Investigation into AMPK phosphatases and identification and characterisation of the interaction between AMPK and PAK1

Shuai (Huza) Zhang

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Cellular Stress Group
Medical Research Council, Clinical Sciences Centre
Imperial College London
Declaration

I declare that the work presented in this thesis is my own work and information derived from published or unpublished work of others has been acknowledged in the text and in the list of references. This work has not been submitted in any form for another degree or diploma at any university or other institute of tertiary education.

Signed: [Signature]  Date: 29th July 2013

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Abstract

AMP-activated protein kinase (AMPK) plays important roles in the regulation of energy homeostasis and has been implicated in a number of diseases including diabetes and cancer. Obtaining a full picture of AMPK signalling is important for the understanding of these disease states and the development of novel therapies.

AMPK is activated by phosphorylation of T-172 within the α catalytic subunit. While a number of AMPK upstream kinases have been identified, identification of the AMPK phosphatase has proved elusive. In the first part of this thesis, the identity of the AMPK phosphatase(s) was investigated. The data presented shows that treatment of mammalian cells with the protein phosphatase (PPP) family inhibitor Calyculin-A caused marked increases in AMPK activity. These results indicate that one or more of the AMPK phosphatases belong to the PPP family, with the concentration profile pointing towards PP1 involvement. However, using siRNA based techniques; it was not possible to pinpoint the specific phosphatase(s) that dephosphorylates AMPK. Our results, combined with recently published studies, suggest that regulation of AMPK dephosphorylation is a complex process with involvement of different protein phosphatases depending on cell-type and/or conditions of cell stress.

Next, quantitative mass spectrometry techniques were utilised in order to identify AMPK interacting proteins. From the list of candidate interactors, the interaction between AMPK and the PAK1 signalling complex was characterised. AMPK directly phosphorylated PAK1 on Ser-21 leading to its activation. Activation of AMPK in mammalian cells led to increased activity and phosphorylation of endogenous PAK1. PAK1 activity/phosphorylation was reduced in cortical neurons derived from AMPK α1 knockout mice compared to wild-type neurons. Furthermore, activation of AMPK with A769662 in melanoma cell-lines significantly increased cell invasion and this effect was abolished by the PAK1 inhibitor, IPA3. These results show that PAK1 is an AMPK substrate and suggest that at least part of the effects of AMPK on axonogenesis and tumour formation is mediated through PAK1 signalling.

In addition to the effects of AMPK on PAK1, PAK1 was shown to directly phosphorylate the AMPK α1 subunit at Thr-377 and Ser-403/405. This phosphorylation was able to further increase the activity of CaMKKβ phosphorylated AMPK. This finding adds a novel layer of control to the regulation of AMPK.
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I want to thank the support of my family throughout my PhD and in the writing up period. I want to thank my mum who has always been there for me and has provided much support, both emotional and practical in these years. I would also like to thank my dad, who although has not been in this country as much I would like has nevertheless provided me with much support. I would also like to thank my girlfriend Danni, who has always been there for me the past few years and who has moved so far from her parents to be in London. Thanks for all the help and advice and all the great cooking! I could not have done this without them.

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC</td>
<td>Acetyl-Coenzyme A carboxylase</td>
</tr>
<tr>
<td>AICAR</td>
<td>5-aminoimidazole-4-carboximide ribonucleoside</td>
</tr>
<tr>
<td>AMPK</td>
<td>5’adenosine monophosphate-activated protein kinase</td>
</tr>
<tr>
<td>AMARA</td>
<td>Synthetic peptide, AMARAASAAALARRR</td>
</tr>
<tr>
<td>CaM KKβ</td>
<td>Ca²⁺/Calmodulin-dependent protein kinase kinase beta</td>
</tr>
<tr>
<td>CAPS</td>
<td>3-[cyclohexylamino]-1-propanesulfonic acid</td>
</tr>
<tr>
<td>CBS</td>
<td>Cystathionine beta synthesize (domains)</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP-response element binding protein</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulphoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra-acetic (ethanoic) acid</td>
</tr>
<tr>
<td>et al.</td>
<td>et alia</td>
</tr>
<tr>
<td>FAS</td>
<td>Fatty acid synthase</td>
</tr>
<tr>
<td>FKHR</td>
<td>The forkhead transcription factor (also known as Foxo)</td>
</tr>
<tr>
<td>GBD</td>
<td>Glycogen binding domain</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GPAT</td>
<td>Glycerol-3-phosphate acyl-transferase</td>
</tr>
<tr>
<td>GS</td>
<td>Glycogen synthase</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>Hepes</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HMG-CoA reductase</td>
<td>3-hydroxy-3-methylglutaryl-CoA reductase</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSL</td>
<td>Hormone-sensitive lipase</td>
</tr>
<tr>
<td>HST</td>
<td>High Salt Tween</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>KD</td>
<td>Kinase dead</td>
</tr>
<tr>
<td>LB medium</td>
<td>Luria Bertani medium</td>
</tr>
<tr>
<td>LKB1</td>
<td>Liver kinase B1</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin basic protein</td>
</tr>
<tr>
<td>PAK1</td>
<td>P21-activated protein kinase 1</td>
</tr>
<tr>
<td>PAK1tide</td>
<td>Synthetic peptide, RRRLSFAEPG</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PEPCK</td>
<td>Phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>Akt/PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonyl fluoride</td>
</tr>
<tr>
<td>PP</td>
<td>Protein phosphatase (e.g. PP1, PP2A, PP5 and PP6)</td>
</tr>
<tr>
<td>PPM</td>
<td>Metal dependent phosphatases</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Reverse transcription polymerase chain reaction (real-time)</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate - Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>S.E.M</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SAMS</td>
<td>Synthetic peptide, HMRSAMSGLHLVKRR</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate buffer</td>
</tr>
<tr>
<td>TCEP</td>
<td>tris(2-carboxyethyl)phosphine</td>
</tr>
<tr>
<td>TORC2</td>
<td>Transducer of regulated CREB activity 2</td>
</tr>
<tr>
<td>TRIS</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>WPW</td>
<td>Wolff-Parkinson-White syndrome</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>ZMP</td>
<td>5-amino-4-imidazolecarboxamide ribonucleoside monophosphate</td>
</tr>
</tbody>
</table>
# Table of Contents

Declaration ........................................................................................................................................... 2  
Abstract ............................................................................................................................................... 3  
Acknowledgements ............................................................................................................................... 4  
Abbreviations ....................................................................................................................................... 5

Table of Contents ................................................................................................................................. 6

1 Introduction ......................................................................................................................................... 14
  1.1 AMP-activated protein kinase ..................................................................................................... 14
    1.1.1 Structure of AMPK ............................................................................................................. 15
    1.1.2 Function and targets of AMPK .......................................................................................... 17
    1.1.3 Regulation of AMPK activity .............................................................................................. 24
    1.1.4 Regulation of AMPK by whole body signals ....................................................................... 35
  1.2 PAK1 ............................................................................................................................................. 38
    1.2.1 Structure and regulation of PAK1 ....................................................................................... 38
    1.2.2 Upstream signals for PAK1 activation ............................................................................... 40
    1.2.3 Function and targets of PAK1 ............................................................................................ 41
    1.2.4 PAK1 function in physiology and pathology ....................................................................... 44
    1.2.5 PAK1 activation and expression in cancer .......................................................................... 46
    1.2.6 PAK1 and AMPK ............................................................................................................... 49
  1.3 Summary and aims ......................................................................................................................... 52

