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).

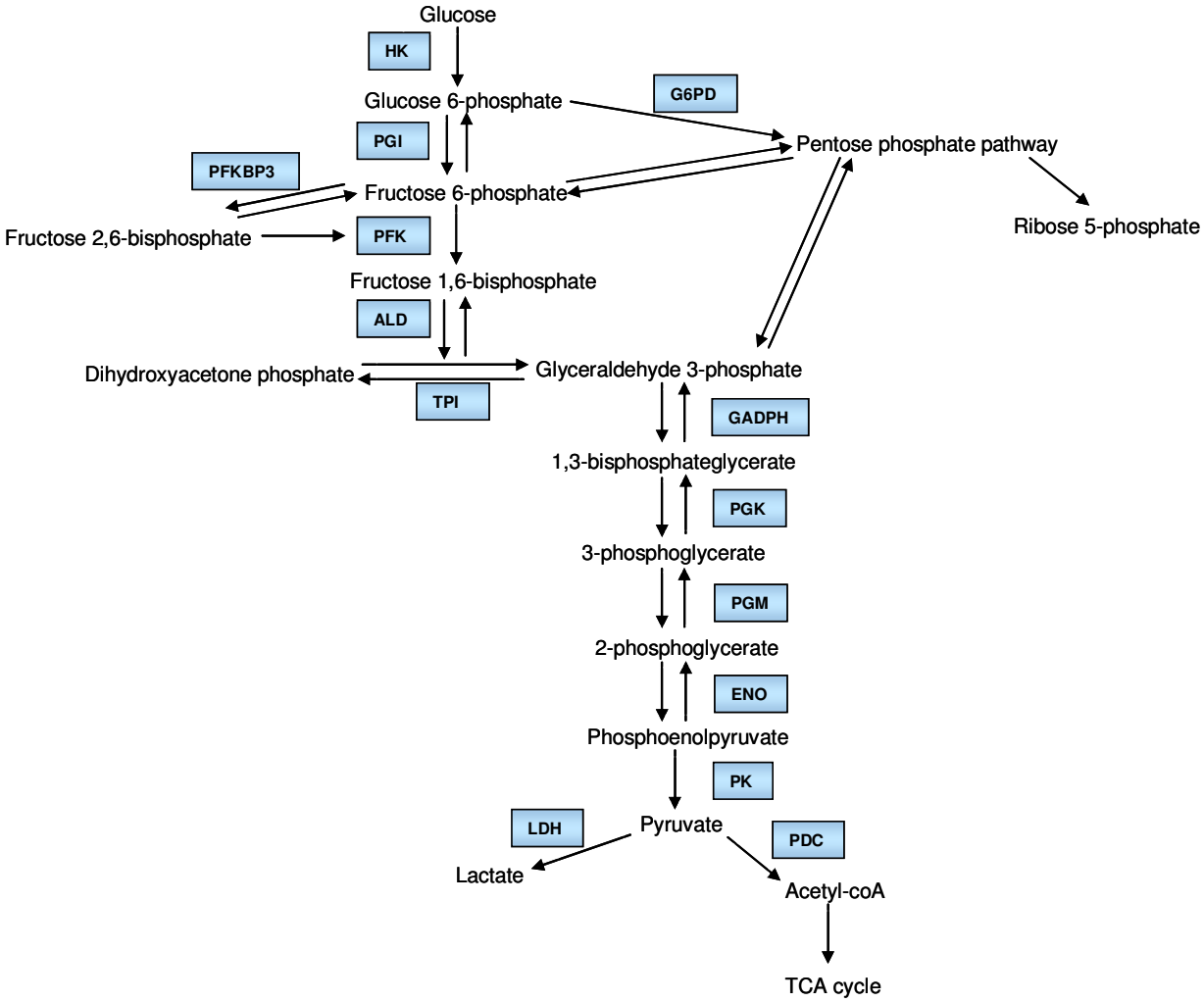

Figure 4-1 The main reactions of glycolysis. Glucose is converted to pyruvate through a series of enzymatic reactions. The resulting pyruvate can then enter the TCA cycle (figure 1-6). A number of glycolysis intermediates can also be diverted to the pentose phosphate pathway to generate ribose sugars 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.

