, et al. 2002). mTORC1 is a rapamycin-sensitive complex consisting of mTOR, GβL, RAPTOR, mLST8 and PRAS40 (Kim, et al. 2002; Kim, et al. 2003; Wang, et al. 2007). mTORC2 is a rapamycin-insensitive complex consisting of mTOR with RICTOR, MSIN1, mLST8 and PROTOR (Sarbassov, et al. 2004; Jacinto, et al. 2004; Yang, et al. 2006; Frias, et al. 2006; Jacinto, et al. 2006; Guertin, et al. 2006; Pearce, et al. 2007). Whilst mTORC1 is involved in the regulation of cell growth via protein synthesis, translation and autophagy in response to cellular energy status and nutrient levels, mTORC2 is not. mTORC2 appears to be directly activated by receptor tyrosine kinases and phosphorylates and activates AKT (Sarbassov, et al. 2005) and the more recently discovered serum glucocorticoid-induced kinase (SGK) (Garcia-Martinez, et al. 2008; Jones, et al. 2009; Soukas, et al. 2009). mTORC2 may also sit upstream of the rho GTPases and regulate the actin cytoskeleton (Jacinto, et al. 2004). mTORC2 may also play a role in fat metabolism and longevity in *C.elegans* (Jones, et al. 2009; Soukas, et al. 2009).

1.2.6.2 Role of mTOR in translation and protein synthesis

Some of the best understood downstream targets of mTOR are the p70S6 kinases (RPS6KB1 (S6K1) and RPS6K2 (S6K2)) and the eukaryotic initiation factor 4E-binding proteins 1, 2 and 3 (4E-BP1, 2 and 3) (Brown, et al. 1995; Brunn, et al. 1997; Hara, et al. 1997). These proteins share a common motif the TOR signalling motif (TOS) which is essential for the binding of RAPTOR, a scaffold protein for TOR phosphorylation of substrates (Schalm, et al. 2003; Choi, et al. 2003; Nojima, et al. 2003). 4E-BP's in their unphosphorylated forms are bound to eukaryotic initiation factor 4E (eIF4E) at the 5' end of mRNAs and prevent translation. Phosphorylation by mTOR on four serine/threonine residues results in the disassociation of 4E-BP1 from eIF4E and subsequent translation (Lin, et al. 1995; von Manteuffel, et al. 1996). eIF4E has been shown to have increased expression in breast and prostate cancers and its expression correlates with patient survival (Graff, et al. 2009; Coleman, et al. 2009). p70S6K and 4E-BP1 co-operate to control cell size and mediate mTOR-dependent cell cycle control (Fingar, et al. 2002; Fingar, et al. 2004).

p70S6K has been found to phosphorylate SKAR (polymerase (DNA-directed), delta interacting protein 3) (Richardson, et al. 2004), a nuclear protein which has been proposed to couple transcription with pre-mRNA splicing and mRNA export (Richardson, et al. 2004). Other p70S6K targets include eIF4B which plays a critical role in recruiting the 40S ribosomal subunit to the mRNA to increase its association with the eukaryotic translation initiation factor 3 (Raught, et al. 2004; Shahbazian, et al. 2006), and the eukaryotic elongation factor 2 kinase (eEF2K). Phosphorylation and inactivation of eEF2K aids in the elongation phase of protein synthesis through activation of eEF2 (Wang, et al. 2001).

1.2.6.3 mTOR as a therapeutic target

Rapamycin is an immunosuppressant drug used to prevent rejection in organ transplantation. Rapamycin and its derivatives have since been used in clinical trials for a variety of malignancies with mixed outcomes possibly highlighting the complexity of mTOR signalling. Some patients have benefited from mTOR inhibition but results have largely been variable. Everolimus (RAD001) was found to prolong progression-free survival in patients with metastatic renal cell carcinoma who had progressed on other targeted therapies (Motzer, et al. 2008) and was approved for treatment of advanced kidney cancer earlier this year. However in gemcitabine-refractory metastatic pancreatic cancer treatment with everolimus yielded no complete or partial responses (Wolpin, et al. 2009). In a trial of RAD001 in advanced B-cell chronic lymphocytic leukaemia patient's treatment was stopped after severe toxicity with everolimous was observed (Decker, et al. 3009). A trial investigating rapamycin activity in *PTEN*-deficient glioblastoma multiforme patients showed a decrease in tumour cell proliferation in half of patients after a week, however in some patients AKT activation was observed and this led to a shorter time to progression (Cloughesy, et al. 2008). RAD001 has been shown to restore gefitinib sensitivity in resistant NSCLC cell lines (Milton, et al. 2007; LaMonica, et al. 2009) and this combination of gefitinib and RAD001 has entered phase II trials for patients with advanced NSCLC (Milton, el. 2007). RAD001 has recently shown modest clinical activity in advanced NSCLC previously treated with chemotherapy or chemotherapy with EGFR inhibitors (Soria, et al. 2009).

