Identification and Functional Characterisation of a Genetic Subset of Non-Small Cell Lung Cancer

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This dissertation is submitted for the degree of Doctor of Philosophy

DECLARATION

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically indicated in the text. The dissertation does not exceed the word limit set by the Biology Degree Committee.

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ABSTRACT

Non-small cell lung cancer (NSCLC) constitutes ~80% of all lung cancer cases. It is often treated with conventional chemotherapy and has survival rates of only 7% at 5 years. Survival rates improve if it is diagnosed early and can be surgically removed, however over half of all cases are at stage IV at diagnosis. There is a need for new therapeutic targets as well as better detection. This project aimed to functionally characterise a new genetic subset of NSCLC characterised by *LKB1* loss and *KRAS* activation (*LKB1null/KRASmut*). The association of mutations is interesting as the signalling pathways are linked by RHEB. Loss of *LKB1* may lead to over activation of RHEB which inhibits wild type BRAF but not mutant BRAF thus blocking MAPK signalling. NSCLC with *LKB1* mutations may therefore have a general requirement for an activation of the MAPK cascade to overcome suppression of MAPK signalling by RHEB. Following from this, subsequent experiments showed this genetic subset to be sensitive to MEK inhibition and mTOR inhibition. The sensitivity to MEK inhibition is not due to downstream effects on cyclin D1 as reported for melanoma. Comparison of gene expression in this genetic subset compared to other NSCLC cell lines revealed a unique expression signature. Analysis of this signature revealed a metabolic profile dominated by truncation of the citric acid cycle at the pyruvate dehydrogenase reaction. We further characterised these cells using ^{13}C and proton NMR spectroscopy. These data confirmed truncation of the citric acid cycle in the *LKB1/KRAS* mutant subset and suggests this genetic subset of lung cancer creates the Warburg Effect through inactivation of the pyruvate dehydrogenase complex. The NMR spectroscopy highlighted further metabolic changes including the reliance of this subset on both glucose and glutamine metabolism despite the ideal growth conditions of cell culture. The *in vitro* phenotypic data presented in this study make a strong case for these changes being “hard-wired” by the mutation states and thus present further opportunities for their investigation as potential avenues for therapeutic development.

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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).

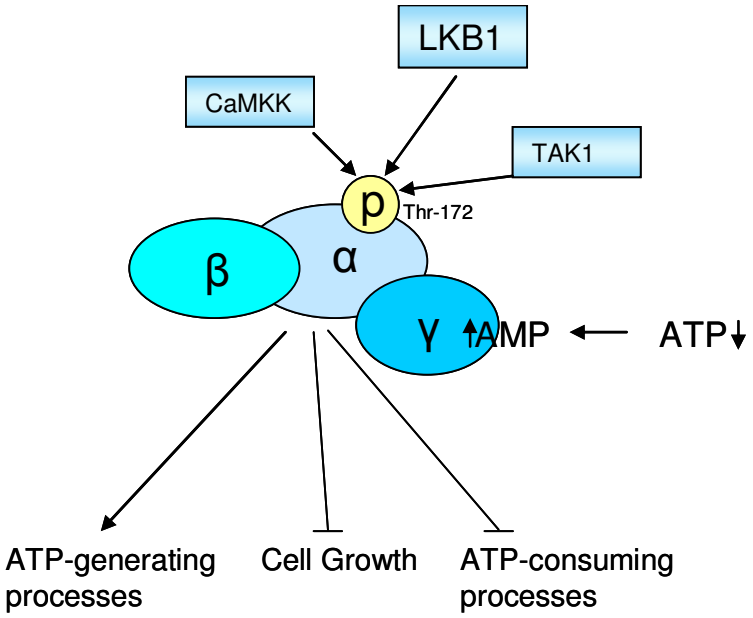

Figure 1-1. AMPK structure and regulation. AMPK is a heterotrimeric protein which consists of a catalytic α-subunit, and regulatory β- and γ-subunits. AMP levels rise under conditions of stress and bind to the γ-subunit of AMPK. This prevents the catalytic subunit from being dephosphorylated. LKB1 is 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.

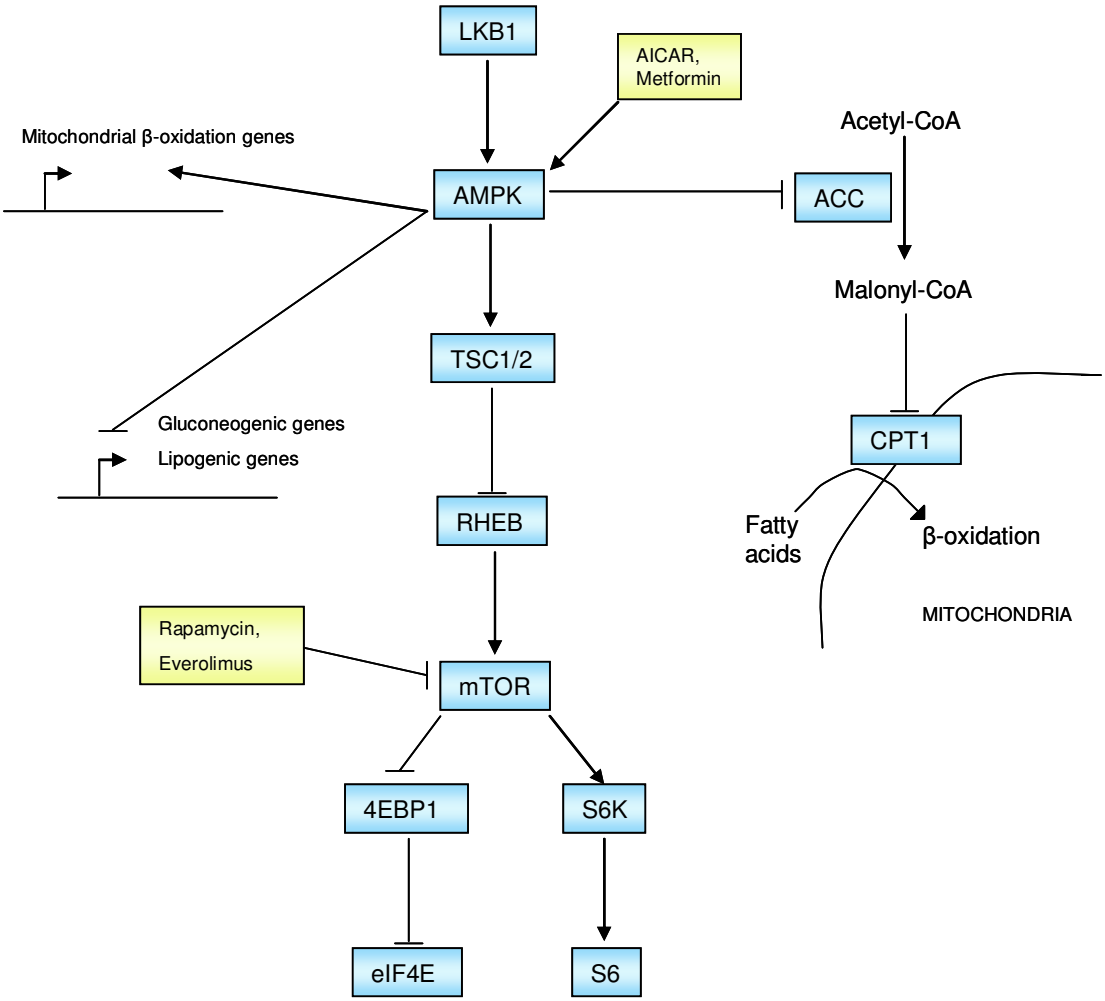

Figure 1-2. A simplified overview of the known components of the LKB1/AMPK/mTOR pathways. LKB1 signals to activate AMPK under conditions of stress. AMPK inhibits translation and protein synthesis through inhibition of mTOR signalling. AMPK also enhances β-oxidation of fatty acids through inhibition of 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).

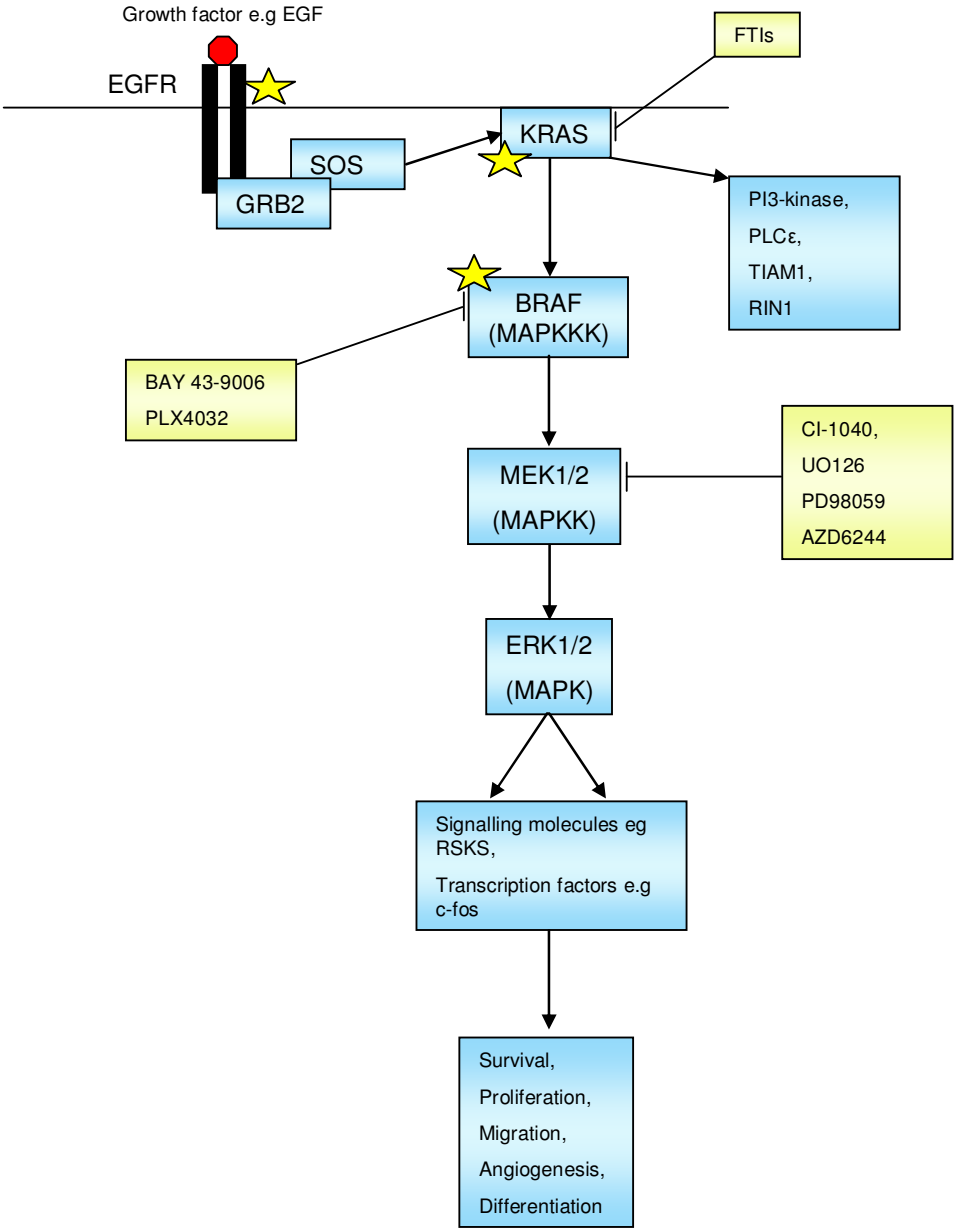

Figure 1-3 Simplified overview of RAS-MAPK signalling. Growth factor binding (e.g. EGF) to receptor tyrosine kinases (epidermal growth factor receptor, EGFR) on the cell surface recruits and activates GRB2; this in turn recruits SOS which activates RAS. KRAS activation signals to a number of pathways 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).

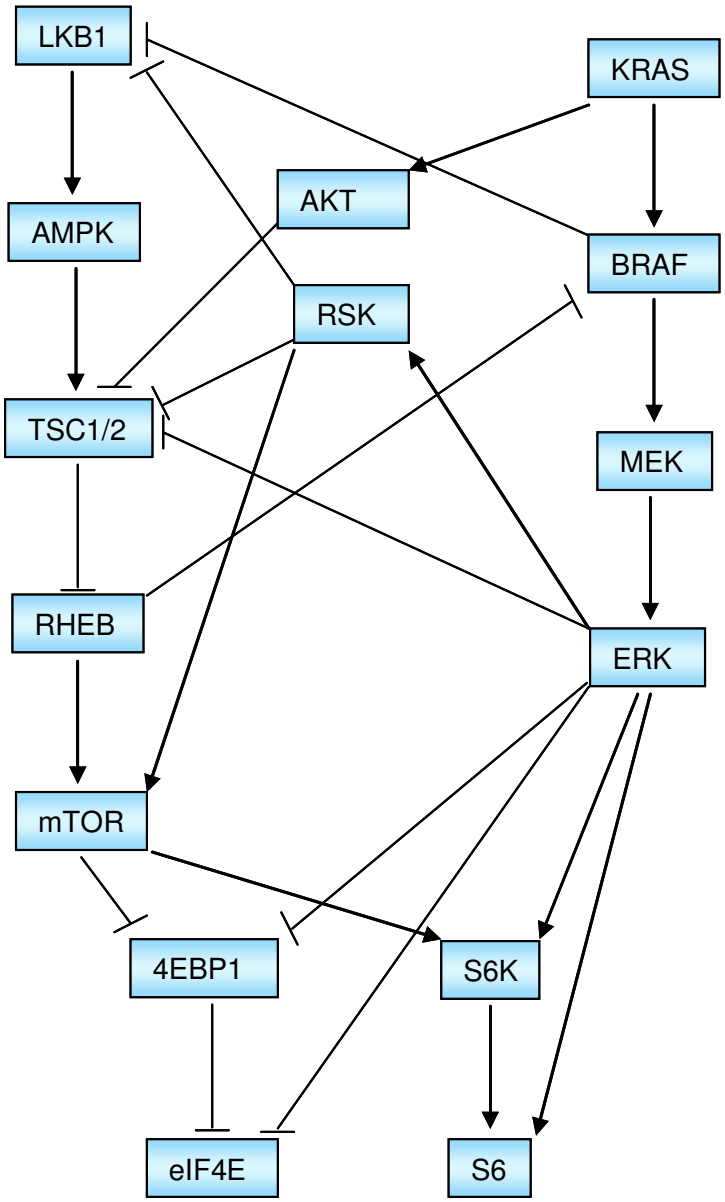

Figure 1-4 Simplified overview of the known signalling crosstalk between the LKB1/AMPK/mTOR pathway and the RAS-MAPK signalling pathway. For references see main text. Over the years a number of interaction points have been found between these two signalling pathways creating a complex network of interactions.

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

Chapter 2: Materials and Methods

2.1 Cell culture

Cell lines were purchased from American Type Culture Collection (ATCC) or the Developmental Therapeutics Program at the National Cancer Institute. Cell lines were maintained RPMI 1640 (Invitrogen) supplemented with 10% foetal bovine serum (Invitrogen) and 1% penicillin-streptomycin-gentomycin (Invitrogen) in a humidified atmosphere at 37°C and 5% CO_2. Cell lines were subcultured twice a week at a ratio of 1:6 and 1:10 dependent on the confluency. Cell culture media was aspirated and the flask washed twice with 15ml phospho buffered saline (PBS). Cells were then detached using 10ml Trypsin-EDTA solution (SIGMA). This was then neutralised with 20ml of media and cells collected by centrifugation for 5minutes at 1500rpm.

2.2 Drug Assays

2.2.1 Cell plating

Cell lines of a known genetic background (Table 2-1) were maintained as stated above (2.1) in T-150cm^2 flasks (Costar). Prior to each experiment one 80-90% confluent flask was trypsinised and centrifuged as described above (2.1), the supernatant was aspirated and the cell pellet resuspended in 2ml of media and gently pipetted to prevent clumping of cells. 10µL of cell solution was pipetted onto a haemocytometer and cells counted. Approximately 1000-2500 cells were seeded in 6 replicates to 48-well tissue culture

plate depending on the growth rates of the cell lines in a volume of 350µl. The plates were then incubated for 24hrs at 37^{o}C.

Cell Line	Histology	LKB1	BRAF	KRAS	PIK3CA	P53	CDNKA	EGFR
		Mutation	Mutation	Mutation	Mutation	Mutation	Mutation	Mutation
SKMEL28	Malignant melanoma		c.1799T>A p.V600E			c.434_435TG>GT p.L145R		c.2257C>T p.P753S
CAL-12T	NSCLC nos	No protein[A]	c.1397G>T p.G466V			c.404G>T p.C135F	c.172C>T p.R58*	
A549	NSCLC nos	c.109C>T p.Q37*		c.34G>A p.G12S			c.1_471del471 p.M1_*157del	
NCI-H1734	NSCLC Adenocarcinoma	c.152_153insCT p.M51fs*14		c.37G>T p.G13C				
NCI-H460	NSCLC Large cell carcinoma	c.109C>T p.Q37*		c.183A>T p.Q61H	c.1633G>A p.E545K		c.1_457del457 p.?	
NCI-H2030	NSCLC Adenocarcinoma	c.949G>T p.E317*		c.34G>T p.G12C		c.785G>T p.G262V		
NCI-H1563	NSCLC Adenocarcinoma	c.816C>A p.Y272*					c.1_471del471 p.M1_*157del	
NCI-H2009	NSCLC Adenocarcinoma			c.35G>C p.G12A		c.818G>T p.R273L		
NCI-H1975	NSCLC Adenocarcinoma				c.353G>A p.G118D	c.818G>A p.R273H	c.205G>T p.E69*	c.2573T>G p.L858R
NCI-H1838	NSCLC Adenocarcinoma					c.818G>T p.R273L	c.1_471del471 p.M1_*157del	
NCI-H1792	NSCLC Adenocarcinoma			c.34G>T p.G12C		c.672+1G>A p.?		
NCI-H358	NSCLC Adenocarcinoma			c.34G>T p.G12C				
NCI-H661	NSCLC Large cell carcinoma					c.644G>T p.S215I c.473G>T p.R158L	c.457+1G>T p.?	
NCI-H226	NSCLC Squamous cell carcinoma						c.1_150del150 p.?	

Table 2-1 Genetic background and histology of the cell lines used in this study Mutation data taken from COSMIC (http://www.sanger.ac.uk/genetics/CGP/CellLines/). (A) No mutation has been found in this sample by sequencing, however immunoblot analysis revealed no protein present. NSCLC nos = NSCLC not otherwise specified.

2.2.2 Drug dilutions

CI-1040 was a kind gift from Richard Marais; 50mg was dissolved in 1ml DMSO (X) to give a stock solution of 110mM. Rapamycin was purchased from Sigma; 1mg was dissolved in 1ml DMSO to give a stock solution of 1.1mM. AMPK compound C was purchased from Merck as a 10mM solution. 6-aminonicotinamide was purchased from Sigma; 250mg were dissolved in 5ml DMSO to give a stock solution of 360mM.

2.2.3 Drug Addition

After 24hrs of incubation of the plated cells, drug dilutions were made up in fresh media. Media was then aspirated from the plated cells and replaced with 350µl of media containing the compound of interest. All drug dilutions were carried had 6 replicates. An equal volume of media was added to the untreated control cells. The plate was then incubated at 37^{o}C for 72hrs.

2.2.4 Proliferation assay

After 72hrs of incubation with the compound of interest cells were subjected to a proliferation assay. This was carried out using the CyQuant® Proliferation Assay Kit (Invitrogen). Assays were carried out according to manufacturer's instructions. Media was shaken off rather than aspirated, so as not to disturb the cells and alter the cell number. Wells washed once with PBS and PBS shaken off rather than aspirated, plates were then frozen at -70^{o}C. The CyQuant® solution was made up as needed on the day of the assay. 20x lysis buffer was diluted with nuclease-free water (Ambion) and CyQuant® fluorescent dye added, this solution was protected from the light using aluminium foil. 500µl of this solution was added to each well and plates were incubated in the dark for 5minutes at room temperature before the fluorescence was measured using Fluorescein filters.

2.2.5 Data Analysis

The 6 replicates were averaged to give a mean value and a standard deviation calculated. The rate of proliferation was then estimated relative to the untreated control. The average and standard deviation from at least 2 independent experiments was calculated for each cell line for each drug concentration.

2.3 siRNA knockdown

2.3.1 Cell plating

Cell lines of a known genetic background (Table 2-1) were maintained as stated above (2.1) in T-150cm^2 flasks. Prior to each experiment one 80-90% confluent flask was trypsinised and centrifuged as described above (2.1), the supernatant was aspirated and the cell pellet resuspended in 2ml of media and gently pipetted to prevent clumping of cells. 10µl of cell solution was pipetted onto a haemacytometer and cells counted. Approximately 500-1000 cells were seeded in quadruplicate to 96-well tissue culture plate depending on the growth rates of the cell lines in a volume of 150µl of antibiotic-free media. The plates were then incubated for 24hrs at 37^{o}C.

2.3.2 Transfection of siRNA

ON-TARGETplus single siRNAs were purchased from Dharmacon. siRNAs were resuspended in 1x siRNA buffer to a stock concentration of 20µM. Aliquots of 2µM were prepared to avoid freeze-thaw degradation. After 24hrs of incubation cells were transfected in triplicate with the siRNA using 0.25µM Lipofectamine reagent in 100µl of antibiotic-free media. Negative controls included ON-TARGETplus Non-targeting siRNAs (Dharmacon) and Lipofectamine only control. These were also carried out in triplicate. After 8hrs of incubation with the siRNA, media was aspirated gently and replaced with antibiotic–free media containing no siRNA. 48hrs later the transfection was repeated.

2.3.3 Proliferation assay

After 7 days of incubation with the compound of interest cells were subjected to a proliferation assay. This was carried out using the CyQuant® Proliferation Assay Kit (Invitrogen) as described in 2.2.4. 200µl of CyQuant® solution was added to each well and plates were incubated in the dark for 5minutes at room temperature before the fluorescence was measured using Fluorescein filters.

2.3.2 Data Analysis

Triplicates were averaged to give a mean value and a standard deviation calculated. Toxicity was estimated by comparing the non-targeting control and Lipofectamine™ 2000 control to the untreated control. The rate of proliferation for the siRNA of interest was estimated relative to the non-targeting control. The average and standard deviation from at least 2 independent experiments was calculated for each cell line.

2.4 Protein Quantification

2.4.1 Cell plating

2.4.1.1 To determine the downstream effects of CI-1040 and rapamycin.

Cells were seeded to 6-well plates and incubated at 37^{o}C for 24hrs. Cells were then treated with a range of concentrations of CI-1040 (0-10µM) or rapamycin (0-200nM) and incubated for 8 or 24hrs and protein harvested.

2.4.1.2 To estimate level of siRNA knockdown

Cells were seeded to 6-well plates and 24hrs later transfected with 10nM, 20nM or 50nM of the siRNA using 0.25µM Lipofectamine™ 2000 in antibiotic-free media. After 6hrs the media was replaced with antibiotic-free media containing 10% FCS. At 48hrs the transfection was repeated. 96hrs after the first transfection the cells were harvested for immunoblot analysis of protein levels.

2.4.2 Harvesting protein

Protein was harvested from cells seeded to 6-well plates. Cells were washed twice with ice-cold PBS and then treated with 200µl RIPA buffer (Sigma) containing protease (Sigma) and phosphatase inhibitors (Roche). The solution was than incubated with gentle shaking on ice for 30mins and then centrifuged at 13200rpm at 4^{o}C for 20minutes. The supernatant was then transferred to a new tube on ice and all samples stored at -70^{o}C.

2.4.2 Quantification of protein

Quantification of protein was carried out using the BCA assay. The working reagent was created by mixing 50 parts bicinchoninic acid with 1 part copper (II) sulphate solution. Bovine Serum Albumin (Sigma) standards were prepared in duplicate (0-1000µg/ml). Unknown samples were diluted 5-fold and 10-fold in a 96-well plate in a volume of 25µL. 200µL of BCA working reagent was added to all samples and incubated at room temperature for two hours or at 37^{o}C for 30mins. The absorbance was measured at

562nm. BSA standards were used to create a standard curve and sample concentrations estimated using this curve.

2.4.3 SDS-PAGE

Samples were diluted to 20µg/well using RIPA buffer (Sigma). 10µl of each sample was denatured and reduced by adding 3.7µL 4x NuPAGE® LDS Sample buffer (Invitrogen), 1.5µL (10x) NuPAGE® Sample Reducing Agent (Invitrogen) and boiled at 95-100^{o}C for 5-10mins. Samples were then cooled on ice and spun down.

14.5µL of samples were loaded into pre-cast NuPAGE® 4-12% Bis-Tris Gel (Invitrogen) alongside 5µL Novex® Sharp Pre-stained Protein Standards (Invitrogen). Samples were run for approximately 1 hour at 200V in 1X NuPAGE® MES SDS Running Buffer (Invitrogen) containing 1ml NuPAGE® antioxidant (Invitrogen) in an Xcell SureLock™ system.

2.4.4 Western blotting

SDS-PAGE gels were transferred to PVDF membranes (Invitrogen) using the following protocol. 1X NuPAGE® Transfer Buffer (Invitrogen) containing 10% methanol and 1ml NuPAGE® Antioxidant was prepared on the day of the transfer. Prior to transfer, blotting pads and filter paper were soaked in the transfer buffer. PVDF membranes were prepared by soaking for 1min in methanol, briefly washed with de-ionised water and finally soaked in transfer buffer. The protein samples were transferred to the PVDF membrane using the Xcell SureLock™ system at 30V for an hour. Membranes were then incubated in 1X blocking buffer (Sigma) for an hour at room temperature.

Membranes were then incubated overnight at 4°C with primary antibodies (see table 2-2). Membranes were then washed 3x for 10mins in TBST (500mM NaCl, 20mM Tris-HCl, 0.01% Tween-20, pH7.6), secondary Anti-rabbit IgG, HRP-linked Antibody (Cell Signalling Technology) was diluted 1:2500 in blocking buffer and membranes incubated at room temperature for 2hrs with the secondary antibody.

Antibody	**Supplier**	**Dilution**
cyclin D1 (C-20): sc-717	Santa Cruz Biotechnology	1:500
p44/42 MAPK (Erk1/2) (137F5) Rabbit mAb	Cell Signalling Technology	1:1000
Phospho-p44/42 MAPK (Erk1/2)(Thr202/Tyr204) Rabbit mAb	Cell Signalling Technology	1:1000
p70 S6 Kinase (49D7) Rabbit mAb	Cell Signalling Technology	1:1000
Phospho-p70 S6 Kinase (Thr389) (108D2) Rabbit mAb	Cell Signalling	1:1000
AKR1B10 (ab62218)	Abcam	Min. 1:20000

Table 2-2 Antibodies used in this study

2.4.5 Visualisation and data analysis

Protein bands were visualised using the SuperSignal West Pico Chemiluminescent Substrate (Pierce). Equal amounts of the two solutions were mixed on the day, membranes were incubated for 5mins at room temperature, excess fluid drained, wrapped in cling film and exposed to X-ray film (Amersham) in a dark room. Relative amounts of protein were estimated using the software ImageJ.

2.5 Analysis of Affymetrix Microarray data

2.5.1 Data analysis

mRNAs were extracted from candidate cell lines and expression profiles were determined using the Affymetrix Human U133 Plus 2 GeneChip by GlaxoSmithKline. Data quality test and normalization were performed using Affy package in R (Irizarry, et al. 2003a). The quality of each array was assessed by checking the mean and standard deviation of over-all expression, percentages of present gene calls and background levels, 3': 5' ratios for spiked-in and control genes specific to the array type and the correlation among replicated samples. Background correction of each array was using robust multi-array average expression measure (Irizarry, et al. 2003; Bolstad, et al. 2003; Irizarry, et al. 2003b). The data were normalized by quantile method and expression level of each gene was log2 transformed. Genetic signature analysis was using Limma package from BioConductor (Smyth, 2004). Paired t-test was used to differences between the expression profiles of between *LKB1/KRASmut* cell lines and wild type cell lines. *P*-values were corrected by Benjamini/Hochberg (BH) paradigm with a false discovery rate (FDR) of 0.01. Unsupervised hierarchical cluster was carried out using Cluster 3.0 (Eisen et al., 1998) and presented by Java TreeView (Saldanha, 2004). The similarities between cell lines were calculated based on uncentered Pearson correlation and used average linkage for clustering. This section of analysis was carried out by Lina Chen.

2.5.2 Bioinformatic analysis of genes with significantly different expression

Genes found to be significant using the BH paradigm with an FDR of 0.01 were subjected to GO term enrichment analysis using the web-based program GoMiner (http://discover.nci.nih.gov/gominer/). Differentially expressed genes were also mapped on to metabolic pathways using the web-based program; Metacyc (http://metacyc.org/).

2.6 Expression analysis of CI-1040, rapamycin and AMPK inhibitor treated cells

2.6.1 Cell plating and drug addition

Cell lines of a known genetic background (Table 2-1) were seeded to 10cm dishes, in triplicate at time 0hrs and in duplicate for all other time points. The following day untreated controls were harvested for time point 0hrs. The remainder of the dishes were treated with 5μM CI-1040, 40nM rapamycin or 7μM AMPK inhibitor compound C. Cells were harvested and RNA extracted 8hrs and 48hrs after treatment.

2.6.2 RNA extraction

RNA extraction was carried out using the Qiagen RNeasy® kit. Approximately $1x10^6$ cells were lysed directly in the dish by addition of 600μL of RLT buffer. The lysate was collected and transferred to an RNase-free tube and vortexed to disperse clumps. Lysate was transferred to a QIAshredder® spin column in a 2ml collection tube and centrifuged for 2mins at full speed. 1 volume of 70% ethanol was added to each sample and pipette mixed. 700μL of the sample was transferred to an RNeasy spin column in a 2ml collection tube and centrifuged for 15 seconds at ≥8000g, flow-through discarded

and the same step repeated with the remainder of the sample. 700µL of RWI buffer was added to the spin column and centrifuged for 15sec at ≥8000g, flow-through discarded. 500µL of RPE buffer was added to the column and centrifuged for 15sec at ≥8000g, flow through discarded; this step was then repeated and centrifuged for 2mins at ≥8000g. The spin column was transferred to a new 2ml collection tube and spun at full speed for 1min before being transferred to a new 1.5ml collection tube and the RNA eluted by adding 50µL of RNase-free water and centrifuged for 1min at ≥8000g. Eluted samples were stored at -70^{o}C.

2.6.3 Assessing quality and quantity of RNA

RNA quality was assessed using the RNA 6000 Nano kit (Agilent). On arrival of the kit the RNA ladder was prepared by transferring to a new RNase-free vial, heat denatured at 70^{o}C for 2mins and immediately cooled on ice. Aliquots with the required amount for typical daily use were prepared in RNase-free tubes and stored at -70^{o}C. RNA 6000 Nano gel matrix was prepared by pipetting 500µL into a spin filter, centrifuging at 1500g for 10mins at room temperature. Aliquots of 65µL were prepares in 0.5ml RNase-free vials and stored at 4^{o}C for 4 weeks.

On the day of the experiment the gel-dye mix was prepared. RNA 6000 Nano dye concentrate was equilibrated at room temperature for at least 30mins, vortexed, spun down and 1µL added into a 65µL aliquot of filtered gel. The gel-dye mix was vortexed thoroughly and spun at 13000g for 10mins at room temperature.

9µL of gel dye mix was loaded into a new RNA 6000 Nano chip on the chip priming station. 1µL of RNA 6000 Nano Marker was added to all sample wells and in the well for

the RNA ladder. 1µL of the prepared RNA ladder was pipetted into the marked well and 1µL of each sample into separate wells. The loaded chip was then vortexted in an IKA vortexer for 1min at 2400rpm. The chip was then loaded straight into the Bioanalyser and gels assessed for RNA quality. RNA concentration was estimated using a Nanodrop spectrophotometer.

2.6.4 Microarray

Samples were diluted to 50ng/µL in a 96-multi well plate and the microarry performed by Peter Ellis of the Microarray team. mRNA was reverse transcribed using oligo(dT) primers for two hours at 42^{o}C which incorporates a T7 RNA polymerase binding site at the 5'-end. The RNA was then digested with RNaseH and the cDNA converted to double stranded cDNA using DNA polymerase for 2 hours at 16^{o}C. The purified cDNA was then incubated overnight at 37oC with T7 RNA polymerase and rNTPs (including biotin-tagged rUTP) to produce biotinylated single-stranded anti-sense RNA (cRNA).

Once quantified and purified 1500ng of the cRNA (per sample/array) was mixed with hybridisation buffer and applied to the array. The arrays were incubated in a humidified atmosphere at 58^{o}C for 16-20hrs followed by washing according to a standard Illumina protocol: 10min at 55^{o}C in wash buffer, 5mins at room temperature in E1BC, 10min in 100% ethanol at room temperature, 2mins at room temperature in E1BC, 10mins in blocking agent (casein in PBS) at room temperature, 10mins at room temperature in blocking agent containing 1µg/ml streptavidin-cy3 and 5mins in E1BC at room temperature. Finally slides were dried by spinning at 275 x g for 4mins. Slides were then scanned using a BeadArray reader and the output exported to BeadStudio

2.6.5 Data Analysis

Expression profiles were determined using the Illumina HumanWG-6_V3 chip. Data quality test and normalization were performed using Lumi package in R (Du, et al. 2008). The quality of each array was assessed by checking the mean and standard deviation of over-all expression, ratio of detectable probe, expression information of control probes (housekeeping genes) and the correlation among replicated samples. Background correction of each array was using variance-stabilizing transformation (Lin, et al. 2008). The data were normalized by quantile method and expression level of each gene was log transformed. Genetic signature analysis was using Limma package from BioConductor (Smyth, 2004). Paired t-test was used to access the significant level of expression change before and after drug treatment and the expression profile between *LKB1/KRAS* mutant cell lines and wild type cell lines. *P*-values were corrected by Benjamini/Hochberg paradigm with a false discovery rate of 0.01. Unsupervised hierarchical cluster has been done by using Cluster 3.0 (Eisen, et al. 1998) and presented by Java TreeView (Saldanha, 2004). The similarities between cell lines were calculated based on uncentered Pearson correlation and used average linkage for clustering. Heatmaps were produced by smcPlot from PGSEA package in BioConductor (http://www.bioconductor.org/packages/2.4/bioc/html/PGSEA.html). This section of analysis was carried out by Lina Chen.

2.6.6 Bioinformatic analysis of genes with significantly different expression

A cut-off of 1.5 for the fold change was used when comparing treated to untreated samples. Gene lists were compiled of significantly altered genes for the treated samples versus the untreated time 0hrs, for each cell line. Genes which only showed

altered expression after compound treatment in *LKB1/KRAS* mutant cell lines were subjected to further analysis. Genes found to be significant and differentially expressed in *LKB1/KRAS* mutant cell lines after compound treatment were subjected to GO term enrichment analysis using the web-based program GoMiner (http://discover.nci.nih.gov/gominer/).

2.7 NMR spectroscopy

2.6.1 Labelling of cells with ^{13}C glucose

Cells were grown in T-150cm^2 flasks until at least 50% confluent. Media was aspirated and replaced with glucose-free media containing 11.11mM ^{13}C-glucose. Cells were incubated at 37^{o}C for 24-48hrs, trypsinised as described in 2.1, ensuring that the media used for neutralising the trypsin was glucose-free.

2.6.2 Preparation of samples for NMR spectroscopy

Carried out by Jules Griffin (Department of Biochemistry, University of Cambridge). Dried aqueous extracts from the aqueous layer of the cell extract were dissolved in 600µl D_2O containing 1mM trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TSP) and transferred to 5mm NMR tubes for analysis. All NMR was performed on a 500MHz NMR spectrometer interfaced with a TXI 5mm probe. For 1H NMR spectroscopy a standard 1D NOESY pre-saturation pulse sequence with pre-saturation applied during the relaxation delay and mixing time was used in order to suppress the residual water signal. The sample temperature was 300K for all experiments. 256 transients were collected

into 64k data points over a spectral width of 20ppm. Spectra were collected with a relaxation delay of 2s, mixing time of 150ms and t1 of 4µs.

For all 1D spectra the 1H-NMR free induction decays (FID) were Fourier transformed after application of an exponential window function. The spectra were then phased, base line corrected, referenced to TSP at 0.00ppm and integrated using ACD labs NMR Processor (version 8, ACD, Toronto, Canada). 1H-NMR FIDs were Fourier transformed with a line broadening of 0.3Hz. The spectra were integrated in 0.01ppm buckets between 0.20 and 9.95ppm, excluding the water region (4.72-5.05ppm)

2.6.3 Pattern Recognition Methods

Carried out by Jules Griffin (Department of Biochemistry, University of Cambridge). Normalised data was imported into SIMCA-P+ version 11 (Umetrics, Umea, Sweden) for multivariate statistical analysis. The data was mean centred and scaled in SIMCA. Data were Pareto scaled to suppress the contribution from regions of the spectra containing only noise. Initially, principal components analysis (PCA) models were built to identify the major trends in datasets and identify outliers. The supervised technique partial least squares discriminate analysis (PLS-DA) was then used to look for differences between control and *LKB1/KRAS* mutant cells and identify changes responsible for the separation. Significantly changed metabolites were identified using loading column plots with error bars set at 95% confidence limit using a jack-knifing routine within the SIMCA software. R2 values were used to assess the variation explained by the models and Q2 values were used to assess the robustness of the models. Additionally, the SIMCA P+ validate function was used to ensure that the PLS-DA models were not over fitted.