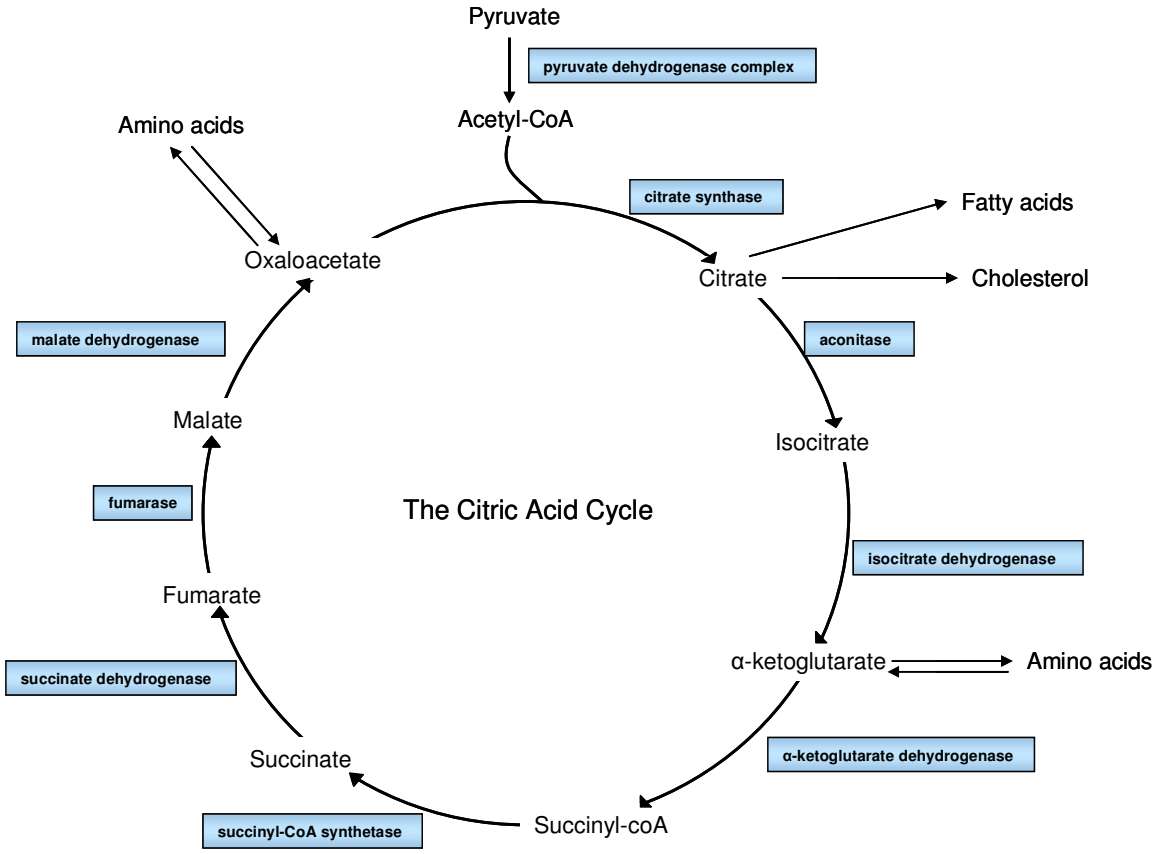

Figure 4-2. An overview of The Citric Acid Cycle. Pyruvate is converted to Acetyl-coA by the pyruvate dehydrogenase complex and enters the TCA cycle where it undergoes a series of enzymatic reactions for the generation of NADH for oxidative phosphorylation and ATP. Amino acids such as glutamate can be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

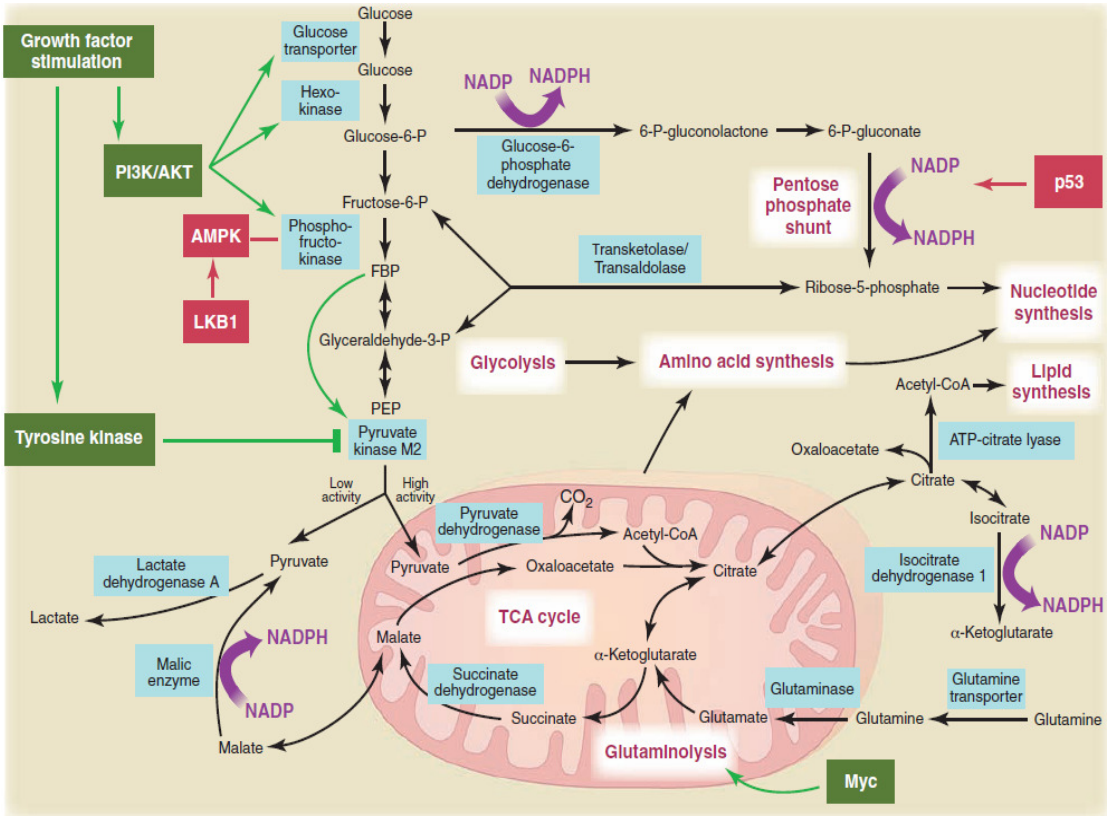

Figure 4-3 Overview of metabolic alterations in proliferating cancer cells (Figure from Vander Heiden et al. 2009). Signalling pathways which contain oncogenes and tumour suppressors directly and indirectly regulate the metabolic pathways within a cell. Shown here are the interactions between glycolysis,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