1.2.7 Role of LKB1 in proliferation and apoptosis

PJS patients develop polyps. These polyps are likely to arise from a shift in balance between apoptosis and proliferation in the cells. There is evidence for the role of LKB1 in both proliferation and apoptosis, however recently this has been weighted towards a role in proliferation.

The role of LKB1 in apoptosis remains unclear. It has been shown to physically interact with p53 and mediate the expression of p53 dependent genes including p21/WAF (Karuman, et al. 2001; Zeng, et al. 2006). LKB1 has also been found to be phosphorylated by ATM, which mediates a DNA damage checkpoint and p53-dependent apoptosis following radiation induced DNA damage (Sapkota, et al. 2002). Work in *LKB1*-deficient mouse embryonic fibroblasts revealed that in spite of a lack of AMPK activation, they are hypersensitive to apoptosis induced by energy stress (Shaw, et al. 2004b). It has also been suggested that LKB1 may play an antiapoptotic role in cells with constitutively active AKT, after it was found that suppression of LKB1 in cells with activated AKT resulted in apoptosis (Zhong, et al. 2008). In addition, it was found that there is a requirement for LKB1 in AKT mediated phosphorylation in a number of proteins which suppress apoptosis including FOXO3A, ASK1, BAD, FOXO1, FOXO4 and GSK3β (Zhong, et al. 2008). Further LKB1 expression in osteosarcoma cells was found to lead to TRAIL-induced apoptosis through association with DAP3 (Takeda, et al. 2007).

LKB1 appears to play a key role in regulating cell proliferation. It has been known for many years that reintroducing wild type LKB1 into *LKB1*-null cancer cell lines results in G1 cell cycle arrest (Tiainen, et al. 1999; Sapkota, et al. 2001; Shen, et al. 2002; Jimenez, et al. 2003; Qiu, et al. 2006). Introduction of kinase dead mutants showed that this growth inhibition is dependent in the kinase activity (Tiainen, et al, 1999). In

some cases growth inhibition correlates with the induction of p21WAF (Tiainen, et al 2002; Shen, et al. 2002). The *C. elegans* orthologs of LKB1 and AMPK are responsible for regulating germline proliferation and for cell cycle quiescence in nutrient poor conditions (Narbonne, et al. 2006). More recent work has linked LKB1 to key regulators of cell proliferation including C-MYC and BRAF (Liang, et al. 2009; Zheng, B et al. 2009; Esteve-Puig, et al. 2009). Re-expression of LKB1 in the *LKB1-null* lung adenocarcinoma cell line A549 was shown to cause a non-transcriptional decrease in the C-MYC levels and was due to MYC protein degradation by the proteasome (Liang, et al. 2009). In melanoma it was shown that oncogenic BRAF phosphorylates LKB1 and prevents it activating AMPK, suggesting that oncogenic BRAF can act as a negative regulator of LKB1 (Zheng, et al. 2009). This work was further expanded by Esteve-Puig et al. 2009 revealing a possible link between cell proliferation in response to mitogenic stimuli and resistance to low energy conditions in tumour cells.

1.3 MAPK signalling

The mitogen-activated protein kinase (MAPK) pathways integrate extracellular signals to co-ordinate cellular response. The signals which they respond to are diverse and include growth factors, stresses, cytokines and toxins. These signals are relayed through a network of kinase reactions that control cellular processes including; proliferation, growth, differentiation, apoptosis and migration. The basic signalling architecture consists of a MAPK which is phosphorylated by a MAPKK, which in turn is phosphorylated by a MAPKKK. There are at least six MAPK protein families in mammals including ERK1/2, ERK3/4, ERK5, ERK7/8, JNK1/2/3 and p38α/β/γ(ERK6)/δ. There are twenty genes, not including splice variants encoding MAPKKK and seven encoding MAPKK. Canonical MAPK and JNK pathways are some of the most commonly deregulated in cancer. The abnormal activities of these MAPK proteins would impinge on most if not all of the six hallmarks of cancer defined by Hanahan and Weinberg in 2000:

1. Unlimited replicative potential
2. Evade apoptosis
3. Independence of proliferative signals
4. Insensitive to anti-growth signals
5. Ability to invade and metastasise
6. Attract and sustain angiogenesis

The most studied pathway and known to be altered in approximately a third of cancer is the RAS-MAPK pathway (http://www.sanger.ac.uk/genetics/CGP/cosmic/), which will be the MAPK pathway focussed on in the work presented in this thesis.

