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).

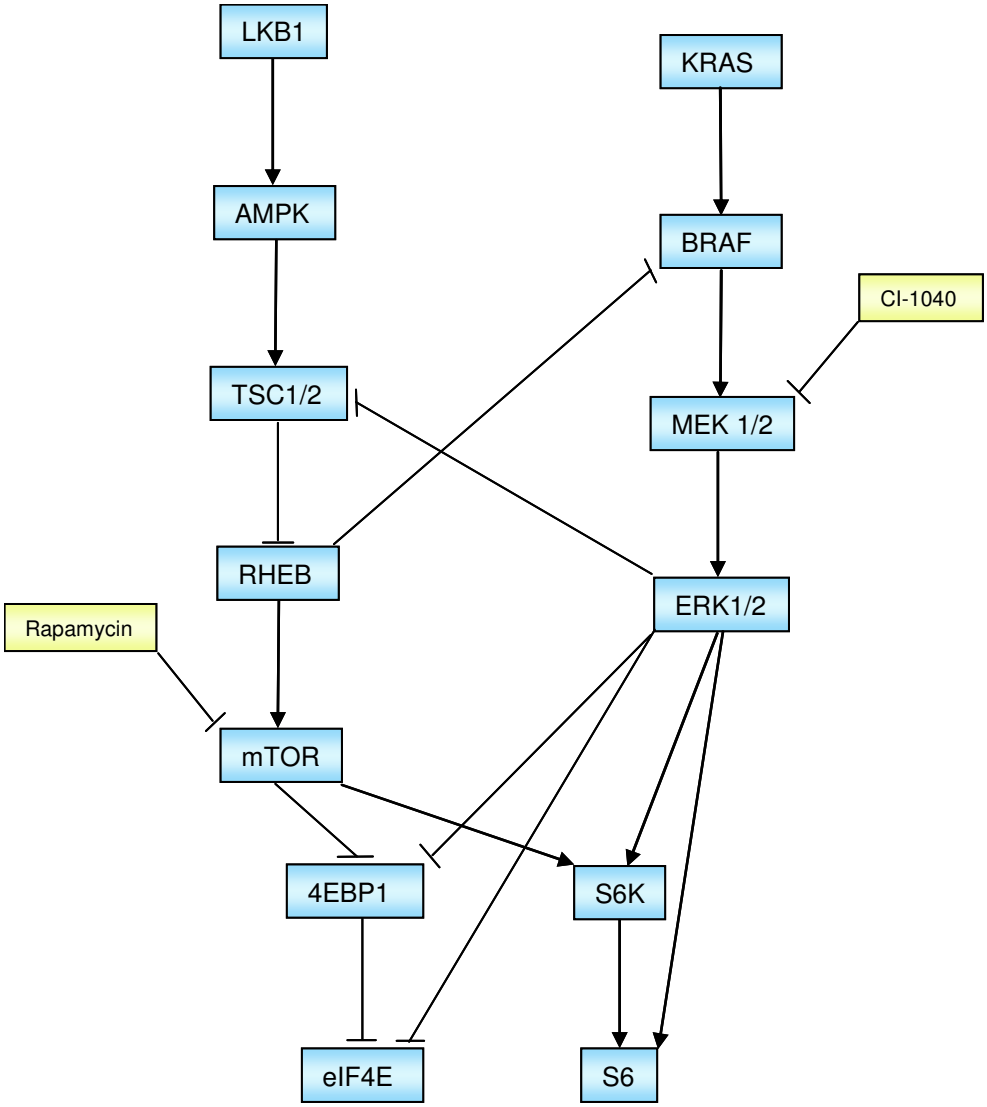

Figure 3-1 Simplified snapshot of crosstalk between LKB1 and RAS-MAPK signalling pathways by the analysis of literature (for references, see Chapter 1). Highlighted in the yellow boxes are possible targets for therapeutic intervention.

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).

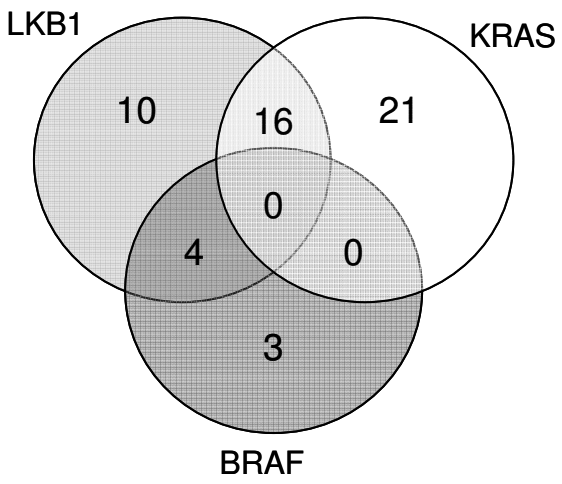

Figure 3-2. Venn diagram showing the overlap of *LKB1, KRAS* and *BRAF* mutations in 87 NSCLC cell lines.