Chapter 3:

LKB1 mutations co-occur with KRAS mutations in NSCLC and confer sensitivity to the MEK inhibitor CI-1040 and the mTOR inhibitor rapamycin.

3.1 Introduction

The aim of this thesis was to functionally characterise a genetic subset of NSCLC. Mutations in the serine/threonine kinase, LKB1 are found in approximately 30% of NSCLC and in the hereditary cancer Peutz-Jeghers Syndrome (PJS) which is characterised by benign hamartomatous polyps, especially in the gastrointestinal tract and marked cutaneous pigmentation of the mucous membranes. The mutations are loss of function mutations and often encompass whole exons or multiple exons. Early work in PJS examined whether LKB1 required co-operation with another cancer gene in order to turn the hamartomatous polyps into adenomatous and carcinomatous lesions (Miyaki, et al. 2000). I wanted to examine whether in NSCLC, LKB1 co-operated with any other known cancer genes and if it did whether this would render the tumour sensitive to targeted therapies.

LKB1 has 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. The majority of mutations in LKB1 in PJS and sporadic lung cancer are loss of function,

including in-frame deletions, splicing mutations, deletions of exons, frameshift mutations, stop mutations and point mutations (reviewed by Alessi, et al. 2006).

We made the observation that *LKB1* loss occurs with *RAS-MAPK* activating mutations in NSCLC (Mahoney, et al. 2009; http://www.sanger.ac.uk/genetics/CGP/CellLines/). A similar finding was made by Matsumoto et al. 2007, where they identified *LKB1* loss with *KRAS* activation in primary lung tumours. A study in mouse embryonic fibroblasts suggested that loss of the LKB1 tumour suppressor provoked intestinal polyposis but resistance to transformation by RAS (Bardeesy, et al. 2002). The statistically significant co-occurrence of these mutations in a number of NSCLC cell lines suggests a biological link where NSCLC cell lines with *LKB1* mutations may have a general requirement for activation of the MAPK cascade to overcome suppression of MAPK signalling by RHEB.

This chapter investigates the functional relationship of the *LKB1* loss with *KRAS* activation in NSCLC cell lines. LKB1 and RAS-MAPK signalling pathways are linked via RHEB, which when active, activates mTOR and inhibits wild type BRAF, but has no inhibitory effect on *V600* mutant *BRAF* (Garami et al., 2003; Im, 2002; Karbowniczek et al., 2004). It was recently shown that the inhibition of RAF1(cRAF) activity by RHEB prevents heterodimerisation of BRAF and RAF1 by phosphorylating BRAF (ser446) and RAF1 (ser338) in a rapamycin-insensitive manner and by inhibiting the association of BRAF with HRAS (Karbowniczek et al., 2006). *LKB1* mutations in NSCLC may therefore have a general requirement for an activation of the MAPK cascade to overcome suppression through RHEB inhibition. This interdependence suggests that inhibition of MAPK signalling may constitute a potential opportunity for therapeutic intervention in this genetic subset of NSCLC (Figure 3-1).

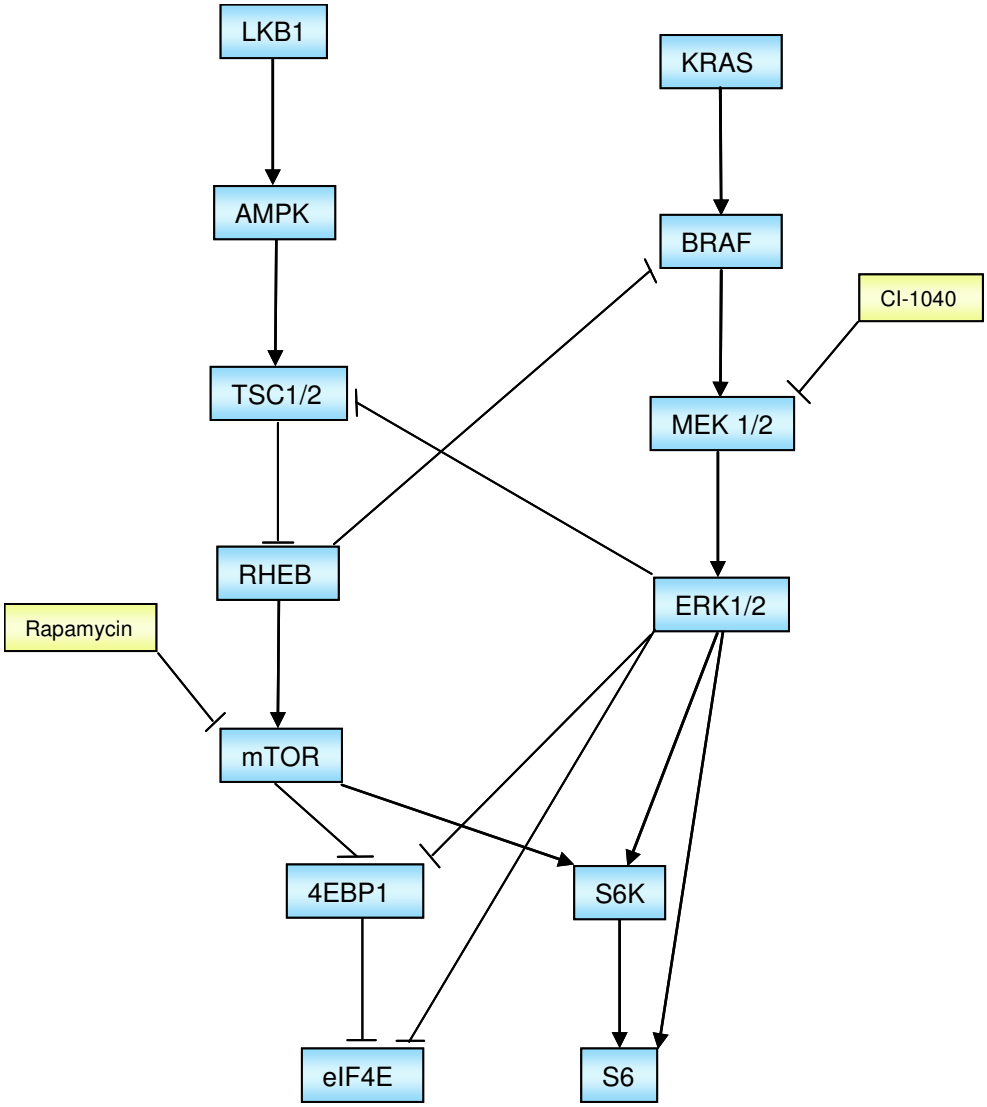

Figure 3-1 Simplified snapshot of crosstalk between LKB1 and RAS-MAPK signalling pathways by the analysis of literature (for references, see Chapter 1). Highlighted in the yellow boxes are possible targets for therapeutic intervention.

3.2 Results

3.2.1 *LKB1* loss co-occurs with *RAS-MAPK* mutations in NSCLC cell lines

Resequencing of known cancer genes in 87 NSCLC cell lines (http://www.sanger.ac.uk/genetics/CGP/CellLines/) found significant association of LKB1 inactivating mutations with KRAS activating mutations (*P*-value=0.03) (Table 3-1). The association between *LKB1* and *KRAS* mutations has been confirmed by another study (Matsumoto, et al. 2007). In addition we also observed association of *LKB1* inactivation with *non-V600 BRAF* mutations (Figure 3-2).

		KRAS/BRAF mutation		Total
		Y	N	
LKB1 mutation	Y	16	10	26
	N	21	40	61
Total		37	50	87
		Two-tailed *P*-value		**0.03**

Table 3-1. Statistical significance of *LKB1* mutations and *RAS-MAPK* pathway mutations in NSCLC. Statistical analysis of 87 NSCLC cell lines was carried out by Fisher's Exact Test (*P*-value=0.03).

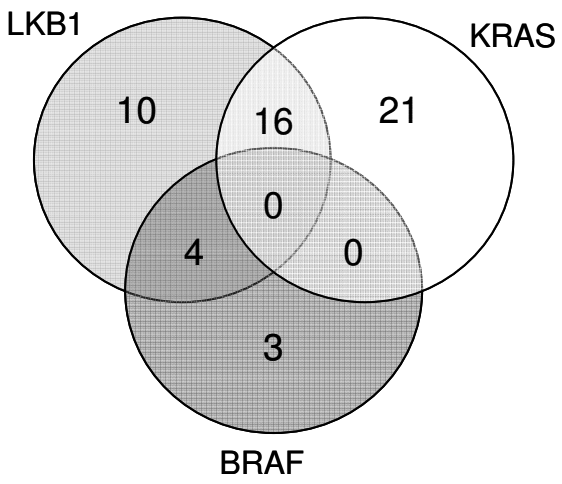

Figure 3-2. Venn diagram showing the overlap of *LKB1, KRAS* and *BRAF* mutations in 87 NSCLC cell lines.

3.2.2 Cell lines with inactivation of *LKB1* and activated *KRAS* are more sensitive to CI-1040

To test the hypothesis that NSCLC cell lines with *LKB1* inactivation may have a general requirement for mutational activation of the MAPK cascade to overcome suppression through RHEB inhibition, NSCLC cell lines of a known genetic background (Table 3-2) were treated with CI-1040 (PD184352) for 72hrs and proliferation rate determined (Figure 3-3). For a positive control the melanoma cell line; SKMEL-28 which is known to be sensitive to CI-1040 was also treated (Solit, et al. 2006).

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Cell Line	Histology	LKB1	BRAF	KRAS	PIK3CA	P53	CDNKA	EGFR
		Mutation	Mutation	Mutation	Mutation	Mutation	Mutation	Mutation
SKMEL28	Malignant melanoma		c.1799T>A p.V600E			c.434_435TG>GT p.L145R		c.2257C>T p.P753S
CAL-12T	NSCLC nos	No protein[A]	c.1397G>T p.G466V			c.404G>T p.C135F	c.172C>T p.R58*	
A549	NSCLC nos	c.109C>T p.Q37*		c.34G>A p.G12S			c.1_471del471 p.M1_*157del	
NCI-H1734	NSCLC Adenocarcinoma	c.152_153insCT p.M51fs*14		c.37G>T p.G13C				
NCI-H460	NSCLC Large cell carcinoma	c.109C>T p.Q37*		c.183A>T p.Q61H	c.1633G>A p.E545K		c.1_457del457 p.?	
NCI-H2030	NSCLC Adenocarcinoma	c.949G>T p.E317*		c.34G>T p.G12C		c.785G>T p.G262V		
NCI-H1563	NSCLC Adenocarcinoma	c.816C>A p.Y272*					c.1_471del471 p.M1_*157del	
NCI-H2009	NSCLC Adenocarcinoma			c.35G>C p.G12A		c.818G>T p.R273L		
NCI-H1975	NSCLC Adenocarcinoma				c.353G>A p.G118D	c.818G>A p.R273H	c.205G>T p.E69*	c.2573T>G p.L858R
NCI-H1838	NSCLC Adenocarcinoma					c.818G>T p.R273L	c.1_471del471 p.M1_*157del	

Table 3-2. Mutation status of oncogenes and tumour suppressors known to be commonly mutated in NSCLC for the cell lines treated. Mutation data taken from COSMIC (http://www.sanger.ac.uk/genetics/CGP/CellLines/). ([A]) No mutation has been found in this sample by sequencing, however immunoblot analysis revealed no protein present. NSCLC nos = NSCLC not otherwise specified.

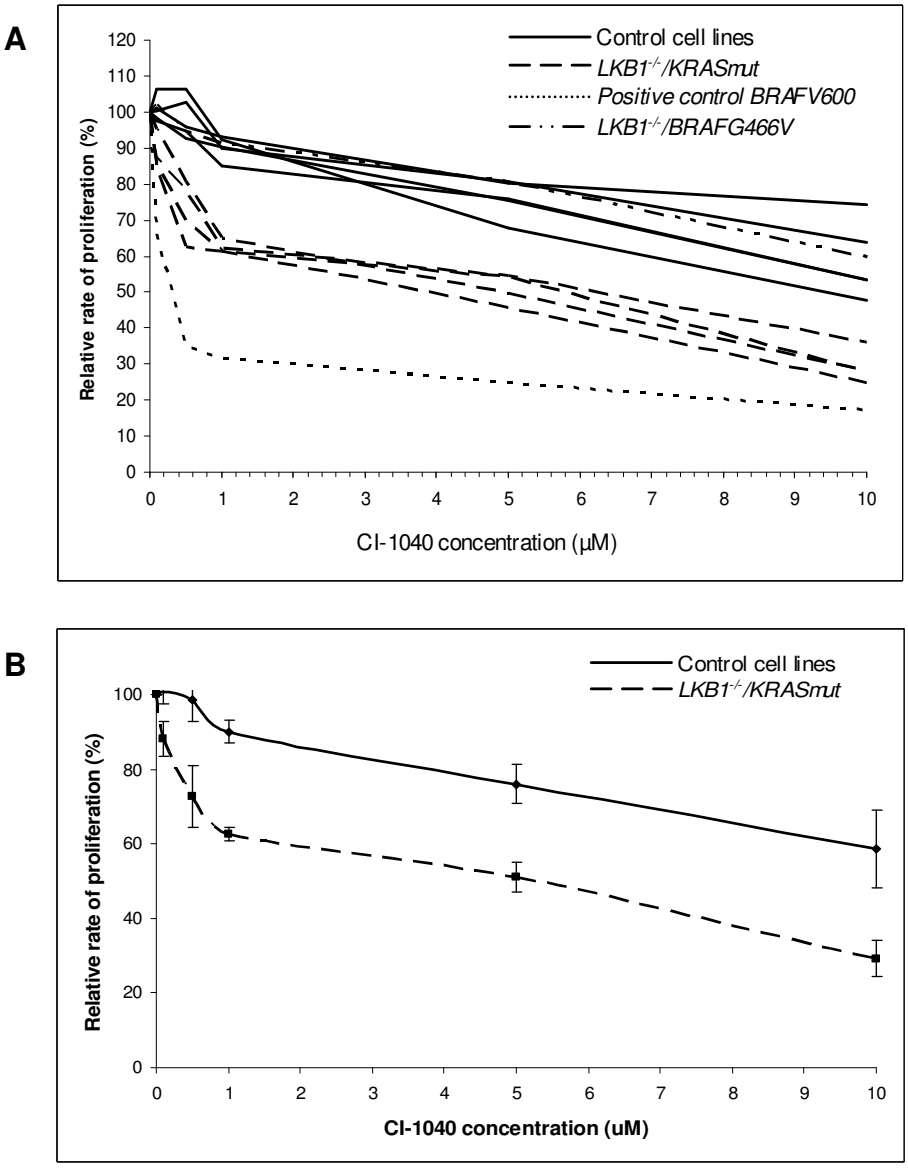


Figure 3-3 Cell lines with inactivated *LKB1* and activated *KRAS* are more sensitive to the MEK inhibitor CI-1040 A) Shows the relative rate of proliferation 72hrs after CI-1040 treatment for all cell lines used in the study (n=12 from 2 independent experiments), cell lines tested: NCI-H460 (*LKB1*$^{-/-}$*/KRASmut*), A549 (*LKB1*$^{-/-}$*/KRASmut*), NCI-H1734 (*LKB1*$^{-/-}$*/KRASmut*), NCI-H2030 (*LKB1*$^{-/-}$*/KRASmut*), collectively labelled *LKB1*$^{-/-}$*/KRASmut* in the figure; NCI-H1838 (*wt*), NCI-H1975 (*wt*), NCI-H2009 (*KRASmut*), NCI-H1563 (*LKB1*$^{-/-}$*),* collectively labelled control cell lines; CAL-12T (LKB1$^{-/-}$/BRAFmut), and SKMEL28 (positive control, *BRAF V600*). B) Values from the 2 clusters in A were averaged to calculate the statistical significance between the clusters, values shown ±s.d between the cell lines within the cluster, n≥4 for each cluster.

LKB1/KRAS mutant cell lines show a uniform enhanced sensitivity to MEK inhibition when compared to wild type cell lines, *LKB1* mutant lines or *KRAS* mutant lines (labelled control cell lines in Figure 3-3A). Interestingly, the *LKB1/BRAF466V* mutant cell line (CAL12T) is insensitive to CI-1040 and falls in the top cluster. The mean relative proliferation rate calculated for *LKB1/KRAS* mutant cell lines and compared to the control cell line cluster was statistically significant (2-tailed unpaired t-test; *P*-value<0.001 at all CI-1040 concentrations > 0) (Figure 3-3B). *LKB1/KRAS* mutant cell lines have a mean IC_{50} value of 5μM compared to the control cell lines which have a mean IC_{50}>10μM. This sensitivity appears to correlate with *LKB1* and *KRAS* mutation status and not any other known genetic alterations (Table 3-2).

3.2.3 Cell lines with inactivation of *LKB1* and activated *RAS-MAPK* pathway are more sensitive to rapamycin

We next investigated the role of mTOR in the genetic subtypes of lung cancers under study using the mTOR inhibitor, rapamycin (Figure 3-4).

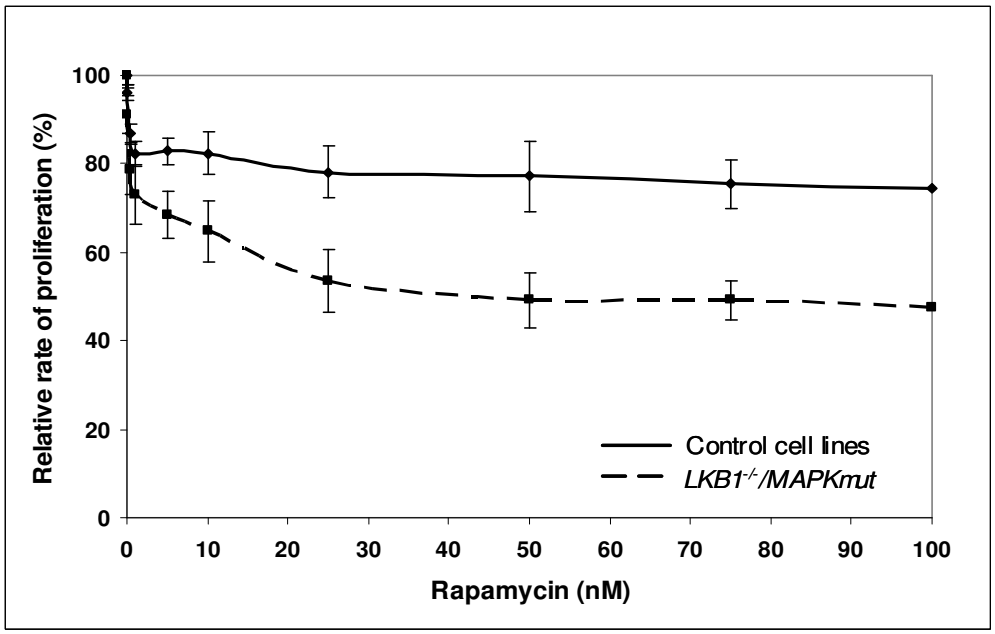


Figure 3-4. Cell lines with inactivated *LKB1* and activated *RAS-MAPK* are more sensitive to mTOR inhibition with rapamycin. Due to the similar nature of the results to the CI-1040 experiment the cell lines were clustered according to their mutation status (*LKB1$^{-/-}$/ras-MAPKmut*) (NCI-H460, A549, CAL12T, NCI-H2030) or control cells (*LKB1$^{-/-}$, WT and KRAS mut*) NCI-H1563, NCI-H1838, NCI-H2009).

Figure 3-4 shows inhibiting mTOR using rapamycin had a more pronounced affect on proliferation in *LKB1/KRAS* mutant cell lines; in this case the sensitive cluster also included the *LKB1/BRAF* mutant cell line CAL12T. The IC_{50} of the *LKB1/MAPK* mutant cluster was significantly different from the control cluster (40nM vs. >100nM, *P*-value ≤0.04). For all rapamycin concentrations ≤10nM the difference in proliferation rate

between *LKB1/MAPK* mutants and controls is statistically significant (Unpaired 2-tailed *t*-test; *P*-value<0.05).

3.2.4 Dual inhibition with rapamycin and CI-1040 is neither additive nor synergistic in *LKB1/KRAS* mutant cell lines.

Due to the sensitivity of *LKB1/KRAS* mutant NSCLC cell lines to single agent treatment with rapamycin or CI-1040 we wanted to determine whether dual agent treatment would be additive/synergistic. The same cell lines were treated with 10nM rapamycin +/- CI-1040 (figure 3-5).

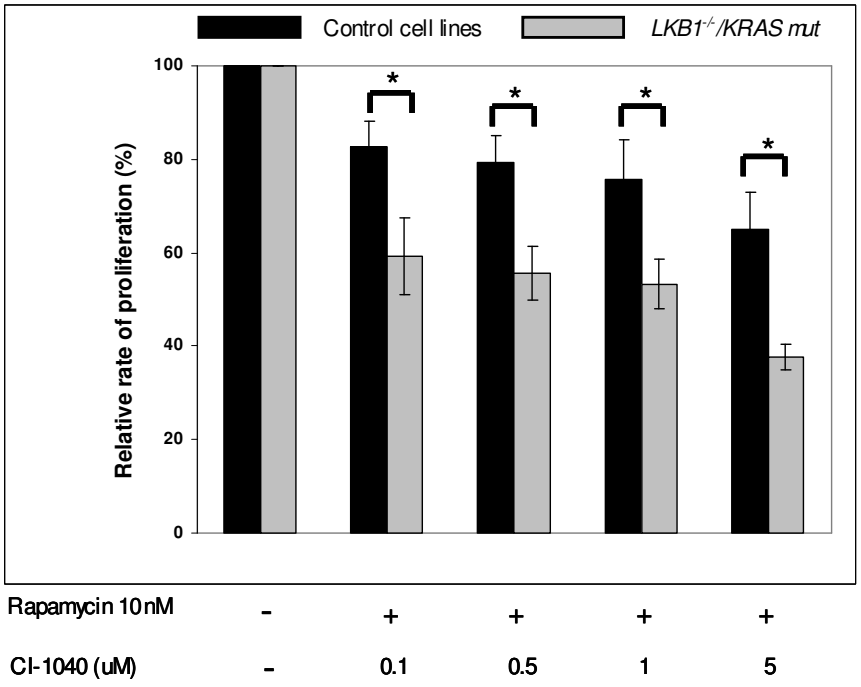


Figure 3-5 The *LKB1/KRAS mutant* NSCLC cell lines are more sensitive to dual inhibition than control cell lines. Dual inhibition experiments were carried out using 10nM rapamycin and a range of CI-1040 concentrations in the same format as single agent experiments; cell lines were again grouped by their mutation status, results from two independent experiments each with 6 replicates means ±s.d.

Dual inhibition of MEK and mTOR leads to a statistically significant decrease in the proliferation rate in the *LKB1/KRAS* mutant cluster versus the control cell lines (2-tailed unpaired *t*-test; P ≤0.01). This effect of dual inhibition did not reach statistical significance for additivity nor being synergistic in either cluster (Figure 3-5). At the highest concentrations (5μM CI-1040 with 10nM rapamycin) in the *LKB1/KRAS* mutant cluster the data were consistent with an additive model (Unpaired 2-tailed *t*-test; **P*-value = 0.005). However this may be due to the combined toxic effects of higher drug concentrations. The non-additive effects of MEK and mTOR inhibition would suggest redundancy in the pathways.

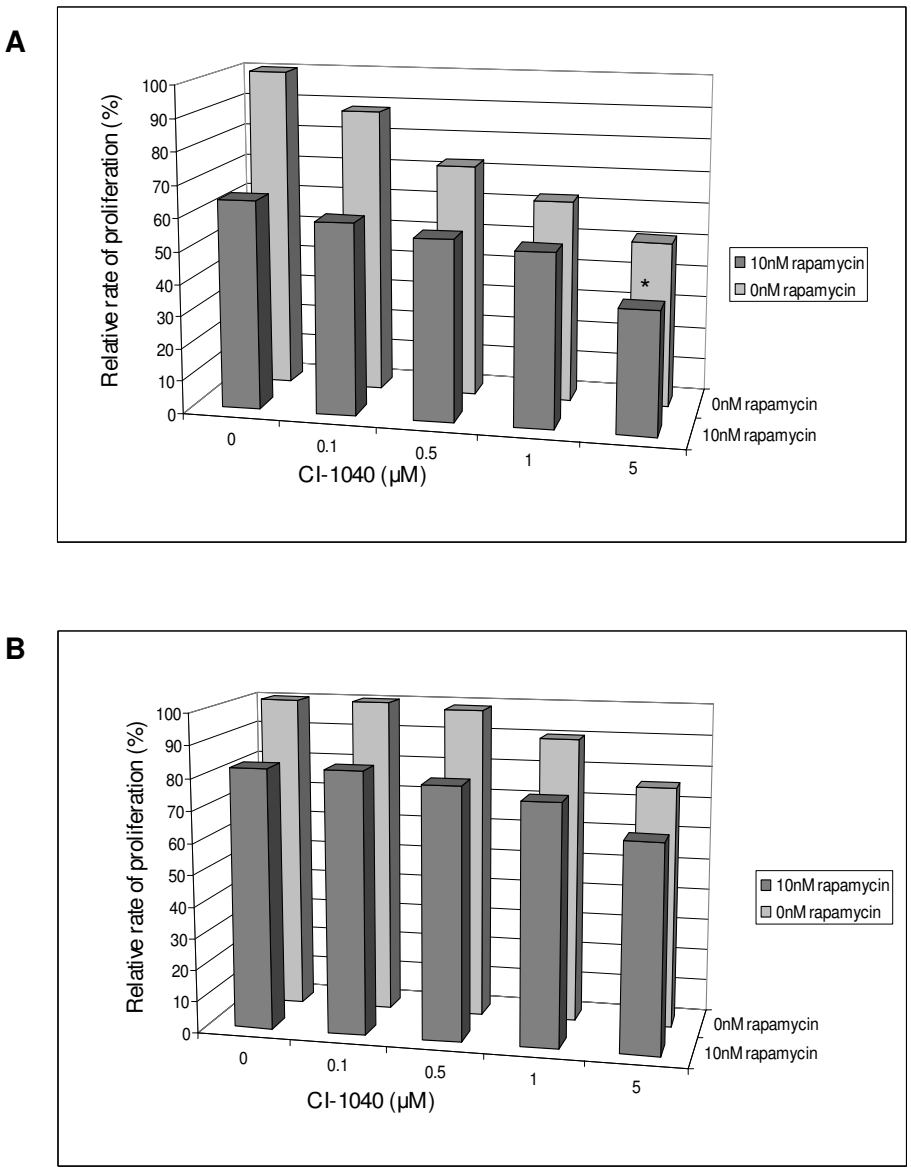


Figure 3-6 Dual inhibition with rapamycin and CI-1040 is neither additive nor synergistic. A) Comparison of dual agent treatment to most potent single agent treatment to determine if the agents are additive/synergistic in the *LKB1*$^{-/-}$ */KRASmut* group. Statistical significance determined using unpaired 2-tailed t-tests between single agent treatment group and each dual treatment group. The only significant value marked * p= 0.005, n=3 means±s.d. B) Comparison of dual agent treatment to most potent single agent treatment in control cell lines to determine if the agents are additive/synergistic in the control group. No statistically significant values were found, n=3, means±s.d

3.2.5 Sensitivity of *LKB1/KRAS* mutant cell lines to CI-1040 is not due to downstream effects on cyclin D1.

Previous work with CI-1040 in *BRAF* mutant melanomas has shown that the growth inhibition is correlated with a decrease in phospho-ERK and cyclin D1 protein levels (Solit, et al. 2006). To confirm that MEK inhibition was being achieved in the cells, we carried out immunoblot analysis of ERK, phosphorylated ERK and cyclin D1 levels at 8 and 24hrs following CI-1040 treatment (Figure 3-6). In all cell lines, levels of phosphorylated ERK decreased with increasing CI-1040 concentration by 8hrs, however cell lines wild type for both genes or *LKB1* mutant required higher concentrations of CI-1040 to prevent phosphorylation of ERK. The effect of MEK inhibition on cyclin D1 levels did not appear to correlate with genetic status and interestingly the *KRAS* mutant cell line NCI-H2009 showed a similar decrease in phosphorylated ERK and perhaps the greatest decrease in cyclin D1 levels, despite the inhibitor having little effect on proliferation. Together these data demonstrate the effects of MEK inhibition on phospho-ERK are driven by the presence or absence of a *KRAS* mutation and are independent of *LKB1* mutation status, whereas the proliferation effects are related to *LKB1/KRAS* combined mutation status. This is result is in contrast to what was observed in melanoma cell lines (Solit, et al. 2006).

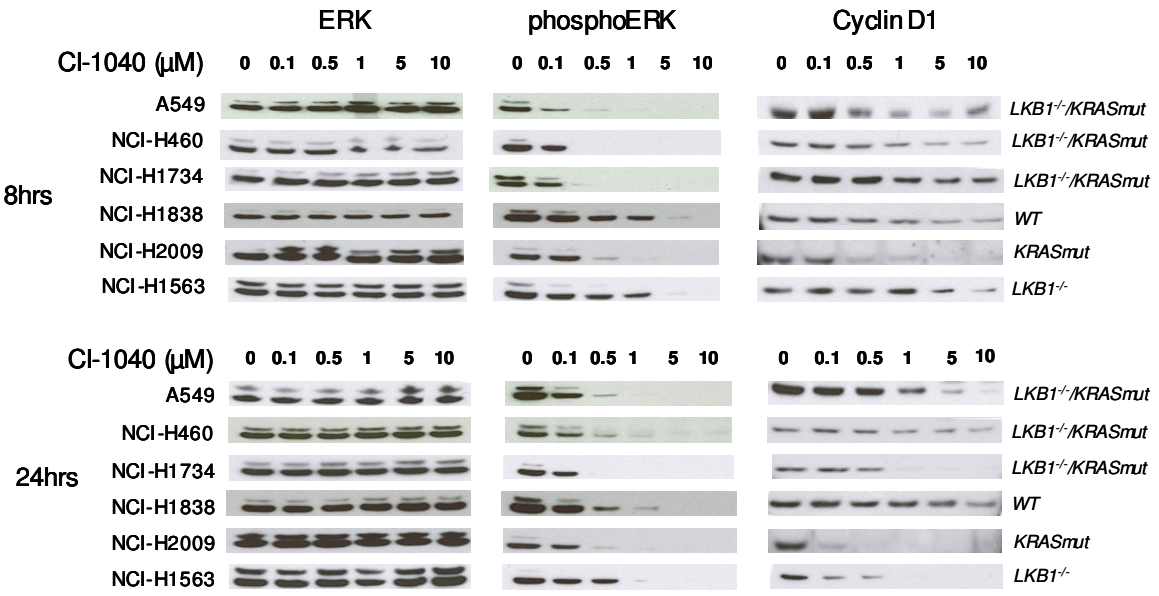


Figure 3-7 Sensitivity to the MEK inhibitor in *LKB1/KRAS* mutant cell lines is not due to downstream effects on cyclin D1. Cell lines were treated with CI-1040 and protein harvested 8 and 24hrs later. CI-1040 treatment leads to inhibition of phosphorylation of ERK as early as 8hrs and continues past 24hrs and the degree of inhibition is dependent on KRAS mutation status. Cyclin D1 protein levels are largely unaffected at 8hrs (apart from NCI-H2009) and although there is a more pronounced effect at 24hrs it does not appear to correlate with genetic status.

3.2.6 Sensitivity of *LKB1/KRAS* mutant cell lines to CI-1040 may be due to downstream effects on p70S6K.

To determine whether rapamycin was inhibiting mTOR and whether MEK inhibition was affecting downstream targets of mTOR, immunoblot analysis of p70S6K and phospho-p70S6K (thr-389) levels was carried out. Phosphorylation of this residue is critical for kinase function (Pullen et al., 1997). Figure 3-7 shows that CI-1040 treatment had no effect on total p70S6K protein levels; however a decrease was observed in phospho-p70S6K (thr-389) levels, specifically in *LKB1/KRAS* mutant cell lines. This decrease in

phosphorylation correlated well with the observed IC_{50} for this genetic subset. Figure 3-7 shows that rapamycin treatment had no effect on cyclin D1 protein levels but had a potent effect on phospho-p70S6K (thr-389) levels in all cell lines regardless of the mutation status.

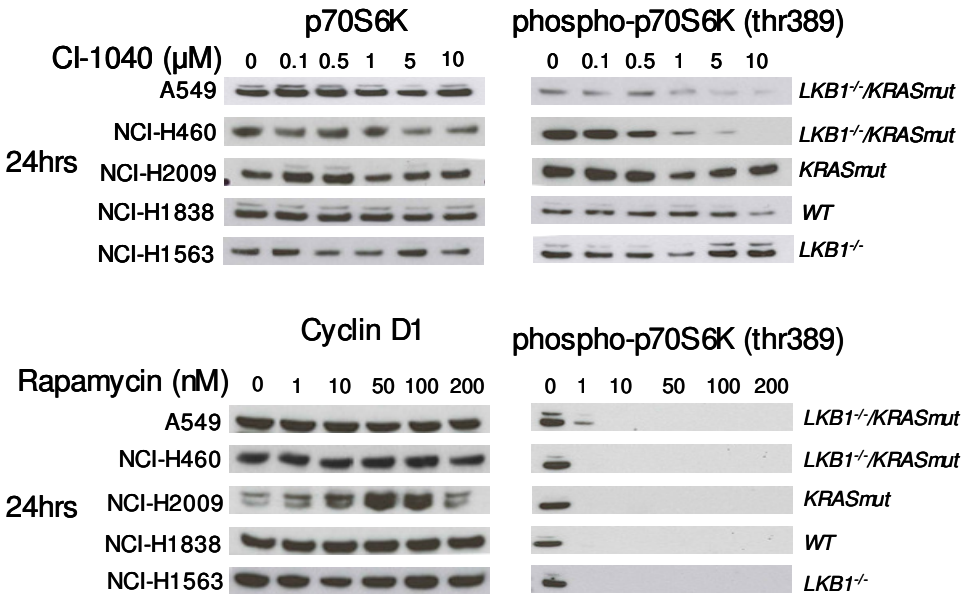


Figure 3-8 Immunoblot analysis of total p70S6K and phospho-p70S6K(thr-389) in CI-1040 treated cell lines and rapamycin treated cell lines. CI-1040 treatment has no effect on total protein levels of p70S6K, however in *LKB1/KRAS* mutant cell lines there is a decrease in phospho-p70S6K (thr-389), which isn't present in the control cell lines. Rapamycin leads to complete loss of phospho-p70S6K (thr-389) at all concentrations but has no effect on cyclin D1 protein levels.

3.3 Discussion

This aim of this chapter was to functionally characterise a newly identified genetic subset of NSCLC, with the aim of using this genetic knowledge to investigate the potential differential activity of targeted therapies. Here I report that *LKB1* inactivation and *KRAS* activation in non small-cell lung cancer denotes a functionally distinct set of lung cancer, which display sensitivity to the single agent treatment with the MEK inhibitor CI-1040 or rapamycin. It has been previously shown in melanomas that treatment with CI-1040 caused a dose dependent reduction in phospho-ERK and cyclin D1 protein levels in *BRAFV600E* mutant cell lines but not in *NRAS* mutant melanomas (Solit, et al. 2006). The results, while showing dose-dependent reduction of phospho-ERK in *KRAS* mutant NSCLC cell lines, demonstrate inhibition of proliferation only in the subset with both *KRAS* and *LKB1* mutations, highlighting the importance of the crosstalk in these pathways and supporting the genetic data that the co-occurrence of these two mutations is non-random.