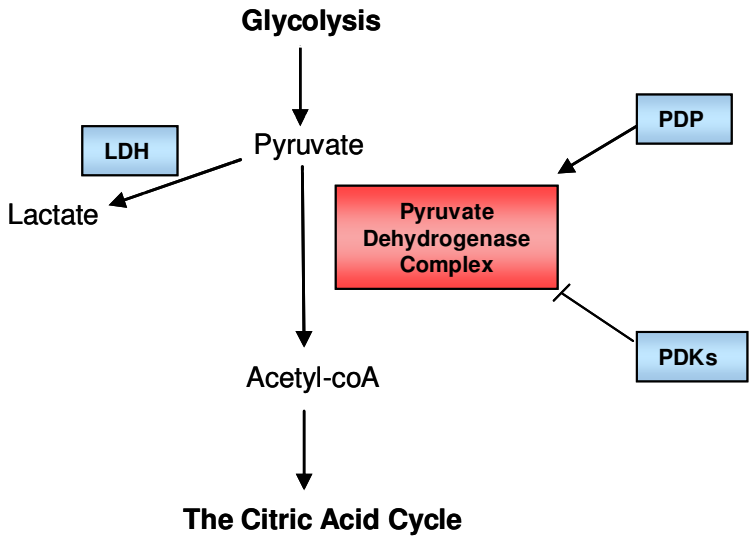

Figure 4-4 Regulation of the Pyruvate Dehydrogenase Complex (PDC). PDC controls the entry of pyruvate into the TCA cycle. It is regulated in a highly specific manner in depending on tissue type. Pyruvate dehydrogenase phosphatases (PDP) dephosphorylate the complex and activate it whilst pyruvate dehydrogenase 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.

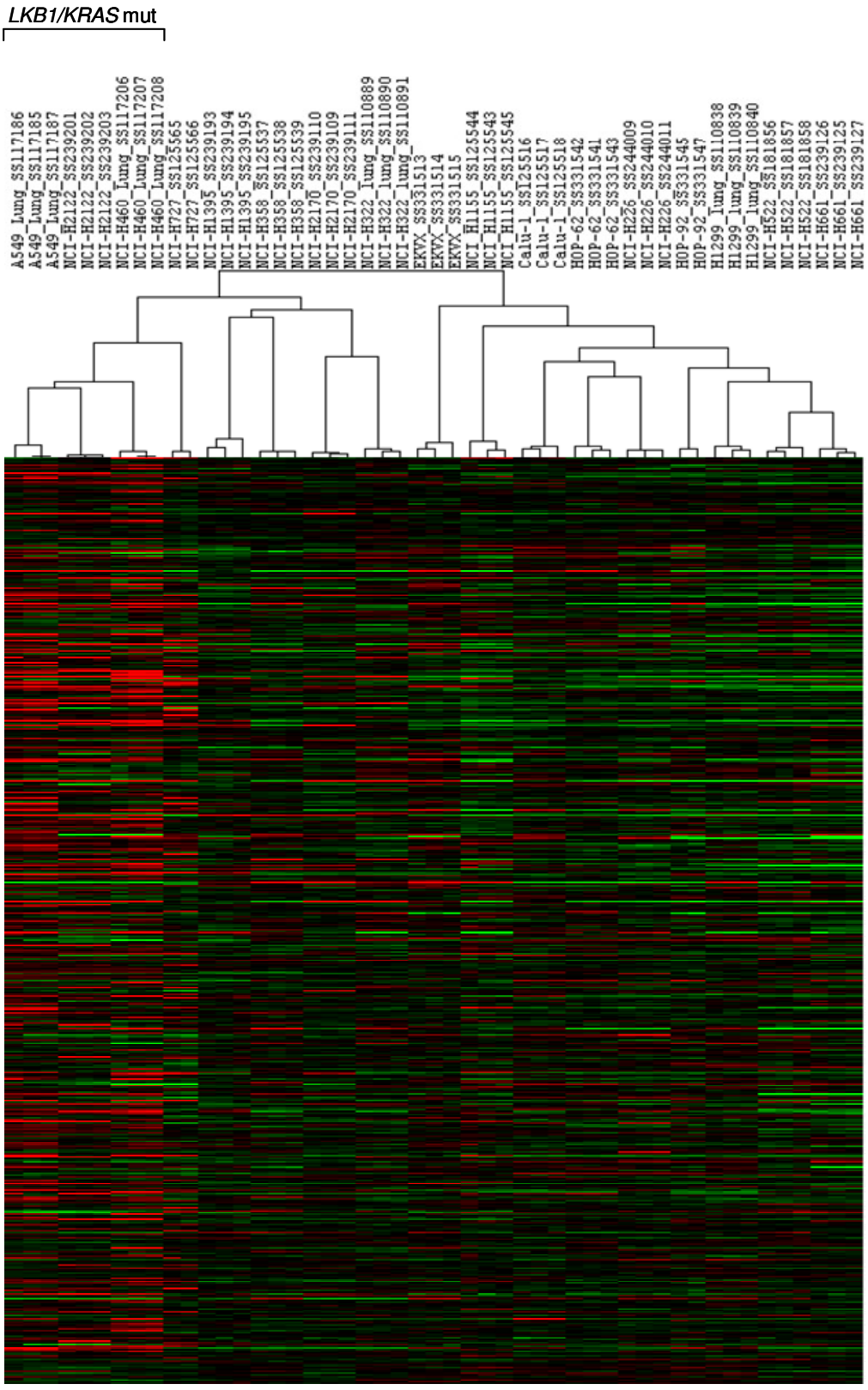
LKB1/KRAS mut
A549_Lung_SS117186
A549_Lung_SS117185
A549_Lung_SS117187
NCI-H2122_SS239201
NCI-H2122_SS239202
NCI-H2122_SS239203
NCI-H460_Lung_SS117206
NCI-H460_Lung_SS117207
NCI-H460_Lung_SS117208
NCI-H727_SS125565
NCI-H727_SS125566
NCI-H1395_SS239193
NCI-H1395_SS239194
NCI-H1395_SS239195
NCI-H358_SS125537
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.

