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.

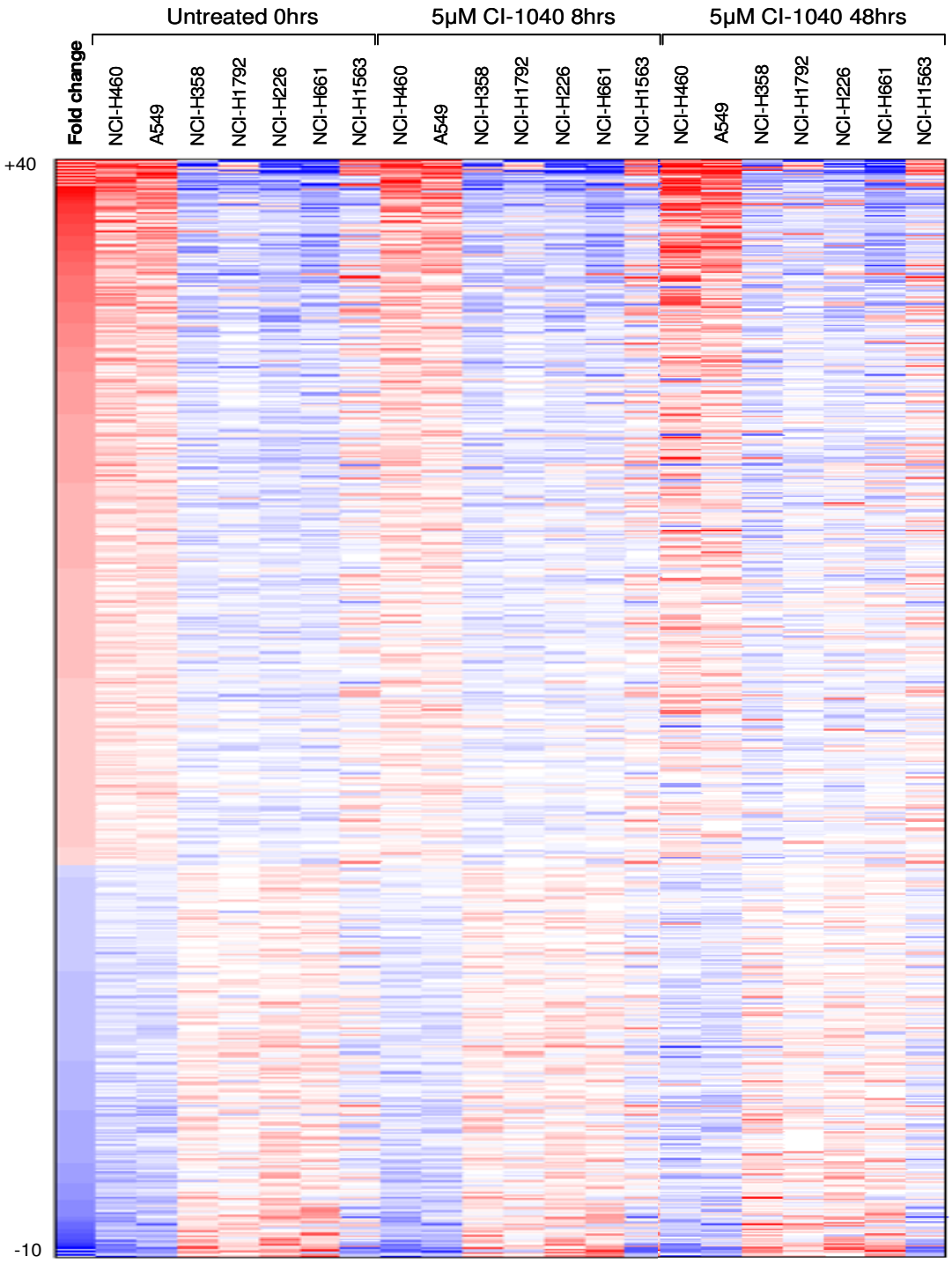


Figure 5-1. Heatmap of differentially expressed genes in *LKB1/KRAS* mutant cell lines before and after CI-1040 treatment. *LKB1/KRAS* mutant cell lines (NCI-H460 and A549) have a unique expression signature with 674 differentially expressed genes which do not show altered expression upon treatment with 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.