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.

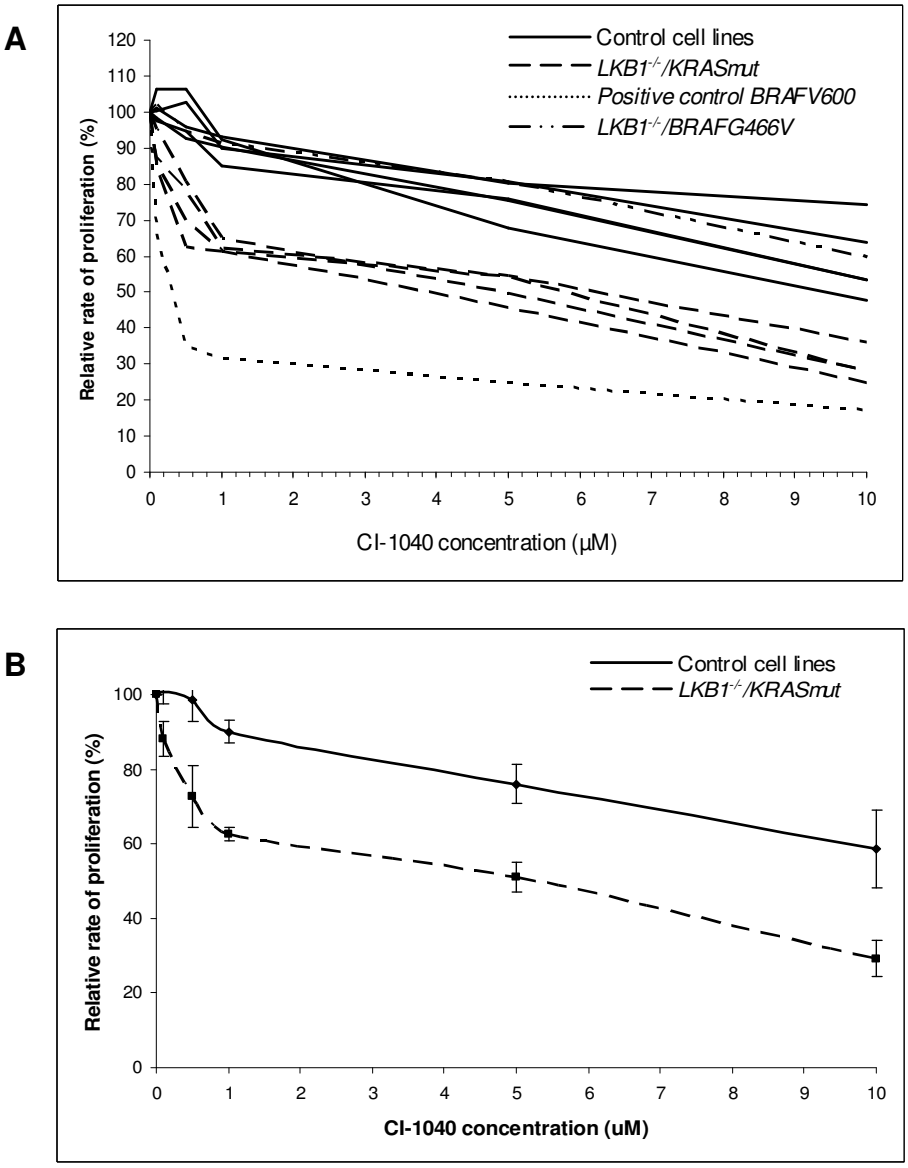

Figure 3-3 Cell lines with inactivated *LKB1* and activated *KRAS* are more sensitive to the MEK inhibitor CI-1040 A) Shows the relative rate of proliferation 72hrs after CI-1040 treatment for all cell lines used in the study (n=12 from 2 independent experiments), cell lines tested: NCI-H460 (*LKB1*$^{-/-}$*/KRASmut*), 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).