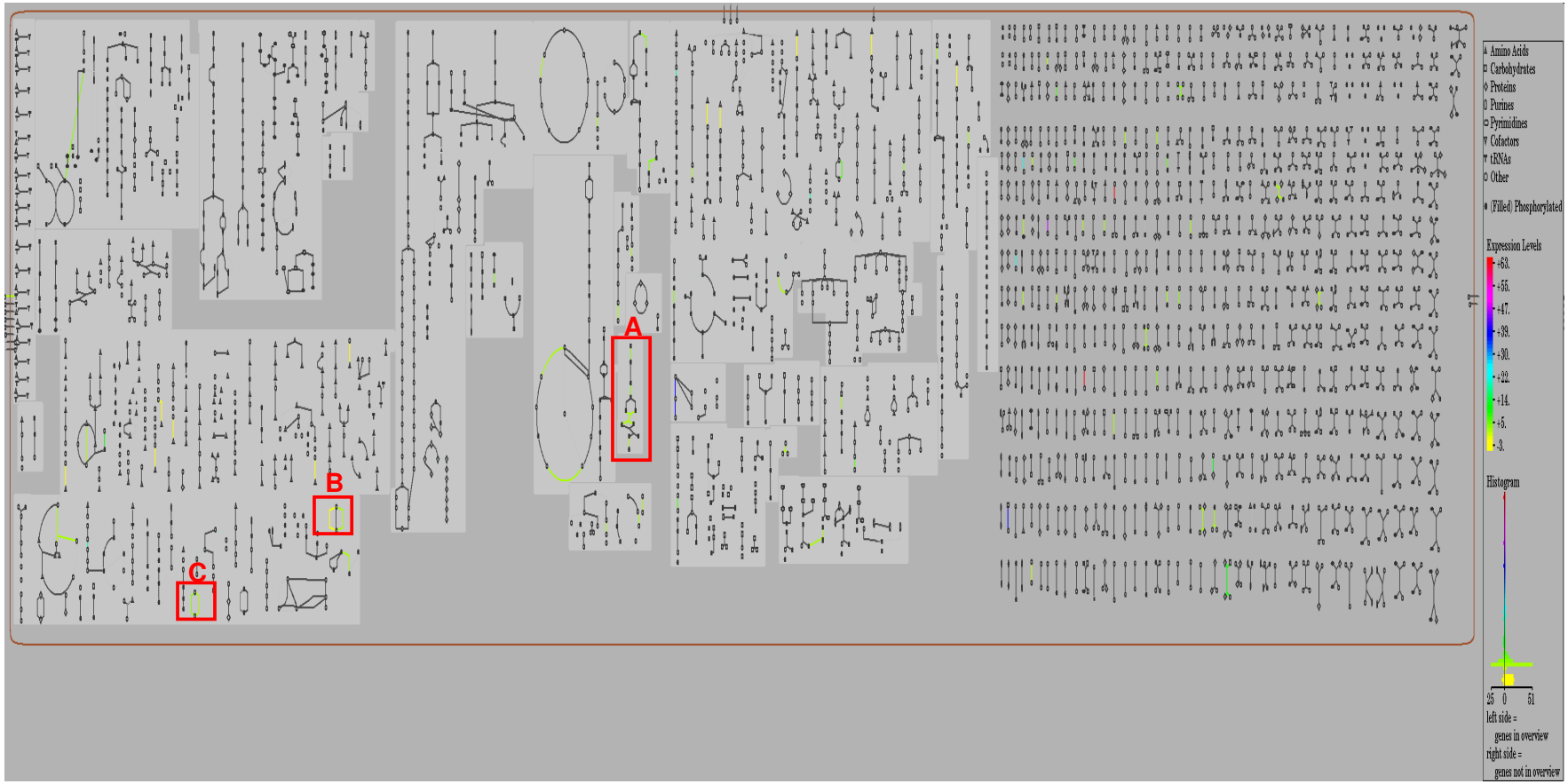

Figure 4-6. Metacyc map of the differentially expressed genes in *LKB1/KRAS* mutant NSCLC cell lines. Red boxes highlight pathways with a number of alterations in them. A indicates the pentose phosphate pathway and B+C glutathione metabolism.