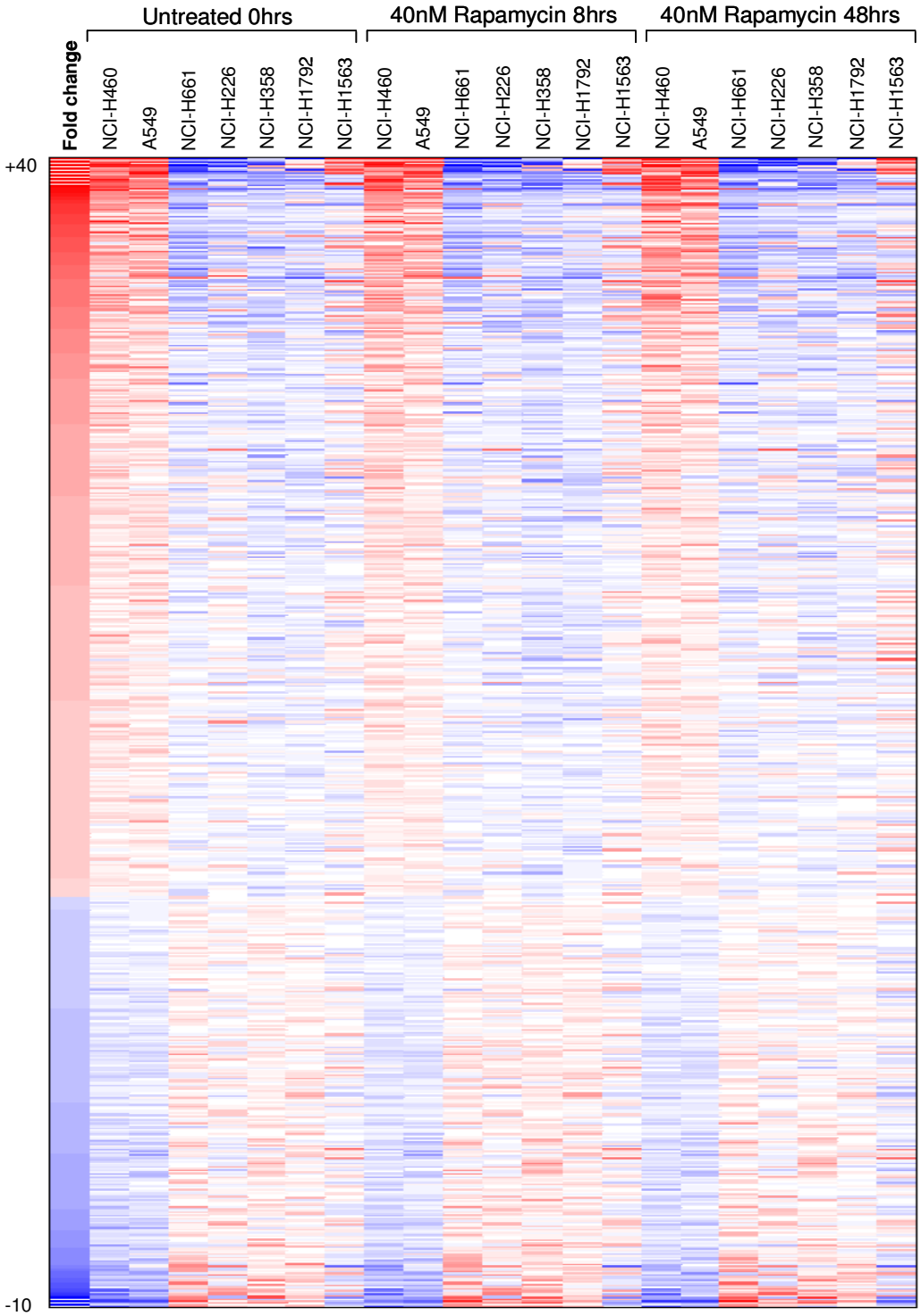


Figure 5-2. Heatmap of differentially expressed genes in *LKB1/KRAS* mutant cell lines before and after rapamycin treatment. *LKB1/KRAS* mutant cell lines (NCI-H460 and A549) have a unique expression signature with 674 differentially expressed genes which do not show altered expression upon treatment 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.