1.3.1 RAS-MAPK signalling

RAS-MAPK signalling is activated by a number of external signals. Ligands such as the epidermal growth factor (EGF) bind to receptor tyrosine kinases on the surface of cells and activate them, receptors dimerise, autophosphorylate and recruit a number of target proteins which they phosphorylate on tyrosine recognition motifs. This includes growth factor receptor-bound protein 2 (GRB2) which binds to EGFR via its SH2 and SH3 domains (Lowenstein, et al. 1992). GRB2 recruits son of sevenless (SOS) a guanine nucleotide exchange factor for RAS proteins (Buday, et al. 1993; Chardin, et al. 1993). This results in activation of RAS through guanosine triphosphate (GTP) loading and active RAS recruits the serine/threonine kinase RAF to the plasma membrane for activation (Moodie, et al. 1993; Warne, et al 1993; Zhang, et al. 1993; Vojtek, et al. 1993). RAF phosphorylates MAPK/extracellular signal-related kinase (ERK) kinase (MEK) (Mcdonald, et al. 1993) which dual phosphorylates ERK1/2 (Crews, et al. 1992). This phosphorylation results in a 50000-fold increase in the K_{cat} of ERK2 (Prowse, et al 2000). ERK1/2 then activate a variety of transcription factors and other signalling molecules such as the RSK family of proteins (Chen, et al. 1992) Most of the alterations in cancer occur at the beginning steps of the pathway; overexpression of mutation of the receptor tyrosine kinase, or activating point mutation of *RAS* or *RAF* genes (Figure 1-3).