2 Materials and Methods ..................................................................................................................... 53
  2.1 Materials ....................................................................................................................................... 53
    2.1.1 General Reagents ............................................................................................................... 53
    2.1.2 Buffers ............................................................................................................................... 54
    2.1.3 Antibodies .......................................................................................................................... 55
    2.1.4 Cells ..................................................................................................................................... 56
    2.1.5 Proteins ................................................................................................................................ 56
    2.1.6 Compounds ........................................................................................................................ 56
    2.1.7 Plasmids ............................................................................................................................. 56
    2.1.8 Mice ..................................................................................................................................... 57
  2.2 Methods ......................................................................................................................................... 57
2.2.1 Agarose gel electrophoresis .............................................. 57
2.2.2 Polymerase chain reaction (PCR) and DNA Cloning ................. 57
2.2.3 Site Directed Mutagenesis .................................................. 57
2.2.4 Bacterial transformation and culture growth .................................. 58
2.2.5 Determining DNA/RNA concentration and DNA sequencing .......... 59
2.2.6 Expression and purification of recombinant proteins in E.coli ............ 59
2.2.7 Radio-labelling of proteins and auto-radiography ........................ 61
2.2.8 Activation of AMPK by CaMKKβ ........................................ 61
2.2.9 PAK1 activation of AMPK .................................................. 61
2.2.10 Kinase activity assays ...................................................... 61
2.2.11 Mammalian Cell culture .................................................... 62
2.2.12 Mammalian cell treatment .................................................. 64
2.2.13 Mammalian cell transfection ............................................... 64
2.2.14 Small interfering RNA (siRNA) silencing .................................. 65
2.2.15 Generation of Mammalian PPM expression constructs .................... 65
2.2.16 Cell lysis .......................................................................... 65
2.2.17 Protein concentration determination for mammalian cell lysates ......... 66
2.2.18 Determination of ratio of nucleotides within cells ......................... 66
2.2.19 Western blotting .................................................................. 66
2.2.20 AMPK Immunoprecipitation from cell lysates .............................. 67
2.2.21 RNA extraction .................................................................. 67
2.2.22 qRT-PCR .......................................................................... 67
2.2.23 Immunoprecipitation of phosphatases and in-vitro dephosphorylation assay ................................................................................ 68
2.2.24 In-vivo Crosslinking and analysis ........................................... 68
2.2.25 Stable isotope labelling by amino acids in cell culture and quantitative mass spectrometry .......................................................... 69
2.2.26 Collagen based invasion assay ............................................... 69
2.2.27 Transwell invasion assay ...................................................... 70
2.2.28 Statistical analyses .............................................................. 70

3 Identification of AMPK phosphatase(s) .......................................... 71
3.1 Introduction: ........................................................................... 71
3.2 Results: .................................................................................. 71
3.2.1 Pharmacological activation of AMPK in HEK293 cells .................... 71
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2.2</td>
<td>Okadaic acid treatment of HEK293 cells</td>
<td>75</td>
</tr>
<tr>
<td>3.2.3</td>
<td>Endogenous expression of PPM family phosphatases in HEK293 cells</td>
<td>80</td>
</tr>
<tr>
<td>3.2.4</td>
<td>Optimisation of siRNA mediated protein knockdown in HEK293 cells</td>
<td>81</td>
</tr>
<tr>
<td>3.2.5</td>
<td>Knockdown of PPM isoforms in HEK293 cells</td>
<td>82</td>
</tr>
<tr>
<td>3.2.6</td>
<td>Effect of PPM knockdown on AMPK activity</td>
<td>83</td>
</tr>
<tr>
<td>3.2.7</td>
<td>Over-expression of PPM isoforms in HEK293 cells</td>
<td>96</td>
</tr>
<tr>
<td>3.2.8</td>
<td>Okadaic acid titration in cytosolic suspensions of HEK293 cells</td>
<td>100</td>
</tr>
<tr>
<td>3.2.9</td>
<td>Calyculin-A treatment of HEK293 cells and mouse hepatocytes</td>
<td>102</td>
</tr>
<tr>
<td>3.2.10</td>
<td>Knockdown of PPP isoforms in HEK293 cells and mouse hepatocytes</td>
<td>111</td>
</tr>
<tr>
<td>3.3</td>
<td>Discussion</td>
<td>116</td>
</tr>
<tr>
<td>4</td>
<td>Identification of AMPK interaction proteins</td>
<td>129</td>
</tr>
<tr>
<td>4.1</td>
<td>Introduction</td>
<td>129</td>
</tr>
<tr>
<td>4.2</td>
<td>Results</td>
<td>129</td>
</tr>
<tr>
<td>4.2.1</td>
<td>Over-expression of AMPK subunits in HEK293 and COS7 cells</td>
<td>129</td>
</tr>
<tr>
<td>4.2.2</td>
<td>Chemical Cross-linking of proteins in HEK293 and COS7 cells</td>
<td>130</td>
</tr>
<tr>
<td>4.2.3</td>
<td>Immunoprecipitation of AMPK and mass spectrometry analysis of cross-linked proteins</td>
<td>131</td>
</tr>
<tr>
<td>4.2.4</td>
<td>Stable isotope labelling by amino acids in cell culture and quantitative proteomics in HEK293 cells</td>
<td>137</td>
</tr>
<tr>
<td>4.3</td>
<td>Discussion</td>
<td>143</td>
</tr>
<tr>
<td>5</td>
<td>Investigation of AMPK regulation of PAK1</td>
<td>147</td>
</tr>
<tr>
<td>5.1</td>
<td>Introduction</td>
<td>147</td>
</tr>
<tr>
<td>5.2</td>
<td>The PAK1/GIT1/βPIX complex interacts with AMPK</td>
<td>147</td>
</tr>
<tr>
<td>5.3</td>
<td>Investigation of the effect of AMPK on PAK1</td>
<td>149</td>
</tr>
<tr>
<td>5.3.1</td>
<td>Phosphorylation of PAK1 by AMPK</td>
<td>149</td>
</tr>
<tr>
<td>5.3.2</td>
<td>Identification of AMPK phosphorylation sites on PAK1</td>
<td>151</td>
</tr>
<tr>
<td>5.3.3</td>
<td>Production of mutant PAK1 protein</td>
<td>155</td>
</tr>
<tr>
<td>5.3.4</td>
<td>AMPK phosphorylation of mutant PAK1 proteins</td>
<td>155</td>
</tr>
<tr>
<td>5.3.5</td>
<td>AMPK activates PAK1 in vitro</td>
<td>157</td>
</tr>
<tr>
<td>5.3.6</td>
<td>Activation of AMPK in HEK293 cells leads to increased PAK1 activity</td>
<td>163</td>
</tr>
<tr>
<td>5.3.7</td>
<td>AMPK regulation of PAK1 activity in cortical neurons</td>
<td>163</td>
</tr>
<tr>
<td>5.3.8</td>
<td>PAK1 phosphorylation and activity are reduced in AMPK α1 knockout</td>
<td>164</td>
</tr>
<tr>
<td>5.3.9</td>
<td>Physiological implications of AMPK regulation of PAK1</td>
<td>173</td>
</tr>
</tbody>
</table>
5.3.10 AMPK enhances cancer cell invasion through PAK1 signalling........176
5.3 Discussion ..................................................................................184

6 Investigating PAK1 regulation of AMPK ......................................194
6.1 Introduction ................................................................................194
6.2 Results ......................................................................................194
   6.2.1 PAK1 phosphorylates AMPK in vitro ..................................194
   6.2.2 The effect of PAK1 on activity of AMPK ............................198
   6.2.3 The effect of PAK1 on activity of pre-phosphorylated AMPK ..198
   6.2.4 PAK1 phosphorylation sites on AMPK α ............................203
   6.2.5 Mutation of PAK1 phosphorylation sites in AMPK ..............205
   6.2.6 Phosphorylation of AMPK mutants by PAK1 .......................205
   6.2.7 The effect of PAK1 phosphorylation on the activity of mutant AMPK...206
   6.2.8 Expression of the AMPK AAA triple mutant and its effects on PAK1
       induced phosphorylation .........................................................206
   6.3 Discussion: ............................................................................212

7 Conclusions and Future perspectives ...........................................219
References .......................................................................................224
Appendices .....................................................................................Error! Bookmark not defined.
List of Figures