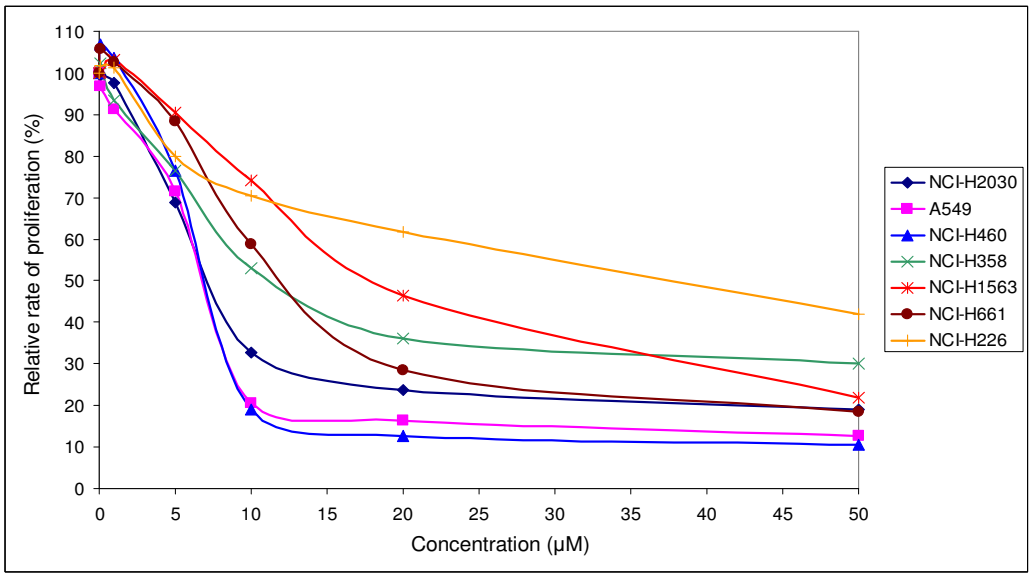
	Fold change in untreated (0hrs)	Fold change after treatment with 40nM rapamycin				
		8hrs		48hrs		
Gene Name	**A549 and H460 vs. controls**	**A549**	**H460**	**A549**	**H460**	**Full Gene name**
CTDSP2	-2.3			1.8	1.7	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase 2
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

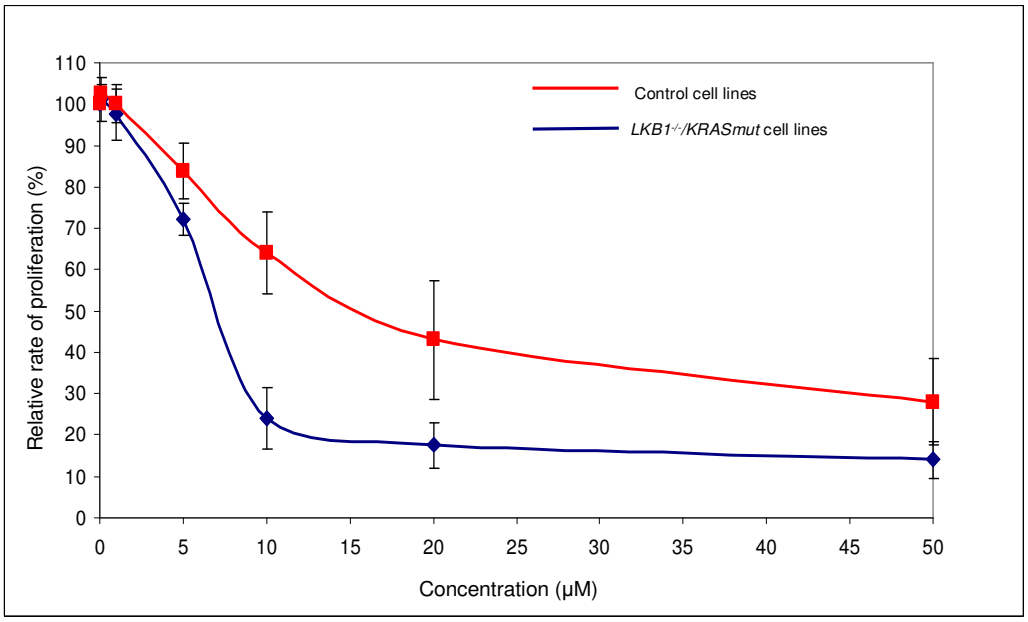


Figure 5-3 *LKB1/KRASmut* cell lines are more sensitive to the AMPK inhibitor compound C A) Shows the relative rate of proliferation 72hrs after Compound C treatment for all cell lines used in the study (n=12 from 2 independent experiments), cell lines tested: NCI-H460 (*LKB1/KRASmut*), A549 (*LKB1/KRASmut*), 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.