Dose dependent decrease in phospho-ERK and reduction in proliferation rate did not result in a corresponding decrease in cyclin D1 levels, suggesting that in this subset of NSCLC the downstream effecter pathways may be different to *BRAFV600E* melanomas. The effects of reduced proliferation in this genetic subset may be due to reduction in the activity of p70S6K which is downstream of both mTOR and ERK1/2, shown by the specific reduction of phosphorylation of thr-389 p70S6K in *LKB1/KRAS* mutant cell lines following MEK inhibition. These data suggest that in this genetic subset RAS-MAPK signalling may also play an important role in control of protein synthesis. These data further highlight the importance of complexities of the crosstalk between these pathways in this genetic subset. MEK inhibition results in a decrease in phosphorylated ERK in all

cell lines tested but inhibition of p70S6K phosphorylation only occurs in *LKB1/KRAS* mutant cell lines. This would suggest that either there is a intermediary signalling protein between ERK and p70S6K specific to *LKB1/KRAS* mutant cell lines or that the phosphorylation of p70S6K is occurring in a MEK dependent/ERK independent manner. Despite being sensitized to CI-1040 and rapamycin, dual agent treatment did not have demonstrably additive or synergistic effects in *LKB1/KRAS* cell lines, suggesting possible redundancy in the pathways. The lack of additivity may be explained by the observation that rapamycin potently inhibits p70S6K phosphorylation at thr-389, therefore precluding any additional effect of CI-1040 on the p70S6K activity, further confirming redundancy of the pathways in this genetic subset of NSCLC. Interestingly, CAL12T the *LKB1/BRAF* mutant cell line is insensitive to CI-1040 yet sensitive rapamycin, suggesting an additional, smaller genetic subset in NSCLC and possibly highlighting the difference of the *non-V600 BRAF* mutations found in NSCLC (Brose et al., 2002). Further work will be required to determine whether this is indeed a smaller genetic subset and the molecular mechanisms underlying the rapamycin sensitivity and CI-1040 insensitivity.

Whilst the data support the hypothesis that *LKB1/KRAS* mutant NSCLC cell lines are a genetically and functionally distinct subset of NSCLC, this study did not examine the effects of compound treatment on apoptosis. The assay used in this study is an indirect measure of cell proliferation through cell numbers, cell number could decrease due to apoptosis. However, no apoptotic effects were visible upon light microscope examination. Further work could examine any apoptotic effects of compound treatment and expand on the proliferation assay carried out in this study using techniques such as BrdU incorporation or metabolic incorporation of tritiated thymidine to measure proliferation rate. The time course examined here was a standard time course used for

proliferation assays of 72hrs - further studies could examine the effects of a compound treatment over a longer period of time, another variable which could affect the data is the density at which the cells are plated, although care was taken to optimise this. Additional studies could examine the links between the pathways to determine whether in the context of NSCLC RHEB does inhibit BRAF as suggested by other groups (Im, et al. 2002; Garami, et al. 2003; Karbowniczek, et al. 2004). siRNA knockdown of RHEB in these cell lines should elucidate the signalling crosstalk between MAPK and mTOR pathways. Recent data in *RAS*-mutated melanoma has found there is a switch from BRAF to RAF1 signalling (Dumaz, et al. 2006). siRNA knockdown of BRAF would determine whether the signalling in this subset of NSCLC is through BRAF or RAF1.

Mutation status in cancers has been shown to predict response to targeted therapies, as exemplified by the efficacy of EGFR inhibitors in EGFR mutant lung cancer (Lynch et al., 2004; Paez et al., 2004). Data presented here suggest that *LKB1/KRAS* mutated tumours are a genetic and functionally distinct subset of NSCLC. This data agrees with findings by Matsumoto, et al. 2007 and Ding et al. 2008, the latter screened a large number of primary lung tumour samples for mutations in cancer genes including *LKB1* and *KRAS* and identified 2 subsets of *LKB1* mutant tumours, one with *KRAS* mutations and one without. Further, these data suggest that investigation of this subset of lung cancers with respect to newer generation inhibitors of MAPK and mTOR signalling pathways may provide a new opportunity for investigation of targeted therapeutics in this common adult malignancy. Recent data from a NSCLC clinical trial revealed RAD001 has displayed modest clinical activity (Soria, et al. 2009) it would be interesting to examine the mutation status of the responders.

Chapter 4:

Investigation of a unique expression profile in *LKB1/KRAS* mutant NSCLC

4.1 Introduction

The previous chapter identified and functionally characterised, a novel genetic subset of NSCLC characterised by *LKB1* loss and *KRAS* activation. This work suggests at the molecular signalling level, this genetic subset differ from other NSCLC. Both the LKB1/AMPK/mTOR and RAS-MAPK pathways control a number of downstream effectors both directly and indirectly through transcriptional changes. Previously, Fernandez, et al. (2004) described how *LKB1* null primary lung cancers had a unique expression profile, characterised by deregulation in signal transduction, cytoskeleton, transcription factors, metabolism of AMP and ubiquitinisation. Utilising microarray expression data, a study was carried out to compare the expression profiles of *LKB1/KRAS* mutant NSCLC to determine if there was a unique expression signature and, if so, what the nature of this signature was. From this it was hoped we would gain further insight into the molecular mechanisms underlying this genetic subset of NSCLC, with the possibility of identifying potential therapeutic targets.

The data revealed an expression signature in *LKB1/KRAS* mutant cell lines dominated by metabolic genes. Here I will give a brief introduction into normal cellular metabolism and cancer cell metabolism.

4.2 Introduction to metabolism

4.2.1 Normal cellular metabolism

Metabolism is generally broken down into two processes catabolism and anabolism. Catabolism is the break down of organic matter to produce energy, whereas anabolism uses energy to construct cellular components. The purpose of catabolism is therefore to produce the energy and components required for anabolism. Metabolism is organised into metabolic pathways where small molecules (metabolites) are produced and converted by a series of enzymes. The basic pathways are highly conserved among species.

Carbohydrates in the form of polysaccharides are broken down to monosaccharides such as glucose and transported into cells. Once in the cell, glucose is converted to pyruvate and a small amount of ATP via the ten enzymatic reactions of glycolysis (Figure 4-1). Pyruvate is then converted to acetyl-coA by the pyruvate dehydrogenase complex (PDC), the acetyl-coA then enters the citric acid cycle (TCA cycle) where a small amount of ATP is produced along with NADH from the oxidation of acetyl-coA (Figure 4-2). The NADH is then used by oxidative phosphorylation to generate large amounts of ATP by passing electrons from NADH to oxygen to pump protons across the mitochondrial membrane, generating an electrochemical gradient. Pumping protons back into the mitochondria via ATP synthase causes the phosphorylation of ADP, creating ATP. Under conditions of anaerobia, pyruvate is converted to lactate by lactate dehydrogenase, this reoxidises NADH to NAD+ which can re-enter glycolysis. An alternative pathway for glucose metabolism is the pentose phosphate pathway which is used to generate pentose sugars such as ribose for nucleotide synthesis. Fats can also be catabolised and produce more energy than carbohydrates; during this process free fatty acids and glycerol are

formed. The glycerol can be used for glycolysis and the fatty acids are broken down through beta-oxidation to form acetyl-coA which can enter the TCA cycle. Amino acids can be oxidised to urea and carbon dioxide and used as a source of energy. Transaminases remove the amino group which leaves a keto acid which can enter the TCA cycle. Glutamate can be deaminated by glutaminase to produce α-ketoglutarate a TCA cycle intermediate. Glucose can also be produced from non-carbohydrate sources such as lactate, glucogenic amino acids and glycerol by the process of gluconeogenesis (Voet & Voet, 2nd Ed. Chapter IV).

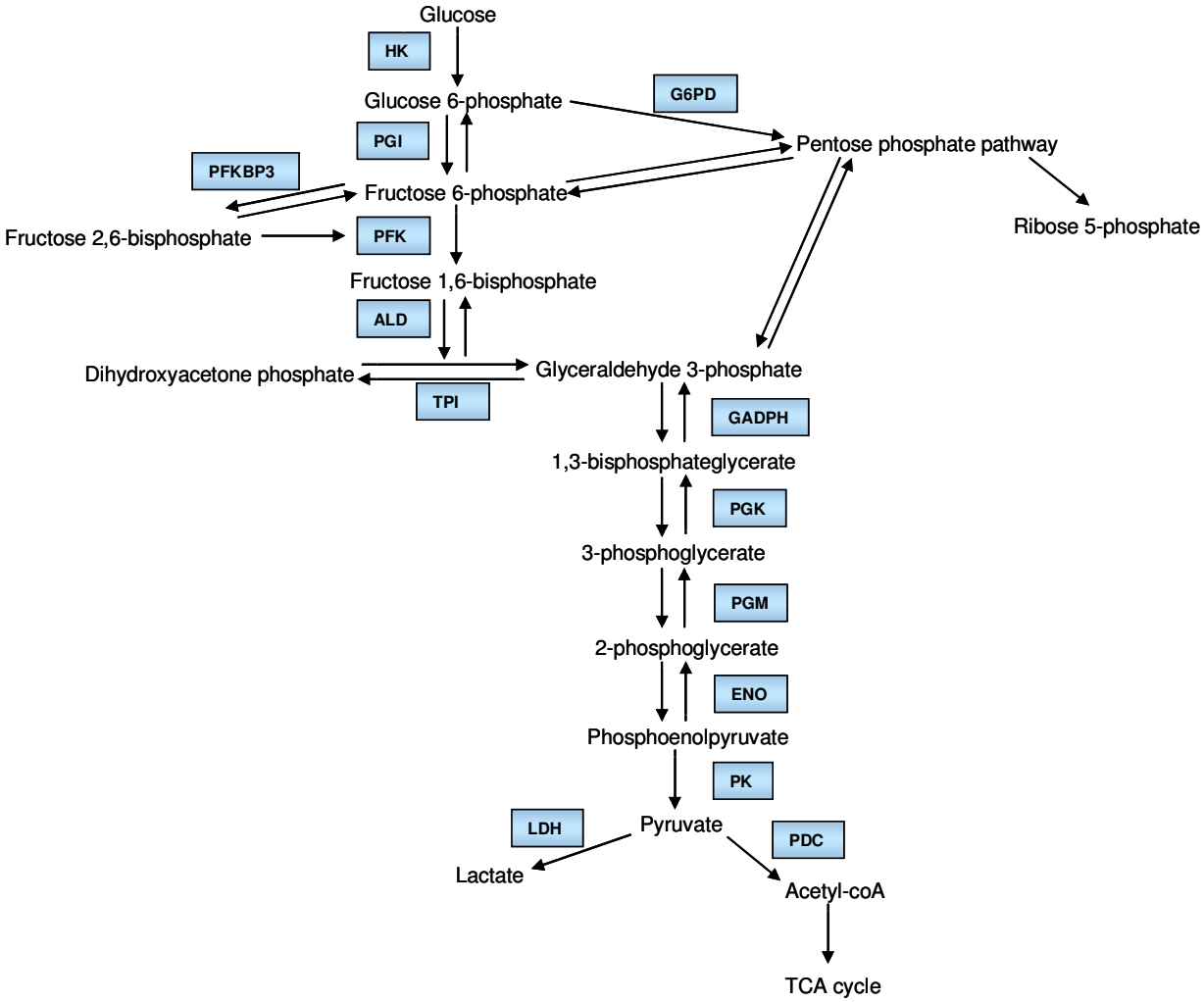


Figure 4-1 The main reactions of glycolysis. Glucose is converted to pyruvate through a series of enzymatic reactions. The resulting pyruvate can then enter the TCA cycle (figure 1-6). A number of glycolysis intermediates can also be diverted to the pentose phosphate pathway to generate ribose sugars for nucleic acid synthesis and reducing equivalents under certain conditions. Abbreviations: HK – hexokinase, PGI – phosphoglucose isomerise, PFKBP3 – 6-phosphofructo-2-kinase/fructose-2,6-bisphosphate 3. PFK– phosphofructokinase, ALD – aldose, TPI – triose phosphate isomerise, GADPH – glyceraldehyde 3-phosphate dehydrogenase, PGK phosphor glycerate kinase, PGM – phosphoglycerate mutase, ENO - enolase, PK – pyruvate kinase, LDH – lactate dehydrogenase, PDC – pyruvate dehydrogenase complex, G6PD – glucose-6-phosphate dehydrogenase.

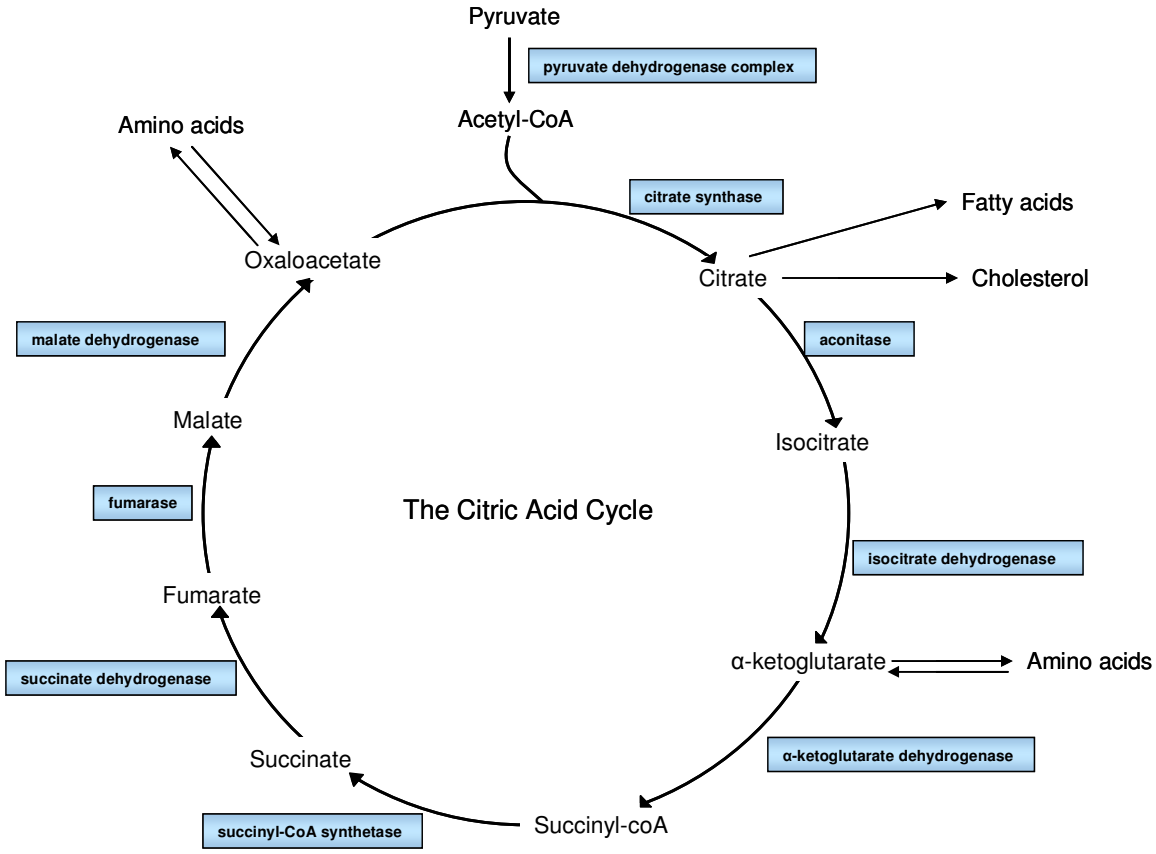


Figure 4-2. An overview of The Citric Acid Cycle. Pyruvate is converted to Acetyl-coA by the pyruvate dehydrogenase complex and enters the TCA cycle where it undergoes a series of enzymatic reactions for the generation of NADH for oxidative phosphorylation and ATP. Amino acids such as glutamate can be used to generate TCA cycle intermediates, fatty acids can be broken down to form acetyl-coA and citrate can be used to build fatty acids.

4.2.2 Cancer cell metabolism

It was first observed by Otto Warburg in 1956 that cancer cells predominantly produce energy through lactic acid fermentation rather than by oxidation of pyruvate in the mitochondria, an effect now know as the Warburg effect or aerobic glycolysis (Warburg, 1956). He postulated that this change in metabolism was the cause of cancer, which became known as the Warburg Hypothesis. It is now known that the cause of cancer is genetic and not metabolic; however mutations in genes which affect metabolic pathways have been found in a number of cancers. Isocitrate dehydrogenases (IDH) couple the interconversion of cytosolic isocitrate and a-ketoglutarate in an NADP+/NADPH-dependent reaction. IDH1 and IDH2 are mutated in almost 80% of gliomas (Parsons, et al. 2008; Balss, et al. 2008; Bleeker, et al. 2009; Yan, et al. 2009). Germline mutations have also been found in succinate dehydrogenase in hereditary paraganglioma and pheochromocytomas (Baysal, et al. 2000; Astuti, et al. 2001). In addition various signalling pathways altered in cancers such as LKB1/AMPK, PI3K and p53 all regulate different aspects of metabolism. For an overview of alterations to metabolism in cancer cells see Figure 4-3.

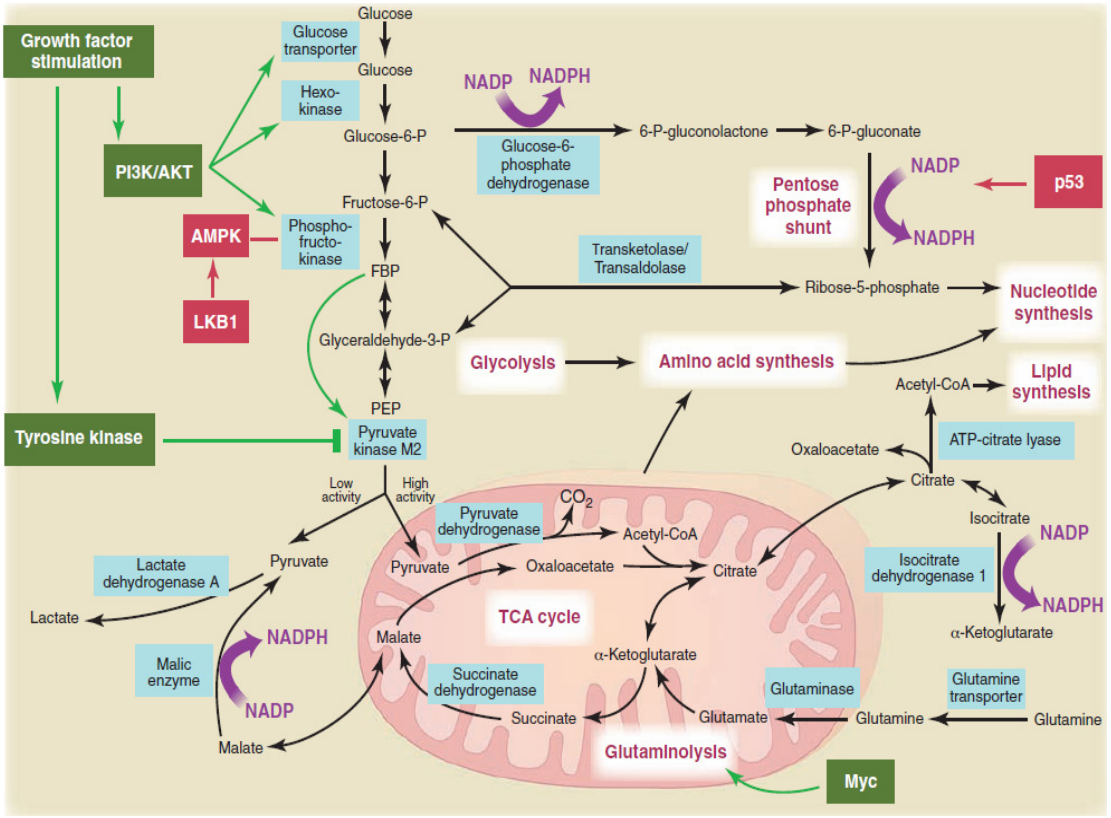


Figure 4-3 Overview of metabolic alterations in proliferating cancer cells (Figure from Vander Heiden et al. 2009). Signalling pathways which contain oncogenes and tumour suppressors directly and indirectly regulate the metabolic pathways within a cell. Shown here are the interactions between glycolysis, oxidative phosphorylation, glutamine metabolism and lipid synthesis.

4.2.2.1 Alterations to glycolysis in cancer cells

There are a number of ways in which cancer cells alter their metabolic pathways. Glycolysis requires glucose and accelerated glycolysis requires greater amounts of glucose, tumour cells achieve this by upregulating different forms of the glucose transporter. For instance head and neck cancers overexpress GLUT1 and 3 (Mellanen, et al. 1994), and breast cancers GLUT1 (Brown, et al. 1993). Glucose is then converted to glucose-6-phosphate (G6P) by hexokinase. Hexokinase has four isoforms, hexokinase I is under the control of HIF1 and MYC transcription factors, both of which play a role in cancer (Jonas, et al. 1999). G6P is then converted to fructose 6-phosphate by phosphoglucoisomerase (also known as autocrine motility factor (AMF)). Secreted phosphoglucoisomerase is thought to increase the motility and metastasis of tumour cells as it is secreted from them (Tsutsumi, et al. 2004; Yanagawa, et al. 2004). Increased expression of phosphoglucoisomerase has also been found along with its extracellular receptor in a number of tumours including NSCLC (Nakamori, et al. 1994; Maruyama, et al. 1995; Takanami, et al. 1998) where it has been associated with poor prognosis. Increased expression of phosphoglucoisomerase has also been observed in response to hypoxia (Niizeki, et al. 2002). Phosphofrucktokinase 1 (PFK1) is an important regulatory enzyme in glycolysis as it can regulate the entry of metabolic intermediates to other pathways such as the pentose phosphate pathway. PFK1 can regulate the rate of glycolysis, and is inhibited by ATP. PFKBP3 an isoform of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphate, creates fructose 2,6-bisphosphate (F2,6BP) which allosterically activates PFK1, thus increasing glycolytic flux. PFKBP3 protein expression has been found to be increased in a number of tumours types including colon, prostate, lung, breast, kidney, ovary, pancreas and thyroid (Atsumi, et al. 2002). PFKBP3 activity has been found to be enhanced by AMPK under conditions of energetic stress and oncogenic RAS (Kole, et al. 1991; Almeida, et al. 2004; Telang et al. 2006).

Pyruvate kinase catalyses the final step of glycolysis. There are four isoforms of pyruvate kinase, L and R, found in liver and red blood cells, M1 which is found in most other adult tissues and M2 which is expressed in foetal and tumour tissues (Mazurek, et al. 2005). Following on from the finding that tumour cells express the embryonic isoform of pyruvate kinase PKM2 (Mazurek, et al. 2005; Dombrauckas, et al. 2005), it was found that this isoform is essential for tumour cell growth and the Warburg Effect (Chirstofk, et al. 2008a). Knockdown of the M2 isoform by shRNA and replacement with the M1 isoform reversed the Warburg Effect and decreased the ability of H1299 cells to form tumours in nude mouse xenografts (Christofk, et al. 2008a). The same group showed that PKM2 is a phosphotyrosine-binding protein. PKM2 binding of phosphotyrosine peptides resulted in release of its allosteric activator fructose-1,6-bisphosphate and inhibition of PKM2 activity (Christofk, et al. 2008b). The phosphotyrosine regulation of PKM2 results in diversion of glucose to anabolic processes after growth factor stimulation (Christofk, et al. 2008b).

4.2.2.2 Other metabolic alterations beyond glycolysis in cancer cells

The end product of glycolysis is pyruvate which can either be converted into acetyl-coA and metabolised by the TCA cycle or converted to lactate. The conversion of pyruvate to acetyl-coA requires the PDC. It is a large multisubunit complex of enzymes and regulatory enzymes. The E1 component catalyses the oxidative decarboxylation of pyruvate, the E2 component transfers the acetyl group to coA and the E3 component catalyses the regeneration of oxidised lipoamide. The PDC is essential for responding to energy demands within a cell. When carbohydrate stores are low the activity of the complex is low. There are dedicated regulatory enzymes which control the complex via phosphorylation in a tissue-specific manner which ensures that the control of the complex is highly adaptable (Patel, et al. 1990). The regulation is carried out by pyruvate dehydrogenase kinases (PDKs) and pyruvate

dehydrogenase phosphatases (PDPs), the balance of phosphorylation and dephosphorylation by these two enzymes decides how much of the complex is in its active non-phosphorylated state. When the complex is phosphorylated and inactive the pyruvate is converted to lactate. There are four PDKs (1-4) which regulate the phosphorylation of the E1α subunit (Figure 4-4) (Roche, et al. 2001; Patel, et al. 2006).

The PDK4 isoform is expressed in order to conserve carbohydrates during starvation in heart, skeletal muscle, kidney and liver (Wu, et al. 1998, 1999 & 2001; Holness, et al. 2000; Roche, et al. 2001; Muoio, et al. 2001, Huang, et al. 2002). PDK4 is under the transcriptional regulation of the glucorticoid receptor, FOXO, peroxisome proliferator activated receptor (PPAR) and the estrogen-related receptor (Huang et al. 2002; Araki, et al. 2006; Zhang, et al. 2006). More recently it has been shown that PDK4 expression is synergistically induced by AMPK and fatty acids (Houten, et al. 2009). The PDK1 and PDK3 isoforms have been shown to be direct targets of HIF1 implicating the PDKs in hypoxia response and drug resistance (Kim, et al. 2006; Lu, et al. 2008). In the review by Roche and Hiromasa in 2007 they state that "Regardless of the rationale, the transition to Warburg metabolism requires shutting down of the PDC reaction". It is therefore thought that PDK inhibitors may be a powerful tool in cancer either for killing or slowing the growth of heavily glycolytic cancer cells.

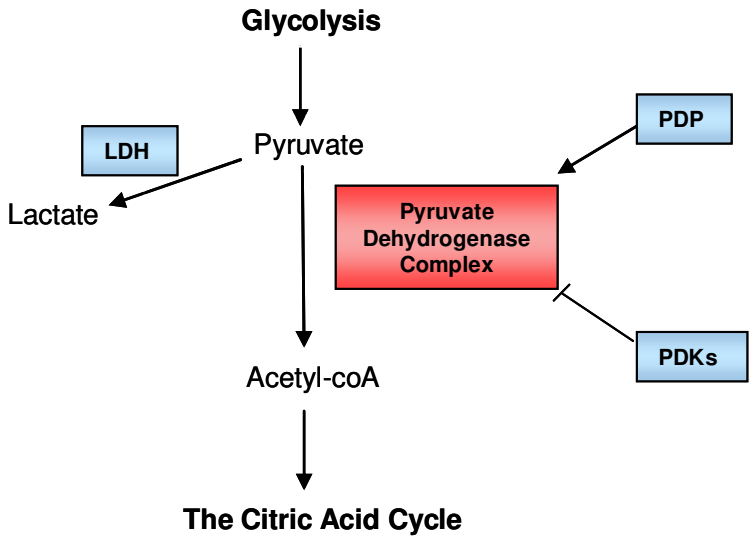


Figure 4-4 Regulation of the Pyruvate Dehydrogenase Complex (PDC). PDC controls the entry of pyruvate into the TCA cycle. It is regulated in a highly specific manner in depending on tissue type. Pyruvate dehydrogenase phosphatases (PDP) dephosphorylate the complex and activate it whilst pyruvate dehydrogenase kinases (PDKs) phosphorylate and inactivate it. In its inactive form it can no longer convert pyruvate to acetyl-coA and the pyruvate is converted to lactate by lactate dehydrogenase (LDH).

The conversion of pyruvate to lactate with concomitant interconversion of NADH and NAD+ is carried out by lactate dehydrogenase (LDH). The LDHA isoform is the most commonly upregulated in cancers and suppression of LDHA by shRNA has been shown to slow the proliferation of cancer cells *in vitro* and severely compromised their tumourigenicity *in vivo* (Fantin, et al. 2006).

The pentose phosphate pathway is required for the synthesis of nucleotides and rapidly dividing cells need to increase the production of nucleotides. As mentioned previously, glucose from the glycolysis pathway can be channelled into the pentose phosphate pathway to synthesise ribose 5-phosphate by two methods either the oxidative arm which uses glucose 6-phospate or the non-oxidative arm which uses

fructose 6-phosphate. As well as increasing nucleotide synthesis the pentose phosphate pathway generates NADPH required for reduction of oxidised glutathione. This increases the anti-oxidant capacity of the cell. Together these processes help cells repair DNA damage. Glucose 6-phosphate dehydrogenase is the first and the rate limiting enzyme in the pentose phosphate pathway and is known to be up-regulated in cancers (Board, et al. 1990).

Glutamine is an alternative energy source for cancer cells and is used for amino acid synthesis, nucleotide synthesis and anaplerotic reactions of the TCA cycle (DeBerardinis et al. 2007). Glutaminase converts glutamine to glutamate by deamination (glutaminolysis) which can be further converted to α-ketoglutarate and enter the TCA cycle. In addition breakdown of other amino acids and oxidation of odd-chain fatty acids can generate succinyl-coA to enter the TCA cycle.

In summary cancer cells display numerous alterations to their metabolic pathways all designed to support their excessive proliferation and need for biosynthetic processes. Some of the key deregulated metabolic enzymes may represent new targets for therapeutic intervention. While there is a much larger body of literature on the other metabolic pathway alterations found in cancer the ones described here are the most pertinent to the research that will be described.

4.3 Results

4.3.1 *LKB1/KRAS* mutant NSCLC cell lines have a unique gene expression profile

Expression profiles were determined using the Affymetrix Human U133 Plus 2 GeneChip (carried out by GlaxoSmithKline). Data quality test and normalization were performed using Affy package in R by Lina Chen (Irizarry, et al. 2003a). The quality of each array was assessed by checking the mean and standard deviation of over-all expression, percentages of present gene calls and background levels, 3': 5' ratios for spiked-in and control genes specific to the array type and the correlation among replicated samples. Background correction of each array was using robust multi-array average expression measure (Irizarry, et al. 2003; Bolstad, et al. 2003; Irizarry, et al. 2003b). The data were normalized by quantile method and expression level of each gene was log2 transformed. Genetic signature analysis was using Limma package from BioConductor (Smyth, 2004). Paired t-test was used to analyse differences between the expression profiles of between *LKB1/KRASmut* cell lines and wild type cell lines. *P*-values were corrected by Benjamini/Hochberg (BH) paradigm with a false discovery rate (FDR) of 0.01. Unsupervised hierarchical cluster was carried out using Cluster 3.0 (Eisen et al., 1998) and presented by Java TreeView (Saldanha, 2004). The similarities between cell lines were calculated based on uncentered Pearson correlation and used average linkage for clustering.

Statistical analysis of the 47000 transcripts using the BH paradigm and a FDR of 0.01 resulted in 550 genes with significantly different expression in *LKB1/KRAS* mutant NSCLC cell lines versus all other genetic subtypes (see Appendix A for complete list). Figure 4-5 shows the heatmap for the 550 differentially expressed genes, revealing a distinct expression pattern in *LKB1/KRAS* mutant cell lines, suggesting that cooperation of these two mutations results in a unique transcriptional output.

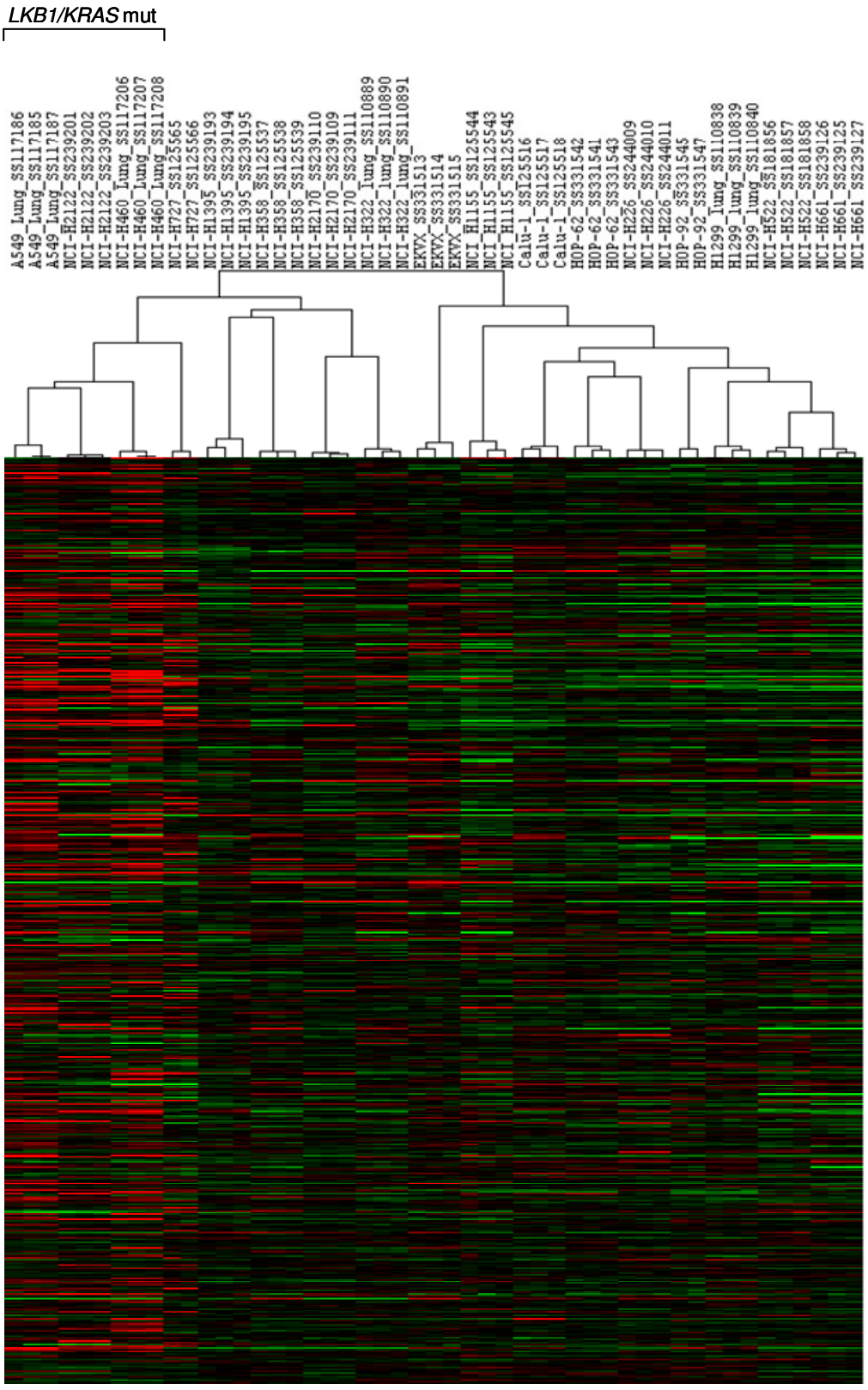

LKB1/KRAS mut
A549_Lung_SS117186
A549_Lung_SS117185
A549_Lung_SS117187
NCI-H2122_SS239201
NCI-H2122_SS239202
NCI-H2122_SS239203
NCI-H460_Lung_SS117206
NCI-H460_Lung_SS117207
NCI-H460_Lung_SS117208
NCI-H727_SS125565
NCI-H727_SS125566
NCI-H1395_SS239193
NCI-H1395_SS239194
NCI-H1395_SS239195
NCI-H358_SS125537
NCI-H358_SS125538
NCI-H358_SS125539
NCI-H2170_SS239110
NCI-H2170_SS239109
NCI-H2170_SS239111
NCI-H322_lung_SS110889
NCI-H322_lung_SS110890
NCI-H322_lung_SS110891
EKVX_SS331513
EKVX_SS331514
EKVX_SS331515
NCI_H1155_SS125544
NCI_H1155_SS125543
NCI_H1155_SS125545
Calu-1_SS125516
Calu-1_SS125517
Calu-1_SS125518
HOP-62_SS331542
HOP-62_SS331541
HOP-62_SS331543
NCI-H226_SS244009
NCI-H226_SS244010
NCI-H226_SS244011
HOP-92_SS331545
HOP-92_SS331547
H1299_lung_SS110838
H1299_lung_SS110839
H1299_lung_SS110840
NCI-H522_SS181856
NCI-H522_SS181857
NCI-H522_SS181858
NCI-H661_SS239126
NCI-H661_SS239125
NCI-H661_SS239127

Figure 4-5 *LKB1/KRAS* mutant cell lines have a distinct expression pattern. Analysis of 47000 transcripts using the BH paradigm and an FDR of 0.01 resulted in 550 genes with differential expression in *LKB1/KRAS* mutant NSCLC cell lines versus other genetic subtypes of NSCLC. Cell lines used in this study: NCI-H460, A549, NCI-H2122, NCI-H1395, NCI-H358, NCI-H727, NCI-H2170, NCI-H322, EKVX, NCI-H1155, Calu-1, HOP-82, NCI-H226, HOP-92, H1299, NCI-H522, NCI-H661.

The most differentially expressed gene was AKR1B10 with 70-fold higher expression in *LKB1/KRAS* mutant cell lines than NSCLC cell lines of other genetic subtypes (Appendix, Table 1). AKR1B10 is an aldo-keto reductase found to be over expressed in ~30% of NSCLC and has been postulated to be a biomarker (Fukumoto, et al. 2005; Kim, et al. 2007). This data raises the possibility it may be a marker for this genetic subset. Further investigation of AKR1B10 and its role in this genetic subset of NSCLC is described in Chapter 6.