Figure 1.1 Cellular effects of AMPK.................................................................23
Figure 1.2 Regulation of AMPK by phosphorylation...........................................34
Figure 1.3 Regulation of AMPK activity..............................................................37
Figure 1.4 Primary structure of PAK1.................................................................40
Figure 1.5 Activation of PAK1 and downstream targets ......................................43
Figure 3.1 Activity of AMPK α1 and α2 isoforms in HEK293 cells.......................73
Figure 3.2 Activity of upstream kinases of AMPK in HEK293 cells.......................73
Figure 3.3 Activation of AMPK in HEK293 cells...............................................74
Figure 3.4 Inhibition of the ACC phosphatase using okadaic acid .......................77
Figure 3.5 Treatment with okadaic acid does not alter the ATP:ADP ratios in HEK293 cells.................................................................78
Figure 3.6 Okadaic acid has no significant effect on the activity of AMPK α1 and α2 isoforms in HEK293 cells.................................................................79
Figure 3.7 Expression of PPM phosphatase isoforms in HEK293 cells.................85
Figure 3.8 Validation of siRNA (SiGLO) transfection in HEK293 cells.................86
Figure 3.9 siControl has no effect on AMPK activity or AMPK activation............87
Figure 3.10 Optimisation of siRNA concentration for PPM knockdown.................88
Figure 3.11 Optimisation of time course for PPM knockdown..............................89
Figure 3.12 Knockdown of PPM isoforms in HEK293 cells.................................90
Figure 3.13 Validation of protein knockdown of PPM1B, PPM1H and ILKAP ......91
Figure 3.14 Knockdown of individual PPM phosphatases does not affect basal AMPK activity.................................................................92
Figure 3.15 Multiplexed knockdown of PPM1F, PPM1H and PPM1L in HEK293 cells.................................................................93
Figure 3.16 Knockdown of individual PPM phosphatases does not affect activity of AMPK α1 or α2 isoforms.................................................................94
Figure 3.17 Knockdown of individual PPM phosphatases does not affect the level of AMPK activation by phenformin.........................................................95
Figure 3.18 Overexpression of PPM isoform constructs in HEK293 cells...............97
Figure 3.19 Validation of the activity of overexpressed PPM phosphatase s.........98
Figure 3.20 PPM overexpression does not alter basal AMPK activity or activation by phenformin.................................................................99
Figure 3.21 Okadaic acid supplementation protects AMPK from dephosphorylation in cytosolic suspensions.................................................................101
Figure 3.22 Inhibition of the ACC phosphatase using calyculin A in HEK293 cells 104
Figure 3.23 Calyculin A treatment activates AMPK α1 and α2 isoforms in HEK293 cells.................................................................105
Figure 3.24 Treatment with calyculin A does not alter the ATP:ADP ratios in HEK293 cells.................................................................106
Figure 3.25 Protection of dephosphorylation of AMPK in HEK293 cells after H2O2 treatment .................................................................107
Figure 3.26 Inhibition of the ACC phosphatases using calyculin A in mouse primary hepatocytes .................................................................108
Figure 3.27 Calyculin A treatment activates both AMPK α1 and α2 isoforms in mouse primary hepatocytes.................................................................109
Figure 3.28 Treatment with calyculin A does not alter the ATP:ADP ratios in mouse primary hepatocytes .................................................................110
Figure 3.29 Knockdown of PPP isoforms in HEK293 cells....................................113
Figure 3.30 Validation of protein knockdown of PP1 and PP2A
Figure 3.31 Knockdown of PPP isoforms does not affect the level of AMPK activation by phenformin
Figure 4.1 Over-expression of AMPK αβ1γ1 in HEK293 and COS7 cells
Figure 4.2 Over-expression of AMPK α1β1γ1 leads to increased AMPK activity in HEK293 and COS7 cells
Figure 4.3 In-vivo cross-linking of proteins in COS7 and HEK293 cells
Figure 4.4 Immunoprecipitation of cross-linked AMPK using pan-β subunit antibodies
Figure 4.5 Reversal of paraformaldehyde cross-linking
Figure 5.1 Detection of endogenous PAK1, GIT1, βPIX in HEK293 cells
Figure 5.2 Validation of AMPK interaction with PAK1, GIT1 and βPIX
Figure 5.3 AMPK phosphorylates PAK1 in vitro
Figure 5.4 Purification of recombinant PAK1
Figure 5.5 AMPK phosphorylation of Kinase dead PAK1
Figure 5.6 Phosphopeptide mapping of PAK1 phosphorylated by AMPK
Figure 5.7 Production of recombinant mutant form of PAK1 protein
Figure 5.8 AMPK phosphorylation of mutant PAK1
Figure 5.9 Incubation of AMPK with PAK1 results in phosphorylation of PAK1 activations sites
Figure 5.10 Validation of PAK1 kinase assay
Figure 5.11 AMPK does not phosphorylate PAKtide efficiently
Figure 5.12 AMPK activates PAK1 in vitro
Figure 5.13 AMPK activation leads to elevated PAK1 activity in HEK293 cells
Figure 5.14 Co-immunoprecipitation of AMPK and PAK1 in mouse primary cortical neurons
Figure 5.15 Stimulation of AMPK leads to PAK1 activation in mouse cortical neurons
Figure 5.16 Pre-treatment with the AMPK inhibitor Compound C prevents M991 induced PAK1 activation
Figure 5.17 Activity of AMPK α1 and α2 complexes in WT and AMPK α1 knockout cortical neurons
Figure 5.18 Overall AMPK activity/phosphorylation in AMPK WT and α1 knockout cortical neurons
Figure 5.19 PAK1 phosphorylation/activity is reduced in AMPK α1 knockout neurons
Figure 5.20 M991 treatment enhances PAK1 phosphorylation/activity in AMPK α1 knockout cortical neurons
Figure 5.21 Effect of pharmacological treatment on dendritic branching of primary cortical neurons
Figure 5.22 Activity of AMPK in melanoma cell-lines
Figure 5.23 Activation of AMPK in melanoma cells
Figure 5.24 AMPK activation stimulates invasion of melanoma cell-lines
Figure 5.25 Treatment of WM1361 cells with A769662 and IPA3
Figure 5.26 (A) A769662 increases invasion of WM1361 melaonma cells via PAK1 signalling
Figure 5.27 (B) A769662 induced AMPK activation increases invasion of WM1361 melaonma cells via PAK1
Figure 6.1 Validation of PAK1 activity
Figure 6.2 PAK1 phosphorylates kinase dead AMPK α1 and α2 in vitro
Figure 6.3 Active PAK1 does not phosphorylate the SAMS peptide
Figure 6.4 PAK1 does not activate non-activated AMPK \textit{in vitro}
Figure 6.5 CaMKK\(\beta\) activation of AMPK \(\alpha_3\beta_1\gamma_1\) or \(\alpha_2\beta_1\gamma_1\) complexes
Figure 6.6 Titration of AMPK in the SAMS peptide assay
Figure 6.7 PAK1 causes further activation of CaMKK\(\beta\) phosphorylated AMPK
Figure 6.8 Phospho-peptide mass spectrometry analysis of PAK1 phosphorylated AMPK
Figure 6.9 Expression of mutant AMPK complexes
Figure 6.10 PAK1 phosphorylation of KD AMPK mutants
Figure 6.11 CaMKK\(\beta\) activation of AMPK complexes
Figure 6.12 PAK1 activation of pre-phosphorylated WT and mutant AMPK
Figure 6.13 PAK1 phosphorylation of KD AMPK mutants
List of tables

Table 1.1 Direct targets of AMPK .......................................................... 24
Table 1.2 Cancers with alterations in PAK1 activity/expression ................. 49
Table 4.1 Antibodies and controls used in SILAC MS .............................. 138
Table 4.2 Potential AMPK interactors identified using SILAC/MS ............ 142

List of Appendices

Appendix 1 .................................................................................................. 250
Appendix 2 .................................................................................................. 251
Appendix 3 .................................................................................................. 252
1 Introduction

Cells require energy to carry out almost every cellular process, including establishing and maintaining ionic concentrations of sub-cellular compartments, transporting molecules across cell membranes and the synthesis of macromolecules (e.g. proteins, glycogen, DNA etc.) necessary for cellular and whole body function. The main unit of energy in the cell is ATP, and the energy generation process involves the hydrolysis of ATP to ADP and if needed to AMP.

In order to maintain cellular nucleotide concentrations at optimum levels, processes which produce ATP, termed catabolic processes (such as glycolysis and fatty acid oxidation) need to be finely balanced with anabolic processes which utilise ATP, termed anabolic processes (such as glycogen and protein synthesis). Cellular mechanisms are required to detect any changes in the ratio of ATP:AMP+ADP and effect changes which will restore balance. For example when the AMP and ADP levels rise in the cell relative to ATP, indicating the cell is entering an energy deprived state, the cell would act to switch on catabolic processes while switching off anabolic processes until the optimum ratio has been restored. Almost all diseases involve some measure of dysregulation of cellular energy status. For example, diseases ranging from diabetes to cancerous solid tumours all involve alterations in the normal regulation of glucose and fatty acid metabolisms. In order to find new methods of treatment of these conditions, it is very important to gain a good understanding of cellular systems which regulate energy balance.