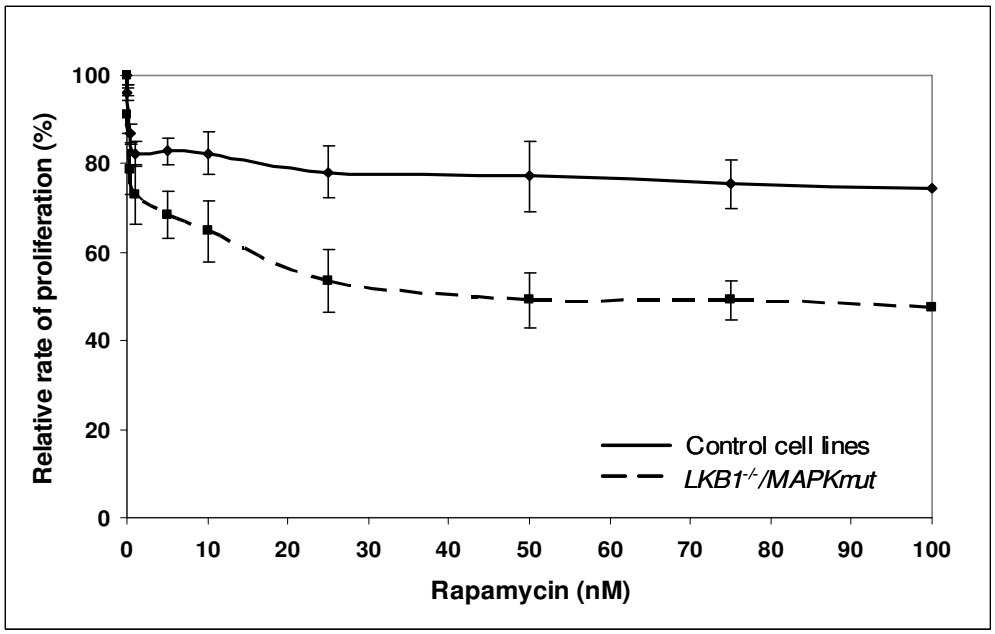

Figure 3-4. Cell lines with inactivated *LKB1* and activated *RAS-MAPK* are more sensitive to mTOR inhibition with rapamycin. Due to the similar nature of the results to the CI-1040 experiment the cell lines were clustered according to their mutation status (*LKB1$^{-/-}$/ras-MAPKmut*) (NCI-H460, A549,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

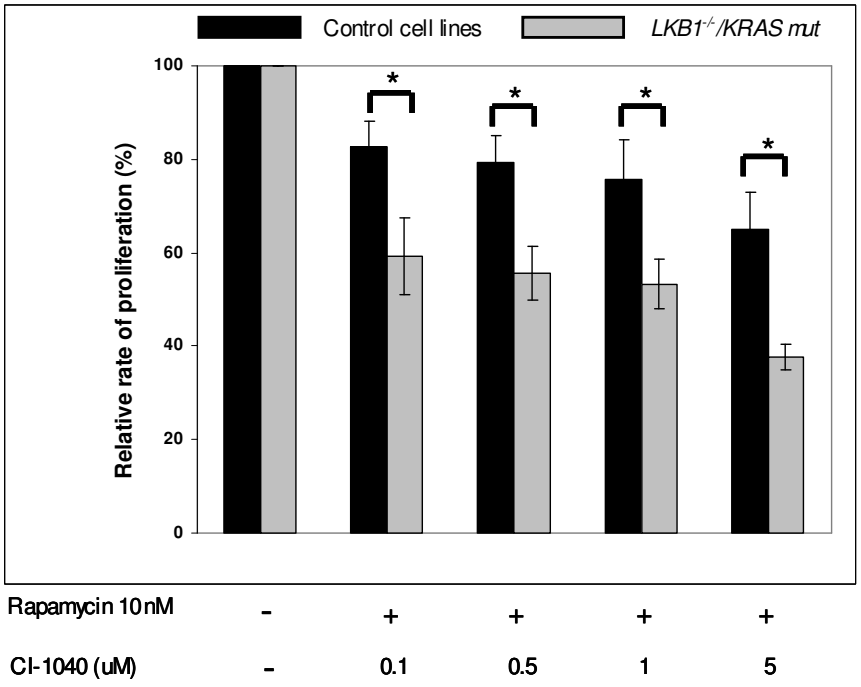

Figure 3-5 The *LKB1/KRAS mutant* NSCLC cell lines are more sensitive to dual inhibition than control cell lines. Dual inhibition experiments were carried out using 10nM rapamycin and a range of CI-1040 concentrations in the same format as single agent experiments; cell lines were again grouped by their 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.