GO term enrichment analysis of 550 differentially expressed genes using GoMiner (http://discover.nci.nih.gov/gominer/htgm.jsp) reveals that the top 10 significantly enriched categories are related to cellular metabolism (Table 4-1). The web-based program Metacyc (http://metacyc.org/) was used to overlay the gene expression data onto a map of the metabolic pathways in a human cell (Figure 4-6). These analyses revealed interesting pathway enrichment associated with *LKB1/KRAS* mutant status (Figures 4-6 & 4-7). There are a number of genes altered in the pentose phosphate pathway and in glutathione metabolism. The pentose phosphate pathway has been implicated in cancer cell metabolism for its role in producing reducing equivalents and nucleotide biosynthesis. It is important for producing the reducing equivalent NADPH which is required as a cofactor for glutathione metabolising enzymes, Figure 4-6 also highlights alterations in the expression of various glutathione metabolic pathways. Glutathione is a tripeptide composed of glutamate, cysteine and glycine and has numerous important functions within cells. It serves as a reductant and plays a role in amino acid transport, drug conjugation, cofactor in enzymatic reactions and generally maintaining the intracellular thiol-redox state. Increased glutathione levels generally increase antioxidant capacity and resistance to oxidative stress, an effect observed in many cancer cells, and also results in increased resistance to chemotherapeutic compounds (Yang, et al. 2006).

GO Category	Total genes	Changed genes	Enrichment	LOG10(p)	FDR
GO:0044237_cellular_metabolic_process	6894	485	1.192683	-10.5533	0
GO:0008152_metabolic_process	7501	500	1.145088	-7.55067	0
GO:0044238_primary_metabolic_process	6853	450	1.154206	-6.98812	0
GO:0044249_cellular_biosynthetic_process	805	97	1.637634	-6.17824	0
GO:0043170_macromolecule_metabolic_process	5884	504	1.164123	-5.95222	0
GO:0009058_biosynthetic_process	1181	127	1.461486	-5.25628	0
GO:0006732_coenzyme_metabolic_process	110	22	2.718135	-4.86271	0.002857
GO:0051186_cofactor_metabolic_process	138	25	2.462079	-4.65466	0.005
GO:0006139_nucleobase__nucleoside__nucleotide_and_nucleic_acid_metabolic_process	3370	301	1.213885	-4.40708	0.005556
GO:0043283_biopolymer_metabolic_process	4585	393	1.164915	-4.19402	0.01

Table 4-1 The most significantly enriched GO categories are related to cellular metabolism. *LKB1/KRASmutant* NSCLC cell lines have 550 differentially expressed genes; these are enriched for genes involved in various metabolic and biosynthetic pathways.

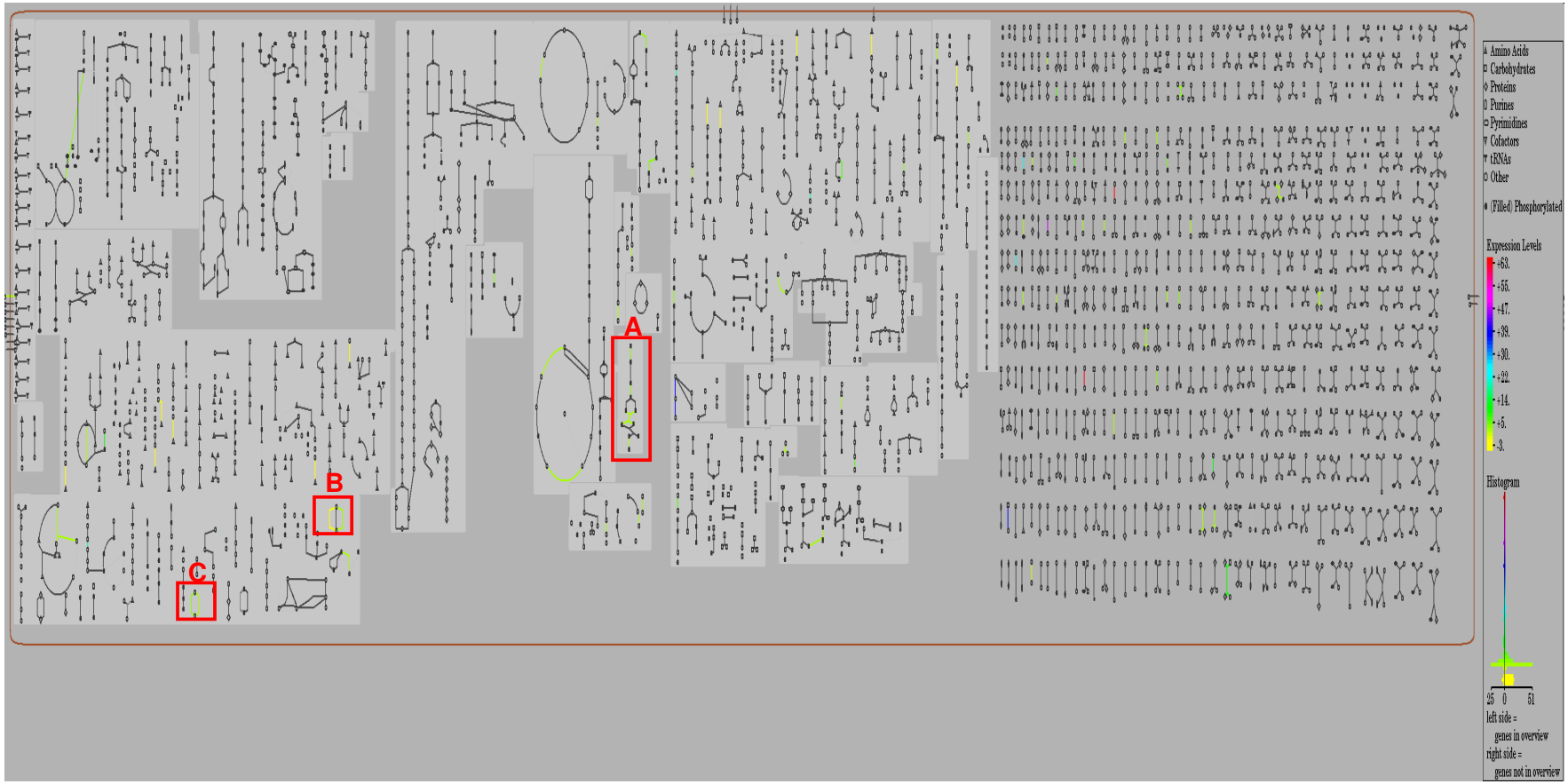


Figure 4-6. Metacyc map of the differentially expressed genes in *LKB1/KRAS* mutant NSCLC cell lines. Red boxes highlight pathways with a number of alterations in them. A indicates the pentose phosphate pathway and B+C glutathione metabolism.

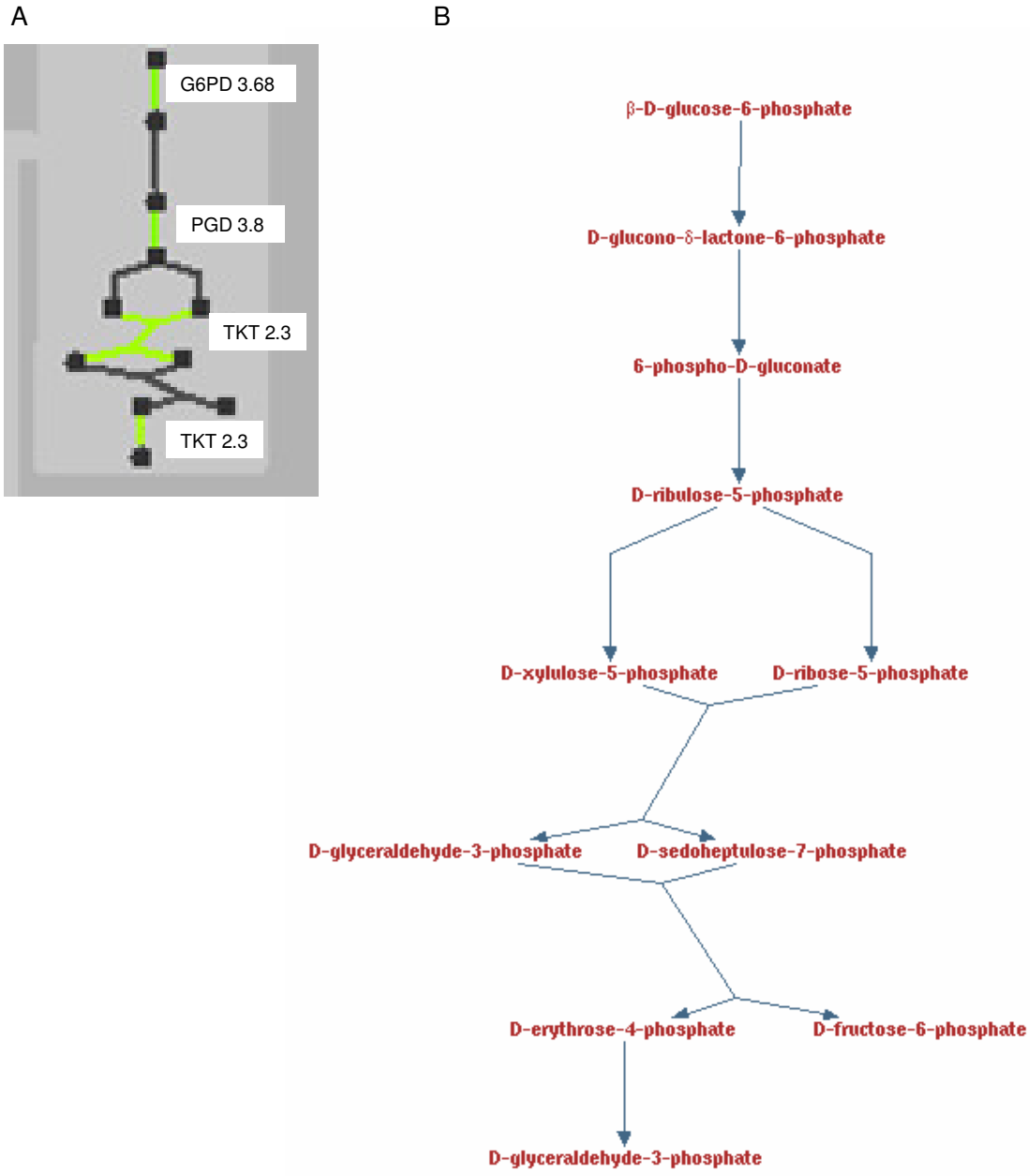


Figure 4-7 *LKB1/KRAS*mutant NSCLC cell lines show overexpression of 3 pentose phosphate pathway genes. A) Overlay of gene expression data onto the Metacyc pentose phosphate pathway. Gene names are in white boxes followed by fold change in expression in *LKB1/KRAS* mutant NSCLC cell lines. B) Graphical representation of the pentose phosphate pathway intermediates from Metacyc.

4.3.2 Investigation into the role of the pentose phosphate pathway in *LKB1/KRAS* mutant NSCLC cell lines

Glucose-6-phosphate dehydrogenase (G6PD) is the rate-limiting enzyme for the pentose phosphate pathway and *LKB1/KRAS* mutant cell lines have approximately 3.4 fold higher expression of the gene encoding G6PD. 6-aminonicotinamide (6-AN) is an inhibitor of G6PD and has been shown to sensitise tumour cell lines (including A549) to cisplatin by increasing the cellular accumulation of cisplatin, this correlated with an increase in the number of platinum adducts in the DNA (Budihardjo, et al. 1998). 6-AN is taken up by cells and transformed into 6-amino-$NADP^+$, an NADP analogue which inhibits G6PD. If *LKB1/KRAS* mutant cell lines were reliant on the pentose phosphate pathway for the production of reducing equivalents and/or nucleotide synthesis, it would be expected that 6-AN treatment might result in greater growth inhibition.

Cell lines were treated for 72 hours with a range of concentration of 6-AN (Figure 4-8). There is no significant difference in the proliferation rate of *LKB1/KRAS* mutant NSCLC cell lines compared to the other cell lines. Cell lines with *RAS-MAPK* pathway alterations (NCI-H460 (*KRAS*), NCI-H2030 (*KRAS*), A549 (*KRAS*), NCI-H1792 (*KRAS*) AND NCI-H1975 (*EGFR*)) appear to be more sensitive to 6-AN than cell lines without *RAS-MAPK* pathway alterations (NCI-H661), suggesting that in this case the *KRAS* mutation is the dominant mutation in predicting response to 6-AN. However, as there is only one cell line in this study without a *RAS-MAPK* pathway alteration this would need to be tested against a bigger panel of cell lines to determine whether *RAS-MAPK* pathway alterations are a determining factor in the response to 6-AN. Additional experiments including siRNA knockdown of enzymes in the pentose phosphate pathway such as G6PD should determine whether this

pathway is important for tumour growth specifically in *LKB1/KRAS* mutant NSCLC or generally in cell lines with *RAS-MAPK* pathway alterations.

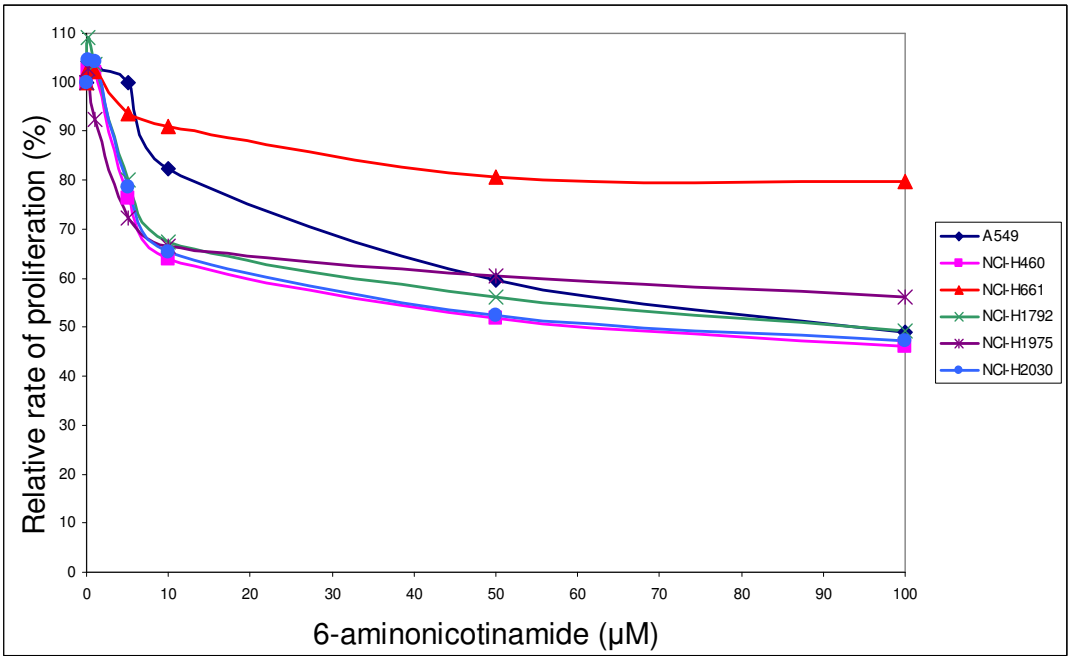


Figure 4-8 Cell lines with *RAS-MAPK* alterations are more sensitive to the G6PD inhibitor 6-AN. The relative rate of proliferation 72hrs after 6-AN treatment was examined for 6 NSCLC cell lines. Cell lines tested: NCI-H460 (*LKB1/KRASmut*), A549 (*LKB1/KRASmut*), NCI-H2030 (*LKB1/KRASmut*), NCI-H661 (wt), NCI-H1975 (*EGFR*mut), NCI-H1792 (*KRAS*mut), n=12 from 2 independent experiments.

4.3.3 PDK4 and investigation of its role in the Warburg Effect through inhibition of the Pyruvate Dehydrogenase Complex

PDK4 has 18-fold higher mRNA expression in *LKB1/KRAS* mutant NSCLC cell lines and is the fifth highest expressed gene on the array. PDK4 plays an important role in controlling the activity of the pyruvate dehydrogenase complex (PDC). PDC acts between glycolysis and the TCA cycle to regulate entry of pyruvate into the TCA cycle by converting it into acetyl-coA through a series of enzymatic reactions (Figure 4-9). The activity of the PDC is controlled through phosphorylation. Pyruvate dehydrogenase kinases (PDK1-4) inactivate the complex by phosphorylation whilst pyruvate dehydrogenase phosphatases (PDP1&2) activate it by dephosphorylation. In addition to the high expression of PDK4, the expression data revealed almost a 6-fold lower expression of PPM2C (pyruvate dehydrogenase phosphatase catalytic subunit 1 (PDP1)) in *LKB1/KRAS* mutant cell lines. Through the altered expression of these two genes it is possible that this would render the PDC inactive, preventing the conversion of pyruvate to acetyl-coA and leaving pyruvate to be converted to lactate (Figure 4-9). The Warburg Effect was an observation made by Otto Warburg over 50 years ago that most cancer cells predominantly produce energy by glycolysis followed by lactate production, rather than by oxidation of pyruvate in mitochondria even though it is energetically less efficient. Inactivation of the PDC has been hypothesised to be a mechanism by which the Warburg Effect could be created and this section concentrates on investigation of whether this mechanism is likely operative in *LKB1/KRAS* mutant NSCLC cell lines.

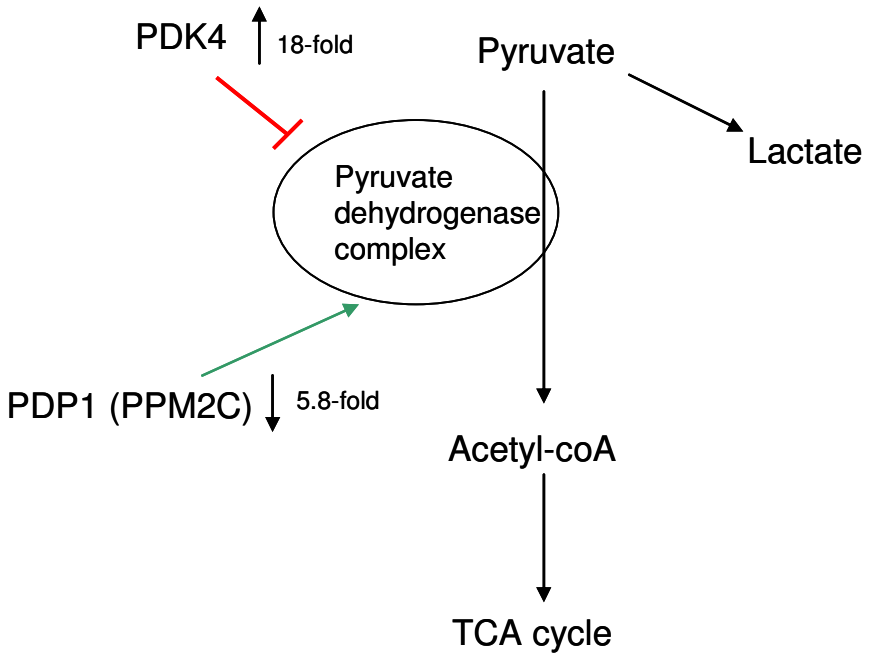


Figure 4-9 Regulation of the Pyruvate Dehydrogenase Complex (PDC). PDC controls the entry of pyruvate into the TCA cycle. It is regulated in a highly specific manner in depending on tissue type. Pyruvate dehydrogenase phosphatases (PDP) dephosphorylate the complex and activate it whilst pyruvate dehydrogenase kinases (PDKs) phosphorylate and inactivate it. In its inactive form it can no longer convert pyruvate to acetyl-coA and the pyruvate is converted to lactate by lactate dehydrogenase (LDH). *LKB1/KRAS* mutant NSCLC cell lines show 18-fold higher expression of PDK4 and almost 6-fold lower expression of PDP1 when compared to NSCLC cell of different genetic subtypes.

If the PDC was inactivated in this genetic subset of NSCLC, these cells would be predicted to produce large amounts of lactate relative to other NSCLC. One way to investigate the levels of metabolites such as lactate is Nuclear Magnetic Resonance (NMR) Spectroscopy. This work was carried out in collaboration with Jules Griffin (Department of Biochemistry, University of Cambridge).

Five cell lines were used in this study: Two *LKB1/KRAS* mutant (A549 and NCI-H460), one *KRASmut* (NCI-H1792), one *LKB1null* (NCI-H1563) and one wild type

(NCI-H1838). Cells were fed media containing ^{13}C labelled glucose rather than normal glucose and after 24-48hrs of incubation in this media, cells were harvested, frozen at -70^{o}C, then processed and prepared for NMR spectroscopy. Two types of analysis were performed proton NMR (1H NMR) (a measure of total metabolite levels) and ^{13}C NMR (an indication of metabolite turnover). Figure 4-10 shows the PLS-DA (partial least squares-discriminant analysis) plots for the two different spectra. These plots show a clear separation between the *LKB1/KRAS* mutant cell lines and control cell lines, suggesting that are different metabolically. The metabolites which cause this separation are shown in table 4-2.

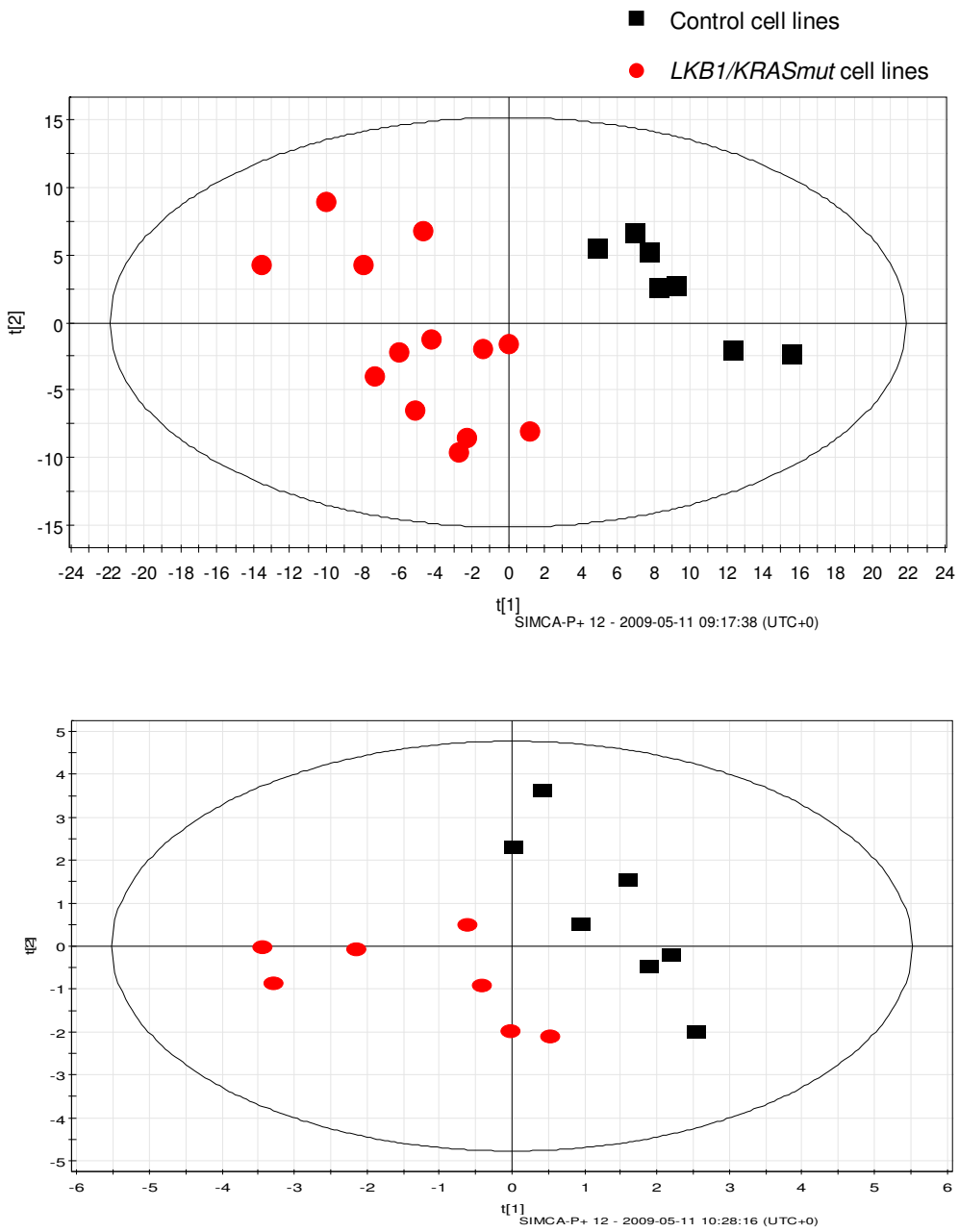


Figure 4-10. A) PLS-DA plot of 1H NMR spectra of control and *LKB1/KRAS* mutant cell lines. R^2=22%. Q^2= 51%. B) PLS-DA plot of 13C NMR spectra of control and *LKB1/KRAS* mutant cell lines. R^2=42%. Q^2= 22%. Both models passed validation by a jack knifing procedure (within the SIMCA software) used to reduce the likelihood of over fitting.

1H NMR		^{13}C NMR	
LKB1/KRASmut	**Controls**	***LKB1/KRASmut***	**Controls**
Lactate	Uridine	Glutamate C3	C1 glucose
Lysine	Myoinositol	Acetate	
Succinate	Phosphocholine	Glutathione	
N-acetyl aspartate	Glutathione	Alanine	
	Glutamine	Glycerol phosphocholine	
		Choline	
		Aspartate C3	

Table 4-2 Metabolites in 1H NMR and ^{13}C NMR spectra which are significantly different between *LKB1/KRAS* mutant cell lines and control cell lines. A metabolite at high levels in one genetic subtype suggests reciprocally that it is low in the other genetic subtype. Significant metabolites were identified using loading column plots with error bars set to 95% confidence limit using a jack-knifing routine within the SIMCA software.

The significantly different metabolites in table 4-2 suggest a number of interesting findings. Perhaps the most telling is the apparently increased levels of lactate in *LKB1/KRAS* mutant cell lines. As shown by figure 4-11 there is a larger amount of lactate in the *LKB1/KRAS* mutant samples versus the control samples. This result would be consistent with the PDC being inactivated as suggested by the expression signature for *LKB1/KRAS* mutant cell lines. From the ^{13}C spectra a peak for C1 glucose is expected, as this is the labelled glucose added to the media. This peak is absent in the *LKB1/KRAS* mutant samples and present in the control samples, suggesting that the *LKB1/KRAS* mutant samples have taken up all of the labelled glucose. This would indicate a much higher rate of metabolism, consistent with the production of large amounts of lactate.

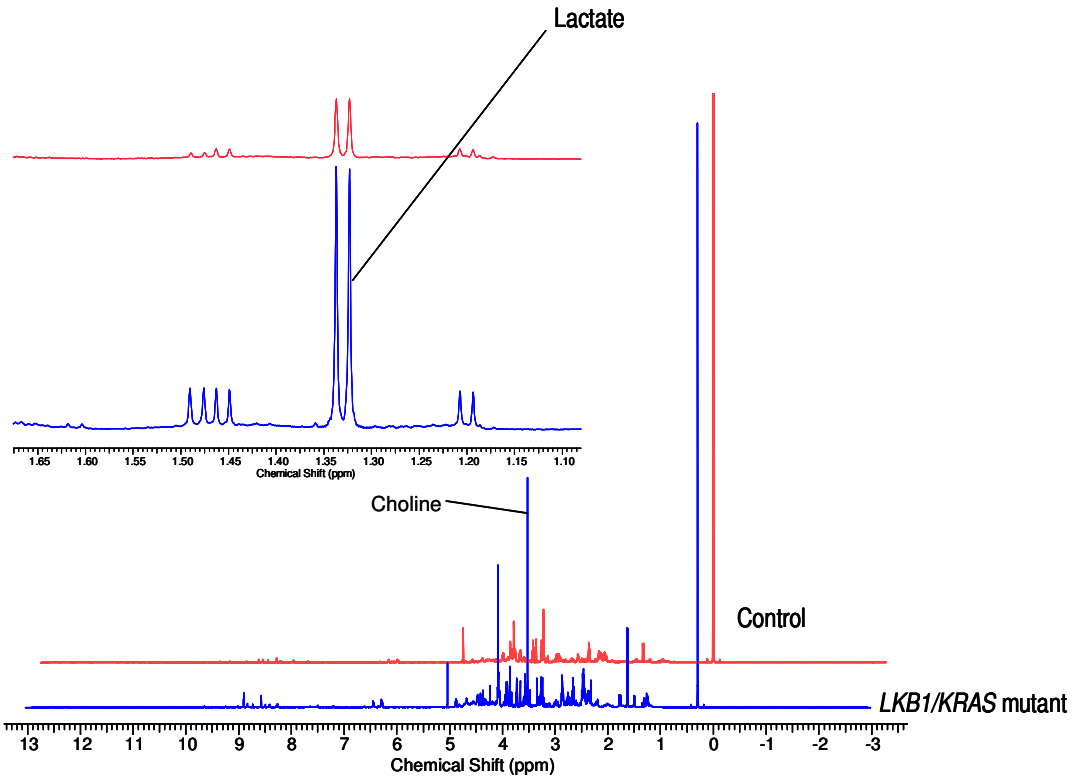


Figure 4-11 *LKB1/KRAS* mutant cell lines produce more lactate than control cell lines. Example 1H NMR spectra for control (red) cell lines and *LKB1/KRAS* mutant cell lines (blue). Highlighted are the peaks for lactate. Data normalised to trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TSP) (chemical shift 0ppm).

The appearance of a number of amino acids such as glutamate and aspartate in the ^{13}C spectra for *LKB1/KRAS* mutant samples as well as acetate, would suggest a higher rate of the TCA cycle. This would at first appear to be paradoxical, as inactivation of the PDC complex would result in a decrease in available acetyl-coA, which would normally enter the TCA cycle. However, glutamate and aspartate pools are often used as indications of TCA cycle rate as they can be converted into TCA cycle intermediates. For example glutamate can be converted into α-ketoglutarate a TCA cycle intermediate. Consistent with this is the finding that the control samples have high amounts of glutamine which is present in the media. Glutamine is

converted to glutamate and is often considered an additional energy source to glucose. Glutamine catabolism can sustain cellular viability via anaplerotic reactions which replenish TCA cycle intermediates (Figure 4-12). High levels of glutamine in control samples would suggest reciprocally that there are low levels in *LKB1/KRAS* mutant samples. The presence of labelled glutamate in these samples likely indicates a fast turnover of glutamine to glutamate for use in the TCA cycle. Accelerated glutamine metabolism also produces greater amounts of reducing equivalents which can be used in glutathione metabolism.

The build up of succinate in the *LKB1/KRAS* mutant samples would indicate that the TCA cycle is indeed progressing to this point, perhaps being replenished by glutamine catabolism. The build up of succinate suggests that there is a block somewhere in the cycle. This could be at the step where oxaloacetate is converted to citrate using the acetyl-coA produced by the PDC. Inactivation of the PDC in the *LKB1/KRAS* mutant samples would result in minimal amounts of acetyl-coA being produced. This would cause a block in the conversion of oxaloacetate to citrate resulting in a build-up of succinate. A potential effect of the build-up of succinate would be aberrant stabilisation of HIFα, an effect observed in tumours with succinate dehydrogenase mutations (Gimenez-Roqueplo, et al. 2001 & 2002). This occurs through inhibition of the HIFα prolyl hydroxylases resulting in activation and stabilisation of HIFα, an effect known as pseudohypoxia (Koivunen, et al. 2007; Selak, et al. 2005). This would imply that this genetic subset of cells could survive in any environment regardless of the oxygen conditions.

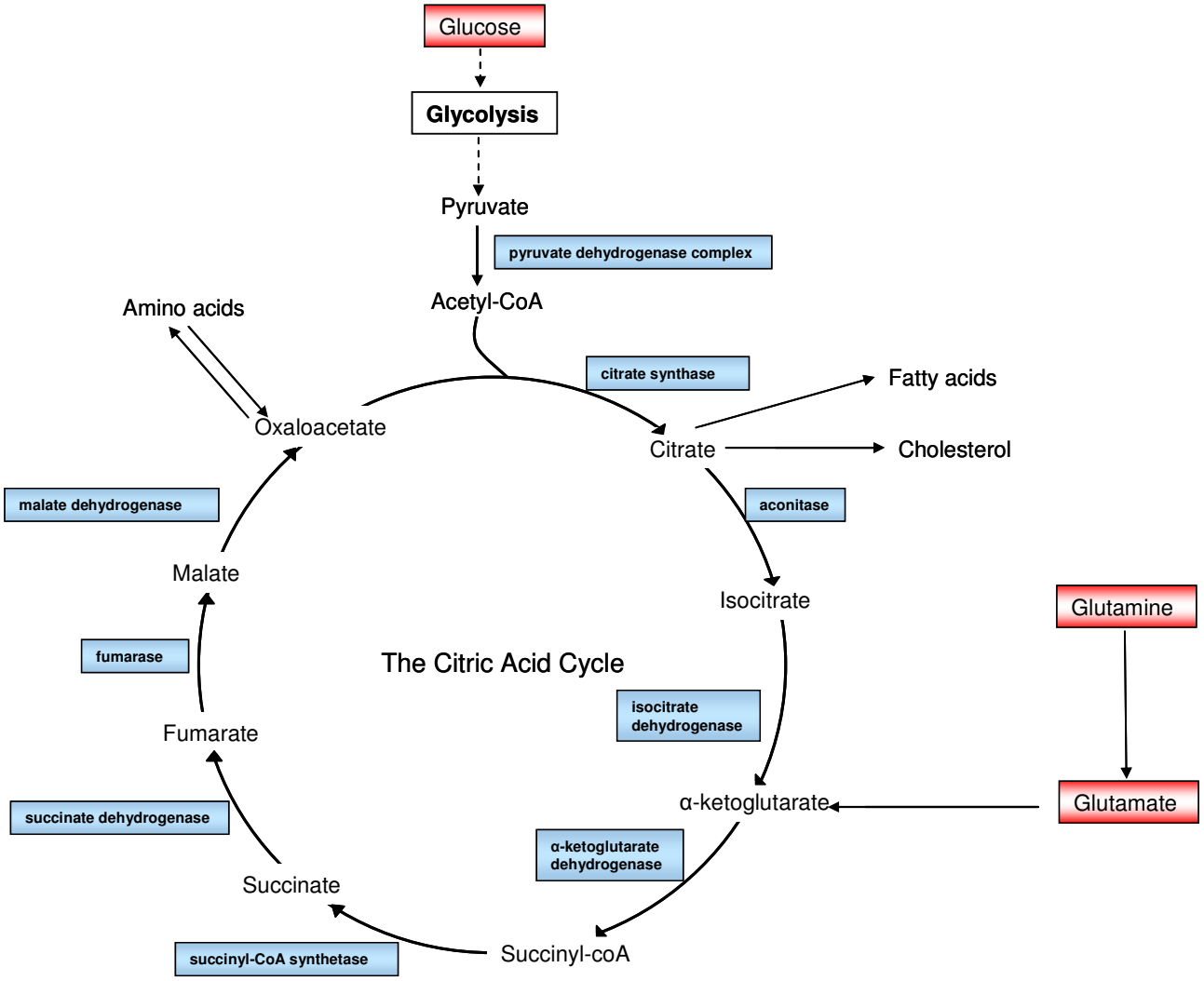

Glucose
Glycolysis
Pyruvate
pyruvate dehydrogenase complex
Acetyl-CoA
Amino acids
citrate synthase
Fatty acids
Oxaloacetate
Citrate
Cholesterol
malate dehydrogenase
aconitase
Malate
Isocitrate
Glutamine
fumarase
The Citric Acid Cycle
isocitrate dehydrogenase
Fumarate
α-ketoglutarate
Glutamate
succinate dehydrogenase
α-ketoglutarate dehydrogenase
Succinate
Succinyl-coA
succinyl-CoA synthetase

Figure 4-12 Simplified overview of glucose and glutamine metabolism. Cancer cells use two main energy sources glucose and glutamine. The data presented here suggests that *LKB1/KRAS* mutant cells rely heavily on both energy sources.

The appearance of various choline and phosphocholine metabolites in the ^{13}C spectra could be an indication of increased membrane turnover, which would be consistent with the fast growth rate observed in *LKB1/KRAS* mutant cell lines. It is however, often difficult to distinguish by NMR the naturally abundant versions of these metabolites and the labelled versions. Therefore caution must be exercised when interpreting the data. Mass spectroscopy is a better method for studying choline and its various metabolites.

Glutathione as mentioned earlier is a marker of oxidative stress. The appearance of glutathione in the ^{13}C spectra would indicate high turnover of glutathione which would be consistent with the observation from the expression data that this genetic subset have altered expression of glutathione metabolic enzymes. A higher turnover of glutathione would suggest that these cells are under higher amounts of oxidative stress, or, they have altered genetically and have been "hard-wired" into a permanently up-regulated glutathione metabolic state, with the likely attendant advantages for growth *in situ* that these changes might afford.

It was suggested through the expression data that the pentose phosphate pathway is upregulated in this genetic subtype of NSCLC. However we were unable to obtain any supporting evidence from the NMR spectra for this. This could be due to the difficulty in measuring the pathway intermediates by NMR spectroscopy (Jules Griffin, personal communication). The pathway is continuous flux like the TCA cycle and therefore the intermediates are rarely at high enough concentrations to be detected.