1.1 AMP-activated protein kinase

AMP-activated protein kinase (AMPK) is a multi-subunit protein kinase. It functions as a cellular energy gauge. AMPK detects changes in the energy state of a cell and acts to alter a variety of parameters in order to maintain energy homeostasis (Carling et al., 2008; Hardie, 2008; Steinberg and Kemp, 2009). AMPK is highly conserved in eukaryotes and is activated by direct phosphorylation by the upstream kinases liver kinase B1 (LKB1), Ca²⁺/calmodulin-dependent protein kinase kinase β (CaMKKβ)
and transforming growth factor-beta-activating kinase 1 (TAK1) (Carling et al., 2008; Momcilovic et al., 2006). However, the physiological role for TAK1 in activating AMPK has not been clearly identified. Although acting directly at a cellular level, AMPK is indirectly activated in response physiological and pathological stimuli at the whole body level, such as hormone stimulation, hypoxia, energy deprivation (e.g. starvation), ischemia and exercise, all of which causes increases in cellular AMP:ATP ratios (Kahn et al., 2005; Steinberg and Kemp, 2009). Once activated, AMPK acts to phosphorylate a number of downstream targets, resulting in a shift away from ATP-consuming processes, (e.g. fatty acid synthesis, glycogen synthesis and protein synthesis) and acts to ‘switch on’ ATP-producing processes, e.g. fatty acid oxidation and glycolysis, glucose uptake, and increased food intake (Fujii et al., 2004; Hutber et al., 1997; Winder, 2000).

AMPK has been reported to play important roles in many disease states such as metabolic syndrome, type II diabetes, various cardiomyopathies etc. (Steinberg and Kemp, 2009). More recently, AMPK has also been implicated to have a role in the proliferation, migration and invasion of cancer cells. AMPK has been recognised as an important therapeutic target in the treatment of these conditions (Fogarty and Hardie; Luo et al., 2010). A number of hormones and cytokines which have effects on whole body metabolism also exert an influence on AMPK activities, in addition many currently used pharmacological agents such as metformin, phenformin and resveratrol have been reported to derive at least part of their therapeutic value from modulation of AMPK activity (Ewart and Kennedy, 2012; Hardie et al., 2012; Zhang et al., 2009).

1.1.1 Structure of AMPK

AMPK is a heterotrimeric enzyme consisting of three subunits, namely the α, β and γ (Carling, 2004). The α subunit of AMPK is the catalytic subunit and β and γ play regulatory/scaffolding roles. These subunits are always found in complex, and AMPK requires all three subunits in order to function as an active kinase both in vivo and in vitro. Several isoforms of each subunit have been discovered in nature (namely α1, α2, β1, β2, γ1, γ2, and γ3) and different combinations of these subunits can form AMPK complexes, allowing 12 combinations in total (Cheung et al., 2000; Kahn et al., 2005). The different AMPK isoforms may play different roles in the cell or be localised in
different sub-cellular compartments, for example the α₂ subunit has been detected in nucleus as well as in the cytoplasm (Salt et al., 1998).

The α subunit contains the kinase domain of AMPK. It has two isoforms (α₁ and α₂) both with a molecular mass of around 63 kDa. Differential expression patterns have been reported for the two isoforms with α₁ being the predominant form expressed in the brain, lungs, kidneys and pancreas, and the α₂ subunit being highly expressed in the heart and skeletal muscle (Steinberg and Kemp, 2009).

The β subunit also has two isoforms (β₁ and β₂) both of which are around 30 kDa in mass (although it migrates at 38 kDa on SDS-PAGE gels). The C-terminal domain of the β subunits binds both the α and γ subunits whereas the N-terminal region binding glycogen and is named the glycogen binding domain (GBD) which suggests that AMPK may be directly involved in glycogen metabolism (Hudson et al., 2003; Polekhina et al., 2003).

There are three isoforms of the γ subunit (γ₁, γ₂ and γ₃), γ₁ and γ₂ are the predominant isoforms in vivo, with ubiquitous expression in all bodily tissues, γ₃ on the other hand only shows significant expression in glycolytic skeletal muscle (Birk and Wojtaszewski, 2006; Cheung et al., 2000). The mass of γ₁ is around 36 kDa, whereas γ₂ and γ₃ possesses large N terminal domains which results in their masses being 63 kDa and 58 kDa respectively. All γ subunit isoforms contains four tandem repeats which are named CBS repeats/motifs due to their similarity to the cystathionine β-synthase enzyme. The CBS motifs are able to bind the nucleotides ATP, ADP and AMP. The binding of ATP under basal conditions helps to keep the activity of AMPK low, whereas the exchange of AMP for ATP would signal cellular stress and cause allosteric activation of the enzyme and protection from dephosphorylation (Carling et al., 1987; Xiao et al., 2011). Several cardiovascular conditions are caused by mutations in the γ₂ subunit gene, such as Wolff-Parkinson-White (WPW) syndrome, as well as some types of hypertrophic cardiomyopathies and glycogen storages diseases of the heart (Arad et al., 2002; Gollob et al., 2001).
1.1.2 Function and targets of AMPK

As stated previously, the main function of AMPK is as a cellular energy regulator. As a kinase, the main effect of AMPK is to phosphorylate other proteins. Analysis of the peptide sequences around known AMPK targets have revealed several common features. Indeed the SAMS peptide (sequence: HMRSAMSLHVLKRR) (see Chapter 2) now used for AMPK kinase assays by most labs are derived from analysis of these features (from the AMPK target Acetyl-Coenzyme A carboxylase: ACC). The consensus motif for AMPK indicates a preference for hydrophobic residues at +4 and -5 residues from the serine or threonine residue to be phosphorylated (Woods et al., 1996). The functions of AMPK are diverse and have been an area of intense research and new effectors continue to be discovered regularly, the following section will provide a brief overview of this topic as it is has been extensively reviewed elsewhere (Carling, 2004; Hardie et al., 2012; Steinberg and Kemp, 2009). The downstream effects of AMPK on various cellular pathways are summarised in Figure 1.1 with specific targets and phosphorylation sites shown in Table 1.1.

Carbohydrate metabolism

At a basic level, carbohydrate metabolism involves either the breakdown of glucose via glycolysis, its synthesis through gluconeogenesis or its longer term storage as glycogen (through glycogenesis and the reverse process of glycogenolysis). As a cellular energy regulator, AMPK is involved in all of these processes, acting to inhibit anabolic processes and to activate catabolic processes (Figure 1.1/Table 1.1).

In order for the cell to utilise carbohydrates, they first need to be transported across the plasma membrane, this is often the rate limiting step in metabolism, especially in working skeletal muscle or in tissues responding to insulin signalling (Kubo and Foley, 1986). One of the main ways glucose is transported into cells is through facilitated diffusion via GLUT transporters, with the insulin sensitive GLUT4 being the predominant form expressed in skeletal muscle. It was found that treatment with the AMPK activator AICAR (5-aminoimidazole-4-carboximide ribonucleoside) stimulated glucose uptake into rat skeletal muscle and heart cells via a Phosphoinositide 3-kinase (PI3K) independent pathway (Bergeron et al., 1999; Hayashi et al., 1998). This effect is mediated through increased GLUT4 translocation
to the cell membrane (Merrill et al., 1997; Russell et al., 1999; Winder and Hardie, 1999). More recently, there have been reports showing that AMPK phosphorylates the protein AS160, which in turn acts to promote the secretion of GLUT4 containing vesicles, thereby facilitating their translocation to the membrane (Geraghty et al., 2007; Kramer et al., 2006). Additionally, chronic AMPK activation (e.g. with AICAR) has been shown to increase GLUT4 expression, thereby further elevating the rate of glucose uptake (Holmes et al., 1999; Zheng et al., 2001). The above described mechanisms appears to be the main ways by which AMPK serves to restore energy balance in exercising muscle. AMPK has been shown to increase insulin sensitivity, and studies have shown that adiponectin mediated AMPK activation leads to improvements in insulin sensitivity in cultured muscle cells. One explanation for this effect comes from the observation of the phosphorylation of insulin receptor substrate 1 by AMPK in these cells (Jakobsen et al., 2001; Wang et al., 2007).

Once glucose is in the cell, it is converted into glucose-6-phosphate before entering glycolysis pathway. This pathway is catalysed by hexokinases and the reverse reaction is catalysed by glucose-6-phosphatase. AMPK has been shown to both increase the activity and transcription of hexokinase-II, and decrease the expression of glucose-6-phosphatase, thereby leading to an increased substrate supply into glycolysis (Holmes et al., 1999; Ojuka et al., 2000; Stoppani et al., 2002; Woods et al., 2000). The enzyme phosphofructokinase-2 (PFK2) is a bi-functional regulator of glycolysis and gluconeogenesis. Phosphorylation of cardiac PFK2 at Ser-466 by AMPK elevates its kinase activity which in turn catalyses the formation of fructose 2,6-bisphosphate, leading to the activation of PFK1 and thereby increasing the rate of glycolysis (Marsin et al., 2000). AMPK has also been shown to activate the bi functional enzyme 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) which is central to the glycolytic flux (Mendoza et al., 2012).