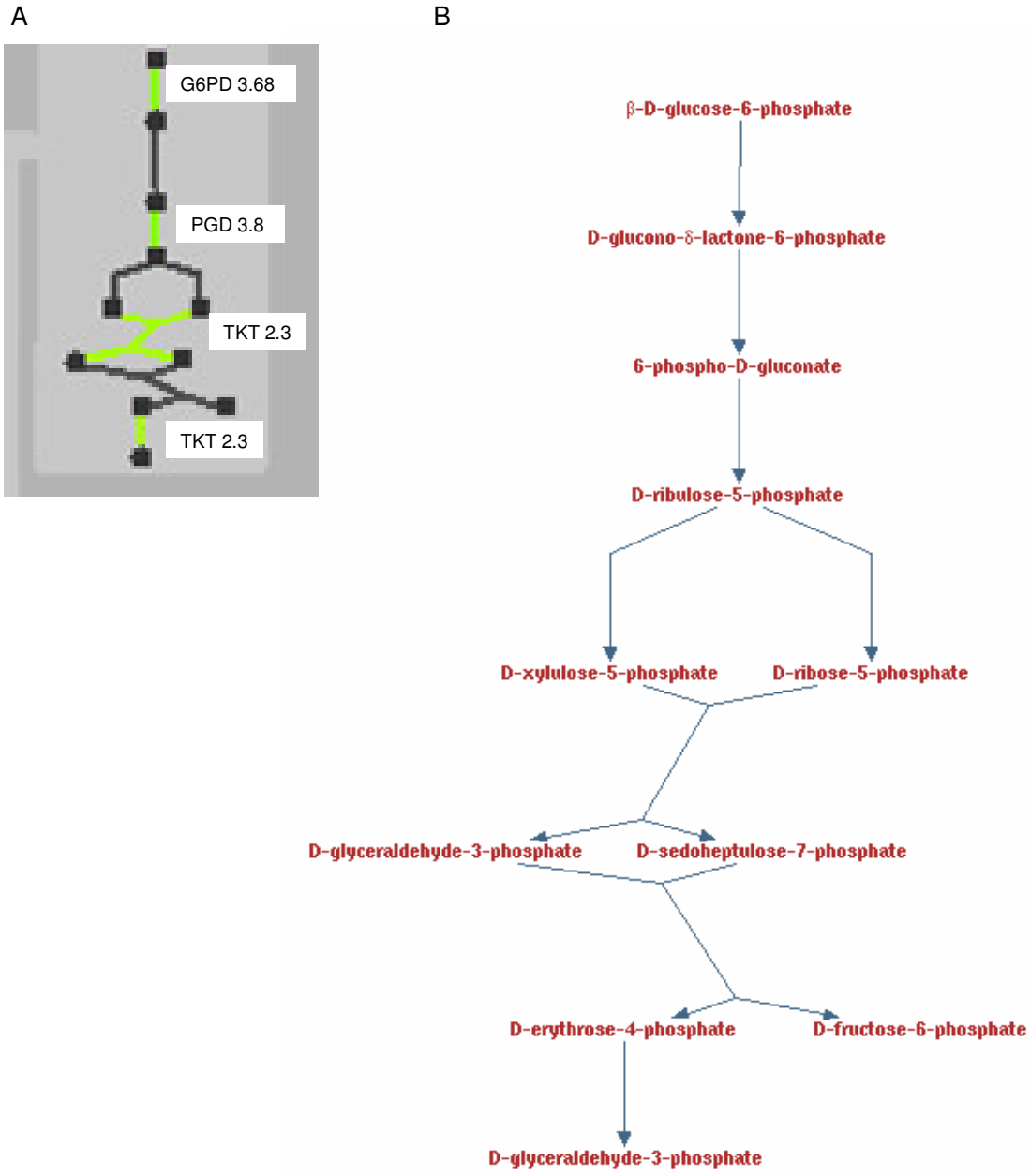

Figure 4-7 *LKB1/KRAS*mutant NSCLC cell lines show overexpression of 3 pentose phosphate pathway genes. A) Overlay of gene expression data onto the Metacyc pentose phosphate pathway. Gene names are in white boxes followed by fold change in expression in *LKB1/KRAS* mutant NSCLC cell lines. B) Graphical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