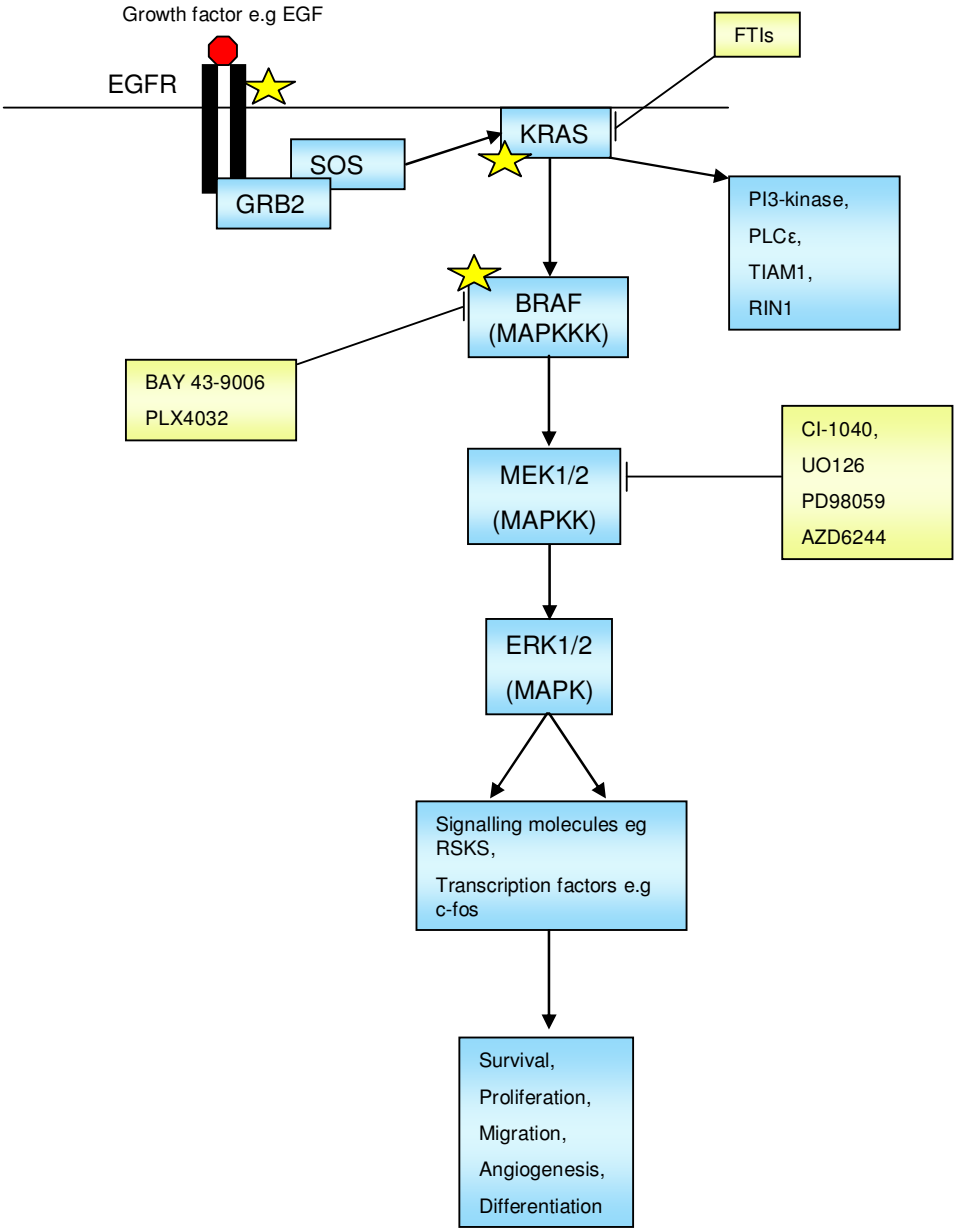

Figure 1-3 Simplified overview of RAS-MAPK signalling. Growth factor binding (e.g. EGF) to receptor tyrosine kinases (epidermal growth factor receptor, EGFR) on the cell surface recruits and activates GRB2; this in turn recruits SOS which activates RAS. KRAS activation signals to a number of pathways including its main effecter BRAF, BRAF activates MEK1/2 which phosphorylates ERK1/2. ERK1/2 have a number of downstream targets including various signalling molecules and transcription factors. For references see main text. In the yellow boxes are targeted inhibitors of the pathway. Yellow stars indicate mutations found in cancer.

1.3.1.1 RAS

The *RAS* genes encode small GTPases mutated in approximately 20% of all cancers (Bos, et al. 1989; http://www.sanger.ac.uk/genetics/CGP/cosmic/). There are three isoforms of RAS in humans; Kirsten sarcoma virus (KRAS), Harvey sarcoma virus associated oncogene (HRAS) and neuroblastoma RAS viral (v-ras) oncogene homolog (NRAS). The cellular homologues of viral Harvey and Kirsten transforming RAS sequences were first identified in the rat genome in 1981 (DeFeo, et al. 1981) and in 1982 in human (Chang, et al. 1982). The mutations in *RAS* are point mutations, altering a single amino acid and occur most commonly at amino acid residues 12, 13 and 61 and 146 (Reddy, et al. 1982; Taparowsky, et al. 1982; Tabin, et al. 1982; Capon, et al. 1983; Edkins, et al. 2006). *NRAS* was identified after HRAS and KRAS after it was found to be mutated in and cloned from neuroblastoma and leukaemia cell lines (Taparowsky, et al. 1983; Hall, et al. 1983; Murray, et al. 1983; Shimizu, et al. 1983). The mutations render RAS constitutively active and in a GTP-bound state. This constitutive GTP-bound state signals to effectors including RAF. In recent years additional effectors have been identified which include the p110 catalytic subunit of the phosphoinositide 3-kinases (PI3Ks) (Rodriguez-Viciana et al. 1994), AF6 (myeloid/lymphoid or mixed-lineage leukaemia (trithorax homolog, Drosophila), translocated to, 4) (Kuriyama, et al. 1996), phospholipase C-ε (PLCε) (Edamatsu, et al. 2006; Bunney, et al. 2006), ras-like small GTPases (White, et al. 1996; Urano, et al. 1996; Chien, et al. 2003), Ras and Rab interactor 1 (RIN1) (Han, et al. 1995), T-cell lymphoma invasion and metastasis 1 (TIAM1) (Lambert, et al. 2002), and Ras association (RalGDS/AF-6) domain family member 2 (RASSF2) (Vos, et al. 2003). *KRAS* mutations are found in number of cancer types; including >90% of pancreatic cancers (Smit, et al. 1988; http://www.sanger.ac.uk/genetics/CGP/cosmic/), and 20% of all lung cancer

(http://www.sanger.ac.uk/genetics/CGP/cosmic/). *NRAS* mutations however are predominately found in lymphoid malignancies and melanoma (Gambke, et al 1985; Janssen, et al. 1985; Needleman, et al 1986; Padua, et al. 1985; van't Veer, et al. 1989; http://www.sanger.ac.uk/genetics/CGP/cosmic/), whilst *HRAS* mutations are found in bladder cancer (Visvanathan, et al. 1988; http://www.sanger.ac.uk/genetics/CGP/cosmic/).

KRAS mutations occur in approximately 30% of NSCLC (Reynolds, et al 1991; Reynolds, et al. 1992; http://www.sanger.ac.uk/genetics/CGP/cosmic/) and it appears to be an early initiating event as mutations can be detected in 25-40% of potential precancerous lesions (Cooper, et al. 1997). Transgenic mice carrying a mutant *KRAS* allele develop a number of tumours, including early-onset lung cancer, suggesting in mice that *KRAS* mutations are important in the initiating phase (Johnson, et al. 2001). Recently a population of bronchioalveolar cells with the properties of stem cells including self-renewal and multipotency were identified (Kim, et al 2005). These cells with oncogenic KRAS give rise to lung tumours *in vivo* (Kim, et al 2005). *KRAS* mutations are present in lung cancers arising in both smokers and never smokers (Riely, et al. 2008). In smokers the most common alteration is a G to T transversion at codon 12 suggesting that carcinogens in tobacco smoke such as benzo(a)pyrene are responsible for this mutation pattern (Westra, et al. 1993). Lung tumours induced in mice using benzo(a)pyrene also show a similar mutation spectrum (You, et al. 1989; Massey et al. 1995; Sills et al. 1999). In never-smokers the G to A transition is more common (Riely, et al. 2008). There is some debate over the significance of *KRAS* mutations in prognosis. It does however seem to be of significance for predicting clinical outcome to EGFR inhibitors. A number of groups have studied the effect of *KRAS* mutations on response to various EGFR-tyrosine kinase inhibitors including erlotinib and gefitinib and found that tumours harbouring a *KRAS* mutation display resistance to EGFR inhibitors (Pao, et al. 2005; Fujimoto, et

al. 2005; Hirsch, et al. 2006; Han, et al. 2006; Jackman, et al. 2007; Massarelli, et al. 2007, Zhu, et al. 2008, Miller, et al. 2008).