In addition to stimulating the catabolic process of glycolysis, AMPK also acts to inhibit gluconeogenesis. One possible mechanism though which this is achieved is via AMPK’s phosphorylation of the CREB-regulated transcription coactivator-2 protein CRTC2 (formerly known as TORC2), which causes its sequestration in the cytoplasm and thereby inhibiting transcription of gluconeogenesis genes such as glucose-6-phosphatase (G-6-Pase), and phosphoenol-pyruvate carboxykinase, (da Silva Xavier
et al., 2000a; da Silva Xavier et al., 2000b; Koo et al., 2005; Leclerc et al., 1998; Lochhead et al., 2000). Furthermore AMPK has been shown to negatively regulate a number of other gluconeogenic genes such as HNF-4α, PCG1α and the carbohydrate response element binding protein (ChREBP) (da Silva Xavier et al., 2000a; da Silva Xavier et al., 2000b; Hong et al., 2003; Jager et al., 2007; Kawaguchi et al., 2002; Leclerc et al., 2001). However, more recently this theory has been called into question with one group demonstrating that inhibition of gluconeogenesis as a result of metformin treatment is independent of the activation of the AMPK pathway (Foretz et al., 2010).

AMPK also negatively regulates the storage of glucose as glycogen. In 1989, AMPK was shown to phosphorylate and inhibit glycogen synthase (GS) (Carling and Hardie, 1989). Additionally AICAR treatment in vivo also led to a decrease in GS activity (Wojtaszewski et al., 2002). However, the above effect could be are overridden by high concentrations of glucose-6-phosphate (Aschenbach et al., 2002).

**Fatty acid metabolism**

Like glucose, fatty acids need to be able to cross the plasma membrane into the cytoplasm in order to be used in cellular metabolism. Here, AMPK has also been shown to be an important regulator, AMPK activation leads to increased fatty acid uptake into both heart and skeletal muscle cells (Figure 1.1/Table 1.1) (Luiken et al., 2003; Shearer et al., 2004a; Shearer et al., 2004b; Steinberg et al., 2006b).

One of the first important cellular targets identified for AMPK is Acetyl-Coenzyme A carboxylase (ACC) (Carling et al., 1987). In mammals, there are two isoforms of ACC, ACC1 and ACC2, both phosphorylated by AMPK on the residues Ser79 and Ser218 respectively (Tong, 2005). ACC1 is a 265 kDa protein which catalyses the irreversible carboxylation of acetyl-CoA to produce malonyl-CoA as a substrate for fatty acid synthesis. Phosphorylation of ACC results in its inactivation and therefore reduced production of malonyl-CoA. This results in a decrease in the rate of fatty acid synthesis. Additionally, the presence of malonyl Co-A also inhibits carnitine:palmitoyl-CoA transferase (CPT1), which transports activated fatty acids into the mitochondria, thereby decreasing the rate of fatty acid oxidation. It can be seen that the actions of AMPK would serve to increase fatty acid oxidation via
reduction in malonyl Co-A levels. Phosphorylation of ACC at Ser79 and Ser218 are now routinely used as indicators for AMPK signalling in vivo (Park et al., 2002b). In addition, AMPK has been shown to positively regulate the activity of malonyl-CoA decarboxylase, which catalyses the conversion of malonyl-CoA into acetyl-CoA (Park et al., 2002a). Furthermore, AMPK may also inhibit the expression of the fatty acid synthase (FAS) enzyme, thereby further inhibiting fatty acid production (An et al., 2007). AMPK also plays a role in inhibiting cholesterol synthesis by negatively regulating the HMG-Co-A reductase (Corton et al., 1994). In addition, both the mitochondrial glycerol-3-phosphate acyl-transferase (which is a rate limiting enzyme in TG synthesis) and hormone-sensitive lipase (which is responsible for hydrolysis of triglycerides into free fatty acids) are inactivated by AMPK (Steinberg et al., 2006a).

**Cell growth, survival and migration**

In line with its role in inhibiting anabolic processes, AMPK is a negative regulator of new cellular growth, appearing to induce cell cycle arrest at G1/S phase (Figure 1.1/Table 1.1). This effect is dependent on the tumour suppressor p53, with AMPK activation inducing phosphorylation of p53 on serine 15 (Jones et al., 2005). AMPK also directly phosphorylate p27 at Thr198 which may act to decrease cell growth while promoting survival of existing cells (Liang et al., 2007).

Evidence has emerged that AMPK activation may help cancer cells survive the nutrient and oxygen deprivation encountered when a solid tumour grows. Pancreatic cancer cells pre-treated with siRNA against AMPK α1 and α2 were found to have much lower tolerances to glucose deprivation compared to non-siRNA treated cancer cells (Kato et al., 2002). Furthermore, in prostate cancer cells, it has been shown that AMPK activity is necessary for the transcription of hypoxia inducible factor 1 (HIF1), which allows cancer cells to metabolically better adapt to anaerobic conditions (Lee et al., 2003). Recently, the expression of the AMPK β1 subunit has been demonstrated to be essential to the survival of numerous prostate cancer cell lines (Ros et al., 2012).

In recent years AMPK has been implicated in the regulation of both normal cell migration and cancer cell invasion. Studies have shown AMPK to be involved in the process of cytoskeletal reorganisation (Brenman, 2007; Miranda et al., 2010). A study in 2003 found that AMPK was a regulator in hypoxia induced angiogenesis, and
suppression of AMPK reduced the migration of human umbilical vein endothelial cells (HUVECs) towards VEGF chemo-attractant (Nagata et al., 2003). Adiponectin was found to increase the motility of human prostate cells, and this action was inhibited by both siRNA directed against AMPK and treatment with the AMPK inhibitor compound C (Chiu et al., 2009; Tang and Lu, 2009). Further evidence published recently has implicated CaMKKβ acting via AMPK, in promoting migration of prostate cancer cells (Frigo et al., 2011). Recently, the pro-migratory effect AMPK has been shown in a number of other cell types, both malignant and non-malignant. In ovarian cancer cells, lysophosphatidic acid (LPA) induced migration was dependent on AMPK expression, with AMPK siRNA knockdown impairing LPA induced migration (Kim et al., 2011a). AMPK inhibitors and siRNA mediated knockdown of AMPK was found to negate ghrelin induced motility in glioma cells (Chen et al., 2011). Additionally, the microRNA-451 has been shown to negatively regulate the activities of LKB1/AMPK, thereby suppressing cell migration (Godlewski et al., 2010a; Godlewski et al., 2010b). Also, it was found that endothelial nitric oxide synthase activation promoted lymphocyte migration in a manner dependent on CaMKKβ/AMPK regulation (Martinelli et al., 2009). A 2010 study found that AMPK directly phosphorylated the cytoplasmic linker protein CLIP-170, which is involved in modulating microtubule dynamics. Inhibition of AMPK in this system disrupted microtubule stabilisation and directional cell migration (Nakano et al., 2010). This could present a possible mechanism for AMPK to exert its pro-migratory effects.

However, there is contention in the field with relation to the role AMPK plays in cell migration and invasion. In the monocyte-like cell line U97, AICAR and phenformin have been shown to decrease random motion of cells as well as migration towards stromal cell-derived factor (SDF) 1α (Kanellis et al., 2006). Similar effects were observed in both vascular smooth muscle and umbilical vein endothelial cells where AICAR treatment reduced injury induced cell migration (Esfahanian et al., 2012; Peyton et al., 2012; Stone et al., 2013). Additionally, both expression of dominant negative AMPK or siRNA knockdown in the prostate cancer cell-line C4-2 led to increased malignant behaviour such as cell migration (Zhou et al., 2009). Similarly, metformin treatment was seen to reduce migration in glioblastoma cells (Ferla et al., 2012). In a 2010 study, adiponectin was found to inhibit Lipopolysaccharide (LPS)
induced migration of myofibroblasts in a mechanism dependent on AMPK expression and activation (Cai et al., 2010).