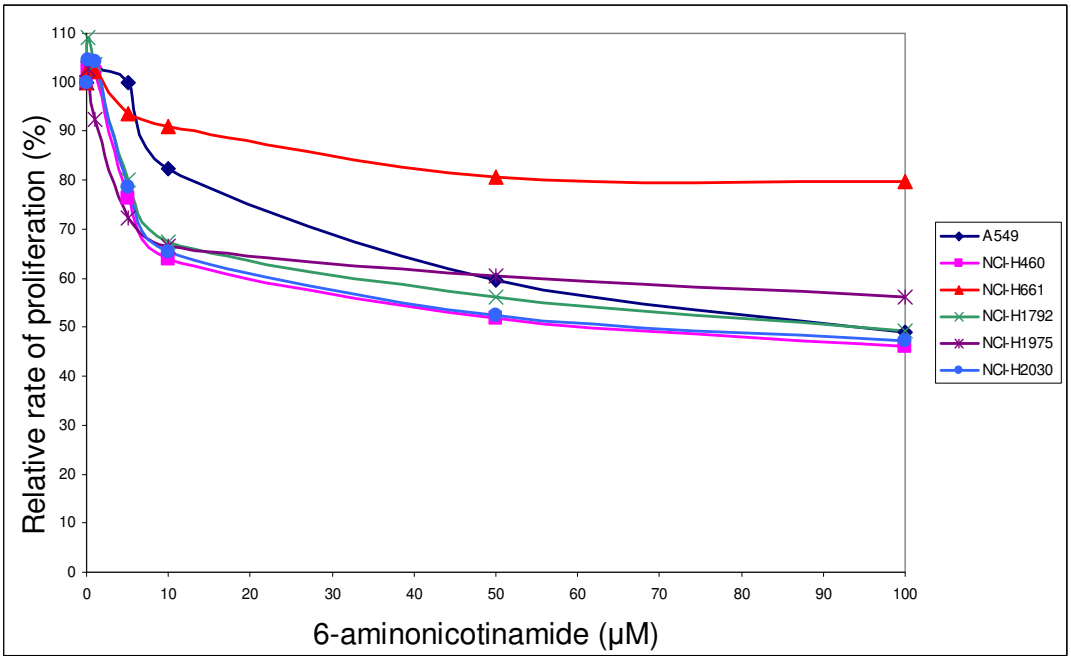

Figure 4-8 Cell lines with *RAS-MAPK* alterations are more sensitive to the G6PD inhibitor 6-AN. The relative rate of proliferation 72hrs after 6-AN treatment was examined for 6 NSCLC cell lines. Cell lines tested: NCI-H460 (*LKB1/KRASmut*), A549 (*LKB1/KRASmut*), NCI-H2030 (*LKB1/KRASmut*), NCI-H661 (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.

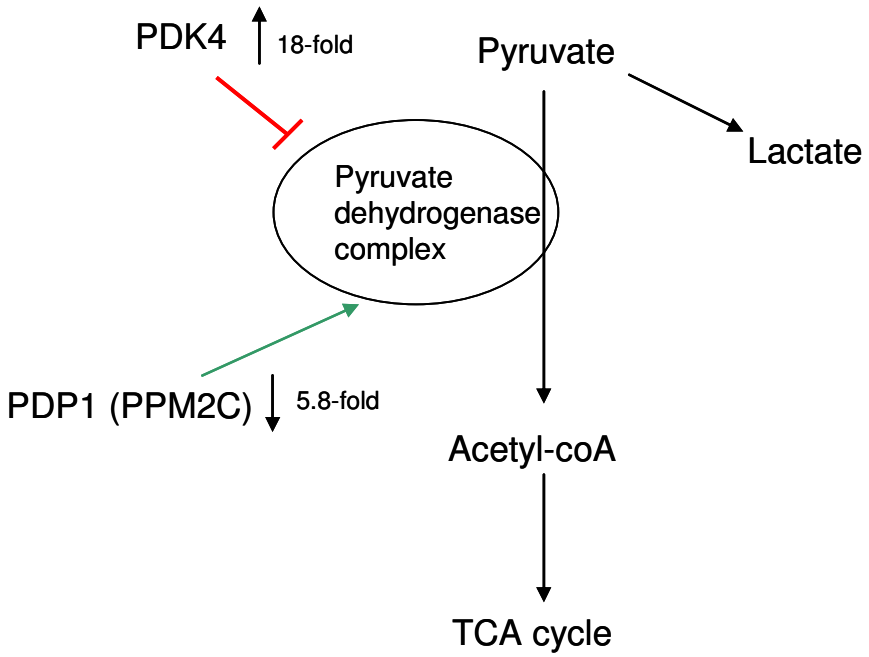

Figure 4-9 Regulation of the Pyruvate Dehydrogenase Complex (PDC). PDC controls the entry of pyruvate into the TCA cycle. It is regulated in a highly specific manner in depending on tissue type. Pyruvate dehydrogenase phosphatases (PDP) dephosphorylate the complex and activate it whilst pyruvate dehydrogenase 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.