Overall, the NMR data suggests that in general *LKB1/KRAS* mutant samples use both glucose and glutamine metabolism at a much higher rate than other genetic subtypes of NSCLC. They produce significantly more lactate due to inactivation of the PDC. This could be the mechanism through which the Warburg Effect is created in this subset of NSCLC. *LKB1/KRAS* mutant cell lines have high flux of glutamate which can be taken in to the TCA cycle. In keeping with the notion of a truncated TCA cycle, this subset of NSCLC have increased levels of succinate, possibly cause by the lack of acetyl-coA entering the TCA cycle caused by inactivation of the PDC, but still being fed with glutamate. In addition they appear to be genetically altered in order to permanently up-regulate glutathione metabolism. This would result in a permanent metabolic state which could cope with conditions of oxidative stress, and perhaps better able to survive in adverse conditions presented in the context of tumour growth *in vivo*.

4.4 Discussion

This chapter examined the differences between *LKB1/KRAS* mutant cell lines and other NSCLC cell lines at the level of gene expression and metabolism via NMR spectroscopy. A strong expression signature in *LKB1/KRAS* mutant cell lines was identified and in Chapter 5 this expression signature is identified again using an additional array – the Illumina HumanWG-6_V3 chip. This expression data was obtained from GlaxoSmithKline and contained a relatively limited number of cell lines, analysis of a larger number would further increase the robustness of the signature. The signature identified in this study is dominated by genes involved in metabolic pathways. In the past it has been suggested that in mouse embryonic fibroblasts that these two mutations cannot coexist, as *LKB1* null mouse embryonic fibroblasts are resistant to transformation by HRAS (Bardeesy, et al. 2002). It was hypothesised that this is due to the metabolic strain a *RAS* mutation would induce in a cell due to rapid proliferation. *LKB1* mutant cells would be unable to sense this strain and alter their metabolism accordingly. The data here would suggest that in NSCLC cell lines these two mutations can coexist and the reason may be that they have "hard-wired" the metabolism into a form that acts permanently as if the cell is under energetic stress.

The Warburg Effect is the observation that most cancer cells predominantly produce energy by glycolysis followed by the production of lactate, rather than by oxidation of pyruvate in mitochondria like most normal cells, even if oxygen is plentiful. This observation was made over 50 years ago by Otto Warburg and it is still not clear why and how cancer cells switch to this inefficient method of producing energy. It has been hypothesised that the glucose is diverted to produce other metabolites, but it still leaves the question of how the cells manage to metabolically support rapid proliferation without using oxidative phosphorylation. The recent discovery that

cancer cells can switch the isoform of pyruvate kinase which they express to an embryonic isoform (Mazurek, et al. 2005), which is essential for tumour growth and maintenance of the Warburg Effect (Chirstofk, et al. 2008a) has re-ignited interest in the Warburg Effect and cancer metabolism.

In this Chapter we suggest that in this genetic subset, transition to the Warburg metabolism maybe through inactivation of the PDC. The PDC regulates the entry of acetyl-coA into the TCA cycle and inactivation would cause a build up of pyruvate, this pyruvate is then converted into lactate. The expression data suggested that two key enzymes involved in the regulation of PDC show alteration at the transcriptional level. PDK4, a kinase which phosphorylates and inactivates PDC had 18-fold higher expression and PDP1 a phosphatase which dephosphorylates and activates PDC showed almost 6-fold lower expression in this genetic subset. NMR spectroscopy revealed that this genetic subset is metabolically distinct from other NSCLC cells lines, confirming the findings from the microarray, and one of the distinguishing metabolites was lactate. In fact, these cell lines metabolised the labelled glucose at a much faster rate than the control cell lines, highlighted by a lack of the labelled glucose in the *LKB1/KRAS* mutant sample spectra and they appear to production of large quantities of lactate. In addition they also metabolised glutamine, an alternative energy source to glucose at an accelerated rate with the resulting glutamate able to enter the TCA cycle. A lack of acetyl-coA from PDC inactivation results in a block on the conversion of oxaloacetate to citrate and this together with glutamate entering the TCA cycle, results in a build up of succinate. The build up of succinate may also lead to aberrant stabilisation of HIFα and pseudohypoxia. Altogether, there are several lines of evidence which suggest that genetic alterations of PDK4 and PDP1 are responsible for a truncated TCA cycle and transition to the Warburg Effect in *LKB1/KRAS* mutant NSCLC.

The data would suggest that reactivation of the PDC may be a therapeutic option in this genetic subset of NSCLC and this could be achieved through inhibition of PDK4. Further work to examine the unique metabolic alterations could include the addition of labelled glutamine and other amino acids to the cell culture media, in order to trace in more detail the alterations to the pathways. Because this subset appears to rely heavily on an accelerated rate of both glucose and glutamine metabolism, this could suggest a weakness which could be therapeutically exploited. Co-targeting glycolysis and glutaminolysis enzymes in patients may not be a therapeutic possibility but *in vitro* studies may yield further targets that would be more feasible in patients.

Although the NMR spectroscopy data confirms the hypothesis that the PDC is inactivated in this subset of NSCLC through increased expression of PDK4 and decreased expression of PDP1, further work should confirm the altered expression of these enzymes and others identified by the array using quantitative RT-PCR and immunoblotting.

The data here has given a unique insight into the metabolic changes in a genetic subset of lung cancer brought about by changes at the transcriptional level, hardwiring these cancers into a form phenotype that may well predict increased survival advantages *in vivo*. This data is further corroborated by the observations made here are under ideal growth conditions with nutrient-rich media, further indicating that even when the cells do not need this exacerbated metabolic phenotype, it is still present. This work has also provided potential new therapeutic targets to explore such as inhibition of PDK4 to reactivate the pyruvate dehydrogenase complex thereby abrogating the preferred energy generating mechanism for this subset of NSCLC. As well, inhibition of glutamine metabolism

should be explored in this subset given the suggestion of reliance on glutamate as a potential energy source.

Chapter 5:

Microarray analysis of cell lines treated with CI-1040, rapamycin and AMPK inhibitor compound C.

5.1 Introduction

The first results Chapter described how NSCLC cell lines with mutations in *LKB1* and *KRAS* are sensitive to MEK inhibition with CI-1040 and mTOR inhibition with rapamycin. Dual inhibition however is neither additive nor synergistic suggesting possible redundancy in the pathways. This subset of NSCLC also has a unique expression profile defined by altered expression in a number of metabolic genes suggesting that NSCLC with *LKB1* inactivation and *KRAS* activation may have survival advantages in the demanding *in vivo* environment. In addition it revealed a novel method of creating the Warburg Effect. This chapter focuses on whether sensitivity to CI-1040 treatment or rapamycin treatment in this genetic subset is due to perturbation of the unique expression profile.

5.2 Outline of experiment plan

Seven cell lines were selected for this experiment, five of which were used for the microarray in the previous chapter (A549, NCI-H460, NCI-H358, NCI-H226, NCI-H661). The cell lines were *LKB1KRAS* mutant (NCI-H460, A549) and five control (NCI-H358, NCI-H1792 (*KRASmut*), NCI-H226, NCI-H661 (wild type), NCI-H1563 (*LKB1null*). mRNAs were extracted from candidate cell lines before and after compound treatment and expression profiles were determined using the Illumina HumanWG-6_V3 chip. Data quality test and normalization were performed using

Lumi package in R (Du, et al. 2008). The quality of each array was assessed by checking the mean and standard deviation of over-all expression, ratio of detectable probe, expression information of control probes (housekeeping genes) and the correlation among replicated samples. Background correction of each array was using variance-stabilizing transformation (Lin, et al. 2008). The data were normalized by quantile method and expression level of each gene was log transformed. Genetic signature analysis was using Limma package from BioConductor (Smyth, 2004). Paired t-test was used to access the significant level of expression change before and after drug treatment and the expression profile between mutation cell lines and wild type cell lines. *P*-values were corrected by Benjamini/Hochberg paradigm with a false discovery rate of 0.01. Unsupervised hierarchical cluster has been done by using Cluster 3.0 (Eisen, et al. 1998) and presented by Java TreeView (Saldanha, 2004). The similarities between cell lines were calculated based on uncentered Pearson correlation and used average linkage for clustering. Heatmaps were produced by smcPlot from PGSEA package in BioConductor (http://www.bioconductor.org/packages/2.4/bioc/html/PGSEA.html).

As this experiment is using the Sanger in-house Illumina array, correlation between the data from this array and the array data in chapter 4 was assessed by comparing the expression profiles from both arrays for the untreated *LKB1/KRAS* mutant cell lines. Despite a different number of probes and smaller number of cell lines in this study, the overlap between the arrays was statistically significant (*P*-value $<2.2\times10^{-16}$). The Illumina array did produce a higher number of significantly altered genes – 674.

5.3 Results

5.3.1 Expression analysis following CI-1040 treatment

To determine whether any significant changes in the gene expression signature occurred upon CI-1040 treatment in sensitive cell lines versus resistant cell lines, the seven cell lines were treated with 5μM CI-1040. RNA was harvested after 8 and 48hrs of CI-1040 treatment and expression profiles determined using the Illumina HumanWG-6_V3 chip. To determine which genes had altered expression in the treated versus the untreated, expression profiles for each time point for each cell line were compared to the untreated time 0hrs for that cell line. Using the Benjamini/Hochberg paradigm to correct *P*-values with a FDR of 0.01, gene lists were compiled of significantly altered genes for each cell line, 8hrs and 48hrs after CI-1040 treatment and compared to the untreated 0hrs sample. To make comparisons more simple and reduce the number of genes to analyse, fold change was calculated for each gene, and only genes with a fold change ≥ 1.5 used in the analysis.

To determine whether there was any effect to the expression signature, heatmaps were constructed using smcPlot from PGSEA package in BioConductor (http://www.bioconductor.org/packages/2.4/bioc/html/PGSEA.html) (Figure 5-1). Figure 5-1 shows the expression changes all the cell lines, before and after treatment with 5μM CI-1040. As the figure shows there is very little effect on the expression profile of *LKB1/KRAS* mutant cell lines when treated with CI-1040. This would suggest that global perturbation of the expression profile is not the reason for the sensitivity to CI-1040.

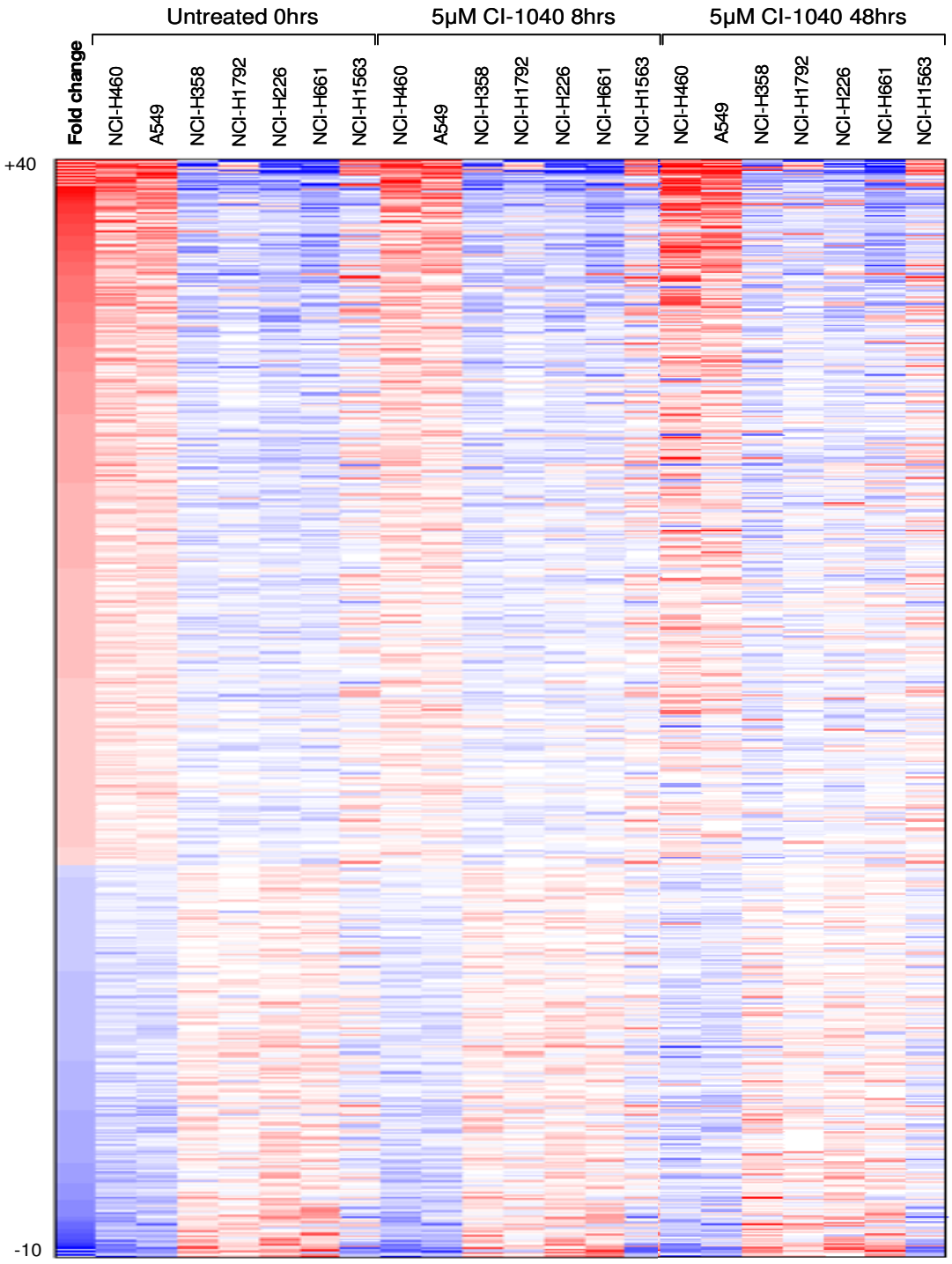


Figure 5-1. Heatmap of differentially expressed genes in *LKB1/KRAS* mutant cell lines before and after CI-1040 treatment. *LKB1/KRAS* mutant cell lines (NCI-H460 and A549) have a unique expression signature with 674 differentially expressed genes which do not show altered expression upon treatment with 5μM CI-1040. CI-1040 does not alter the expression of the same genes in control cell lines (NCI-H358, NCI-H1792, NCI-H226, NCI-H661, and NCI-H1563).

As CI-1040 treatment did not affect the expression profile of *LKB1/KRAS* mutant cell lines we examined which genes were specifically altered by CI-1040 treatment in this genetic subset. Gene lists from control cell lines were compared to *LKB1/KRAS* mutant cell lines to identify genes significantly altered only in the *LKB1/KRAS* mutant subset. This gave a list of 212 genes specifically altered by CI-1040 treatment in *LKB1/KRAS* mutant cell lines. GO term enrichment analyses were performed on this list using GoMiner and the results are shown in Table 5-1. There were no significantly enriched GO terms with an FDR of less than 0.1.

The majority of the genes that are differentially expressed in LKB1/KRAS mutant cell lines only show change after 48hrs of CI-1040 treatment and are likely to be a result of the cells undergoing growth inhibition and cell cycle changes. To examine the specific effects of CI-1040 treatment on *LKB1/KRAS* mutant cell lines it may well be more informative to examine the genes altered 8hrs after CI-1040 treatment as this timepoint is more likely to capture the direct transcriptional response to CI-1040 (Table 5-2). Only 26 genes show significant altered expression after 8hrs, the majority of which are down-regulated following CI-1040 treatment. It includes a number of ribosomal proteins including rpS6 which may together with the decrease in phosphorylation of p70S6K upon CI-1040 treatment highlight the importance of the RAS-MAPK signalling pathway on protein synthesis. This set includes angiogenin which has increased expression in this genetic subset before CI-1040 treatment and CI-1040 treatment appears to increase this further. NEDD9, a protein implicated in metastasis and invasion and already suggested to have high expression in a mouse model of *LKB1/KRAS* mutant lung cancer (Ji, et al. 2007), has decreased expression following CI-1040 treatment. GLS the gene encoding glutaminase the enzyme responsible for the conversion of glutamine to glutamate shows decreased expression after CI-1040 treatment. Further work will be needed to establish whether

any of these genes are responsible for the growth inhibitory effects of CI-1040 in this genetic subset of lung cancer.

Go Category	Total genes	Changed genes	Enrichment	LOG10(p)	FDR
GO:0010517_regulation_of_phospholipase_activity	3	2			
GO:0010518_positive_regulation_of_phospholipase_activity	3	2			
GO:0032429_regulation_of_phospholipase_A2_activity	3	2			
GO:0032430_positive_regulation_of_phospholipase_A2_activity	3	2			
GO:0032431_activation_of_phospholipase_A2	3	2			
GO:0006541_glutamine_metabolic_process	15	3	15.6131	-3.07632	0.64
GO:0006520_amino_acid_metabolic_process	203	9	3.461031	-2.91585	0.39
GO:0032365_intracellular_lipid_transport	5	2	31.22619	-2.79851	0.231667
GO:0032366_intracellular_sterol_transport	5	2	31.22619	-2.79851	0.231667
GO:0032367_intracellular_cholesterol_transport	5	2	31.22619	-2.79851	0.231667

Table 5-1 GO term analysis of genes differentially expressed in *LKB1/KRAS* mutant cell lines after CI-1040 treatment. Treatment with 5μM CI-1040 results in altered expression of 212 genes specifically in *LKB1/KRAS* mutant cell lines. GO term enrichment analysis reveals there is no GO term enrichment with an FDR less than 0.1.

	Fold change in T0 Untreated	Fold change after treatment with 5μM CI-1040				
		T8		T48		
Gene Name	A549 and H460 vs. controls	A549	H460	A549	H460	Full Gene Name
ANG	3.9	1.7	1.6	5.3	6.0	angiogenin, ribonuclease, RNase A family, 5
C14ORF142	No difference	-1.6	-1.6	No change	No change	chromosome 14 open reading frame 142
C14ORF166	No difference	-1.5	-1.5	No change	No change	chromosome 14 open reading frame 166
C21ORF63	2.9	-2.4	-2.3	No change	-2.1	chromosome 21 open reading frame 63
CAST	2.1	-1.6	-1.5	1.5	1.6	calpastatin
CLK1	No difference	2.1	2.3	2.0	2.4	CDC-like kinase 1
ECT2	2.0	-1.9	-2.1	-2.4	-3.2	epithelial cell transforming sequence 2 oncogene
FAM89A	2.9	-2.3	-1.8	-1.8	-1.8	family with sequence similarity 89, member A
GLRX	1.5	-1.5	-1.8	2.0	1.9	glutaredoxin (thioltransferase)
GLS	1.7	-1.6	-1.6	2.0	No change	glutaminase
LOC401019	No difference	-1.8	-1.7	-1.6	No change	ribosomal protein S15 pseudogene 4
LOC441282	14	-2.0	-1.5	No change	No change	aldo-keto reductase family 1, member B10-like
LOC645138	No difference	-1.5	-1.5	No change	No change	
LOC653314	2.1	-1.8	-1.7	No change	No change	ribosomal protein L19 pseudogene 9
LOC654194	No difference	-1.9	-1.8	-1.8	No change	
LOC728505	-1.5	1.6	1.5	No change	No change	WD repeat domain 82 pseudogene 1
NDUFB10	2.7	-1.8	-1.7	No change	No change	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10, 22kDa
NEDD9	1.6	-1.8	-1.5	No change	No change	neural precursor cell expressed, developmentally down-regulated 9
NIF3L1	No difference	-1.6	-1.5	-1.6	No change	NIF3 NGG1 interacting factor 3-like 1 (S. pombe)
NPAS2	1.6	-1.8	-1.5	No change	No change	neuronal PAS domain protein 2
PPIL3	No difference	-1.6	-1.7	No change	-1.6	peptidylprolyl isomerase (cyclophilin)-like 3
RPL37A	No difference	-1.7	-1.7	No change	No change	ribosomal protein L37a
RPS15A	2.1	-1.6	-2.0	No change	No change	ribosomal protein S15a
RPS27A	1.8	-1.7	-1.6	No change	No change	ribosomal protein S27a
RPS6	1.8	-1.6	-1.6	No change	No change	ribosomal protein S6
SEPHS2	No difference	-1.5	-1.5	No change	No change	selenophosphate synthetase 2

Table 5-2 5μM CI-1040 treatment results in altered expression of 26 genes in *LKB1/KRAS* mutant cell lines 8hrs after treatment. No difference indicates at T0 there is no difference in expression between LKB1/KRAS mutant cell lines and controls. No change indicates that at that timepoint there is no difference in expression between T0 untreated and the treated timepoint

5.3.2 Expression analysis following rapamycin treatment

To determine whether any significant changes in the gene expression signature occurred upon rapamycin treatment in sensitive cell lines versus resistant cell lines, the seven cell lines were treated with 40nM rapamycin. The data were analysed identically to the CI-1040 data. To determine whether there was any effect to the expression signature, a heatmap was constructed using smcPlot from PGSEA package in BioConductor (http://www.bioconductor.org/packages/2.4/bioc/html/PGSEA.html) (Figure 5-2). As Figure 5-2 shows there is very little effect on the expression signature of *LKB1/KRAS* mutant cell lines when treated with 40nM rapamycin. This would suggest that global perturbation of the expression signature and moreover the metabolic genes in the expression profile are not responsible for the growth inhibitory effects of rapamycin.

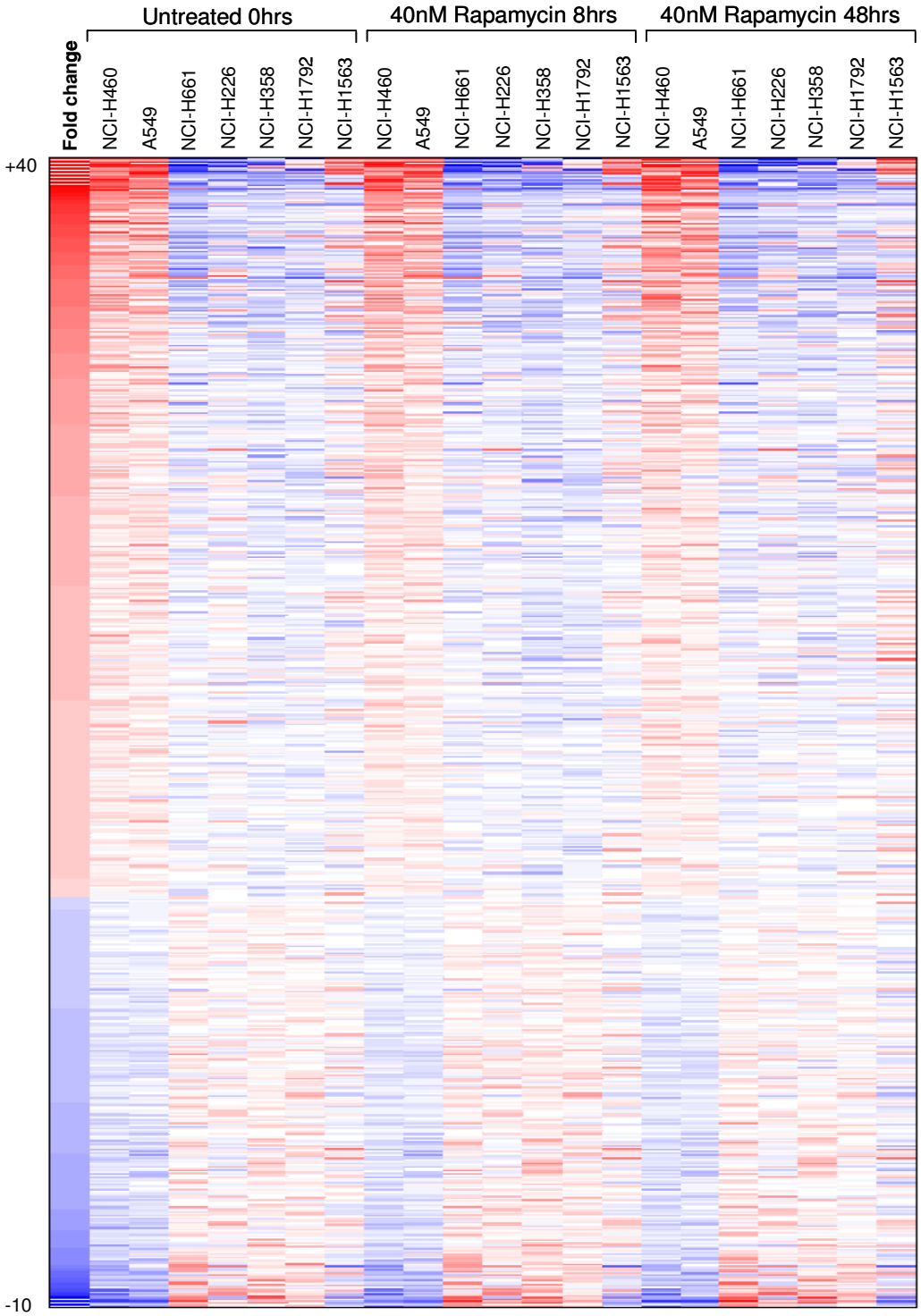


Figure 5-2. Heatmap of differentially expressed genes in *LKB1/KRAS* mutant cell lines before and after rapamycin treatment. *LKB1/KRAS* mutant cell lines (NCI-H460 and A549) have a unique expression signature with 674 differentially expressed genes which do not show altered expression upon treatment with 40nM rapamycin. Rapamycin does not alter the expression of the same genes in control cell lines (NCI-H358, NCI-H1792, NCI-H226, NCI-H661, and NCI-H1563).

To determine which genes were specifically altered by rapamycin treatment in *LKB1/KRAS* mutant cell lines, gene lists from control cell lines were compared to *LKB1/KRAS* mutant cell lines and any genes which overlapped at either time point discarded. This gave a list of only 18 genes specifically altered by rapamycin treatment at all time points in *LKB1/KRAS* mutant cell lines, this is too small a number to perform GO term enrichment analyses on. Again the genes altered 48hrs after rapamycin treatment may be due to the effects of growth inhibition and not due to direct effects of rapamycin. Only three genes show alteration 8hrs after treatment ephrin-A1 (EFNA1), glutaminase (GLS) and N-myc downstream regulated 1 (NDRG1). Interestingly GLS, the gene encoding the enzyme responsible for the conversion of glutamine to glutamate also shows decreased expression after CI-1040 treatment. Given the apparent reliance on glutamate metabolism detailed in chapter 4, it would be interesting to examine further whether down-regulation of GLS is contributing to growth inhibition upon CI-1040 and rapamycin treatment and could potentially account for the lack of non-additive/synergistic effects observed with combined treatment.

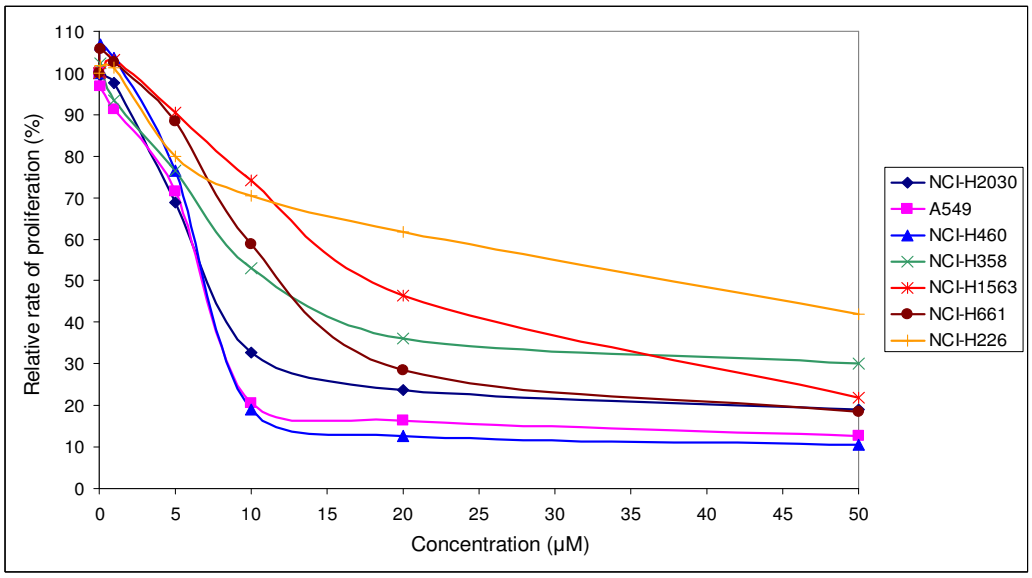
	Fold change in untreated (0hrs)	Fold change after treatment with 40nM rapamycin				
		8hrs		48hrs		
Gene Name	**A549 and H460 vs. controls**	**A549**	**H460**	**A549**	**H460**	**Full Gene name**
CTDSP2	-2.3			1.8	1.7	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase 2
CYBA	No difference	No change	No change	1.5	1.5	cytochrome b-245, alpha polypeptide
EFNA1	No difference	1.5	1.5	2.2	2.4	ephrin-A1
GLS	1.7	-1.7	-1.7	-1.6	-1.7	glutaminase
GNL2	No difference	No change	No change	-1.5	-1.6	guanine nucleotide binding protein-like 2 (nucleolar)
GPR56	No difference	No change	No change	1.7	-1.6	G protein-coupled receptor 56
HIST2H2AA3	No difference	No change	1.6	1.6	1.9	histone cluster 2, H2aa3
HTRA1	No difference	No change	No change	1.7	1.7	HtrA serine peptidase 1
ISG20	No difference	No change	No change	1.8	1.8	interferon stimulated exonuclease gene 20kDa
NDRG1	No difference	1.8	1.5	No change	No change	N-myc downstream regulated 1
PDK4	3.2	No change	No change	1.7	1.9	pyruvate dehydrogenase kinase, isozyme 4
PXMP2	No difference	No change	No change	1.8	1.7	peroxisomal membrane protein 2, 22kDa
RPL22	No difference	No change	No change	1.6	1.8	ribosomal protein L22
RPL28	No difference	No change	No change	1.7	2.1	ribosomal protein L28
SEPW1	No difference	No change	No change	-1.6	-2.0	selenoprotein W, 1
TAX1BP3	No difference	No change	No change	-1.5	-1.7	Tax1 (human T-cell leukemia virus type I) binding protein 3
TRIML2	6.0	No change	No change	-2.2	-1.8	tripartite motif family-like 2
TUBB2A	No difference	No change	No change	-1.6	-1.7	tubulin, beta 2A

Table 5-3 Genes with differential expression in *LKB1/KRAS* mutant cell lines after treatment with 40nM rapamycin. In total 18 genes show altered expression after rapamycin treatment with only three of these altered at 8hrs. No difference indicates at T0 there is no difference in expression between *LKB1/KRAS* mutant cell lines and controls. No change indicates that at that timepoint there is no difference in expression between T0 untreated and the treated timepoint.

5.3.3 Effects of AMPK inhibition on proliferation and gene expression

Due to the complexities in the signalling pathways and the many signalling inputs received by mTOR, we used direct inhibition further upstream in the pathway to determine the contribution to the expression phenotype. An AMPK inhibitor was used to determine whether creating a *LKB1*-null like environment in a *KRAS* mutated background recapitulated the expression signature. To determine the concentration of AMPK inhibitor compound C to use for the microarray and also to examine the effects of the inhibitor, a simple proliferation assay was performed (Figure 5-3).

A



B

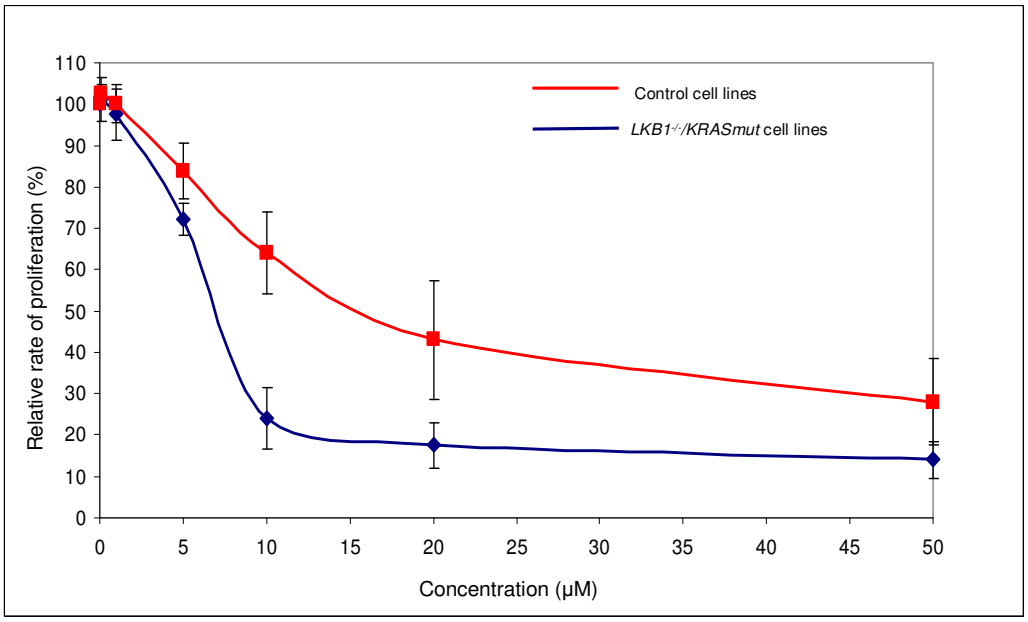


Figure 5-3 *LKB1/KRASmut* cell lines are more sensitive to the AMPK inhibitor compound C A) Shows the relative rate of proliferation 72hrs after Compound C treatment for all cell lines used in the study (n=12 from 2 independent experiments), cell lines tested: NCI-H460 (*LKB1/KRASmut*), A549 (*LKB1/KRASmut*), NCI-H2030 (*LKB1/KRASmut*), NCI-H226 (wt), NCI-H661 (wt), NCI-H358 (*KRASmut*), NCI-H1792 (*KRASmut*) and NCI-H1563 (*LKB1null).* B) Values from the 2 clusters in A were averaged to calculate the statistical significance between the clusters, values shown ±s.d between the cell lines within the cluster, n≥3 for each cluster.

It was hypothesised that the inhibitor would have no effect on cells with *LKB1* loss as they are already unable to phosphorylate and activate AMPK. Surprisingly, AMPK inhibition had a much greater effect on *LKB1/KRAS* mutant cell lines with an IC_{50} of approximately 7μM versus an average IC_{50} of 15μM in the control cell lines (Figure 5-3B) and a significant difference in proliferation rate between *LKB1/KRAS* mutant cell lines (P-value<0.003). There was some apoptosis observed in *LKB1/KRAS* mutant cell lines at lower concentrations of Compound C than observed in control cell lines. Importantly amongst the less sensitive cell lines tested is an *LKB1* mutant without *KRAS* activation, showing that it is the combination of *LKB1* loss and *KRAS* activation again determining the sensitivity.

To further understand the seemingly paradoxical role of AMPK inhibition in *LKB1/KRAS* mutant cell lines, gene expression was examined following treatment with the AMPK inhibitor. Five cell lines (NCI-H460, A549, NCI-H358, NCI-H226 and NCI-H1563) were treated with 7μM AMPK inhibitor Compound C and RNA extracted 8 and 48hrs after treatment. To determine whether there was any effect to the expression signature, a heatmap was constructed using smcPlot from PGSEA package in BioConductor (http://www.bioconductor.org/packages/2.4/bioc/html/PGSEA.html) (Figure 5-4) for the genes with a fold change greater than 1.5 in the untreated samples. As Figure 5-4 shows there is very little effect on the expression profile of *LKB1/KRAS* mutant cell lines when treated with 7μM AMPK inhibitor compound C. This would suggest that the sensitivity to the AMPK inhibitor is not due to perturbation of the expression profile.

108 genes had altered expression after treatment with the AMPK inhibitor (fold change≥ than 1.5) specifically in *LKB1/KRAS* mutant cell lines. The majority of these genes were altered after 48hrs of AMPK treatment, with only 12 changed 8hrs after

treatment (Table 5-4). GO term enrichment analysis was used to determine whether the list of 108 genes was enriched for any GO terms (Table 5-5), as the table shows there were no GO terms with a FDR below 0.1. The GO categories which are enriched but do not reach statistical significance are related to apoptosis, this is in agreement with the observation from the proliferation assay that *LKB1/KRAS* mutant cell lines undergo apoptosis at lower concentrations of Compound C. Interestingly, the expression of glutaminase was unaffected by AMPK inhibition. Together these data suggest the mechanism of growth inhibition by Compound C is different to the mechanism of growth inhibition by CI-1040 and rapamycin.

AMPK inhibitor Compound C is known to have off-target effects on the BMP signalling pathway (Hao, et al. 2008). It is possible that the results observed here could be due to effects on BMP signalling rather than AMPK itself. If true this would be an interesting finding and provide further therapeutic opportunities in this genetic subset of NSCLC. However further work is required to definitively establish the target(s) of this inhibitor in NSCLC.