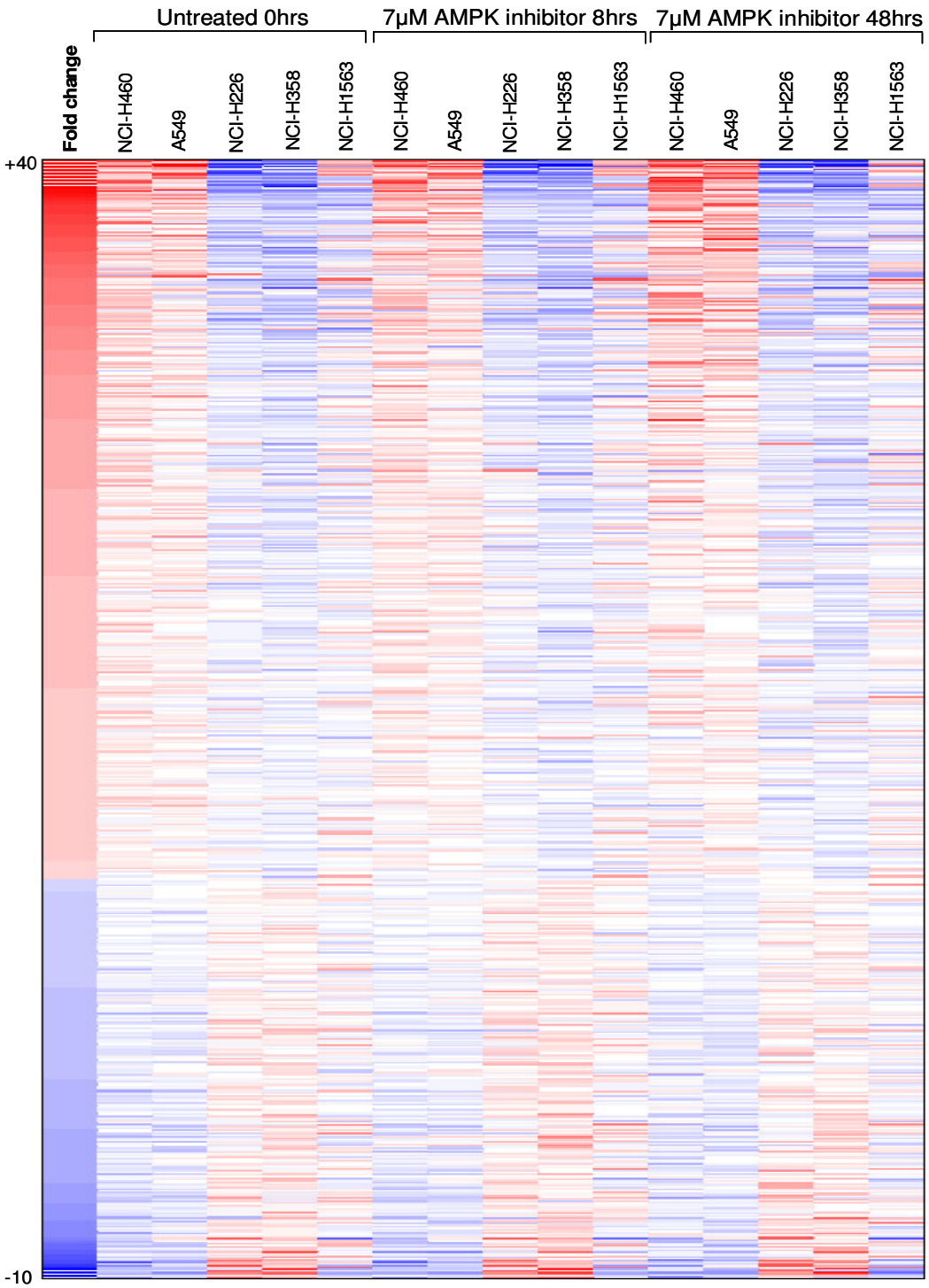


Figure 5-4. Heatmap of differentially expressed genes in *LKB1/KRAS* mutant cell lines before and after Compound C treatment. *LKB1/KRAS* mutant cell lines (NCI-H460 and A549) have a unique expression signature with 674 differentially expressed genes which do not show altered expression upon treatment 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.