From the information presented above, it can be appreciated that the current picture of AMPK regulation of cell migration is a contradictory one, with AMPK being shown to both stimulate and inhibit cell migration. One possibility for this discrepancy is that AMPK exerts different effects on different tissue/cell types. The effects of AMPK may also depend on whether the cell is cancerous or not. The reliability of studies using some AMPK activators or inhibitors needs to be taken into account as molecules such as metformin and compound C have been shown to have off target effects independent of AMPK. A potential explanation for the pro-migratory effects of AMPK is that in some cells, such as those in cancer, following nutrient restriction or hypoxia (and the resulting AMPK activation), there will be a need for the cells to migrate to a more nutrient/oxygen rich area. Clearly more research is needed before any clear conclusion can be drawn.
Figure 1.1 Cellular effects of AMPK
As a regulator of cellular energy homeostasis, AMPK responds to conditions of cellular energy depletion by phosphorylation of downstream targets which leads to inhibition of anabolic processes (Red arrows) and stimulation of catabolic processes (Green arrows). Recently, AMPK has also been shown to regulate both the migration of cell under physiological conditions and the invasion of cancer cells through adjacent tissues. However, evidence for these processes are conflicting, with some sources suggesting a positive role for AMPK and other a negative role. See section 1.1.2 for details.
Regulation of AMPK activity

As AMPK is involved in many physiological and pathological processes, it is very important for the cell to exert strict regulation of its activity. Currently, there are two known methods for direct regulation of AMPK activity: the first and most potent being the regulation of phosphorylation of AMPK by upstream kinases and phosphatases (Steinberg and Kemp, 2009). Secondly, AMP binding to the γ subunit is able to elicit allosteric activation of AMPK (Adams et al., 2004). This also represents an important method for the regulation of AMPK activity. Additionally, recently, our group have identified that ADP binding the γ subunit helps to protect AMPK from dephosphorylation (Xiao et al., 2011). The regulation of AMPK by these processes will be discussed in more detail in the following section (an overview of these processes is provided by Figure 1.3).

Table 1.1 Direct targets of AMPK
Table showing direct protein targets of AMPK, categorised into various different cellular functions. From (Towler and Hardie, 2007).

<table>
<thead>
<tr>
<th>AMPK Target (P-site)</th>
<th>Effect on Target</th>
<th>Effect on pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid metabolism</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACC1 (S80)</td>
<td>↓ Activity</td>
<td>↓ FA synthesis</td>
</tr>
<tr>
<td>ACC2 (S212)</td>
<td>↓ Activity</td>
<td>↑ FA oxidation</td>
</tr>
<tr>
<td>HMG-CoA reductase (S872)</td>
<td>↓ Activity</td>
<td>↓ cholesterol synthesis</td>
</tr>
<tr>
<td>Hormone-sensitive lipase (S554)</td>
<td>↓ Activity</td>
<td>↓ lipolysis</td>
</tr>
<tr>
<td>Carbohydrate metabolism</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycogen synthase (S8)</td>
<td>↓ Activity</td>
<td>↓ glycogen synthesis</td>
</tr>
<tr>
<td>6-phosphofructo-2-kinase (cardiac, S466)</td>
<td>↑ Activity</td>
<td>↑ glycolysis</td>
</tr>
<tr>
<td>6-phosphofructo-2-kinase (insulin, S461)</td>
<td>↑ Activity</td>
<td>↑ glycolysis</td>
</tr>
<tr>
<td>Cell Signalling</td>
<td></td>
<td></td>
</tr>
<tr>
<td>endothelial NO synthase (S1177)</td>
<td>↑ Activity</td>
<td>↑ NO, ↑ blood flow</td>
</tr>
<tr>
<td>TSC2 (S1387)</td>
<td>↑ Activity Rheb-GAP</td>
<td>↓ protein synthesis, cell growth</td>
</tr>
<tr>
<td>Insulin Receptor Substrate 1 (S794)</td>
<td>↑ PI3K binding</td>
<td>↑ Insulin signalling</td>
</tr>
<tr>
<td>AS160 (unknown)</td>
<td>↑ Rab-GAP</td>
<td>↑ glucose uptake</td>
</tr>
<tr>
<td>Transcription</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p300 (S89)</td>
<td>↓ Interaction</td>
<td>↓ Transcription</td>
</tr>
<tr>
<td>HNF-4α (S313)</td>
<td>↓ DNA binding; ↑ degradation</td>
<td>↓ Transcription</td>
</tr>
<tr>
<td>CHREBP (S568)</td>
<td>↓ DNA binding</td>
<td></td>
</tr>
<tr>
<td>TORC2 (S171)</td>
<td>↑ Cytoplasmic localisation</td>
<td>↓ Transcription</td>
</tr>
</tbody>
</table>
Phosphorylation and dephosphorylation of AMPK

The phosphorylation and dephosphorylation of AMPK represent the major mechanisms by which AMPK activity is regulated. The phosphorylation of the key residue threonine-172 (Thr-172), in the activation loop of the kinase domain in the α subunit is essential for AMPK to become active (Davies et al., 1995; Hawley et al., 1996; Stein et al., 2000). Indeed when this residue is altered to an alanine using site directed mutagenesis, AMPK activity is almost completely abolished. Although other residues within AMPK can become phosphorylated, for example serine-485, they do not appear to have any direct effect on AMPK activity (Woods et al., 2003b). A schematic representation of the AMPK activation by upstream kinases and deactivation by upstream phosphatases is shown in Figure 1.2.

Upstream kinases

Phosphorylation of Thr-172 in vivo is mainly achieved by up-stream kinases LKB1 and CaMKKβ (Figures 1.2 and 1.3). LKB1 is a tumour suppressor involved in the development of Peutz-Jeghers Syndrome (PJS) (Hawley et al., 2003; Woods et al., 2003a) and CaMKKβ plays important roles in the CaM-kinase signalling cascade (Hawley et al., 2005; Hurley et al., 2005; Woods et al., 2005).

The functional LKB1 complex is made of 3 proteins, the LKB1 kinase, STE20-related adaptor (STRAD) protein and mouse protein 25 (MO25) (Boudeau et al., 2003; Boudeau et al., 2004; Zeqiraj et al., 2009). The binding of STRAD increases LKB1 activity by 4 to 5 fold and MO25 binding increases the stability of the complex and results in its localisation in the cytoplasm (Boudeau et al., 2004). Expression of this enzyme is ubiquitous and LKB1 seems to be the main AMPK upstream kinase in most metabolic tissues (Carling et al., 2008). Indeed, in LKB1 knockout mouse tissues, a drastic reduction in AMPK activity is seen (Sakamoto et al., 2005; Shaw et al., 2005; Woods et al., 2011).

Although a number of phosphorylation sites have been identified within LKB1, many reports have shown the enzyme as constitutively active (Collins et al., 2000; Sapkota et al., 2002). Mutations of these phosphorylation sites to alanine residues has no effect
on LKB1 activity, and treatment of purified LKB1 in-vitro with phosphatases does not decrease its activity either (Denison et al., 2009).

CaMKKβ, as suggested by its name is activated by increases in intracellular calcium levels. It was first identified as an upstream kinase of AMPK by two groups at almost the same time (Hawley et al., 2005; Woods et al., 2005). The discovery of this upstream kinase showed that AMPK was involved in calcium mediated signalling. Subsequently many hormones and cytokines which lead to activation of the G protein coupled receptor pathway (the Gq-phospholipase C axis), has been shown to mediate their effects at least partially through AMPK. CaMKKβ is expressed in more limited set of tissues compared to LKB1. It is highly expressed in many areas of the brain, (such as the hippocampus, dentate gyrus, hypothalamus, and cerebellum) where it regulates important functions such as memory and cerebellar development. It is also expressed at low levels many other organs including skeletal and smooth muscle, kidneys, and the thymus (Racioppi and Means, 2012).

A third, more controversial up-stream kinase candidate is TAK1 (transforming growth factor-β (TGFβ) -activated kinase-1. However the physiological role of this regulation is not clearly defined (Momcilovic et al., 2006; Xie et al., 2006).