1.3.1.2 RAS as a therapeutic target

Given the high percentage of cancers containing *RAS* mutations and the importance of RAS in cellular signalling, it therefore follows that RAS proteins would be good molecular target. The biochemical characteristics of RAS however have so far prevented this. Mutant RAS displays defective enzymatic rather than increased activity, it would be difficult to design an agent that would restore normal activity and not cause systemic toxicity. One avenue that has been pursued are farnesyl transferase inhibitors. In order to carry out its signalling functions normal and mutant RAS associates with the plasma membrane which requires lipid modification, such as farnesylation (Willumsen, et al. 1984). Farnesyltransferase inhibitors were developed and they entered clinical trials but the results in pancreatic adenocarcinoma showed a lack of efficacy (MacDonald, et al. 2005). This may be due to alternative lipid modifications being substituted for farnesylation, allowing RAS to reach the membrane. There has been more success targeting the downstream effectors of RAS.

1.3.1.3 RAF

RAF is one of the effectors of RAS. There are three RAF proteins (RAF1 (also known as CRAF), BRAF and ARAF) which are similar in structure but differ in regulation, activation, ability to activate the downstream MEK and tissue distribution. RAF1 is the cellular proto-oncogene homologue of v-RAF, (Rapp, et al. 1983) and mutations in *RAF1* and *ARAF* are rare (Emuss, et al. 2005; http://www.sanger.ac.uk/genetics/CGP/cosmic/). *BRAF* on the other hand is mutated

in almost two thirds of melanomas (Davies, et al. 2002) and at a lower frequency in other malignancies (http://www.sanger.ac.uk/genetics/CGP/cosmic/). Most of the mutations in *BRAF* affect the same amino acid - V600E which results in constitutive activation of BRAF (Wan, et al. 2004). *Non-V600 BRAF* mutations (K438Q, K438T, T439P and V458L) have been identified in a small number of human NSCLC (Brose, et al. 2002) however the role of these is unclear. They do surround a known AKT phosphorylation site Thr-439. A study investigating the properties of these mutations found no elevation of the MEK/ERK cascade and no differences in AKT activation between the mutant *BRAF* and wild type *BRAF* (Ikenoue, et al. 2005). A study into colon tumour associated mutations in the glycine-rich loop (G-loop) of BRAF found that only one (G468A) increased MEK/ERK signalling and increased transformation of NIH3T3 cells, F467C moderately increased MEK/ERK signalling and transformation of NIH3T3 cells (Ikenoue, et al. 2004). The remaining mutations (R461I, I462S, G463E, G468E) had no effect on MEK/ERK signalling and did not increase NIH3T3 transformation, G468E actually decreased MEK/ERK signalling and NIH3T3 transformation (Ikenoue, et al. 2004). The role of these *non-V600 BRAF* mutations remains to be determined. They could be passenger mutations which play no role in driving the cancer. They do however form a distinct cluster specifically in NSCLC which would imply they may play a role in driving NSCLC and warrants further investigation. The main role of RAF is to activate MEK1/2 by phosphorylating serines 218 and 222 in the activation loop. BRAF is the strongest MEK activator and ARAF the weakest activator, with a preference for MEK1. RAF1 can activate both MEK1 and 2 (Wu, et al. 1996; Marais, et al. 1997). BRAF has been suggested to be the main RAF effecter for RAS as it requires no other signals for activation, while the other two isoforms are only weakly activated by oncogenic RAS and more strongly by SRC (Marais, et al. 1997). It has been reported that RAF1 and BRAF heterodimerise and can signal through RAF1 (Garnett, et al. 2005; Rushworth, et al. 2006), although the mechanisms remain unclear.