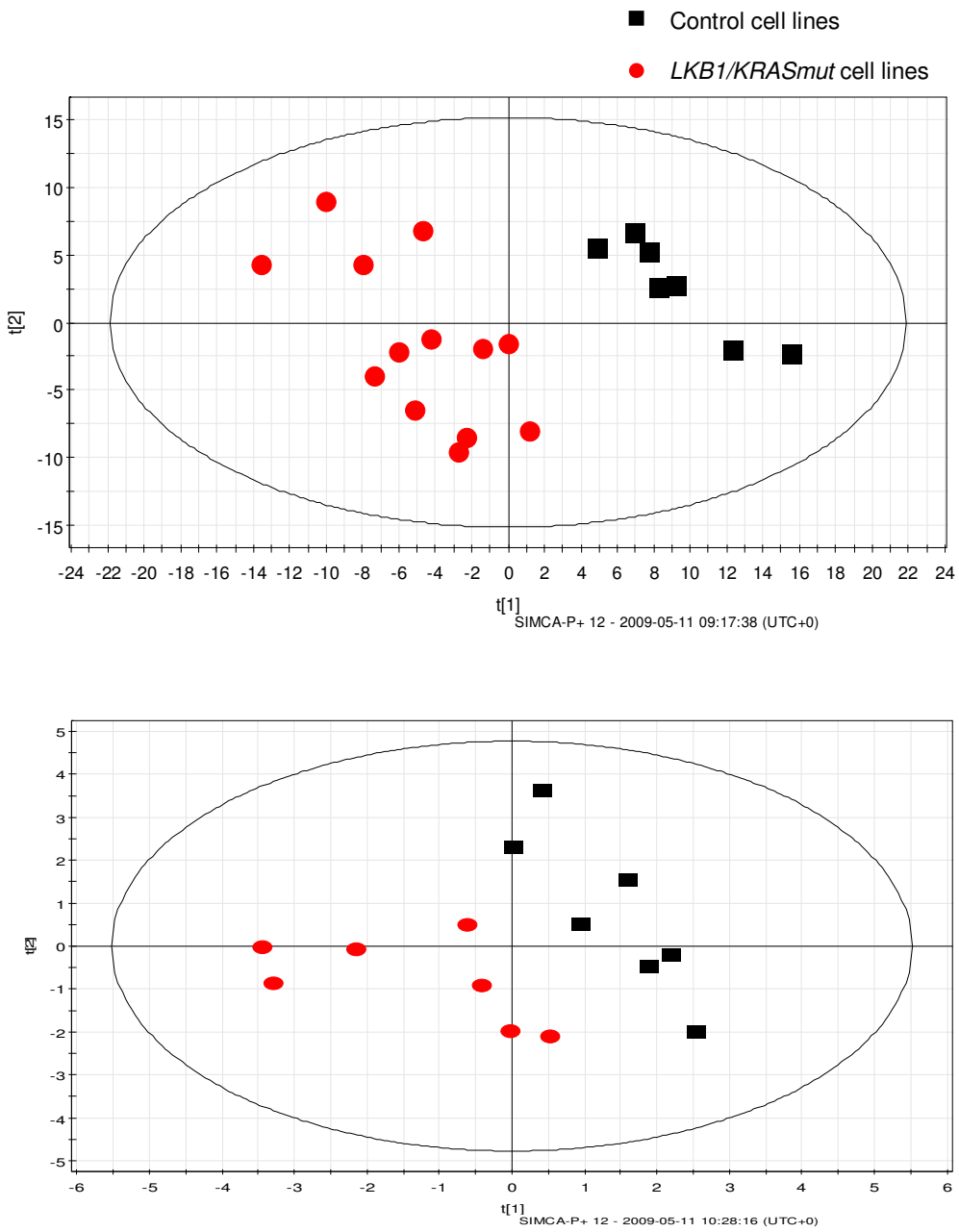

Figure 4-10. A) PLS-DA plot of 1H NMR spectra of control and *LKB1/KRAS* mutant cell lines. R^2=22%. Q^2= 51%. B) PLS-DA plot of 13C NMR spectra of control and *LKB1/KRAS* mutant cell lines. R^2=42%. Q^2= 22%. Both models passed validation by a jack knifing procedure (within the SIMCA software) used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