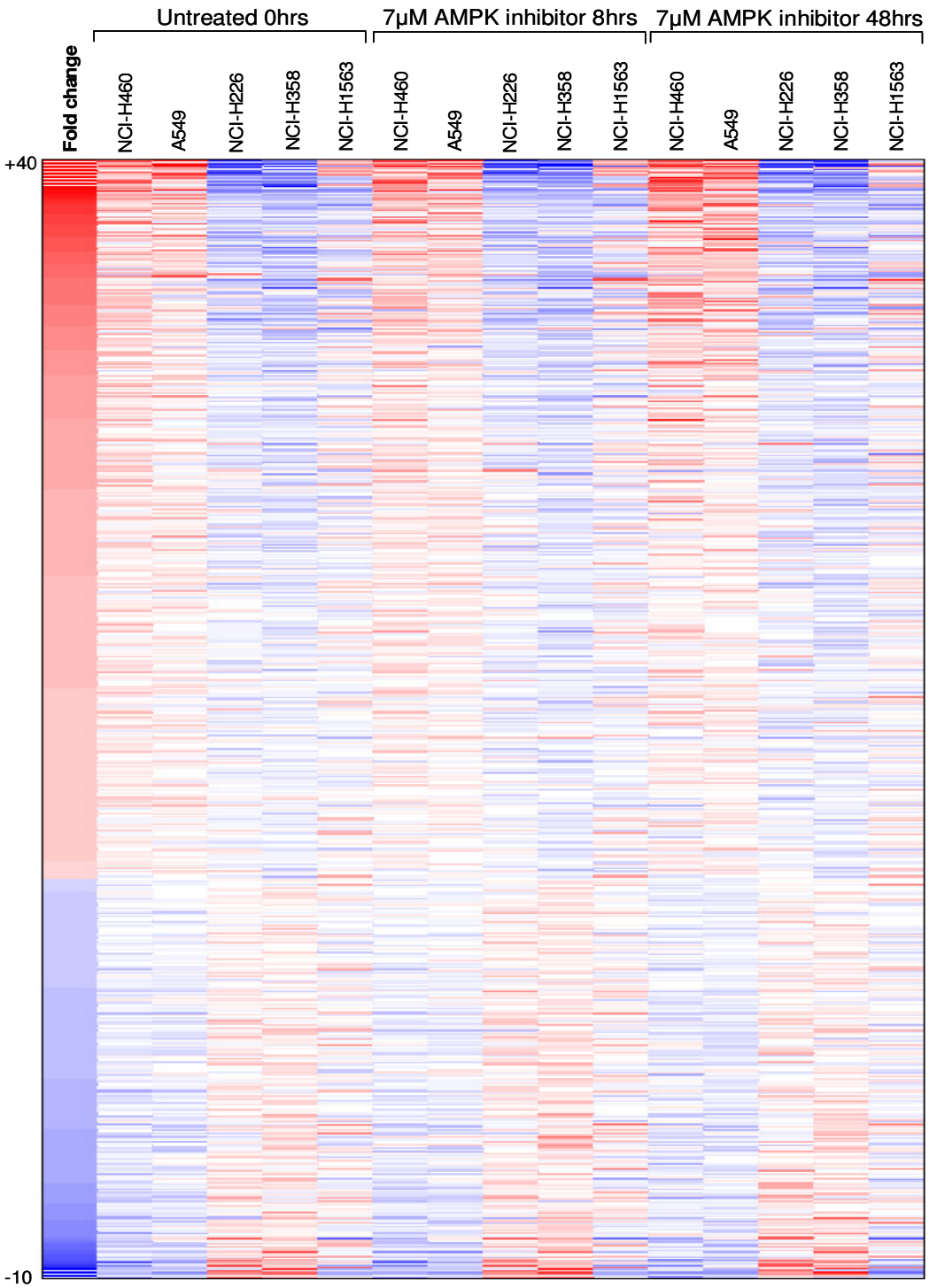


Figure 5-4. Heatmap of differentially expressed genes in *LKB1/KRAS* mutant cell lines before and after Compound C treatment. *LKB1/KRAS* mutant cell lines (NCI-H460 and A549) have a unique expression signature with 674 differentially expressed genes which do not show altered expression upon treatment with 7μM AMPK inhibitor, Compound C. Compound C does not alter the expression of the same genes in control cell lines (NCI-H358, NCI-H1792, NCI-H226, NCI-H661, and NCI-H1563).

	Fold change in untreated (0hrs)	Fold change after treatment with 7μM Compound C				
		8hrs		48hrs		
Gene Name	A549 and H460 vs. controls	A549	H460	A549	H460	Full gene name
CCDC32	No difference	-1.5	-1.9	No change	No change	chromosome 15 open reading frame 57
CTGF	No difference	1.5	1.7	No change	3.0	connective tissue growth factor
CUTC	2.4	-1.6	-1.6	No change	No change	cutC copper transporter homolog (E. coli)
EID3	No difference	1.8	2.4	No change	No change	EP300 interacting inhibitor of differentiation 3
ELK4	No difference	1.5	1.6	No change	No change	ELK4, ETS-domain protein (SRF accessory protein 1)
GNB1L	No difference	-1.5	-1.8	No change	No change	guanine nucleotide binding protein (G protein), beta polypeptide 1-like
HERC4	No difference	-1.8	-1.7	-1.5	No change	hect domain and RLD 4
HMOX1	No difference	1.5	2.4	No change	No change	heme oxygenase (decycling) 1
HS.66187	4.5	-2.2	-1.7	No change	No change	
IGFBP3	No difference	1.7	1.5	No change	4.0	insulin-like growth factor binding protein 3
OKL38	No difference	2.0	2.0	No change	No change	oxidative stress induced growth inhibitor 1
UBE2G2	No difference	-1.6	-1.5	No change	No change	ubiquitin-conjugating enzyme E2G 2 (UBC7 homolog, yeast)

Table 5-4 Genes differential expression in *LKB1/KRAS* mutant cell lines after treatment with 7μM Compound C. In total 12 genes show altered expression 8hrs after Compound C treatment. No difference indicates at T0 there is no difference in expression between *LKB1/KRAS* mutant cell lines and controls. No change indicates that at that timepoint there is no difference in expression between T0 untreated and the treated timepoint.

Go category	Total genes	Changed genes	Enrichment	LOG10(p)	FDR
GO:0006916_anti-apoptosis	160	6	6.071759	-3.339034	0.3
GO:0019377_glycolipid_catabolic_process	6	2	53.971193	-3.254824	0.225
GO:0042981_regulation_of_apoptosis	488	10	3.317901	-3.088931	0.243
GO:0043067_regulation_of_programmed_cell_death	495	10	3.270981	-3.041646	0.188
GO:0010466_negative_regulation_of_peptidase_activity	8	2	40.478395	-2.987245	0.147
GO:0043154_negative_regulation_of_caspase_activity	8	2	40.478395	-2.987245	0.147
GO:0007584_response_to_nutrient	37	3	13.128128	-2.819345	0.181
GO:0048523_negative_regulation_of_cellular_process	1039	15	2.33754	-2.805979	0.159
GO:0009725_response_to_hormone_stimulus	83	4	7.803064	-2.766015	0.174
GO:0043066_negative_regulation_of_apoptosis	218	6	4.456337	-2.644705	0.2

Table 5-5 GO term analysis of differentially expressed genes in *LKB1/KRAS* mutant cell lines after Compound C treatment. Treatment with 7μM Compound C results in altered expression of 108 genes specifically in *LKB1/KRAS* mutant cell lines. GO term enrichment analysis reveals there is no GO term enrichment with an FDR less than 0.1.

5.4 Discussion

The aim of this chapter was to determine whether CI-1040 treatment or rapamycin treatment was causing growth inhibition through alteration of the expression profile observed in the previous Chapter, which is dominated by genes involved in metabolic pathways. The data here would suggest that perturbation of the expression signature is unlikely to be responsible for the growth inhibitory effects of CI-1040 or rapamycin or that the dominant effect observed here is due to LKB1 and not due to its interaction with RAS pathways. As the data suggests it is not a direct transcriptional response further investigation should perhaps focus on direct signalling pathway alterations. Interestingly, there was one gene with decreased expression after both treatments – glutaminase, given the apparent reliance on glutamate metabolism observed in Chapter 4 this may warrant further investigation. Inhibition and siRNA knockdown of glutaminase in *LKB1/KRAS* mutant cell lines should determine the importance of this enzyme in tumour growth in this genetic subset.

Due to the complexities in the signalling pathways and inputs that are received by mTOR we decided to attempt direct inhibition upstream of mTOR. It was hoped that inhibiting AMPK in a *KRAS* mutant background would mimic the effects of *LKB1* loss. As the effects of this inhibitor were unknown a simple proliferation assay was performed. Surprisingly, *LKB1/KRAS* mutant cell lines are more sensitive to AMPK inhibition. The sensitivity was restricted to *LKB1/KRAS* mutant genetic subset *a LKB1* null cell line (NCI-H1563) has an IC_{50} almost three times that of the *LKB1/KRAS* mutant cell lines. There are several possible explanations for this finding. Although *LKB1* loss leads to a decrease in phosphorylation and activation of AMPK, it is possible that there is residual activity in AMPK. Groups have found basal phosphorylation of thr-172 AMPK in NSCLC cell lines including A549 (Carretero, et

al. 2007). The enhanced sensitivity observed here in *LKB1/KRAS* mutant cell lines would suggest this residual activity, if present is somehow essential for survival of the cells. Alternatively it could be a relatively specific *LKB1/KRAS* mutant "off-target" effect. AMPK inhibitor, Compound C (also known as dorsomorphin) inhibits BMP signalling (Yu, et al. 2008). Further examination of the genes altered across all cell lines upon compound treatment did reveal a few BMP target genes such as inhibitor of DNA binding 1, 2 and 3 (Darby, et al. 2008; Lorda-Diez, et al. 2008; Shepherd, et al. 2008). GO term enrichment analysis did not show enrichment for BMP signalling or related processes and caution should be exercised when examining gene lists for specific targets. The expression data would suggest that it is not necessarily an AMPK mediated response as known transcriptional targets of AMPK such as fatty acid synthase and hexokinase are not altered by treatment with the inhibitor. The GO category enrichment analysis did reveal enrichment for genes related to apoptosis but this data did not reach statistical significance. This is consistent with the apoptotic effects of the inhibitor observed at lower concentrations in *LKB1/KRAS* mutant cells. The mechanism of growth inhibition from AMPK inhibition would appear to be different to that observed through MEK/mTOR inhibition with the data presented here suggesting a trend for an apoptotic response to AMPK inhibition compared to the cytostatic response observed from MEK/mTOR inhibition. In addition, the expression data in chapter 4 paradoxically revealed 18-fold higher expression in *LKB1/KRAS* mutant cell lines of PDK4, a gene known to be promoted by AMPK and fatty acids in skeletal muscle (Houten, et al. 2009). Further work is needed to determine the target of the AMPK inhibitor, through examination of the effects of the inhibitor on downstream AMPK and BMP pathway components to determine whether there is any therapeutic potential in this genetic subset of NSCLC.

Chapter 6:

Functional analysis of AKR1B10 expression in *LKB1/KRAS* mutant NSCLC.

6.1 Introduction

As discussed in Chapter 4 we identified 70-fold higher steady state expression of AKR1B10 in *LKB1/KRAS* mutant cell lines compared to the control cell lines. AKR1B10 is not normally expressed in lung tissue but its expression has been found in approximately 30% of NSCLC, this led to the suggestion it may be a biomarker for NSCLC (Fukumoto, et al. 2005; Kim, et al. 2007).

Aldo-keto reductases are a superfamily of NADPH linked oxidoreductases which reduce aldehydes and ketones to their corresponding primary and secondary alcohols (Jez, et al. 1997a, b & 2001). They functionalise carbonyl groups by forming alcohols for conjugation reactions and release $NADP^+$ (Aksonas, et al. 1991; Grimshaw, et al. 1995). They are monomeric proteins of between 34-37kDa and exist across all phyla with 151 members in fifteen families. In humans there are thirteen members which act on a number of substrates including reactive lipid aldehydes, sugar aldehydes, steroids and ketoprostaglandins (reviewed by Jin & Penning 2007). As well they are implicated in xenobiotic metabolism as they metabolise various drugs and chemical carcinogens (Barski, et al. 2008). The three families in humans are AKR1, 6 and 7.

AKR1B10 belongs to the AKR1 family of aldo-keto reductases and is also known as small intestine like aldose reductase due to its limited expression, mainly in the small

intestine (Cao, et al. 1998). AKR1B10 has been found to overexpressed in a small number of cancers, in particular lung cancer with almost 30% of adenocarcinomas showing expression (Fukumoto, et al. 2005; Kim, et al. 2007). Following this it has been postulated to be a biomarker for NSCLC.

The role of AKR1B10 in cancer and indeed normal cells still remains unclear. It has been suggested that it is involved in vitamin A metabolism and retinoic acid signalling due to its high affinity for retinol, with a K_{cat}/K_m 100-fold higher than AKR1B1 and other family members (Crosas, et al. 2003; Gallego, et al. 2006; Gallego, et al. 2007; Ruiz, et al. 2009). Retinoic acid (RA), the oxidised form of vitamin A (retinol) is essential for a broad range of biological processes including development differentiation and growth (Lotan, 1981). RA exerts its functions by binding to nuclear receptors (Heyman, et al. 1992). It has been suggested that in cancer AKR1B10 metabolises retinal back to retinol and prevents the formation of retinoic acid, the ligand for RXR (Penning, et al. 2007) (Figure 6-1). However evidence for this has yet to be shown. There are natural retinoids which include 9-*cis* retinoic acid (9-*cis*-RA) and *trans*-retinoic acid (*trans*-RA) which can function as ligand inducible transcription factors of retinoic acid receptors 9-*cis*-RA can interact with both retinoic acid receptors (RARα and β) and retinoid X receptor (RXR) whereas *trans*-RA can only interact with RAR (Zhang, et al. 1993). There are 3 isoforms of each receptor α, β and γ, which show different expression patterns through development and differentiation. The receptors heterodimerise to modulate gene expression through recognition of retinoic acid response elements (RAREs) (Zhang, et al. 1993). They also autoregulate their expression (Hoffmann, et al. 1990).

Retinoids have been identified as potential anti-cancer agents due to their apoptotic and differentiation effects (Lotan, et al. 1991; Hofmann, 1992), however results thus far have been mixed. One major trial, the CARET (The Carotene and Retinol

Efficacy Trial) trial was halted early (Omenn, et al. 1996). The CARET trial began in 1985 and stopped in 1996, it was a randomized, double-blind, placebo-controlled trial of the cancer prevention efficacy and safety of a daily combination of beta-carotene and retinyl palmitate in almost twenty thousand persons at high risk for lung cancer. It was found that not only was there no evidence for benefit in lung cancer, there was evidence that it had had a harmful effect on lung cancer incidence and mortality (Omenn, et al. 1996). Given the possible role of AKR1B10 in retinoic acid metabolism, one could hypothesise that expression of AKR1B10 in persons with lung cancer would lead to an exacerbation of their condition upon treatment with beta-carotene. Other studies have implicated AKR1B10 in proliferation and DNA synthesis (Yan, et al. 2007), metabolism of daunorubicin and oracin (Martin, et al. 2006) and fatty acid synthesis, through the stabilisation of acetyl-coA carboxylase in breast cancer cells (Ma, et al. 2007).

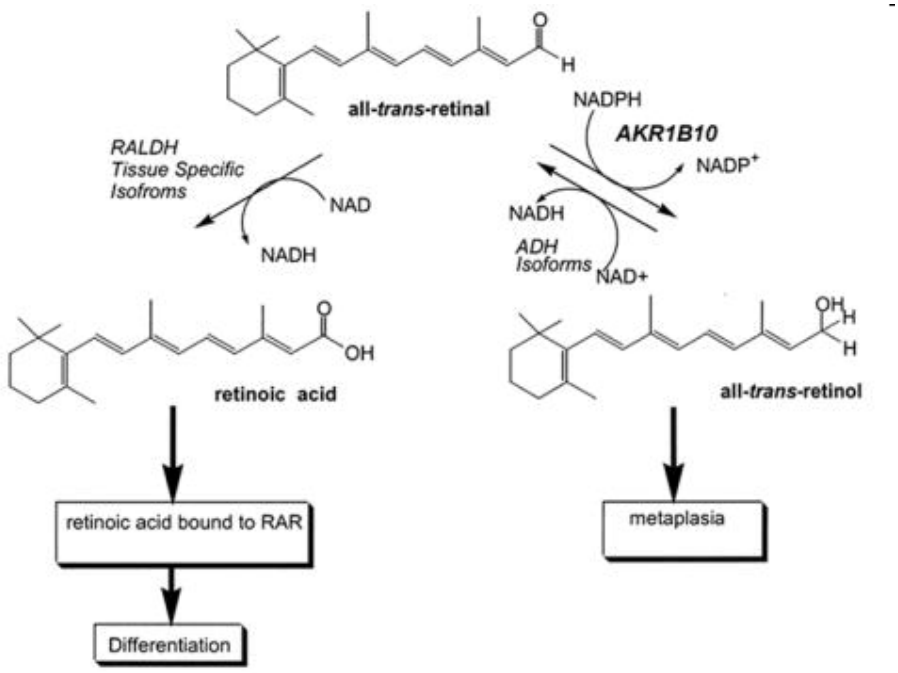


Figure 6-1. The hypothesised role of AKR1B10 in retinoic acid signalling (Figure from Penning et al. 2007). AKR1B10 has been shown to have the highest affinity of all aldo-keto reductases for retinal. Retinoic acid, the oxidised form of vitamin A is essential for differentiation and a number of cellular functions and exerts its functions by binding to nuclear receptors and modulating gene expression. It has been suggested that in cancer it metabolises retinal back to retinol and prevents the formation of retinoic acid, the ligand for RXR.

In this Chapter I examine whether AKR1B10 is an important functional determinant of the *LKB1/KRAS* mutant phenotype by investigating the possible role of AKR1B10 in retinoic acid signalling and cellular proliferation in NSCLC cell lines.

6.2 Results

6.2.1 AKR1B10 protein expression in *LKB1/KRAS* mutant NSCLC cell lines

To determine whether AKR1B10 mRNA expression resulted in over-expression of the protein in *LKB1/KRAS* mutant NCSLC cell lines, immunoblot analysis of AKR1B10 was carried out on a panel of NSCLC cell line protein lysates. This antibody recognises a single band at approximately 36kDa (Figure 6-2).

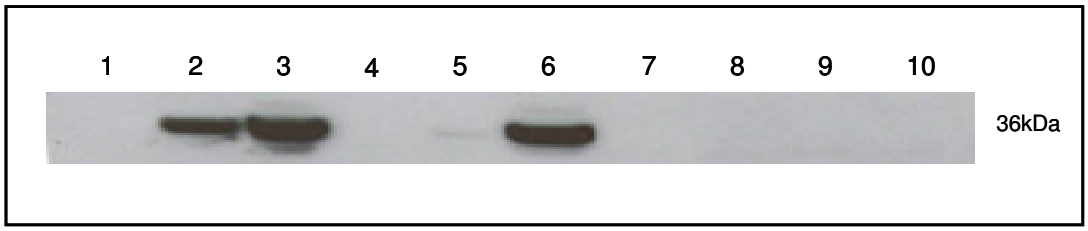


Figure 6-2. AKR1B10 is expressed in *LKB1/KRAS* NSCLC cell lines. Immunoblot analysis of AKR1B10 expression in 10 NSCLC. 1 – CAL-12T (*LKB1/BRAFmut*), 2 - NCI-H460 (*LKB1/KRASmut*), 3 – A549 (*LKB1/KRASmut*), 4 – NCI-H1437 (*LKB1null*), 5 – NCI-H1563 (*LKB1null*), 6 – NCI-H1734 (*LKB1/KRASmut*), 7 – NCI-H1838 (*WT*), 8 – NCI-H1975 (*WT*), 9 – NCI-H1993 (*LKB1null),* 10 – NCI-H2009 (*KRASmut*).

As figure 6-2 shows expression of AKR1B10 protein is restricted to *LKB1/KRAS* mutant NSCLC cell lines, confirming the result from the expression data. There is no expression of AKR1B10 in the *LKB1BRAF* mutant cell line (CAL-12T) consistent with this being a unique genetic subset of NSCLC

6.2.2 siRNA knockdown of AKR1B10 does not affect proliferation rate in AKR1B10 expressing NSCLC cell lines

To determine the role of AKR1B10 on proliferation we used siRNA to knockdown AKR1B10 expressio. Previous work by Yan, et al. 2007 in colorectal cancer cell lines showed significant effects of AKR1B10 knockdown on cell proliferation, 7 days after knockdown. It was therefore decided to assess proliferation rate in the NSCLC cell lines after 7 days of AKR1B10 knockdown. To estimate the level of knockdown being achieved 96hrs after transfection, cells were seeded to 6-well plates and double transfected with four single siRNAs (S1, S2, S3 and S4) to AKR1B10, 24 and 72hrs after cell seeding and the following day cells harvested and protein extracted using RIPA buffer (Figure 6-3).

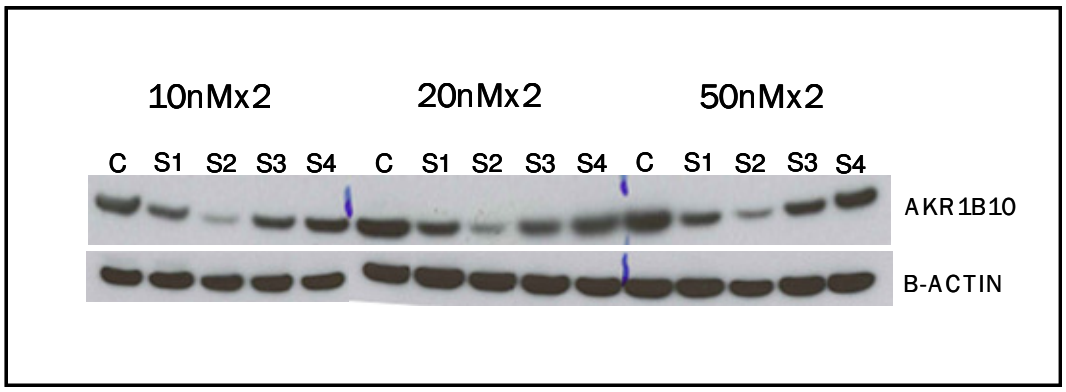


Figure 6-3 Immunoblot of siRNA knockdown of AKR1B10 in NCI-H460 cells. Cells were treated with; 2x10nM siRNA, 2x20nM siRNA or 2x50nM siRNA to AKR1B10 24 and 72hrs after cell seeding, protein was then harvested the day after the final treatment. C – Control not transfected with siRNA, S1-4 - single siRNAs to AKR1B10.

S2 was the only siRNA to have a significant effect on the AKR1B10 protein levels, with knockdown estimated to be 80% using ImageJ software. Increasing the amount

of siRNA from 10nm to 50nm did not increase the level of knockdown. This is despite a <95% transfection efficiency in all cell lines used (data not shown).

To estimate the proliferation rate after AKR1B10 knockdown using siRNA S2, cells were seeded to 96-well plates in quadruplicate and double transfected as described above with 10nM siRNA to AKR1B10 and proliferation rate assessed 7days after knockdown. Controls to estimate the level of toxicity were non-targeting siRNA, lipofectamine™ 2000 control and a media untreated control. Proliferation rate was estimated using CyQuant® reagent 7 days after the first transfection, averages taken from the quadruplicates and relative proliferation rate estimated by comparison to non-targeting siRNA treated cells (Figure 6-4). Five cell lines were used, three expressing AKR1B10 (A549, NCI-H460 and NCI-H2030) and two without AKR1B10 expression (NCI-H1792, NCI-H1838). Figure 6-4 shows there was no significant effect on the proliferation rate of cell lines transfected with 10nM siRNA to AKR1B10 7 days after transfection, suggesting that AKR1B10 may not play a role in NSCLC cell proliferation. Alternatively it could be that the residual 20% expression of AKR1B10 is sufficient for normal growth in these cell lines.

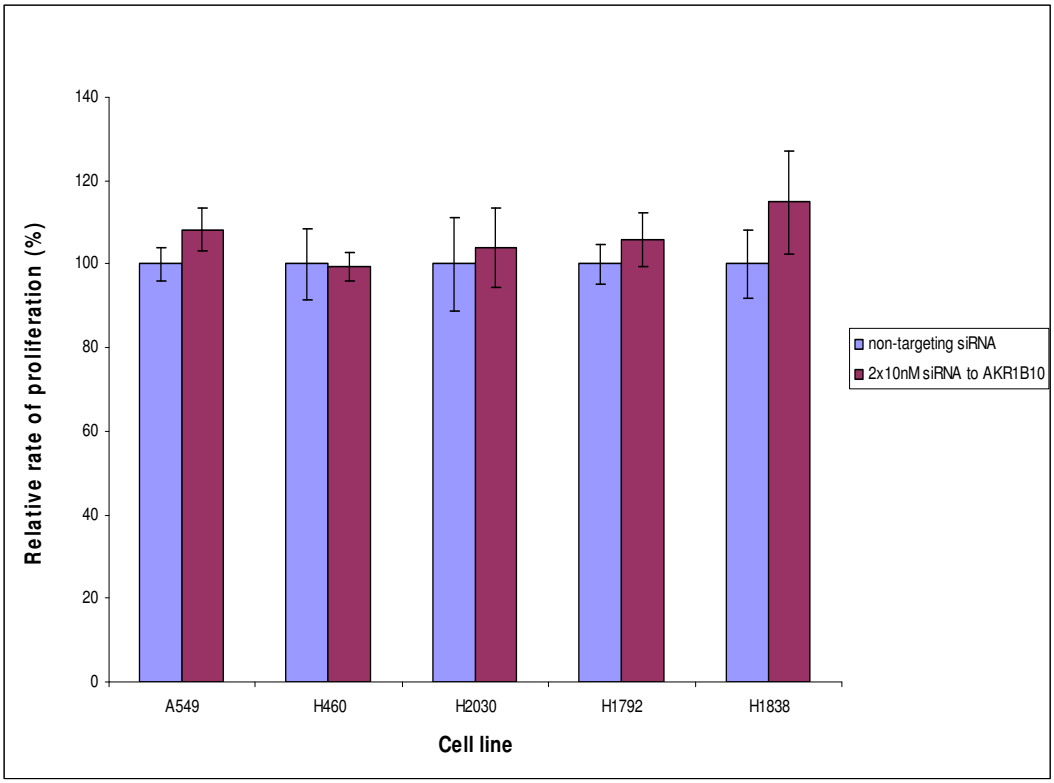


Figure 6-4 Relative proliferation rate of NSCLC cell lines 7 days after transfection with 10nM siRNA to AKR1B10. Values shown here are ± stdev, n=8 from 2 independent experiments. There are no significant differences in proliferation rate of cells treated with non-targeting siRNA (blue bars) and cells treated with 10nm siRNA to AKR1B10 (purple bars). AKR1B10 positive cell lines (A549, NCI-H460 and NCI-H2030), AKR1B10 negative cell lines (NCI-H1792 and NCI-H1838).

6.2.3 AKR1B10 does not play a role in retinoic acid signalling in NSCLC cell lines

AKR1B10 has been found to have high affinity for the retinal family of metabolites (Crosas, et al. 2003; Gallego, et al. 2006; Gallego, et al. 2007; Ruiz, et al. 2009) and is therefore thought to play a role in retinoic acid signalling by preventing the formation of retinoic acid which would normally bind to nuclear receptors. Therefore we hypothesised that AKR1B10 expression in cells would normally prevent the formation of retinoic acid, thus leading to a block on differentiation and increase cell proliferative potential. Treating *LKB1/KRAS* mutant cell lines with retinoic acid derivatives (9-*cis* and ATRA) would therefore be expected to cause a decrease in proliferation rate in cells expressing AKR1B10 (*LKB1/KRAS* mutant cell lines), whereas addition of beta-carotene would have little effect (Figure 6-5). Six cell lines, three with AKR1B10 expression (NCI-H460, A549, NCI-H2030) and 3 without (NCI-H1838, NCI-H1792, NCI-H2009) were treated with beta-carotene (0-40μM, all *trans*-retinoic acid (ATRA) and 9 *cis*-retinoic acid (9 *cis*-RA) (0-20μM) for 72hrs. After 72hrs cells were assayed for proliferation rate using CyQuant reagent.

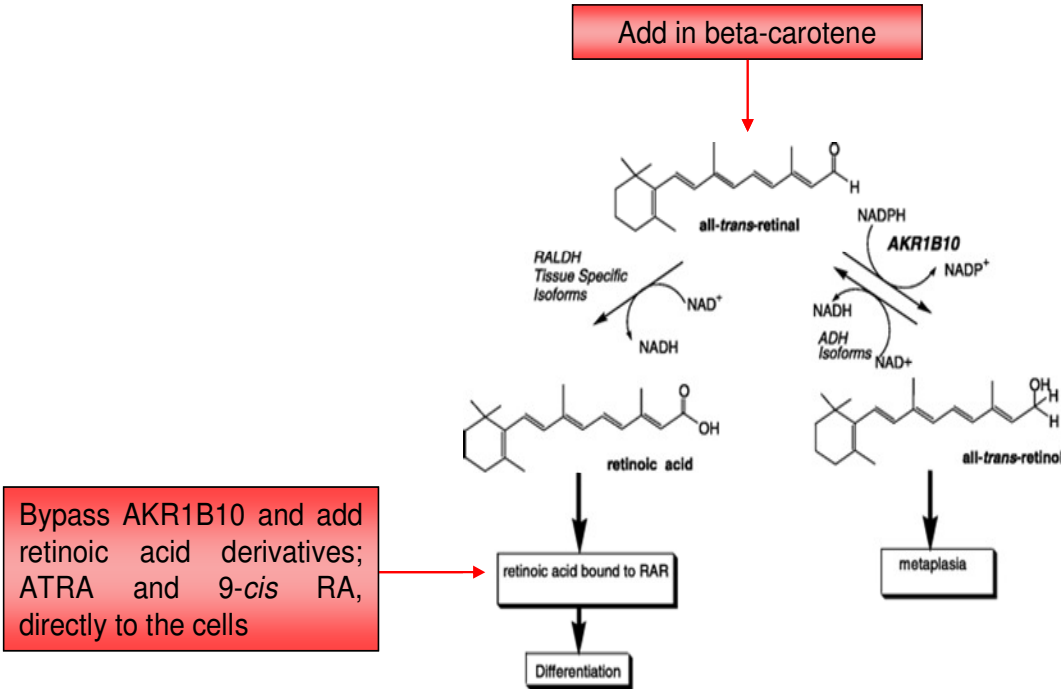


Figure 6-5 Experimental plan to examine whether AKR1B10 plays a role in retinoic acid signalling in NSCLC cell lines. It was hypothesised that addition of retinoic acid derivatives to the cells would bypass AKR1B10 metabolism and induce growth inhibition specifically in *LKB1/KRAS* mutant cell lines, whereas addition of beta-carotene should have little effect on cell proliferation.

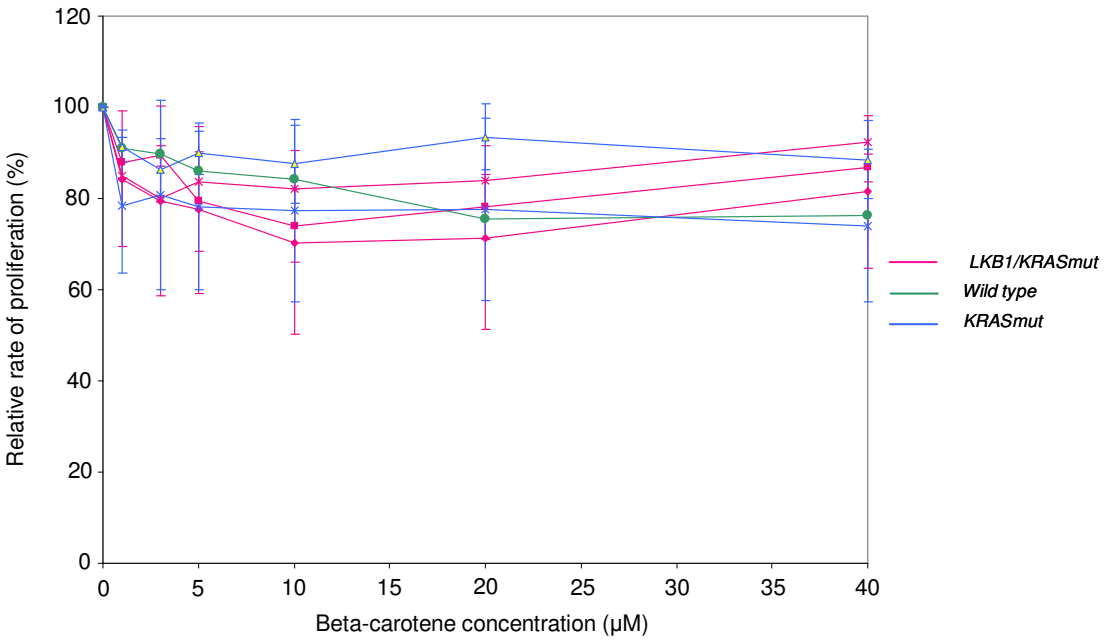


Figure 6-6 Treatment with beta-carotene has no significant effect on proliferation rate of NSCLC cell lines. NSCLC cell lines positive for AKR1B10 expression (NCI-H460, A549, NCI-H2030) or negative (NCI-H1792, NCI-H2009, NCI-H1838) were treated with beta-carotene (0-40μM) for 72hrs. Relative rate of proliferation was then calculated using CyQuant® dye and measured relative to the untreated control. Results shown here are from at least 2 independent experiments (n≥8) ± stdev.

Figure 6-6 shows the relative proliferation rate after 72hrs of beta-carotene treatment. This experiment was hampered by the insolubility of beta-carotene, which is well documented (Palozza, et al. 2006). However, the data suggests that addition of beta-carotene to the cells has no discernable effects on proliferation rate regardless of AKR1B10 expression.

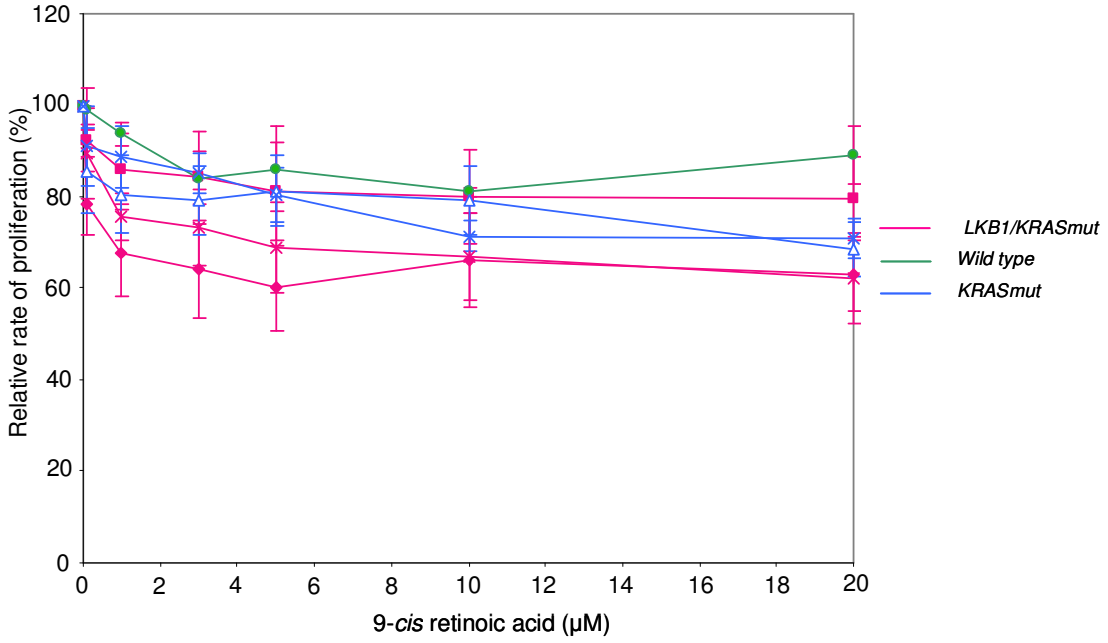


Figure 6-7 Treatment with 9-*cis*-retinoic acid has no significant effect on proliferation rate of NSCLC cell lines. NSCLC cell lines positive for AKR1B10 expression (NCI-H460, A549, NCI-H2030 (pink lines)) or negative (NCI-H1792, NCI-H2009, NCI-H1838 (blue and green lines)) were treated with 9-*cis*-retinoic acid (0-20μM) for 72hrs. Relative rate of proliferation was then calculated using CyQuant® dye and measured relative to the untreated control. Results shown here are from at least 2 independent experiments (n≥8) ± stdev.