**Protein Phosphatases acting on AMPK**

As described above, in the last ten years there have been great advances made in the identification of upstream kinases of AMPK. However, this is only half the story, the reverse reaction in which AMPK is inactivated involves the dephosphorylation of Thr-172 and is mediated by protein phosphatases. Due to the characterisation of LKB1 as constitutively active, this dephosphorylation step could be more important in the regulation of AMPK activity, compared to that of phosphorylation (Lizcano et al., 2004; Woods et al., 2005; Woods et al., 2003a). More recent data showing AMP + ADP binding to AMPK protects the enzyme from dephosphorylation also add further importance to this step (Figure 1.2) (Davies et al., 1995; Sanders et al., 2007b; Suter et al., 2006; Xiao et al., 2011).
Compared to our understanding of the AMPK upstream kinases, much less is known of the phosphatases which act to dephosphorylate it. In recent years, a number of potential AMPK phosphatases have been reported, these studies will be discussed later in this section. First, brief descriptions of the main phosphatase classes are provided below.

**Overview of protein phosphatases**
Protein phosphorylation is a reversible post translational modification catalysed by protein kinases and reversed by protein phosphatases. Protein phosphatases are divided into two major classes according to their substrate specificity: tyrosine specific and serine/threonine phosphatases (Shi, 2009; Zhang, 2002). As the major activation site of AMPK is the Thr-172 residue, this section will focus on the Serine/Threonine phosphatases. Of note, there are also a class of dual specificity phosphatases which are able to dephosphorylate all three afore mentioned residues (although generally classed under protein tyrosine phosphatases).

The serine/threonine phosphatase family consists of three distinct classes, divided according to their structure: The phospho-protein phosphatase (PPP) class, the metal-dependent (Mg$^{2+}$) phosphatase (PPM) class and aspartate-based phosphatases (represented by FCP class phosphatases) (Shi, 2009). As the only known substrate for FCP phosphatases is the C-terminal domain (CTD) of RNA polymerase II, it is of no relevance to this study and will not be discussed further.

Surprisingly, the number of phosphatases appears to be 2-3 times fewer than those of kinases. This may suggest comparison to kinases, phosphatases have a greater dependence on interaction with regulatory other cellular proteins in order to confer specificity.

**The Phospho-Protein Phosphatase (PPP) class**
The PPP class of phosphatases consists of multi-subunit enzymes, and are split into a number of sub-classes. The limited number of catalytic subunits in each sub-class are able to associate with a much larger number of regulatory subunits. Main members of the PPP class include PP1, PP2A, PP2B and other members include PP4, PP5, PP6, and PP7 for which less is known about (Shi, 2009; Virshup and Shenolikar, 2009).
This class of phosphatases account for the majority of phosphatase activity in an *in-vivo* environment.

PP1 is the major subclass of the PPP family and is expressed in all eukaryotic cells. In cells, an active PP1 complex consists of a catalytic subunit and a single regulatory subunit. There are three different PP1 catalytic subunit isoforms, which are highly conserved among eukaryotes, namely PP1Cα, PP1Cβ, and PP1Cγ, all three of which are around 30 kDa in mass and show high levels of similarity apart from the domains which allow it to bind with different PP1 regulatory subunits. The free catalytic subunits of PP1 have high activity and low specificity, and require the binding of regulatory subunits to confer specificity (Cohen, 2002; Terrak et al., 2004).

There are over 50 known regulatory subunits of PP1, these act to confer substrate specificity, as well as assisting the sub-cellular targeting of the various complexes (Ceulemans and Bollen, 2004; Cohen, 2002). These regulatory subunits share little homology apart from a common PP1 catalytic unit binding motif. An example of the regulatory subunits influencing the specificity of PP1c is highlighted here, when the regulatory subunit: myosin phosphatase targeting subunit (MYPT1) binds to PP1C, it causes a change in conformation of in the catalytic cleft which results in a substantial increase in activity for the myosin light chain and a concurrent decrease in activity to other potential substrates (Gallego and Virshup, 2005; Terrak et al., 2004). This dependency on regulatory subunits for specificity creates difficulties for the study of PP1 targets in vitro, as in many studies, the catalytic subunits used to dephosphorylate potential targets without regulatory subunits, and potentially producing misleading results (Davies et al., 1995; Sanders et al., 2007b).

Apart from the regulatory subunits, another layer of regulation for PP1 is achieved via endogenous inhibitory proteins such as Inhibitor-1 (I-1) (Cohen and Nimmo, 1978), inhibitor-2 (I-2) (Foulkes and Cohen, 1980), DARPP32 and CPI-17 (Eto et al., 1997; Walaas and Greengard, 1991). The inhibition of PP1 by these proteins is thought to be achieved through binding to the phosphatase catalytic site.

The PP2A class of phosphatases have much in common with that of PP1 in that they also exist predominantly in multi-subunit forms. PP2A is an extremely abundant
protein, accounting for 0.1% - 1% of total cellular protein content (Gallego and Virshup, 2005). In cells they mostly have two conformations: as a hetero-dimeric core enzyme or a hetero-trimeric holoenzyme. The two subunits which make up the dimeric core enzymes is a catalytic subunit (of around 36 kDa) and a scaffolding subunit (around 65 kDa, also known as PR65), both of which has two different isoforms (named as α and β isoforms (Shi, 2009). This association with the scaffolding subunit is unique for among PPP enzymes, although other PPP members share significant sequence homology with PP2A. Like PP1, the PP2A catalytic subunits have relatively little substrate specificity and will dephosphorylate many potentially non-physiological substrates in vitro. Like PP1, there are a plethora of regulatory proteins that interact with PP2A. They are split into four main families, B55, B56, PR48 and PR93, with the numbers corresponding with their molecular masses, each of which includes multiple isoforms. In most cases, in order to confer specificity the enzyme would need to be in the trimeric conformation attached to a regulatory subunit. Indeed, although purification of the core dimer have been made from various tissues, some studies have suggested that these are in fact artefacts produced by the purification process and only the trimeric complex exists in vivo (Cohen, 1989; Depaoli-Roach et al., 1994). This conclusion would be logical, as based on the activity of the PP2A catalytic subunit in vitro, it would be very difficult to see how the enzyme’s activity can be contained in vivo without a regulatory subunit, and unrestricted/unspecific phosphatase activity could be toxic to cells (Gallego and Virshup, 2005). According to current knowledge, the PP2A scaffolding subunit binds tightly to the catalytic subunit, thereby forming a structure to which the regulatory subunits can bind.

The metal dependent (PPM) class
Compared to PPP family members, PPM (previously named PP2C) isoforms have little sequence homology and are thought to be essentially self-directed in terms of targeting and substrate specificity, conferred via various structural domains. These enzymes are thought to have evolved independently from the PPP family (Barford et al., 1998). As implied by the name, the activities of these phosphatases are dependent on the binding of Mg$^{2+}$ or Mn$^{2+}$ ions. Up to now, there has been at least 17 members of the PPM class identified (Lu and Wang, 2008).
In contrast to the PPP family, PPM phosphatases are single subunit enzymes, with no regulatory or scaffolding subunits, but they do possess domains within the catalytic subunit which may confer substrate specificity, which is rather similar to the situation seen with tyrosine phosphatases. In addition, these enzymes are also resistant to okadaic acid and calyculin A and other classical inhibitors of the PPP family (Swingle et al., 2007).

Members of the PPM family are involved in a wide range of cellular processes. A important process regulated by the PPM phosphatases are the stress signalling pathways, such as the Mitogen-activated protein kinase (MAPK) pathway. Several reports have shown the critical function that PPM isoforms play in correct MAPK signalling (Hanada et al., 2001; Takekawa et al., 1998). In addition, PPM has also been implicated in PI3Ks and Protein Kinase B (PKB/Akt) signalling, protein ubiquitination and cell metabolism, as well as signalling in death/survival. This topic has been the subject of excellent recent reviews, which provides more details on the regulation and function of PPM isoform (Lu and Wang, 2008; Stern et al., 2007).

Candidates for the AMPK phosphatase

The first indications for the identity of the AMPK phosphatase came from studies performed in the late 1980s and early 1990s. Studies performed in vitro, where PP2C/PPM1A or the catalytic subunits of PP2A or PP1 was incubated with pre-activated AMPK (or AMPK isolated from mammalian cell extracts), showed that all these phosphatases are able to dephosphorylate AMPK at Thr-172 and decrease its activity (Davies et al., 1995; Sanders et al., 2007b; Suter et al., 2006). From the research by Davies et al, it can been seen that treatment with PPM1A results in a more pronounced reduction of AMPK activity when compared to that of PP2A (Davies et al., 1995). It was possible to infer from these results that PPM1A could be the in vivo AMPK phosphatase. Indeed PPM1A has become the defacto phosphatase to use when conducting the in vitro dephosphorylation of AMPK. As explained in the previous section, the studies quoted above have inherent problems. As although PPM phosphatases are single subunit enzymes, members of the PPP family almost always require the binding of regulatory subunits to confer target specificity, thus it would
appear that these results for PP2A and PP1 have limited in vivo relevance due to the lack of the multitude of regulatory subunits.