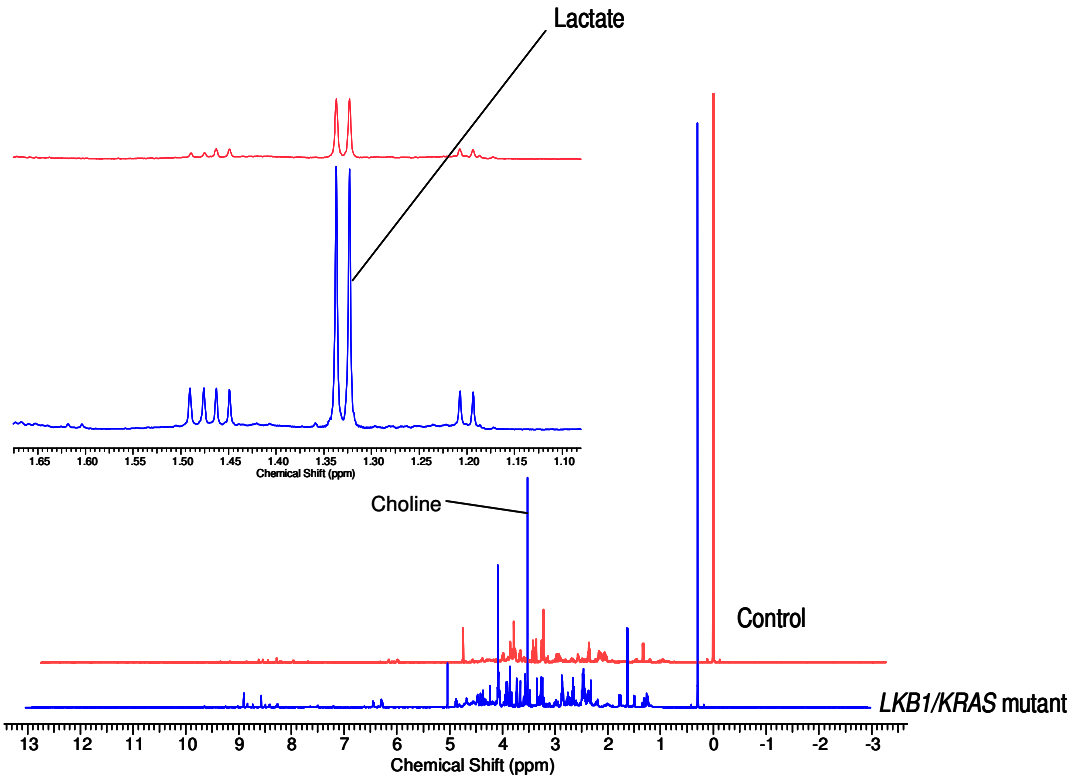

Figure 4-11 *LKB1/KRAS* mutant cell lines produce more lactate than control cell lines. Example 1H NMR spectra for control (red) cell lines and *LKB1/KRAS* mutant cell lines (blue). Highlighted are the peaks for lactate. Data normalised to trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TSP) (chemical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

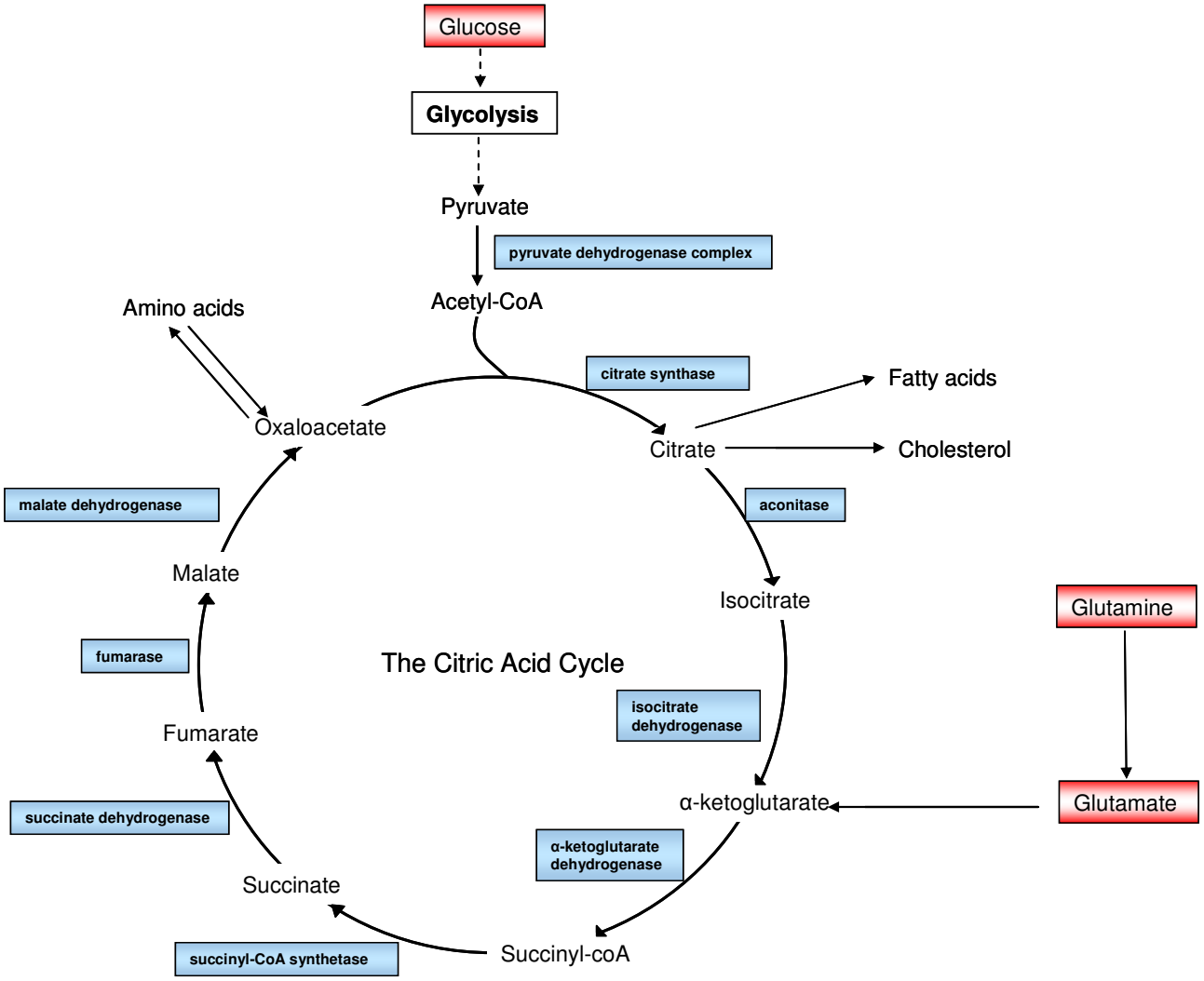
Glucose
Glycolysis
Pyruvate
pyruvate dehydrogenase complex
Acetyl-CoA
Amino acids
citrate synthase
Fatty acids
Oxaloacetate
Citrate
Cholesterol
malate dehydrogenase
aconitase
Malate
Isocitrate
Glutamine
fumarase
The Citric Acid Cycle
isocitrate dehydrogenase
Fumarate
α-ketoglutarate
Glutamate
succinate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