Figure 6-7 shows the relative rate of proliferation rate of NSCLC cell lines after 72hrs of 9-*cis*-RA treatment, which might be expected to cause a decrease in proliferation rate in *LKB1/KRAS* mutant cell lines. There is no significant difference in the proliferation rate of NSCLC cell lines expressing AKR1B10 (*LKB1/KRASmutant*) upon treatment with 9-*cis*-RA which acts as a ligand for both RAR and RXR receptors. There is perhaps a trend for the *KRAS* mutant cell lines regardless of AKR1B10 expression to be more sensitive to 9-*cis* RA (blue and pink lines in Figure 6-7), however this is difficult to determine as there is only one cell line without a *KRAS* mutation (green line in Figure 6-7).

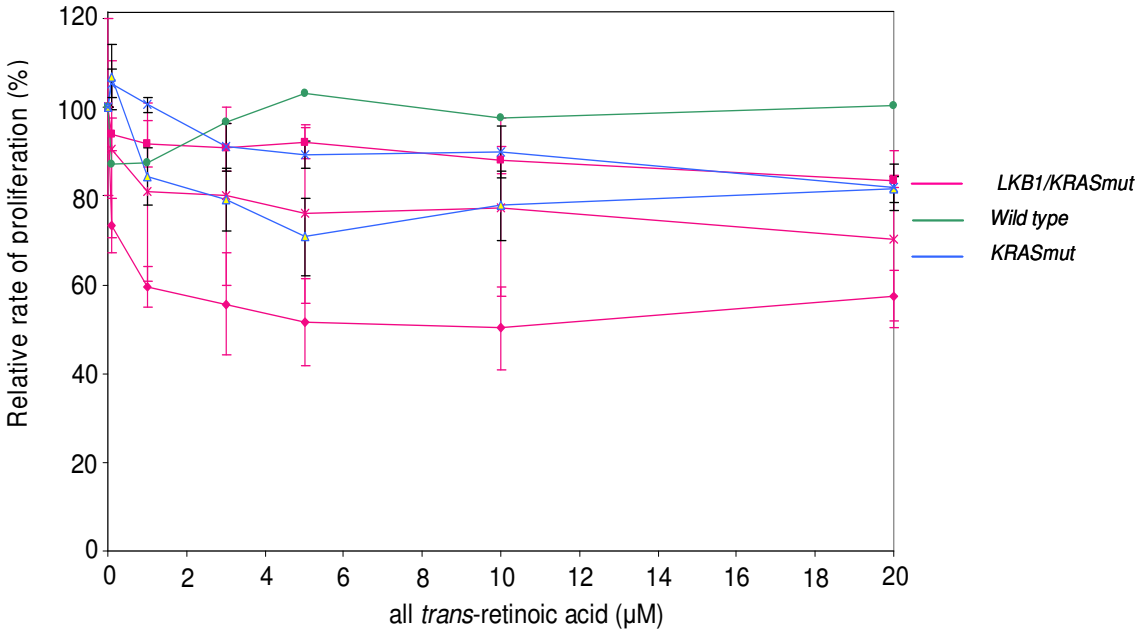


Figure 6-8 Treatment with All-*trans*-retinoic acid treatment (ATRA) has no significant effect on proliferation rate of NSCLC cell lines. NSCLC cell lines positive for AKR1B10 expression (NCI-H460, A549, NCI-H2030) or negative (NCI-H1792, NCI-H2009, NCI-H1838) were treated with ATRA (0-20µM) for 72hrs. Relative rate of proliferation was then calculated using CyQuant® dye and measured relative to the untreated control. Results shown here are from at least 2 independent experiments (n≥8) ± stdev.

Figure 6-8 shows the relative rate of proliferation rate of NSCLC cell lines after 72hrs of ATRA treatment, which might be expected to cause a decrease in proliferation rate in *LKB1/KRAS* mutant cell lines. Treatment with ATRA has no significant effect on the proliferation rate of NSCLC cell lines expressing AKR1B10 (*LKB1/KRASmutant*). Again, there is a trend for the *KRAS* mutant cell lines to be more sensitive to ATRA regardless of AKR1B10 expression and the effect is more pronounced with ATRA than 9-*cis* RA (blue and pink lines in Figure 6-7), however this is difficult to determine as again there is only one cell line without a *KRAS* mutation (green line in Figure 6-7).

6.3 Discussion

AKR1B10 is an aldo-keto reductase implicated in retinoic acid signalling, proliferation, fatty acid metabolism and xenobiotic metabolism (Penning et al. 2007; Yan, et al. 2007; Martin, et al. 2006; Ma, et al. 2007; Martin, et al. 2009). It has also been postulated to be a biomarker for NSCLC after expression was observed in approximately 30% of primary tumour samples (Fukumoto, et al. 2005; Kim, et al. 2007). We observed a correlation of AKR1B10 expression both at the mRNA level and at the protein level with *LKB1/KRAS* mutant NSCLC cell lines specifically.

We sought to determine the role AKR1B10 expression might be playing in the proliferation of NSCLC cell lines using siRNA knockdown. Double transfection of 10nM siRNA only gave 80% knockdown at the protein level and increasing the siRNA concentration did not increase the level of knockdown. In all cell lines tested there was no obvious effect of AKR1B10 knockdown on proliferation rate after 7 days, which suggests AKR1B10 does not play an important role in NSCLC proliferation. However, there was still approximately 20% expression of AKR1B10 protein and this could still be enough to carry out the functions of AKR1B10 in proliferation in NSCLC cell lines. Yan, et al. 2007 used two siRNAs to knockdown AKR1B10 expression in colorectal cancer cell lines, 60-95% knockdown at the protein level resulted in a 50% decrease in cell growth, indicating that in colorectal cancer cell lines AKR1B10 expression is important for cell proliferation.

AKR1B10 has also been proposed to play a role in retinoic acid signalling. Investigation of this in NSCLC cell lines expressing AKR1B10 has suggested that it does not. Treating cell lines with retinoic acid derivatives ATRA and 9-cis-RA did not have any significant effects on proliferation rate of cells expressing AKR1B10, nor did it affect the morphology of the cells (data not shown). There was a trend for *KRAS*

mutant cell lines to be sensitive to retinoic acid treatment regardless of AKR1B10 expression, however the data is not conclusive for this and a bigger panel of cell lines would need to examined.

Altogether these data suggest AKR1B10 does not play a role in the proliferation of NSCLC cell lines nor does it appear to play a role in retinoic acid signalling. Further work is required to determine the function of AKR1B10 expression in NSCLC and identify its substrate(s). Recent data has suggested a role in xenobiotic metabolism and metabolism of toxic lipid aldehydes which occur as a result of reactive oxygen species damaging cell membranes (Zhong, et al. 2009; Martin, et al. 2009).

Chapter 7: General Discussion

This thesis has focussed on the functional characterisation of a genetic subset of NSCLC characterised by loss of the tumour suppressor *LKB1* and activation of the oncogene *KRAS*. NSCLC has poor survival rates (~7% at 5years) (Coleman, et al. 2004). There is therefore a need for early detection and more targeted therapies. Identification of genetic subsets and full characterisation of consequences of the driver mutations should enable targeting of therapies to groups of patients defined by genetics. Through a combination of targeted approaches, expression analysis and NMR spectroscopy this study has shown that *LKB1* inactivation/*KRAS* activation are the key driver mutations in these cancers.

I identified a statistically significant association of *LKB1* inactivating and *KRAS* activating mutations in a subset of NSCLC cell lines (Mahoney, et al. 2009). Due to the crosstalk between the LKB1/AMPK/mTOR and RAS-MAPK pathways I hypothesised that this genetic subset may be more sensitive to targeted inhibition of MEK and mTOR. This genetic subset was more sensitive to MEK and mTOR inhibition compared to NSCLC with either *LKB1* or *KRAS* mutations alone or NSCLC wild type for both genes (Mahoney, et al. 2009). However the effects of MEK inhibition were not due to downstream effects on cyclin D1 as has been shown in melanoma (Solit, et al. 2006) but were possibly due to downstream effects on p70S6K. Dual inhibition was surprisingly neither additive nor synergistic which may be explained by the potent inhibition of phosphorylation of p70S6K by rapamycin, precluding any additional effect of CI-1040, confirming likely redundancy in the pathways specifically in this subset of NSCLC. The data suggests that *LKB1* loss and *KRAS* mutations appear to be the key driver mutations in this subset of NSCLC and newer generation MEK and/or mTOR inhibitors may represent therapeutic

options in the subset of patients with *LKB1/KRAS* mutant tumours. The work presented also suggested that the smaller subset of NSCLC characterised by *LKB1* loss and *BRAF* (*non-V600)* mutations which are insensitive to MEK inhibition but sensitive to mTOR inhibition. There are only a small number of cell lines with this combination of mutations and given the unclear role of *non-V600 BRAF* mutations in NSCLC, Therefore larger studies are needed to more definitely address this potentially important issue.

We further investigated the combination of *LKB1* loss and *KRAS* activation in NSCLC by examining the expression profile of this subset of NSCLC compared to non-*LKB1/KRAS* mutant NSCLC. The *LKB1/KRAS* mutant cell lines showed a distinct expression pattern characterised by alteration to the expression of a large number of metabolic genes. One finding of particular significance was the 18-fold higher expression of PDK4 and almost 6-fold lower expression of PDP1 in *LKB1/KRAS* mutant cell lines versus other NSCLC cell lines. PDK4 and PDP1 control the activation state of the pyruvate dehydrogenase complex (PDC) by phosphorylation. PDK4 phosphorylates and inactivates the complex, PDP1 dephosphorylates and activates it. The role of the PDC is to regulate entry of pyruvate into the TCA cycle. It has been hypothesised that PDC inactivation is a mechanism to create the Warburg Effect (Roche & Hiromasa 2007). The Warburg Effect is the switch to aerobic glycolysis, whereby cancer cells produce energy via lactate production rather than oxidative phosphorylation (Warburg, 1956). It was first observed in the 1950's but only recently are the mechanisms behind it being uncovered. I hypothesised that the altered expression of PDK4 and PDP1 would lead to inactivation of the PDC and production of increased amounts of lactate. The observation that the PDC could be being inactivated through altered expression of PDK4 and PDP1 apparently driven by presence of *LKB1* and *KRAS* mutations suggested a novel mechanism for creating the Warburg Effect. We further investigated this using NMR spectroscopy with

labelled glucose to identify significantly different metabolites between *LKB1/KRAS* mutant cell lines and control cell lines. The data presented in this thesis suggests that these cells produce significantly larger amounts of lactate. In addition they produce significantly more succinate which further indicates that the PDC is inactive. The PDC converts pyruvate to acetyl-coA through a series of enzymatic reactions, PDC inactivation would restrict entry of acetyl-coA into the TCA cycle resulting in a block on the conversion of oxaloacetate to citrate, leading to the build up of succinate. The build up of succinate has also been linked to pseudohypoxia through the inactivation of prolyl hydroxylases and consequent HIFα activation and stabilisation (Koivunen, et al. 2007; Selak, et al. 2005). This would likely give the cells a survival advantage in the demanding *in vivo* conditions.

The NMR data also revealed that this genetic subset would appear to rely heavily on both glucose and glutamine metabolism and indeed the expression data showed increased expression of glutamate metabolism genes such as glutaminase which is responsible for the conversion of glutamine to glutamate. Glutamine metabolism is an alternative to glucose metabolism and the resulting glutamate is converted to α-ketoglutarate to replenish TCA cycle intermediates (DeBerardinis, et al. 2007). Accelerated glutamine metabolism is associated with production of increased amounts of reducing equivalents which can be used in detoxification processes such as glutathione metabolism to deal with oxidative stress from reactive oxygen species (ROS). The expression data also showed altered expression in glutathione metabolic genes suggesting an overall increased capacity to deal with reactive oxygen species under conditions of oxidative stress. Overall the NMR spectroscopy data revealed this genetic subset differed greatly from the other NSCLC cell lines tested at the metabolic level. Interestingly the NMR spectroscopy data could be used to create a metabolic profile-based model of *LKB1/KRAS* mutant cancers that could be used to classify cancers of unknown genetic status into an *LKB1/KRAS* mutant “like”

category. The finding in this study that alterations at the transcriptional level to PDK4 and PDP1 could be the cause of the Warburg Effect has important therapeutic implications. Tumours have been shown to express the embryonic specific isoform of pyruvate kinase (PKM2) (Mazurek, et al. 2005; Dombrauckas, et al. 2005). Replacement of PKM2 with the PKM1 adult isoform reverses the Warburg phenotype and decreases the tumourgenicity of H1299 cells in nude mouse xenografts (Christofk, et al. 2008). Further work is needed to explore whether PDK4 inactivation or PDC reactivation are possible therapeutic targets in this subset of NSCLC to reverse the Warburg Effect. It would also be interesting to study whether *in vivo* these *LKB1/KRAS* mutant lung cancers have the same metabolic phenotype. The additional finding that these tumours rely heavily on both glutamine and glucose metabolism may suggest additional therapeutic opportunities such as inhibition of glutaminase. Whilst these may not be ideal targets in patients, targeting them *in vitro* may help identify more suitable *in vivo* targets.

The data presented here was obtained from cell lines which are grown in ideal nutrient and growth conditions. Even under ideal growth conditions the cells have an accelerated metabolic phenotype, suggesting that *in vivo* they would have a survival advantage. Further the *in vitro* phenotypic data presented make a strong case for these changes being "hard-wired" by the mutation states. Further investigation of the metabolic profile using other labelled metabolites such as glutamine would enable greater depth of knowledge into the metabolic profile and may provide further insights into potential therapeutic avenues. It has recently become apparent that *BRAF(V600)* or *RAS* activated melanomas, LKB1 is phosphorylated and AMPK signalling inactivated (Esteve-Puig, et al. 2009; Zheng, et al. 2009). It would be interesting to study whether the metabolic phenotype is recapitulated in different tumour types which appear to have *RAS-MAPK* activation and LKB1 protein inactivation through signalling mechanisms. Initial work examining

the expression profiles of these tumours should give an indication as to whether they are similar at the transcriptional level to the *LKB1/KRAS* mutant NSCLC discussed here.

We next investigated whether the sensitivity to CI-1040 and rapamycin was due to alteration to the expression signature described above. CI-1040 and rapamycin treatment had very little effect on the expression profile suggesting that the mechanism of compound sensitivity is separate to the expression profile/metabolic phenotype. A small number of genes in *LKB1/KRAS* mutant cell lines did show alterations in expression, including glutaminase which showed decreased expression upon CI-1040 and rapamycin treatment. Glutaminase was one of only three genes which showed alteration to expression after both treatments and given the metabolic data described above may warrant further investigation. In particular it might be informative to carry out similar NMR experiments in CI-1040/rapamycin treated NSCLC cell lines to more clearly address the role of glutamate in *LKB1/KRAS* mutant metabolic phenotype.

Due to the complexities in the signalling pathways and the many inputs mTOR receives we sought to directly inhibit the LKB1/AMPK/mTOR pathway upstream of mTOR to determine the contribution to the expression phenotype. Treatment of cell lines with the AMPK inhibitor, Compound C, revealed it had a paradoxically greater inhibitory effect on the proliferation rate of *LKB1/KRAS* mutant cell lines than other cell lines tested. This was not the case for an *LKB1null/KRAS* wild type cell line indicating that the effect was specific to the *LKB1/KRAS* mutant subtype. There are a number of possible explanations for this provocative finding. It is possible that despite *LKB1* inactivation there could be residual AMPK activity necessary for cell growth. For example other kinases such as TAK1 and CaMKK phosphorylate AMPK and some residual phosphorylation is observed in *LKB1* null cell lines (Carretero, et

al. 2007). If such is the case, then inhibition of AMPK in *LKB1/KRAS* mutant cancers may be an area that warrants further investigation as a potential therapeutic target. An alternative explanation is that the inhibitor has "off-target" effects that are reasonably specific to *LKB1/KRAS* mutant cancers. Compound C is also known as dorsomorphin and is a known inhibitor of BMP signalling (Hao, et al. 2008). Expression analysis of AMPK inhibitor treated cells did not yield any obvious explanations and showed no alteration to characterised AMPK targets such as fatty acid synthase (Zhou, et al.2001). GO term enrichment analysis did not reveal any enrichment for genes involved in BMP signalling but several known BMP targets such as ID1, 2 and 3 (Darby, et al. 2008; Lorda-Diez, et al. 2008; Shepherd, et al. 2008) showed decreased expression upon Compound C treatment. This alteration in expression however was not restricted to *LKB1/KRAS* mutant cell lines. The mechanism of growth inhibition upon Compound C treatment did involve apoptosis at lower concentrations in *LKB1/KRAS* mutant cell lines than control cell lines. Although the GO term enrichment analysis of AMPK inhibited cell lines did not reach statistical significance, the categories enriched were related to apoptosis. Thus, it would appear that the mechanism of growth inhibition from AMPK inhibition is different to that observed from MEK/mTOR inhibition as it involves an apoptotic response rather than a cytostatic response. In addition, contrary to expectation based on current signalling models PDK4 has higher expression in *LKB1/KRAS* mutant NSCLC cell lines than in control cell lines. The expression of PDK4 is normally promoted by AMPK and fatty acids, as observed in skeletal muscle (Houten, et al. 2009) therefore expression would be expected to be decreased in *LKB1null* cell lines. Further work is required to determine the mechanism of the inhibitor and whether it has any therapeutic potential, bearing in mind the role of AMPK in cellular energy homeostasis and whole-organism energy homeostasis.

The expression data suggested that *LKB1/KRAS* mutant cell lines had 70-fold higher expression of an aldo-keto reductase AKR1B10. Overexpression was further confirmed at the protein level. AKR1B10 is an aldo-keto reductase, an NADPH linked oxidoreductase, expressed normally in the small intestine. It metabolises aldehydes and ketones to their corresponding primary and secondary alcohols. AKR1B10 has been implicated in a number of cellular processes, two of which were investigated here. Penning et al. 2007 suggested that AKR1B10 plays a role in cancer through deregulation of retinoic acid signalling. We did not find any evidence for this in AKR1B10 expressing NSCLC cell lines. In addition Yan et al. 2007 reported that siRNA inhibition of AKR1B10 in colorectal cancer cells lead to a decrease in cell proliferation. When siRNA knockdown of AKR1B10 in NSCLC cell lines was carried out as part of the work presented here we did not see any effect on the proliferation rate of the cells. Thus role for AKR1B10 in NSCLC is still not clear. It should be noted that complete knockdown of AKR1B10 at the protein level was not achieved, therefore the interpretation of these data are tentative as there could still be sufficient residual activity to mitigate any knockdown effects. AKR1B10 has been suggested to play roles in chemoresistance and fatty acid metabolism through interactions with ACC and recent data has suggested AKR1B10 expression in the intestine is physiologically important for protecting against dietary and lipid-derived cytotoxic carbonyls (Zhong, et al. 2009). It may therefore play a more important role in metabolism of cytotoxic/chemotherapeutic compounds in NSCLC.

While work presented here has identified a new genetic subset of NSCLC, there are still a number of questions remaining. One such question is the link between *LKB1/KRAS* mutations and the expression signature. Further work on this subset of cancers should perhaps include investigation as to the role of MYC, as both RAS and LKB1 regulate the stability of the MYC protein. MYC is a well known downstream target of ERK1/2 (Sears, et al. 1999) and phosphorylation of serine-62 in the N-

terminus of MYC by ERK1/2 stabilises the MYC protein (Sears, et al. 2000). It has recently been reported that LKB1 may regulate the proteasomal degradation of the MYC protein (Liang, et al. 2009). Given the role of MYC in regulating metabolism, particularly in cancer (reviewed by Gordan, et al. 2007), it may provide the missing link between the alterations at the signalling level and how they result in the expression signature dominated by metabolic genes. Examination of the gene expression data suggests that MYC does not have increased expression specifically in this subset of NSCLC. In addition, *in vivo* work is now of particular importance, to determine which findings are relevant *in vivo* and how they can be exploited therapeutically.

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 cell lines with therapeutic response will gradually categorise cancer into subsets defined by genetics and therapeutic response. This is of particular importance in lung cancer; with poor survival rates and a lack of early detection methods and an urgent need for more targeted therapies and biomarkers. Starting with the identification of genetic subset of lung cancer this project has yielded insights into the functional consequences of the mutations. Through targeted approaches, expression analysis and metabolic profiling it has provided greater understanding of a genetic subset of lung cancer. Although the picture is not complete the work presented here points to a number of promising avenues for further study which will hopefully contribute to the development of new molecular targets for therapy in NSCLC.

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Appendix

Gene	ID	logFC	t	P.Value	adj.P.Val	B	Fold change
AKR1B10	206561_s_at	6.169	14	5.03E-18	2.75E-13	29.64	71.944475
AKR1C3	209160_at	5.244	5.2	4.62E-06	0.252422	4.066	37.8991975
PRG1	201858_s_at	4.473	5	7.34E-06	0.400872	3.629	22.2090766
PDK4	225207_at	4.168	9.6	1.00E-12	5.49E-08	18.52	17.9776393
PLA2G4A	210145_at	3.946	5.6	1.14E-06	0.062511	5.385	15.4100761
OAS1	205552_s_at	3.812	9.9	4.38E-13	2.39E-08	19.29	14.046708
AKR1C2	1562102_at	3.67	5.4	2.43E-06	0.132549	4.674	12.7273635
CNTN1	227209_at	3.563	8.2	1.42E-10	7.78E-06	13.89	11.8190925
AKR1C1	216594_x_at	3.411	4.9	1.15E-05	0.627828	3.206	10.6356285
ALDH3A1	205623_at	3.4	5	8.51E-06	0.464783	3.489	10.5542625
NTS	206291_at	3.393	5.4	2.39E-06	0.130772	4.687	10.5029746
NRCAM	204105_s_at	3.355	8.5	4.35E-11	2.38E-06	15	10.2343796
CXCL1	204470_at	3.208	6	2.95E-07	0.016135	6.668	9.24155384
S100A4	203186_s_at	3.127	4.3	9.40E-05	1	1.231	8.73818478
TESC	218872_at	3.063	13	4.42E-17	2.41E-12	27.7	8.35886087
CA12	214164_x_at	2.833	8.6	2.85E-11	1.56E-06	15.4	7.12720774
SNF1LK	208078_s_at	2.815	5.7	6.71E-07	0.036637	5.891	7.03850069
LXN	218729_at	2.774	4.5	5.23E-05	1	1.781	6.84138568
NR0B1	206645_s_at	2.739	6.6	2.99E-08	0.001634	8.836	6.67585534
DNAJC12	223721_s_at	2.729	5.6	1.17E-06	0.064117	5.361	6.6322299
CD55	1555950_a_at	2.719	4.2	0.0001218	1	0.988	6.58609736
F2RL2	230147_at	2.71	9.1	5.23E-12	2.86E-07	16.98	6.54321232
IGFBP3	210095_s_at	2.704	4	0.0001964	1	0.542	6.51823529
KLF4	221841_s_at	2.639	4	0.0002266	1	0.409	6.22764963
RGS2	202388_at	2.618	6.5	4.25E-08	0.002319	8.505	6.13865462
SERPINB1	213572_s_at	2.605	5.3	3.34E-06	0.182507	4.372	6.08289755
CPLX2	225815_at	2.593	7.8	5.88E-10	3.21E-05	12.55	6.03530148
CYP4F11	206153_at	2.578	3.9	0.0002643	1	0.266	5.96931539
PAK3	214078_at	2.558	4.4	5.55E-05	1	1.725	5.88916923
GCLM	236140_at	2.55	7.6	9.45E-10	5.16E-05	12.1	5.85646108
GPX2	202831_at	2.524	4.1	0.000176	1	0.644	5.75183462
CP	227253_at	2.511	5.7	8.22E-07	0.04489	5.699	5.69992791
TSPAN7	202242_at	2.486	5.7	7.36E-07	0.040198	5.803	5.60348395
ABCG1	204567_s_at	2.476	4.2	0.000106	1	1.118	5.562526
DKFZP686A01247	212325_at	2.469	4	0.0002527	1	0.307	5.53687056
RSPO3	228186_s_at	2.454	4.6	3.08E-05	1	2.276	5.48105624
SLC7A11	217678_at	2.44	4.9	1.38E-05	0.754695	3.032	5.427377
CSTA	204971_at	2.44	4.3	8.12E-05	1	1.367	5.42501127
MGC34646	225008_at	2.414	5.1	5.89E-06	0.321807	3.836	5.33002368
SLC27A2	205768_s_at	2.354	4.3	7.89E-05	1	1.395	5.11261083
PDE1C	239218_at	2.353	4.1	0.000149	1	0.8	5.10988728
RFK	203225_s_at	2.343	13	7.64E-17	4.17E-12	27.21	5.07283259
SCARA5	229839_at	2.271	4.6	2.85E-05	1	2.351	4.82644482
PAQR5	242871_at	2.265	4.8	1.40E-05	0.764003	3.021	4.80635344
SOX7	228698_at	2.236	5.5	1.34E-06	0.072956	5.239	4.70959447

GDA	224209_s_at	2.207	4.3	8.56E-05	1	1.318	4.61755245
SLC7A2	225516_at	2.133	5.1	5.67E-06	0.309472	3.873	4.38756385
FRMD4B	213056_at	2.069	4.4	5.75E-05	1	1.692	4.19685266
GPR133	232267_at	2.055	4.1	0.0001879	1	0.583	4.15621123
PTGS2	1554997_a_at	2.017	5.1	6.82E-06	0.372405	3.698	4.0473447
CCDC68	220180_at	2.004	5.4	2.16E-06	0.118182	4.783	4.01041195
ASB9	205673_s_at	2.002	5.9	3.32E-07	0.018126	6.557	4.00477736
SERPINE2	212190_at	1.999	4.1	0.0001661	1	0.698	3.99664845
PELI2	219132_at	1.98	5.9	3.57E-07	0.019477	6.489	3.94467148
DEFB1	210397_at	1.978	6.8	1.60E-08	0.000876	9.426	3.93992356
GLCE	213552_at	1.973	6.3	8.97E-08	0.004898	7.797	3.92515254
TM4SF4	209937_at	1.96	4.5	3.79E-05	1	2.083	3.89063044
ABCC3	208161_s_at	1.937	5.1	6.47E-06	0.353373	3.748	3.83032829
FLJ33297	1569378_at	1.934	5.4	2.26E-06	0.123264	4.743	3.82187627
ASPH	205808_at	1.908	5.8	5.99E-07	0.032702	5.999	3.75364835
PIR	207469_s_at	1.876	5.3	2.68E-06	0.1461	4.582	3.67169943
TM4SF20	220639_at	1.876	4.2	0.0001037	1	1.139	3.66936472
SRXN1	225252_at	1.849	5.7	8.82E-07	0.048157	5.632	3.60245582
FZD10	219764_at	1.847	4.2	0.0001359	1	0.886	3.59843252
HAL	206643_at	1.843	6.7	2.11E-08	0.001153	9.166	3.58804737
SLC12A2	204404_at	1.823	6.7	2.56E-08	0.001397	8.985	3.5378875
UGDH	203343_at	1.821	5.5	1.51E-06	0.082621	5.121	3.53241256
HTRA1	201185_at	1.812	5.5	1.32E-06	0.071919	5.253	3.51109696
TNFAIP2	202510_s_at	1.808	4.7	2.30E-05	1	2.551	3.50226541
ITPR1	203710_at	1.805	6.4	7.31E-08	0.003991	7.991	3.49492542
RAPGEF4	205651_x_at	1.764	4.1	0.0001829	1	0.609	3.39597624
SLC4A11	223748_at	1.717	5.1	6.60E-06	0.360621	3.729	3.28799087
NUP62CL	220520_s_at	1.708	3.9	0.0002802	1	0.211	3.26813476
PCSK5	205560_at	1.699	7.1	6.67E-09	0.000364	10.26	3.24595601
FAM55C	235030_at	1.674	4.4	7.11E-05	1	1.492	3.19015894
MGST2	204168_at	1.664	4.6	3.38E-05	1	2.191	3.16862755
KCNE4	1552507_at	1.659	4.2	0.000134	1	0.899	3.15838565
GRAMD1B	212906_at	1.644	7.6	1.06E-09	5.81E-05	11.99	3.12472664
ST8SIA4	242943_at	1.637	7.6	9.03E-10	4.93E-05	12.15	3.10987774
HHEX	204689_at	1.63	5.9	3.53E-07	0.019281	6.499	3.09451297
ABCB6	203192_at	1.603	6.9	1.06E-08	0.000581	9.814	3.03858858
RP5-875H10.1	242626_at	1.597	7.5	1.51E-09	8.23E-05	11.66	3.02588349
FLJ32549	1554067_at	1.584	7.4	2.01E-09	0.00011	11.39	2.99772298
CFH	213800_at	1.578	4.2	0.0001273	1	0.947	2.9865818
GALNT14	219271_at	1.577	4.6	3.33E-05	1	2.204	2.98332651
MEGF9	212830_at	1.567	5.9	3.64E-07	0.019864	6.471	2.96190545
GNAI1	227692_at	1.564	4.2	0.0001288	1	0.936	2.95594531
GSR	205770_at	1.563	5.3	3.12E-06	0.170483	4.436	2.95393513
TMEM144	228624_at	1.551	4.1	0.0001762	1	0.643	2.93121269
FXYD2	205674_x_at	1.548	5.2	3.70E-06	0.202098	4.276	2.92406299
MAP2K6	205698_s_at	1.542	6.4	7.84E-08	0.004283	7.924	2.91239622
RPL22L1	225541_at	1.538	7.1	6.23E-09	0.00034	10.32	2.90370053
AGTR1	205357_s_at	1.52	4.7	2.15E-05	1	2.616	2.86812286

LTB4DH	228825_at	1.51	5.4	2.38E-06	0.130148	4.692	2.84732719
XIST	221728_x_at	1.508	4	0.0002419	1	0.348	2.84367919
FSTL4	1564511_a_at	1.506	7.1	5.04E-09	0.000275	10.52	2.83938206
NQO1	201467_s_at	1.505	4	0.0002496	1	0.319	2.83854389
GGT1	207131_x_at	1.451	6.1	1.67E-07	0.009111	7.209	2.73339687
RAB3B	205925_s_at	1.443	4.5	4.67E-05	1	1.887	2.71909951
APOH	205216_s_at	1.435	4.2	0.0001338	1	0.9	2.70320952
EFNA5	1559360_at	1.424	5.9	3.26E-07	0.017798	6.575	2.6837734
SMOX	1555680_a_at	1.405	8.3	7.62E-11	4.16E-06	14.47	2.64804062
SERPIND1	205576_at	1.402	4	0.0002285	1	0.401	2.64211623
GPRIN3	1556698_a_at	1.397	7.5	1.50E-09	8.19E-05	11.67	2.63302765
SPATA7	219583_s_at	1.377	5.3	3.28E-06	0.178947	4.391	2.59726866
CDH17	209847_at	1.373	4.9	1.02E-05	0.557896	3.317	2.5901899
BTBD11	228570_at	1.356	4.9	1.13E-05	0.617177	3.222	2.55887585
AVPI1	218631_at	1.348	5	7.61E-06	0.415775	3.594	2.54582665
MUC5AC	217187_at	1.344	5.2	4.00E-06	0.218712	4.201	2.53829549
TBC1D8	204526_s_at	1.339	4.2	0.000134	1	0.899	2.52995072
GALNT13	234472_at	1.329	4.1	0.0001858	1	0.594	2.51286635
TST	209605_at	1.325	6	2.55E-07	0.013935	6.806	2.50572847
ETV4	1554576_a_at	1.316	4.9	1.29E-05	0.704358	3.097	2.48892948
ATF7	228829_at	1.309	7.5	1.20E-09	6.53E-05	11.88	2.47725465
SLC44A1	224595_at	1.299	5	9.58E-06	0.523371	3.377	2.46073358
CPNE4	228796_at	1.298	5.8	4.76E-07	0.025987	6.216	2.45806797
ZBTB38	225512_at	1.297	4	0.0002491	1	0.321	2.45783223
ATP2B1	212930_at	1.29	6.5	4.42E-08	0.002415	8.466	2.44590451
MAP7	215471_s_at	1.275	5.3	3.06E-06	0.167377	4.454	2.42023388
SLC9A2	211116_at	1.272	9.5	1.53E-12	8.36E-08	18.13	2.41506095
AADAC	205969_at	1.27	7.4	1.82E-09	9.96E-05	11.48	2.41226903
CA8	220234_at	1.26	5.8	5.32E-07	0.029081	6.11	2.39571254
GLA	214430_at	1.256	7.9	3.29E-10	1.80E-05	13.1	2.38909645
ATP2A3	213042_s_at	1.256	4.7	2.36E-05	1	2.528	2.38883067
PITX1	209587_at	1.234	4.1	0.0001743	1	0.653	2.35140072
NEIL3	219502_at	1.227	6.3	1.04E-07	0.00566	7.66	2.34099942
LPGAT1	1555058_a_at	1.224	4.9	1.34E-05	0.73348	3.059	2.33600946
MATR3	242260_at	1.199	4.9	1.24E-05	0.67676	3.135	2.29605279
CASP8	207686_s_at	1.185	5.6	1.05E-06	0.057238	5.469	2.27393093
RASSF6	235638_at	1.182	4.7	2.07E-05	1	2.653	2.26896539
RAN	200749_at	1.175	4.7	2.23E-05	1	2.582	2.25779488
ETFB	202942_at	1.163	8.1	1.93E-10	1.05E-05	13.6	2.23878746
ST6GAL2	1555123_at	1.16	4.4	5.85E-05	1	1.675	2.2351768
TNFSF15	221085_at	1.158	4.5	4.43E-05	1	1.935	2.23126612
NQO2	203814_s_at	1.156	6.5	4.25E-08	0.00232	8.504	2.22827587
CLMN	225759_x_at	1.151	6	3.06E-07	0.016737	6.633	2.22098698
HR	241355_at	1.133	5.3	3.30E-06	0.180282	4.384	2.19259416
TRPC6	206528_at	1.128	4.5	4.77E-05	1	1.867	2.1849124
DMXL2	212820_at	1.12	4.4	7.08E-05	1	1.496	2.17395811
ANKRD5	220144_s_at	1.11	5.9	3.52E-07	0.01923	6.501	2.15888481
SLC26A9	242271_at	1.094	4.5	5.08E-05	1	1.807	2.13438684

EDEM3	223243_s_at	1.093	4.3	7.33E-05	1	1.464	2.13307552
CBLB	227900_at	1.085	5	7.52E-06	0.410893	3.606	2.1209673
ARRDC2	226055_at	1.072	7	9.30E-09	0.000508	9.942	2.10221957
IDH1	1555037_a_at	1.056	4.3	7.90E-05	1	1.394	2.07904435
TMEM71	238429_at	1.053	4.3	9.12E-05	1	1.259	2.07544881
NT5C2	243158_at	1.044	5.1	5.74E-06	0.313311	3.862	2.06214454
SMOC2	223235_s_at	1.043	4	0.0002435	1	0.342	2.06080163
TALDO1	201463_s_at	1.028	4.8	1.90E-05	1	2.731	2.03932841
TSKU	218245_at	1.028	4	0.0001988	1	0.531	2.0391851
ZFP62	227796_at	1.023	5	7.92E-06	0.432719	3.557	2.03188311
USP3	221654_s_at	1.015	5.2	4.50E-06	0.245658	4.091	2.02026482
CDKL5	227004_at	0.998	4.1	0.0001525	1	0.778	1.99762638
ZNF207	1556035_s_at	0.994	6.5	5.27E-08	0.002878	8.3	1.99151326
NAP5	239650_at	0.991	5	7.60E-06	0.415169	3.596	1.98744653
TXN	208864_s_at	0.988	5.2	3.93E-06	0.214625	4.219	1.98391947
CRTC3	232879_at	0.982	5.8	4.56E-07	0.024901	6.257	1.97579147
IL18R1	206618_at	0.981	4	0.0002201	1	0.436	1.97424002
TDO2	205943_at	0.981	4.3	9.77E-05	1	1.194	1.97376085
SALL1	229273_at	0.969	4.7	2.21E-05	1	2.589	1.95737202
HDDC3	227008_at	0.953	5.5	1.77E-06	0.096834	4.971	1.93601127
TRPA1	217590_s_at	0.952	4.1	0.0001719	1	0.666	1.93440022
BMP6	206176_at	0.948	4.9	1.32E-05	0.720564	3.076	1.92898217
ABCC1	202804_at	0.946	4.1	0.0001523	1	0.78	1.92632252
MKKS	222530_s_at	0.94	5.4	2.38E-06	0.129763	4.694	1.91788365
DTWD1	236649_at	0.938	4.9	1.23E-05	0.67048	3.144	1.91563859
NLN	225944_at	0.937	4.9	1.25E-05	0.684425	3.124	1.91438264
ZSWIM6	226208_at	0.936	4.9	1.36E-05	0.740653	3.05	1.91336654
NEDD1	234984_at	0.933	5.4	2.46E-06	0.134483	4.661	1.90952009
CD99	201029_s_at	0.932	3.9	0.0002602	1	0.28	1.90768435
UBXD6	215983_s_at	0.931	5	8.57E-06	0.468267	3.482	1.90696395
LEO1	235096_at	0.929	6	2.34E-07	0.012778	6.888	1.90344729
IL15	217371_s_at	0.923	4	0.0002279	1	0.403	1.89594962
GALNT4	220442_at	0.921	4.2	0.0001063	1	1.116	1.89332999
DET1	219641_at	0.919	5.6	1.21E-06	0.066327	5.329	1.89127215
PART1	205833_s_at	0.917	4.1	0.0001437	1	0.834	1.88836049
GGTLA4	211416_x_at	0.908	5.7	6.62E-07	0.036129	5.904	1.87644229
PPP1R3C	240187_at	0.904	5	7.64E-06	0.417493	3.591	1.87092118
MAST4	40016_g_at	0.902	5.2	4.79E-06	0.261623	4.032	1.86925072
ATRN	211852_s_at	0.899	6.3	9.49E-08	0.00518	7.744	1.86474788
SLC23A2	211572_s_at	0.896	4.7	2.38E-05	1	2.521	1.86027859
NUP88	202900_s_at	0.895	4.8	1.72E-05	0.941295	2.824	1.85985342
CDS2	212864_at	0.886	4.1	0.0001567	1	0.753	1.84816306
TNFRSF10B	210405_x_at	0.873	5.4	2.14E-06	0.116846	4.794	1.83192089
FLJ31958	232803_at	0.86	4.8	1.56E-05	0.849595	2.921	1.81547992
MTFMT	235689_at	0.857	5.1	5.56E-06	0.303536	3.891	1.81135136
BRI3BP	231810_at	0.855	4.4	6.99E-05	1	1.509	1.80883781
FLJ31951	226077_at	0.852	4.4	7.17E-05	1	1.484	1.80442076
CLN8	222874_s_at	0.851	4.6	3.77E-05	1	2.089	1.80382936