1.3.1.4 RAF as a therapeutic target

As mentioned above *BRAF* mutant melanomas are sensitive to MEK inhibition (Solit, et al. 2006) and MEK inhibitors will be discussed in detail in the next section. Given that *BRAF* mutations are common in melanoma and BRAF is downstream of RAS a great deal of work has gone into the research of inhibitors. BAY 43-9006 (Sorafenib) is an ATP-competitive inhibitor of RAF1 and has been one of the most studied RAF inhibitors. It displayed a lack of efficacy against melanomas in the clinical setting (Wilhelm, et al. 2006) but did show some efficacy against renal tumours (Ahmad, et al. 2004), this has been explained by the multi-targeted nature of the compound as it inhibits a number of other kinases besides RAF1, these include vascular endothelial growth factor receptor (VEGFR)-2, VEGFR-3, platelet-derived growth factor receptor beta, Flt-3, and c-KIT (Wilhelm, et al. 2006). Sorafenib was also tested in a phase II monotherapy trial for previously treated NSCLC where it demonstrated activity with a survival rate and disease control rate comparable to other small molecule inhibitors (reviewed by Blumenschein 2008). In addition preliminary data suggests it may show activity in combination with chemotherapy and EGFR inhibitors (reviewed by Blumenschein 2008). Newer compounds are now undergoing clinical evaluation and these include CHIR-265 a dual vascular endothelial growth factor receptor inhibitor/RAF kinase inhibitor and PLX4032 a *V600 BRAF* mutant specific agent. PLX4032 has recently exhibited antitumour activity in *V600 BRAF* mutant tumours in phase I trials (Flaherty, et al. 2009).

1.3.1.5 Signalling downstream of RAF through MEK/ERK and RSK

Active RAF kinases phosphorylate and activate MEK1/2 (Mcdonald, et al. 1993), which phosphorylate and activate ERK1/2 (Crews, et al. 1992). ERK1/2 have numerous cytoplasmic and nuclear targets which they phosphorylate in response to

MEK activation. These include kinases, transcription factors, cytoskeletal proteins and phosphatases (Yoon, et al. 2006). ERK signalling impacts on a diverse range of processes including migration, proliferation, survival, chromatin remodelling, differentiation and angiogenesis and it does this specifically and dependent on cell type (Yoon et al. 2006). This context dependence is less well understood and may be due in part to where ERK is localised in the cell and temporal differences in the signal strength (Murphy, et al. 2006). ERK1/2 were the first described MAPKs in mammalian cells and are activated by most growth factor receptors (Rossomando, et al. 1989). ERK signalling is subjected to negative feedback as very high levels of ERK can lead to cell cycle arrest (Sewing, et al. 1997; Woods, et al. 1997; Mirza, et al. 2004). Downstream effectors of ERK signalling include RSKs (Chen, et al. 1992), c-FOS (Monje, et al. 2003), TP53 (Wang, et al. 2001), SMADs 1, 2, 3 and 4 (Kretzschmar, et al. 1997 &1999; Roelen, et al. 2003), SP1 (Milanini-Mongiat, et al. 2002), c-MYC (Sears, et al. 1999), and ELK1 (Marais, et al. 1993; Yang, et al. 1998).

The RSK family of proteins are serine threonine kinases downstream of MAPK signalling. There are four family members in humans (RSK1-4) and two structurally related homologues (MSK1 and 2). They are activated by ERK1/2 in response to a number of factors including growth factors (Chen, et al. 1992). At the amino acid level they have approximately 75-80% sequence identity and have two kinase domains (Jones, et al. 1988, Fisher, et al. 1996). Like ERK1/2 they phosphorylate a range of targets including CREB (Ginty, et al. 1994; Xing, et al. J. 1996; Bonni, et al. 1999) and a number of targets in the mTOR signalling pathway such as RAPTOR (Carriere et al. 2008), TSC2 (Roux, et al. 2004), eIF4B (Shahbazian, et al. 2006), rpS6 (Wang, et al. 2001) and LKB1 (Sapkota, et al. 2001). Through these targets RSKs can regulate pathways involving protein synthesis and gene expression. RSKs also play a role in preventing apoptosis through phosphorylation of the pro-apoptotic protein BAD, constitutive activation of RSK results in constitutive BAD

phosphorylation which is enhanced by BAD binding of 14-3-3 in the cytosol and results in protection from BAD-modulated cell death (Shimamura, et al. 2000; Tan, et al. 1999). In addition, RSK phosphorylates and inactivates death-associated protein kinase (DAPK) implicated in cancer (Anjum, et al. 2005).