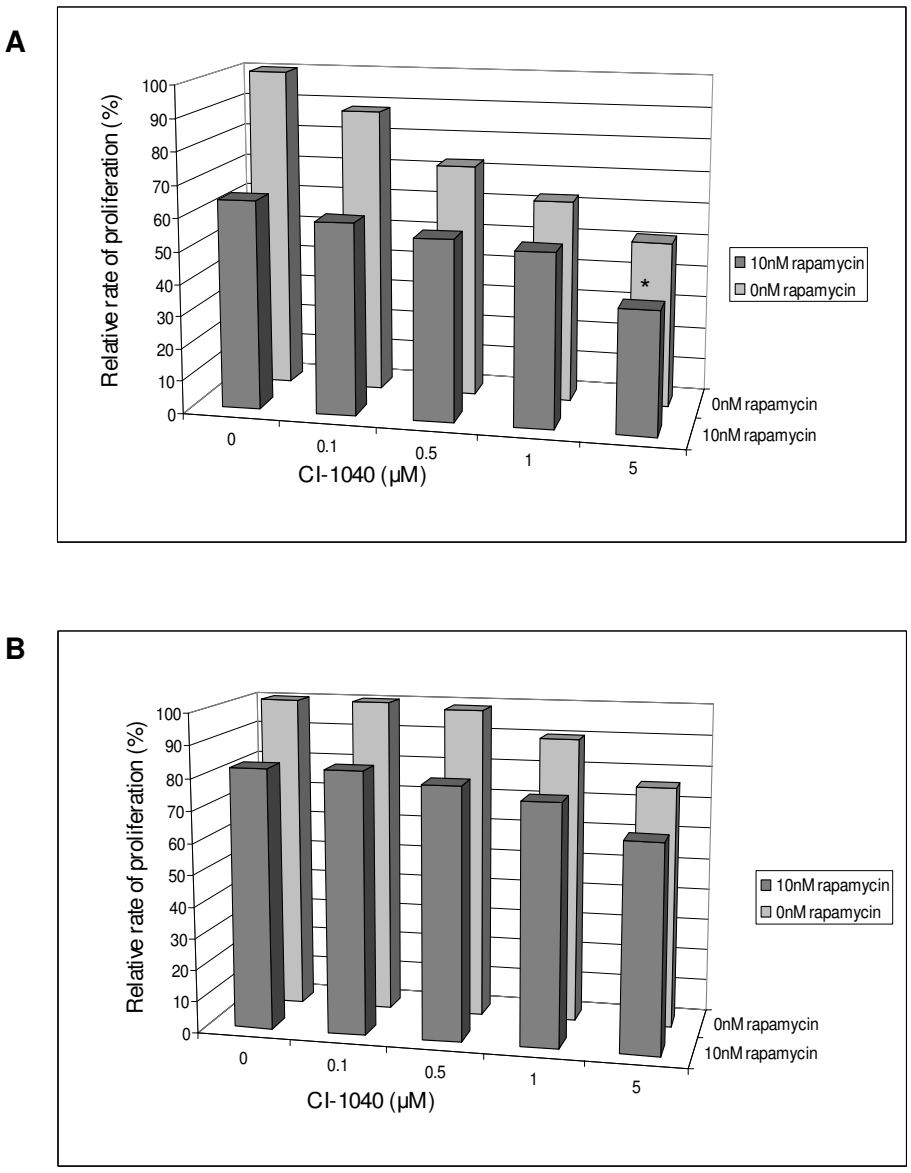

Figure 3-6 Dual inhibition with rapamycin and CI-1040 is neither additive nor synergistic. A) Comparison of dual agent treatment to most potent single agent treatment to determine if the agents are additive/synergistic in the *LKB1*$^{-/-}$ */KRASmut* group. Statistical significance determined using 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).