CALM1	213688_at	0.85	3.9	0.00026	1	0.281	1.80275445
CSRP2BP	228543_at	0.845	4.5	4.07E-05	1	2.015	1.79651538
CNTNAP3	233202_at	0.845	4	0.0002147	1	0.459	1.79588183
FGF14	221310_at	0.844	4	0.0002088	1	0.485	1.79542115
UNC5D	231325_at	0.844	5.1	5.28E-06	0.288547	3.939	1.79445971
PCBD1	203557_s_at	0.839	4.2	0.0001106	1	1.079	1.78873088
TCN1	205513_at	0.833	4.7	2.20E-05	1	2.596	1.78184846
PDE4B	222326_at	0.825	6	2.86E-07	0.015603	6.699	1.77207523
KCNJ2	206765_at	0.822	4.2	0.0001246	1	0.967	1.76799626
AKR1C4	210558_at	0.817	5.9	4.21E-07	0.022979	6.333	1.76205363
RND1	210056_at	0.815	5.1	6.00E-06	0.327889	3.819	1.75949124
TM4SF5	206242_at	0.813	4.4	7.07E-05	1	1.498	1.75659555
OSBPL5	223464_at	0.81	4.2	0.0001089	1	1.093	1.75375627
TGIF	1566901_at	0.809	3.9	0.0002689	1	0.249	1.75200819
FASTKD5	219016_at	0.805	5.7	8.29E-07	0.04525	5.691	1.7469311
TP53AP1	210886_x_at	0.801	4.1	0.0001737	1	0.657	1.74262529
PBEF1	236495_at	0.801	5.7	7.46E-07	0.040725	5.791	1.74217606
ARSD	232423_at	0.794	5.3	2.63E-06	0.143584	4.599	1.73329878
EPB49	204505_s_at	0.789	4.7	2.28E-05	1	2.561	1.72838048
CHMP1B	218177_at	0.789	4.7	2.50E-05	1	2.475	1.72728715
MED11	226958_s_at	0.787	6	2.76E-07	0.01506	6.733	1.72518379
MRPL50	225581_s_at	0.786	4.5	4.86E-05	1	1.849	1.72412827
MGC4562	235005_at	0.785	4.1	0.0001745	1	0.652	1.72310072
FAM84A	234335_s_at	0.785	4.2	0.0001014	1	1.16	1.72297733
TMEM177	230830_at	0.779	5.7	7.50E-07	0.040986	5.785	1.71593514
KCTD6	238077_at	0.777	4	0.0002095	1	0.482	1.71319835
RAB37	228113_at	0.772	4.9	1.27E-05	0.693755	3.112	1.70773421
BTAF1	243996_at	0.772	4.2	0.0001093	1	1.089	1.70739059
STAT4	206118_at	0.771	4.9	1.34E-05	0.733105	3.06	1.70614996
NRG4	242426_at	0.769	5.5	1.62E-06	0.0886	5.055	1.70464674
FBXL15	218938_at	0.757	4.3	9.82E-05	1	1.189	1.68993174
THSD7A	230008_at	0.752	4.6	3.70E-05	1	2.106	1.68468738
COL4A6	210945_at	0.746	4.3	7.72E-05	1	1.415	1.67656315
VPS33B	44111_at	0.744	5.3	2.82E-06	0.153743	4.534	1.67513318
IDE	203327_at	0.742	4.8	1.81E-05	0.988716	2.778	1.67227099
ACADSB	226030_at	0.734	4	0.0002327	1	0.384	1.66274417
OAS3	218400_at	0.732	4.5	4.13E-05	1	2.003	1.66149703
LEPROTL1	202595_s_at	0.732	4.4	7.13E-05	1	1.49	1.6604443
CLINT1	201769_at	0.728	4.4	5.33E-05	1	1.763	1.65684357
RPGR	207624_s_at	0.726	5.7	8.11E-07	0.044276	5.712	1.65396959
CUTC	218970_s_at	0.72	4.2	0.0001068	1	1.111	1.64730485
PIGO	209998_at	0.71	4.5	4.07E-05	1	2.016	1.6356834
DGKG	206395_at	0.709	10	1.76E-13	9.60E-09	20.14	1.63505871
SRA1	224864_at	0.708	5.1	5.19E-06	0.283414	3.956	1.63409282
COX15	219547_at	0.707	5.2	4.68E-06	0.255824	4.053	1.63285479
TNPO1	209225_x_at	0.706	4.5	4.99E-05	1	1.824	1.63147389
RAB18	223336_s_at	0.705	4.4	6.77E-05	1	1.538	1.62986878
EPS8L3	219404_at	0.703	4.4	5.61E-05	1	1.714	1.62832896

IREB2	242261_at	0.703	4.8	1.56E-05	0.8509	2.919	1.62772968
CA9	205199_at	0.702	4.3	9.46E-05	1	1.225	1.62701612
C1QBP	214214_s_at	0.701	6.1	2.20E-07	0.011996	6.948	1.62602284
ITGB1	216178_x_at	0.694	5.5	1.75E-06	0.095609	4.983	1.61755933
DSG1	1554345_a_at	0.694	5.6	9.15E-07	0.049993	5.597	1.61728026
RPAIN	228183_s_at	0.682	4.2	0.0001255	1	0.96	1.60396778
DGKD	208072_s_at	0.668	4.7	2.31E-05	1	2.547	1.58898083
NPAS1	231089_at	0.663	4.7	2.47E-05	1	2.483	1.58359323
SBNO1	218737_at	0.663	4	0.0002086	1	0.486	1.58313209
HCLS1	202957_at	0.662	4.1	0.0001413	1	0.849	1.58255923
SIM2	206558_at	0.662	5.2	4.06E-06	0.221759	4.188	1.58249845
DSG1	219467_at	0.661	5.7	8.63E-07	0.047155	5.652	1.58130603
DDX3X	212515_s_at	0.659	4.5	4.62E-05	1	1.897	1.57892487
RCCD1	226488_at	0.655	4.1	0.0001454	1	0.823	1.57488861
MRPL24	218270_at	0.653	4.7	2.07E-05	1	2.652	1.57237087
PTPN4	205171_at	0.651	3.9	0.0002857	1	0.193	1.56993275
ACTR6	218395_at	0.644	4	0.0001925	1	0.561	1.56259661
NSUN3	219458_s_at	0.634	6.5	5.20E-08	0.002838	8.313	1.55180205
MRRF	225126_at	0.633	4.1	0.0001503	1	0.792	1.55073419
LYPLAL1	239516_at	0.632	5.4	2.45E-06	0.133926	4.665	1.54959561
ALDH16A1	227592_at	0.622	4.6	3.47E-05	1	2.165	1.53946146
ACBD6	225317_at	0.618	4	0.0002248	1	0.416	1.53450236
TRIM7	239694_at	0.614	4.4	6.57E-05	1	1.566	1.53049679
SHH	236263_at	0.612	5.5	1.57E-06	0.085868	5.085	1.52859014
CRADD	209833_at	0.608	7.8	4.61E-10	2.52E-05	12.78	1.52426271
TSEN2	219581_at	0.607	4	0.0002117	1	0.472	1.5230163
CRTAP	227138_at	0.599	4.8	1.52E-05	0.827459	2.945	1.51424316
CRLS1	223978_s_at	0.598	4.4	6.41E-05	1	1.589	1.51390189
TMEM37	227190_at	0.592	4	0.0002432	1	0.343	1.50751267
HOXA13	231786_at	0.591	4.6	3.46E-05	1	2.167	1.50621207
COQ5	223114_at	0.589	4.2	0.0001068	1	1.112	1.50377887
EPB41L4A	228259_s_at	0.586	4.8	1.69E-05	0.923722	2.842	1.50094974
SCARNA15	228930_at	0.583	4.1	0.0001612	1	0.726	1.49813111
GPRIN3	1556697_at	0.577	5.7	7.32E-07	0.039956	5.809	1.49152329
FLJ23556	206548_at	0.568	4.6	2.75E-05	1	2.384	1.48269394
SHC4	235238_at	0.567	4.4	5.49E-05	1	1.735	1.48161333
TUBG1	244880_at	0.564	5	9.14E-06	0.498895	3.422	1.47862867
ZC3HAV1	213051_at	0.56	5.4	1.81E-06	0.099053	4.95	1.47447591
F2RL2	206795_at	0.56	5.8	4.74E-07	0.025884	6.22	1.47414409
RPL23AP13	207283_at	0.554	5.2	4.15E-06	0.226409	4.168	1.46806371
MIS12	221559_s_at	0.55	3.9	0.000277	1	0.222	1.46410186
MRPL46	219244_s_at	0.547	4	0.0002367	1	0.368	1.46066117
OPRS1	201692_at	0.536	4	0.0002484	1	0.323	1.45016139
SNHG4	1564911_at	0.533	4.2	0.0001185	1	1.014	1.44702921
SRC	1558211_s_at	0.523	6	2.78E-07	0.015161	6.726	1.43727398
SLC29A3	219344_at	0.521	4.4	6.06E-05	1	1.643	1.43472043
PSCDBP	209606_at	0.515	4.4	5.87E-05	1	1.672	1.42908097
TMEM5	204808_s_at	0.509	4	0.0001908	1	0.569	1.42299296

CNIH	201653_at	0.498	4.3	8.86E-05	1	1.286	1.41241749
SUCNR1	223939_at	0.491	4.4	6.90E-05	1	1.52	1.40538873
COASY	201913_s_at	0.491	4.2	0.0001105	1	1.08	1.40525845
SMYD3	239784_at	0.489	5	7.16E-06	0.391203	3.652	1.40304784
INVS	210114_at	0.475	4	0.0002187	1	0.442	1.39018102
ELP3	221094_s_at	0.463	4.6	3.08E-05	1	2.278	1.37869132
RABL3	226089_at	0.462	4.3	7.43E-05	1	1.451	1.37737571
APEX1	210027_s_at	0.459	4.1	0.0001714	1	0.669	1.37461194
HARSL	209252_at	0.457	4.3	9.86E-05	1	1.186	1.37307596
EIF4A1	201530_x_at	0.452	5.5	1.77E-06	0.096414	4.975	1.36753393
TMEM93	221255_s_at	0.45	4.4	5.36E-05	1	1.757	1.36644447
MMS19L	229226_at	0.444	4.8	1.63E-05	0.891031	2.876	1.3607069
KRT222P	244111_at	0.443	4.6	3.36E-05	1	2.195	1.35923363
TUBAL3	220310_at	0.435	5	8.68E-06	0.474066	3.471	1.35221925
SRP19	205335_s_at	0.432	4.2	0.000127	1	0.949	1.34929607
SLC35C1	222647_at	0.422	4	0.000197	1	0.539	1.33950502
TM4SF1	238168_at	0.421	4.1	0.0001595	1	0.736	1.33867953
RBM33	226732_at	0.419	4.3	8.76E-05	1	1.297	1.33656919
MRPS5	224877_s_at	0.416	3.9	0.0002843	1	0.198	1.33456356
HNF4A	230914_at	0.409	4	0.0002505	1	0.315	1.32808463
METAP2	209861_s_at	0.409	4	0.0002198	1	0.437	1.32789878
MGC42174	238602_at	0.397	4.3	7.90E-05	1	1.393	1.31670164
KCNN3	205903_s_at	0.394	4.8	1.62E-05	0.882842	2.884	1.31446164
ATE1	234584_s_at	0.382	6.1	1.87E-07	0.010232	7.099	1.30319528
SERPINA6	206325_at	0.379	4	0.0002541	1	0.302	1.30087319
BCL11B	219528_s_at	0.37	4.1	0.0001559	1	0.757	1.2919458
SCRN3	1555595_at	0.366	4.5	4.29E-05	1	1.966	1.28849437
ACBD4	219413_at	0.351	4.3	8.11E-05	1	1.369	1.27579544
GPR157	220901_at	0.351	4.2	0.0001151	1	1.041	1.27521428
NOC4L	218860_at	0.345	4.2	0.0001285	1	0.938	1.27049956
ATP8B4	220416_at	0.343	4.8	1.68E-05	0.918026	2.848	1.26814118
FBXW8	237317_at	0.328	4.4	6.61E-05	1	1.561	1.25566852
EN2	207060_at	0.323	4	0.0001918	1	0.564	1.25110137
RPS17	212578_x_at	0.317	4.1	0.0001564	1	0.755	1.24599739
WDFY2	227490_at	0.315	4.7	2.63E-05	1	2.425	1.2439828
RPL23A	203012_x_at	0.287	4.8	1.56E-05	0.851951	2.918	1.22026091
ZNF343	207296_at	0.287	4	0.0001986	1	0.532	1.22021784
SEC63D1	241469_at	0.262	5	9.24E-06	0.504893	3.411	1.19913305
RIN2	233811_at	0.251	4.1	0.0001441	1	0.831	1.19025617
SCARF1	206995_x_at	-0.17	-4.3	9.75E-05	1	1.197	-1.12806453
LHX9	1565407_at	-0.18	-4	0.0001927	1	0.56	-1.12934422
CLLU1	1558186_s_at	-0.18	-4.1	0.0001523	1	0.779	-1.13368169
CHST11	1570165_at	-0.19	-4.1	0.0001599	1	0.734	-1.14370647
ZNF70	1554578_at	-0.2	-4	0.0002138	1	0.463	-1.14672556
SOS2	217575_s_at	-0.21	-4.5	4.27E-05	1	1.971	-1.15550198
TTBK2	1561705_at	-0.22	-4.1	0.0001571	1	0.751	-1.16141488
DNAH10	1565339_at	-0.22	-4.8	1.59E-05	0.867028	2.901	-1.16284563
CCDC46	1557886_at	-0.23	-4.1	0.0001887	1	0.58	-1.17367198

SIX4	231797_at	-0.23	-3.9	0.0002739	1	0.232	-1.1739554
CEP68	1559159_at	-0.24	-4	0.0002533	1	0.305	-1.18144307
DEFB119	1553093_a_at	-0.24	-4.2	0.0001226	1	0.982	-1.18308839
ATP9B	1564344_at	-0.24	-4	0.0002174	1	0.447	-1.18491902
ZNF501	1562386_s_at	-0.25	-4	0.0002374	1	0.365	-1.18846913
HHLA2	234673_at	-0.25	-4.1	0.0001804	1	0.622	-1.18848111
FMN1	243516_at	-0.25	-4	0.0002453	1	0.335	-1.18995212
CMTM8	234263_at	-0.26	-4.9	1.10E-05	0.600244	3.248	-1.19642087
APOL5	220478_at	-0.26	-3.9	0.0002704	1	0.244	-1.1971998
IGHMBP2	31861_at	-0.26	-4.2	0.0001295	1	0.931	-1.20047473
SEMA5A	1567705_at	-0.27	-4.1	0.0001591	1	0.739	-1.20825665
GOLGA3	1554987_at	-0.28	-4.2	0.000118	1	1.018	-1.2103434
ZNF490	231988_x_at	-0.28	-4	0.0002151	1	0.457	-1.21541965
ALS2CR11	1553260_s_at	-0.28	-4	0.0002047	1	0.504	-1.21709724
OR2M4	1566289_at	-0.28	-4	0.0002471	1	0.328	-1.21789807
NODAL	230916_at	-0.29	-4.3	7.93E-05	1	1.39	-1.21935269
GRIP1	241761_at	-0.29	-4.3	9.59E-05	1	1.212	-1.2202636
FLOT1	213819_s_at	-0.3	-4.5	4.92E-05	1	1.838	-1.23006925
CLCNKA	207047_s_at	-0.3	-4.1	0.0001721	1	0.665	-1.23121614
RAB43	229894_s_at	-0.31	-4	0.0002182	1	0.444	-1.23626042
SLC35E1	79005_at	-0.31	-4.2	0.0001326	1	0.909	-1.23778198
DPY19L2	1557290_at	-0.31	-4	0.0002079	1	0.489	-1.23928892
MT4	217395_at	-0.32	-4.5	3.81E-05	1	2.079	-1.24690637
TBC1D1	1568713_a_at	-0.32	-4.6	3.05E-05	1	2.286	-1.25057278
LSM14B	1553304_at	-0.32	-4.1	0.00016	1	0.733	-1.25234368
DISC1	1563744_a_at	-0.33	-4.2	0.0001175	1	1.022	-1.25404448
DKFZp564H213	234787_at	-0.33	-4	0.0001939	1	0.554	-1.26137413
NGB	236677_at	-0.34	-5.1	5.52E-06	0.301358	3.898	-1.26196544
FLJ31033	1559585_at	-0.34	-4	0.0002032	1	0.51	-1.26371583
PRKAR2A	213052_at	-0.35	-3.9	0.0002694	1	0.248	-1.2730627
CCDC73	237084_at	-0.35	-4.3	8.92E-05	1	1.28	-1.27653579
SLC4A3	205918_at	-0.36	-4	0.0002069	1	0.494	-1.27979782
CTA-216E10.6	219309_at	-0.36	-4.2	0.000134	1	0.899	-1.28005011
FLJ16124	1559939_at	-0.36	-4.1	0.0001671	1	0.693	-1.28067534
NMBR	207333_at	-0.36	-4.2	0.0001029	1	1.146	-1.28335534
ZNF75A	239204_at	-0.36	-4.9	1.13E-05	0.615852	3.224	-1.28728057
ZNF470	234921_at	-0.37	-4.1	0.0001878	1	0.584	-1.2889506
ZNF160	1567032_s_at	-0.37	-4	0.0002191	1	0.44	-1.28981761
PRDM4	1565838_at	-0.37	-4.2	0.0001024	1	1.151	-1.29250822
COQ2	232126_at	-0.37	-4.4	6.33E-05	1	1.601	-1.29286905
SIN3B	39705_at	-0.38	-4.3	8.14E-05	1	1.366	-1.29707981
PRRT3	1556308_at	-0.38	-4.1	0.0001733	1	0.659	-1.30033625
OCIAD1	235537_at	-0.38	-4.2	0.0001134	1	1.055	-1.30560868
TMEM14C	223105_s_at	-0.39	-4.6	2.95E-05	1	2.318	-1.30975369
TRIM39	222732_at	-0.39	-5.2	3.95E-06	0.215945	4.213	-1.31019851
STK19	36019_at	-0.39	-4.5	4.75E-05	1	1.87	-1.31405954
DVL3	201907_x_at	-0.41	-4	0.0002001	1	0.525	-1.32588564
CEMP1	227841_at	-0.42	-5.4	2.02E-06	0.110258	4.848	-1.33519818

PCDHB18	234724_x_at	-0.42	-4	0.0002466	1	0.33	-1.3373416
TRIM62	58308_at	-0.42	-4.3	7.50E-05	1	1.442	-1.34195265
ZFP37	207068_at	-0.43	-4.2	0.0001022	1	1.153	-1.34403864
MCART1	232092_at	-0.43	-4.5	4.74E-05	1	1.873	-1.35109224
TJAP1	47608_at	-0.44	-4.8	1.53E-05	0.834732	2.937	-1.35857907
HSDL1	223248_at	-0.45	-4.2	0.0001368	1	0.88	-1.36212267
FANCB	243597_at	-0.45	-4.4	7.01E-05	1	1.506	-1.36930538
FNIP1	1559060_a_at	-0.47	-4.4	5.45E-05	1	1.741	-1.38064032
ABCC10	215873_x_at	-0.47	-5.1	6.96E-06	0.380305	3.679	-1.38123473
GTF2B	208066_s_at	-0.47	-4.2	0.0001325	1	0.91	-1.38466704
AARSL	231845_at	-0.47	-4.4	7.25E-05	1	1.474	-1.38645563
RASAL2	234343_s_at	-0.47	-4.9	1.20E-05	0.653134	3.168	-1.38797499
UBE4B	202316_x_at	-0.49	-4.7	2.27E-05	1	2.566	-1.40827419
TESK2	205486_at	-0.49	-4.1	0.0001483	1	0.804	-1.40903252
ATN1	40489_at	-0.49	-4.3	7.91E-05	1	1.393	-1.4091281
KLHL12	219931_s_at	-0.5	-4.1	0.0001448	1	0.826	-1.41135746
TBC1D22B	216549_s_at	-0.5	-4.3	8.05E-05	1	1.376	-1.41238595
PCYT1A	204209_at	-0.5	-4.2	0.0001089	1	1.093	-1.4127454
HIST1H2AI	214542_x_at	-0.51	-4.5	4.05E-05	1	2.02	-1.42201761
RSF1	222541_at	-0.51	-4.1	0.0001529	1	0.776	-1.42340306
PBX4	230536_at	-0.51	-4.2	0.0001065	1	1.114	-1.42397116
BRD2	208686_s_at	-0.52	-4.6	3.30E-05	1	2.213	-1.42923798
ANKRD13C	1556361_s_at	-0.52	-4.6	3.37E-05	1	2.194	-1.43188007
ALKBH8	235713_at	-0.52	-4	0.0001987	1	0.531	-1.43332849
INPPL1	201598_s_at	-0.52	-4.3	9.93E-05	1	1.179	-1.43483439
CUL7	36084_at	-0.52	-4.2	0.0001059	1	1.119	-1.43595663
ABCF1	200045_at	-0.54	-6.3	1.11E-07	0.006083	7.591	-1.44925989
FBXL12	220127_s_at	-0.54	-4.2	0.0001054	1	1.123	-1.45169542
HIST1H2AJ	208583_x_at	-0.54	-4.4	6.65E-05	1	1.555	-1.45435998
DHX16	203694_s_at	-0.54	-4.3	7.47E-05	1	1.446	-1.45762434
PUSL1	228733_at	-0.55	-5.9	3.36E-07	0.018351	6.546	-1.46608468
NAGS	229432_at	-0.55	-4.3	8.79E-05	1	1.294	-1.46807906
CC2D1B	225323_at	-0.55	-4	0.0002389	1	0.359	-1.46812615
BAT3	210208_x_at	-0.56	-4.3	9.82E-05	1	1.19	-1.46919351
CPSF3L	232437_at	-0.56	-4.6	3.04E-05	1	2.29	-1.47021806
ARMC8	203487_s_at	-0.56	-4.7	2.02E-05	1	2.675	-1.47553964
SENP2	222523_at	-0.56	-4.5	4.18E-05	1	1.99	-1.47554362
VAC14	218169_at	-0.56	-4.2	0.0001004	1	1.169	-1.47633394
TYW3	227141_at	-0.57	-4.2	0.0001124	1	1.064	-1.48361137
CENPT	218148_at	-0.57	-4	0.0002531	1	0.306	-1.48385348
FLJ37798	227298_at	-0.57	-4.6	2.95E-05	1	2.317	-1.48785542
CDC5L	209055_s_at	-0.58	-4.3	8.77E-05	1	1.296	-1.49199735
RP11-217H1.1	224899_s_at	-0.58	-4.2	0.0001212	1	0.993	-1.49640902
PLXNA3	203623_at	-0.58	-4.7	2.71E-05	1	2.399	-1.49917785
DDX19A	202577_s_at	-0.59	-4.6	3.64E-05	1	2.121	-1.50146939
DR1	207654_x_at	-0.59	-4.1	0.0001399	1	0.858	-1.5020665
PRKACA	216234_s_at	-0.59	-4.1	0.0001727	1	0.662	-1.50337475
MGC19604	226451_at	-0.59	-4.8	1.48E-05	0.807595	2.968	-1.50485086

ZNF599	228093_at	-0.59	-4.7	1.95E-05	1	2.706	-1.50502096
TCEA2	241428_x_at	-0.59	-4.3	9.59E-05	1	1.212	-1.50665839
SH3GL1	201851_at	-0.59	-4.6	3.30E-05	1	2.213	-1.50729822
KDELR2	200700_s_at	-0.6	-4.9	1.33E-05	0.725909	3.069	-1.51103784
SIAH2	209339_at	-0.6	-4.2	0.0001345	1	0.895	-1.51152918
NCOA6	208979_at	-0.6	-4.1	0.0001427	1	0.84	-1.51174914
DDEFL1	222236_s_at	-0.6	-5.8	5.79E-07	0.031629	6.03	-1.51609692
FAF1	224217_s_at	-0.6	-4.4	5.32E-05	1	1.764	-1.51771117
CORO1B	221754_s_at	-0.6	-4.2	0.0001221	1	0.986	-1.52030549
HABP4	232341_x_at	-0.61	-4.3	7.81E-05	1	1.404	-1.52352051
THAP8	228500_at	-0.61	-4.7	2.25E-05	1	2.574	-1.52526681
DR1	216652_s_at	-0.61	-4	0.0002544	1	0.301	-1.52988299
FOXJ2	203734_at	-0.62	-4.9	1.13E-05	0.61577	3.224	-1.5352174
ZGPAT	221848_at	-0.62	-4.5	4.18E-05	1	1.991	-1.53725339
MFN2	216205_s_at	-0.63	-4.5	4.83E-05	1	1.855	-1.5471791
DAXX	201763_s_at	-0.63	-5.5	1.70E-06	0.092803	5.012	-1.54741891
DGKZ	239342_at	-0.63	-5.5	1.30E-06	0.071019	5.265	-1.54907162
ZNF571	206648_at	-0.63	-4	0.0002401	1	0.355	-1.55145141
CDH24	230157_at	-0.63	-4.2	0.0001213	1	0.992	-1.55234522
KCNJ4	208359_s_at	-0.64	-5.1	6.63E-06	0.362334	3.724	-1.55357268
PPP1R15A	37028_at	-0.64	-4.3	9.53E-05	1	1.218	-1.55622307
PIM1	209193_at	-0.64	-4.1	0.00017	1	0.677	-1.55702746
BCAP29	225677_at	-0.65	-4.3	7.53E-05	1	1.439	-1.56386823
ZFYVE1	223388_s_at	-0.65	-4	0.0002221	1	0.428	-1.56619116
CDC14B	211348_s_at	-0.65	-4.9	9.99E-06	0.545394	3.338	-1.57394943
TRAPPC2L	218354_at	-0.66	-5.3	3.26E-06	0.178212	4.395	-1.57710123
MIB2	226644_at	-0.66	-5.3	3.38E-06	0.184517	4.362	-1.58398657
SLC7A6OS	232057_at	-0.66	-5.4	2.47E-06	0.134739	4.659	-1.58417811
LYPLA3	204458_at	-0.66	-4	0.0002129	1	0.467	-1.58472293
TEAD4	204281_at	-0.67	-5.1	6.89E-06	0.376322	3.689	-1.58902377
EIF4G1	208625_s_at	-0.68	-4	0.0002467	1	0.33	-1.59924751
FAM53A	1569139_s_at	-0.68	-4.4	5.63E-05	1	1.711	-1.59999961
RP11-535K18.3	219626_at	-0.68	-4.6	2.79E-05	1	2.37	-1.60512708
OTUD3	213216_at	-0.68	-4.7	2.38E-05	1	2.521	-1.6061306
RP9	227849_at	-0.69	-4.2	0.0001203	1	0.999	-1.60886589
PHF3	217952_x_at	-0.69	-4.2	0.0001005	1	1.168	-1.61333121
DVL3	201908_at	-0.71	-4.8	1.68E-05	0.919144	2.846	-1.63796681
DAPK3	203890_s_at	-0.71	-4.2	0.0001176	1	1.021	-1.64105934
TPMT	203672_x_at	-0.72	-4.6	3.38E-05	1	2.19	-1.65154402
ZNF318	203521_s_at	-0.72	-4.3	9.81E-05	1	1.19	-1.65170422
HHLA3	220387_s_at	-0.72	-4.1	0.0001462	1	0.818	-1.65189488
WWC2	222738_at	-0.72	-3.9	0.0002887	1	0.183	-1.65251383
KIFC2	226791_at	-0.73	-4.1	0.000165	1	0.705	-1.65907286
MOSPD1	218853_s_at	-0.73	-4.3	8.68E-05	1	1.305	-1.659792
PHF1	40446_at	-0.73	-4.6	3.26E-05	1	2.224	-1.66331442
DFFA	223518_at	-0.74	-4.7	2.03E-05	1	2.669	-1.67174764
ZNF382	1561687_a_at	-0.74	-4.1	0.0001601	1	0.733	-1.67532924
ZDHHC8	225744_at	-0.75	-3.9	0.000275	1	0.228	-1.68408035

FAM50A	203262_s_at	-0.75	-4	0.0002254	1	0.414	-1.68532035
TIAM2	222942_s_at	-0.75	-4.1	0.00016	1	0.733	-1.68665296
BRI3	223376_s_at	-0.78	-4.4	6.63E-05	1	1.557	-1.71288741
MRAS	225185_at	-0.78	-4	0.0002189	1	0.441	-1.7136797
PAK1IP1	218886_at	-0.78	-4	0.0002175	1	0.447	-1.71581309
INTU	228946_at	-0.78	-4.3	8.97E-05	1	1.274	-1.72045368
MED28	218438_s_at	-0.79	-4	0.000255	1	0.299	-1.72836496
DNAJB2	202500_at	-0.79	-4.9	1.19E-05	0.650669	3.172	-1.73030129
ZBTB46	227329_at	-0.8	-5	8.99E-06	0.491192	3.437	-1.74139636
SLC7A6	203579_s_at	-0.8	-4.3	7.57E-05	1	1.433	-1.74342246
CLEC2D	1560156_at	-0.81	-4.1	0.000175	1	0.65	-1.75246628
TRIM37	213009_s_at	-0.81	-4.8	1.64E-05	0.895038	2.871	-1.75386005
SERTAD1	223394_at	-0.81	-4.4	6.11E-05	1	1.635	-1.75719545
FYTTD1	224642_at	-0.82	-4.2	0.0001352	1	0.89	-1.7711217
RANBP9	216125_s_at	-0.83	-5	8.90E-06	0.486128	3.447	-1.77188766
DGCR2	214198_s_at	-0.86	-4	0.0002317	1	0.388	-1.8093959
MAP1LC3B	208785_s_at	-0.86	-6.3	9.03E-08	0.004932	7.79	-1.81710679
ZNF788	244552_at	-0.87	-3.9	0.0002635	1	0.268	-1.82656404
ZNF347	238819_at	-0.87	-4.8	1.85E-05	1	2.755	-1.83098951
MAP1LC3B	208786_s_at	-0.91	-4.9	1.30E-05	0.710262	3.089	-1.87477877
DDAH2	215537_x_at	-0.92	-4.4	6.15E-05	1	1.628	-1.8899167
HIST1H2BC	236193_at	-0.94	-4.4	5.69E-05	1	1.701	-1.91735955
RERE	221643_s_at	-0.94	-4.7	2.63E-05	1	2.426	-1.91750547
NOTCH2NL	214722_at	-0.94	-4.5	5.21E-05	1	1.785	-1.91978681
SLC35F2	218826_at	-1.01	-4.1	0.0001402	1	0.857	-2.01094948
RNF166	227726_at	-1.01	-6	2.29E-07	0.012488	6.91	-2.01588513
DAGLBETA	225832_s_at	-1.02	-4	0.0002169	1	0.449	-2.02312657
TIGD7	224365_s_at	-1.03	-5	7.42E-06	0.405032	3.619	-2.04363907
SPHK1	219257_s_at	-1.04	-5.4	2.37E-06	0.129514	4.696	-2.06005576
TMEM142B	218812_s_at	-1.05	-4.9	1.18E-05	0.646357	3.178	-2.07544024
BRPF3	225217_s_at	-1.06	-5.8	5.55E-07	0.030335	6.07	-2.08366225
LSS	202245_at	-1.08	-4.8	1.41E-05	0.767474	3.016	-2.10709161
PSCD3	225147_at	-1.08	-4.2	0.000114	1	1.05	-2.11926925
GAB2	203853_s_at	-1.09	-4.1	0.0001883	1	0.581	-2.13189379
CAND2	213547_at	-1.12	-4.5	4.80E-05	1	1.86	-2.1741476
MICALL1	55081_at	-1.13	-6.2	1.53E-07	0.008375	7.289	-2.18171245
STAU2	227179_at	-1.13	-4.2	0.0001364	1	0.882	-2.19102924
CSDE1	219939_s_at	-1.14	-5.1	5.13E-06	0.280259	3.967	-2.20030298
SIPA1L1	202254_at	-1.14	-6	2.37E-07	0.012925	6.878	-2.2008839
DDAH1	209094_at	-1.16	-5.2	3.99E-06	0.217974	4.204	-2.2289298
TWSG1	219201_s_at	-1.17	-4.7	2.61E-05	1	2.432	-2.25778985
TM7SF2	210130_s_at	-1.18	-4.4	6.19E-05	1	1.622	-2.26561724
JUN	201464_x_at	-1.19	-4.6	3.74E-05	1	2.094	-2.2850761
PANX1	204715_at	-1.2	-4.6	3.05E-05	1	2.288	-2.29709039
PPIC	204518_s_at	-1.22	-4.3	9.95E-05	1	1.177	-2.33029847
SERPINH1	207714_s_at	-1.27	-4	0.0002587	1	0.286	-2.40753786
GPR176	227846_at	-1.3	-4.5	3.99E-05	1	2.034	-2.4579692
PLEKHC1	209209_s_at	-1.35	-4.6	3.65E-05	1	2.117	-2.54108015

NEU1	208926_at	-1.36	-6.7	2.03E-08	0.00111	9.203	-2.56011743
PCDHB2	231725_at	-1.36	-4.3	7.83E-05	1	1.402	-2.56510811
MGC16291	233562_at	-1.37	-4.7	2.49E-05	1	2.477	-2.58080356
OGFRL1	219582_at	-1.4	-4.5	4.70E-05	1	1.881	-2.63277976
ZNF320	229614_at	-1.4	-3.9	0.000278	1	0.218	-2.6335836
ZFAND3	222493_s_at	-1.4	-7.5	1.31E-09	7.18E-05	11.79	-2.63447152
ZNF600	242463_x_at	-1.41	-4.8	1.50E-05	0.816497	2.958	-2.66046443
PEA15	200787_s_at	-1.42	-5.2	3.96E-06	0.216235	4.212	-2.68047725
SLIT2	209897_s_at	-1.47	-4.7	2.08E-05	1	2.646	-2.76419191
HIST1H2AG	207156_at	-1.52	-5.5	1.33E-06	0.072727	5.242	-2.86792982
ZNF738	229700_at	-1.72	-3.9	0.0002775	1	0.22	-3.29856106
LIMA1	222457_s_at	-1.73	-4.2	0.0001338	1	0.9	-3.32149411
TTMB	227386_s_at	-1.77	-5.6	1.06E-06	0.057896	5.458	-3.41389858
IGF2BP2	218847_at	-1.96	-5.3	2.63E-06	0.143478	4.599	-3.89879878
SCRN1	201462_at	-2.02	-5.8	4.61E-07	0.025162	6.247	-4.04423786
CDKN2B	236313_at	-2.05	-4.3	1.00E-04	1	1.173	-4.14582555
ODZ3	219523_s_at	-2.05	-5.1	6.94E-06	0.379121	3.682	-4.1475774
COTL1	224583_at	-2.22	-5.3	3.43E-06	0.187411	4.347	-4.65016252
MICB	206247_at	-2.3	-4.3	7.42E-05	1	1.452	-4.93135234
HDAC9	205659_at	-2.4	-5.8	4.61E-07	0.025156	6.247	-5.29453089
PPM2C	222572_at	-2.53	-7	9.43E-09	0.000515	9.929	-5.7928031
PEG10	212094_at	-2.82	-4.6	3.44E-05	1	2.174	-7.04494909
MAGEA2	214603_at	-3.46	-4	0.0002501	1	0.317	-11.0366623