1.3.1.6 MEK as a therapeutic target

As mentioned earlier *BRAF* mutant melanomas have been shown to be sensitive to MEK inhibition (Solit, et al. 2006). MEK inhibitors were the first small molecule inhibitors of the MAPK pathway to enter clinical trials and there are a number of them at various stages (reviewed by Wong, 2009). The first inhibitors were UO126 and PD098059 both of these have been used extensively in cell systems to study MEK inhibition, UO126 is the more potent inhibitor of the two (Dudley, et al. 1995; Favata, et al. 1998). UO126 has shown a lack of oral activity and PD098059 is fairly insoluble making both of them unsuitable for clinical testing, however they are useful compounds *in vitro*. CI-1040 (PD184352) was the next generation MEK inhibitor and the first to show activity *in vivo* in mice (Sebolt-Leopold, et al. 1999). Based on this evidence CI-1040 entered clinical trials for patients with advanced solid tumours. The phase I results where it demonstrated antitumour activity and target suppression (Lorusso et al. 2005) and it entered phase II of clinical trials. It was tested in advanced breast cancer, NSCLC, colon cancer and pancreatic cancer in phase II (Rinehart, et al. 2004). Even though it was well-tolerated, there were no partial or complete responses but disease stabilisation was observed in eight out of sixty-seven patients (including 3 NSCLC). Due to the lack of clear anti-tumour activity CI-1040 development was discontinued in favour of the newer more potent second generation MEK inhibitor; PD0325901. The structure of PD0325901 is similar to that of CI-1040 but it displays greater than 90-fold increase in potency compared to CI-1040 for suppression of phosphoERK and *in vivo* showed a thirty-fold increase in

efficacy (Wang, et al. 2007). In phase I clinical trials there was suppression of phosphoERK in all tumour types at all doses (Sebolt-Leopold 2008). Disease stabilisation was achieved in eight out of twenty seven patients and partial responses were seen in two melanoma patients however the toxicities were more severe than those observed with CI-1040 (reviewed in Sebolt-Leopold 2008). ARRY-142886 (AZD6244) is another highly selective potent MEK inhibitor and in phase I trials has induced disease stabilisation of 49% of patients and has now entered phase II trials (Sebolt-Leopold 2008).

1.4 LKB1/AMPK/mTOR and RAS-MAPK pathway crosstalk

Although so far I have described the MAPK signalling pathway and mTOR pathways separately, a large amount of crosstalk and feedback exists between the two (Figure 1-4). Active RHEB activates mTOR but inhibits wild type, but not the common mutant form of BRAF (Im, et al. 2002; Garami, et al. 2003; Karbowniczek, et al. 2004). RHEB activity is associated with decreased phosphorylation of BRAF (ser446) and RAF1 (ser338) in a rapamycin-insensitive manner, concomitant with a decrease in the activities of both kinases and inhibition of BRAF and RAF1 heterodimerisation and inhibition of the association of BRAF with HRAS (Karbowniczek, et al. 2006). Another point of crosstalk is at TSC1/2 described previously, TSC1/2 is phosphorylated and activated by AMPK but inhibited by AKT, ERK and RSK phosphorylation (Inoke, et al. 2003; Tee, et al. 2003b; Ma, et al 2005 & 2007; Roux, et al. 2004). RSK phosphorylates LKB1 in BRAF V600 mutant melanoma cells and suppress LKB1-AMPK signalling (Sapkota, et al. 2001; Esteve-Puig, et al. 2009; Zheng, et al. 2009). RSK also phosphorylates raptor, activating mTOR (Carriere, et al. 2008). ERK signalling also effects components downstream of mTOR, including 4EBP1 and eIF4E through the MAP kinase interacting serine/threonine kinase 1 and 2 (MNK1 and 2) (Bhandari, et al. 2001; Scheper, et al. 2001; Knauf, et al. 2001; Herbert, et al. 2002; Duncan, et al. 2005) and rpS6 and S6K (Wang, et al. 2001; Iijima, et al. 2002; Bessard, 2007; Roux, et al. 2007; Huynh, et al. 2009). mTOR has also been shown to alter cyclin D1 levels in a 4EBP1-dependent manner (Averous, et al 2008). As mentioned previously, intriguing work this year by two groups has revealed a new link between RAS-MAPK signalling and LKB1-AMPK signalling in melanoma where RAS pathway activation including BRAF(V600E) mutation promotes the uncoupling of AMPK from LKB1, likely through phosphorylation of LKB1 by ERK and RSK (Esteve-Puig, et al. 2009; Zheng, et al. 2009).