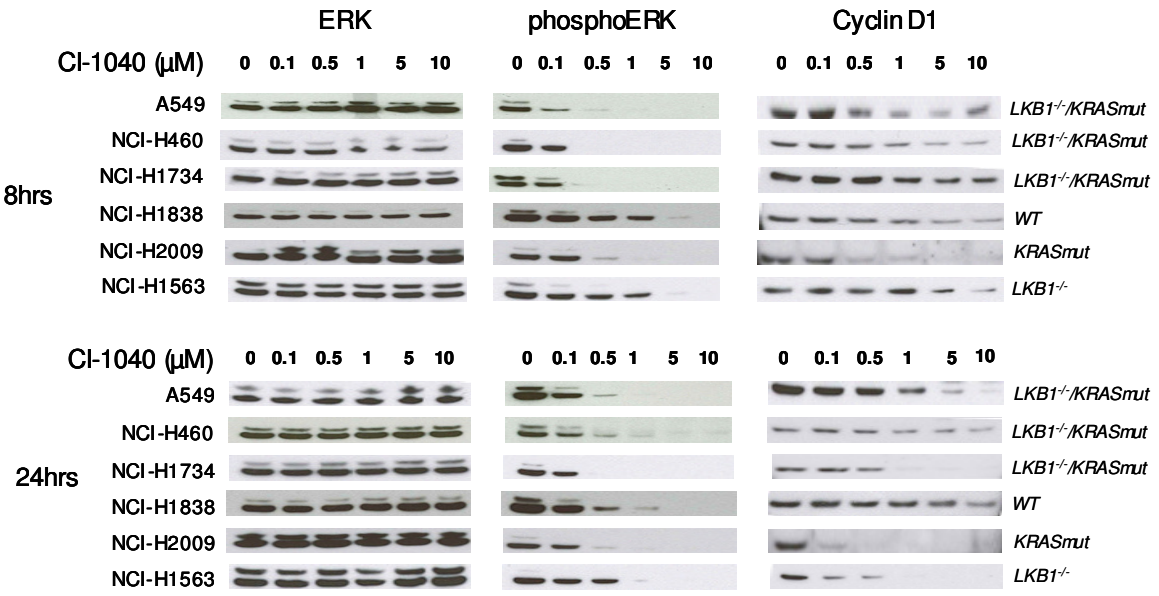

Figure 3-7 Sensitivity to the MEK inhibitor in *LKB1/KRAS* mutant cell lines is not due to downstream effects on cyclin D1. Cell lines were treated with CI-1040 and protein harvested 8 and 24hrs later. CI-1040 treatment leads to inhibition of phosphorylation of ERK as early as 8hrs and continues past 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.

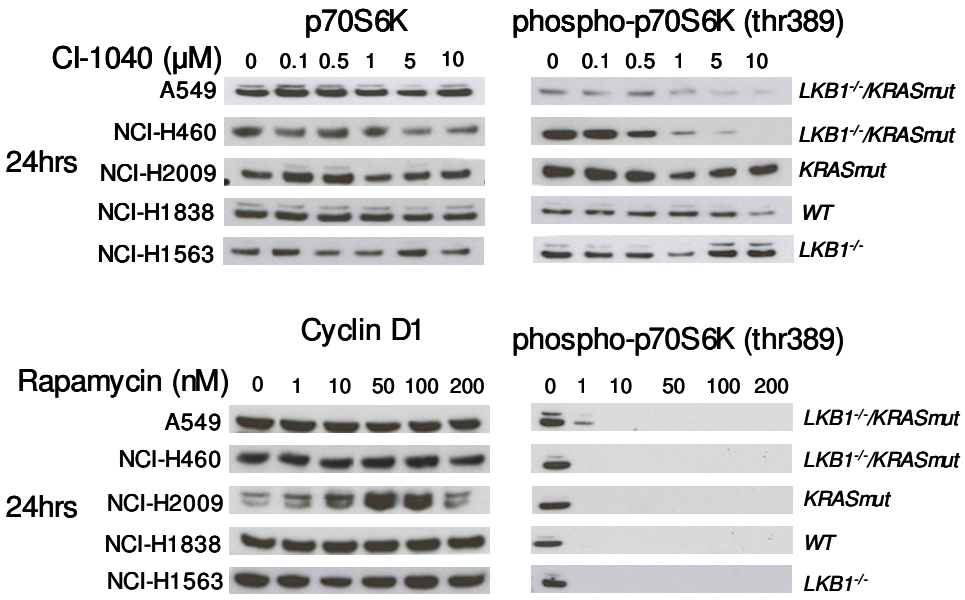

Figure 3-8 Immunoblot analysis of total p70S6K and phospho-p70S6K(thr-389) in CI-1040 treated cell lines and rapamycin treated cell lines. CI-1040 treatment has no effect on total protein levels of p70S6K, however in *LKB1/KRAS* mutant cell lines there is a decrease in phospho-p70S6K (thr-389), which 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.