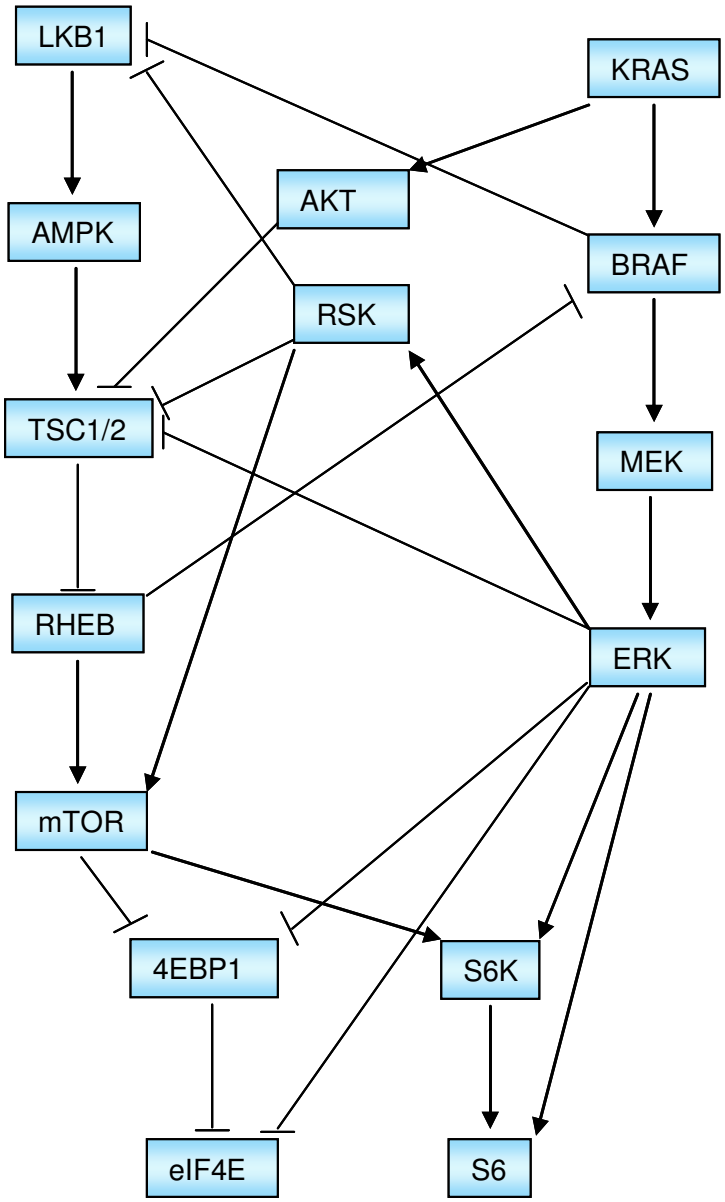

Figure 1-4 Simplified overview of the known signalling crosstalk between the LKB1/AMPK/mTOR pathway and the RAS-MAPK signalling pathway. For references see main text. Over the years a number of interaction points have been found between these two signalling pathways creating a complex network of interactions.

1.5 Cell lines as model systems for developing therapeutics

Human cancer cell lines have been used for many decades as a model system in which to study cancers. They are easy to grow and manipulate and there is now a large body of data concerning the genetic and genomic alterations in each cell line (http://www.sanger.ac.uk/genetics/CGP/CellLines/). This makes them an ideal tool for studying the relationships between drug response and the molecular profiles of cancer. They are a good starting point for sub-classifying tumours based on genetic variations which can be related to therapeutic response, findings from cell lines can then be taken forward and studies in animal models and primary tumour samples and eventually a small number will reach the clinic.

One such example of this is in melanoma. *BRAF* mutant melanoma cell lines were found to be sensitive to the MEK inhibitor CI-1040 when compared to cell lines with wild type *BRAF* or *RAS* mutations (Solit, et al. 2006). Further work in mouse xenografts revealed that the MEK inhibitor CI-1040 inhibited tumour growth in *BRAF* mutant xenografts, whereas *RAS* mutant xenografts exhibited only partial responses (Solit, et al. 2006). This data suggests MEK inhibitors targeted specifically to *BRAF*-mutant melanomas may be an effective treatment .

With the advent of high-throughput drug screening and the knowledge of cancer genomes becoming more complete, research is beginning to deliver on the promise of “personalised medicine”. Projects which combine complete genomic information of cancer cell lines with therapeutic response will gradually categorise cancer into subsets defined by genetics and therapeutic response. This is of particular importance in NSCLC with poor survival rates and a lack of early detection methods and an urgent need for more targeted therapies and biomarkers.

1.6 Introduction to thesis project

The aim of this thesis was to functionally characterise a new genetic subset of NSCLC I identified in mutation data from the Cancer Genome Project. A significant association of *LKB1* inactivating and *KRAS* activating mutations was observed (Mahoney, et al. 2009). The overlap and crosstalk between the pathways implicated by these mutations made it an interesting subset with potential therapeutic implications. The initial hypothesis was centred on RHEB and how loss of *LKB1* could lead to hyperactivation of RHEB. This in turn would lead to inhibition of MAPK signalling through RHEBs interaction with BRAF blocking the proliferative MAPK signalling pathway. Thus LKB1 mutant cells would require pro-activating mutations on the MAPK pathway to overcome this inhibition. If this was the case this subset could be sensitive to MAPK pathway inhibition. Further examination of this genetic subset of NSCLC identified a unique expression signature dominated by metabolic genes which suggested a mechanism by which this subset could create the Warburg Effect.

My thesis research has focussed on the following key areas:

- Targeted inhibition and the effects on the downstream pathway components.
- Analysis of the differences in gene expression in this genetic subset versus other NSCLC cell lines.
- NMR spectroscopy to confirm a possible mechanism by which this genetic subset of lung cancer creates the Warburg Effect (switch to aerobic glycolysis).
- Characterisation of the expression of genes altered by targeted inhibitors.
- Functional characterisation of AKR1B10