The first study in a more physiological ex vivo system was published by Moore et al in 1991. It was noted by researchers that when hepatocytes were isolated from rats they had very high initial AMPK activities, which decreased over time as they were cultured. This was most likely due to the hypoxia induced increase in AMP:ATP or ADP:ATP ratios in the cells as they were isolated. Next, okadaic acid, a selective phosphatase inhibitor of the PPP family phosphatases, but not the PPM family was used to treat newly isolated hepatocytes. It was found that while okadaic acid inhibited the decrease in phosphorylation in acetyl-CoA carboxylase (ACC), the decrease in AMPK activity/phosphorylation was not blocked (Moore et al., 1991). This result suggests that while the ACC-phosphatase maybe a member of the PPP family (most likely PP2A), the AMPK phosphatase is likely to be one or more members of the PPM family. Additional evidence supporting this hypothesis was published more recently. In L6 myotubes, it was observed siRNA knockdown of PPM1A abolished the ability of TNF-α to decrease AMPK activity, although no effect on basal activity was reported (Steinberg et al., 2006b). PPM1A siRNA also reversed the downstream effects of TNF-α on AMPK, such as the suppression of ACC phosphorylation, fatty acid oxidation and insulin mediated glucose uptake. More recently, quercetin, a naturally occurring flavonoid was reported to activate AMPK in mice, while at the same time depressing PPM1A and PPM1B expression (Lu et al., 2010). In 2012, it was reported that N-myristoylation of PPM1A and PPM1B is essential to their ability to dephosphorylate AMPK Thr-172, siRNA mediated knockdown of PPM1A and PPM1B both increased AMPK phosphorylation in cells over-expressing AMPK LKB1, whereas knockdown of the other PPM members had no effect (Chida et al., 2013). Furthermore, the authors identified that PPM1A and PPM1B must be N-myristoylated in order to dephosphorylate AMPK. While this thesis was in progress, another study was published where shRNA was used to create stable knockdowns of PPM isoforms in HEK293 cells. PPM1E silencing caused a significant elevation of AMPK Thr-172 phosphorylation levels. PPM1E depletion also augmented phenformin induced increase in AMPK phosphorylation. Additional data from this study also suggest PPM1F contributed to the regulation of AMPK.
phosphorylation. This study and its relevance to my results will be discussed in more detail in **Chapter 3** (Voss et al., 2011).

Aside from the PPM family, in recent years, members of the PPP family have also been implicated as AMPK phosphatases, making the field more contentious. When various purified phosphatases were used to dephosphorylate protein homogenate fractions from cortical neurons, it was found that PP1, PP2A and PPM1A all reduced AMPK phosphorylation at Thr-172, whereas PP2B had no effect (Kuramoto et al., 2013). In a yeast two-hybrid screen, deletion of PP2A was found to affect the glucose-regulated interaction between the α2 and γ1 subunits of AMPK. It was also found that PR65 (the scaffolding subunit of PP2A) interacted with AMPK αδ (Gimeno-Alcaniz and Sanz, 2003). A 2005 study where rat hepatocytes were treated with increasing concentrations of various PPP inhibitors (including okadaic acid, calyculin A, microcystin and tautomycin) all resulted in significant increases in both AMPK Thr-172 and ACC phosphorylation with dose response characteristics indicating PP2A/PP1 contribution. The inhibitors do not seem to affect motility of the upstream kinase LKB1 on SDS-gels, indicating its phosphorylation was not altered. The authors however think that the effect of the inhibitors on AMPK phosphorylation is indirect and suggest the phosphorylation at multiple sites (in addition to Thr-172) maybe increased by these phosphatase inhibitors, due to the presence of multiple low mobility bands of phosphorylated AMPK (Samari et al., 2005). Exposure of bovine aortic epithelial cells to palmitoleic acid, which activates PP2A, reduced AMPK-Thr-172 phosphorylation. Furthermore, use of okadaic acid and siRNA knockdown of PP2Ac are able to abolish the effect of palmitate on AMPK (Wu et al., 2007).

In another study published during my project, PP1 and its R6 regulatory subunit were shown to regulate the glucose induced dephosphorylation of AMPK in mouse pancreatic beta cells (MIN6) (Garcia-Haro et al., 2010). The implications of this paper will be discussed in more detail in **Chapter 3**.

As can be seen, much of the evidence discussed above was published after this thesis commenced in 2009, and the consensus at that point was that the AMPK phosphatase most likely belonged to the PPM family, with little siRNA based studies having been
published. It was against this backdrop that my work to identify the AMPK phosphatase was started. However, it can be appreciated that the picture today is a rather more complex one. There is no consensus as to the identity of the AMPK phosphatase(s), with all major classes of serine/threonine phosphatases having been put forward as potential candidates.
The activity of AMPK is predominantly determined by phosphorylation of the Thr-172 residue of the α subunit. This phosphorylation is catalysed by upstream kinases, LKB1 and CaMKKβ, more recently TAK1 was shown to phosphorylate and activate AMPK (there are some dispute within the field). At the time of starting this thesis, PP2A and PPM/PP2C have been shown to dephosphorylate AMPK in vitro, however, the protein phosphatase regulating AMPK activity in vivo is unknown.

AMP/ADP: allosterically activates AMPK (step labelled as 1) as well as protecting the enzyme from dephosphorylation (step labelled as: 2)

Additionally, recent studies have implicated AMP and ADP in promoting the phosphorylation of AMPK dependent upon β-subunit myristoylation (Oakhill et al., 2010; Oakhill et al., 2011).
1.1.4 Regulation of AMPK by whole body signals

As well as being regulated by immediate imbalances in the AMP or ADP to ATP ratios, calcium fluxes and other intracellular signals, AMPK activity has been found to be influenced by a number of hormonal signals. These signals induce AMPK to respond to changes in the energy state of tissues or at the whole organism level. A schematic representation of the intracellular and whole body signals which serve to regulate AMPK is shown in Figure 1.3.

AMPK plays an important role in appetite regulation by acting as a relay for various whole body signals. Injection of a virus encoding a constitutively active form of AMPK into rat hypothalamus caused increases in appetite and body weight of these animals, whereas injection of dominant negative AMPK caused the opposite effect (Minokoshi et al., 2004).

AMPK also plays important roles downstream of the appetite regulating hormones ghrelin and leptin. Ghrelin is an important hunger signal, being secreted before meals to increase appetite, leptin on the other hand is secreted by adipocytes to signal satiety, acting to decrease feeding. In the hypothalamus, leptin injection caused a decrease in hypothalamic AMPK activity, whereas ghrelin administration caused an opposite effect, this was then associated with an increase in feeding (Andersson et al., 2004; Minokoshi et al., 2004). Unexpectedly, in contrast to the CNS, leptin exerts an opposite effect on AMPK in peripheral tissues, with leptin acting to increase AMPK activity and thereby stimulating fatty acid oxidation in skeletal muscle (Minokoshi et al., 2002). This rather paradoxical result perhaps reflects the differing roles that AMPK plays in these tissues, with AMPK acting to elevate intracellular ATP levels in the short term, via increased fatty acid oxidation in peripheral tissues while concurrently increasing feeding (which will not yield an immediate energy benefit). Recently a study has shown Intra-peritoneal (IP) administration short-chain fatty acid acetate into mice caused reductions in pAMPK (at Thr-172) and pACC levels (S79) in hypothalamic tissues, these effects were associated with a reduction in appetite in these mice (Frost et al., 2013, accepted for publication in Nature Communications, Apr 2014).
Like leptin, the hormone adiponectin is secreted by adipose tissue and regulates a number of metabolic processes such as carbohydrate metabolism and fatty acid oxidation. Adiponectin has been shown to play important roles in counteracting the detrimental effects of a number of metabolic conditions such as type II diabetes, diet induced obesity, and fatty liver. Combined treatment of leptin and adiponectin has been shown to completely reverse insulin resistance in mouse models (Yamauchi et al., 2001). In skeletal muscle, adiponectin activated AMPK via signalling through adiponectin receptor 1 (AdipoR1), leading to increased anabolic production of ATP via increased fatty acid oxidation and glycolysis (Yamauchi et al., 2002; Yamauchi et al., 2007).

Aside from the hormones described above, AMPK activity/expression has been found to be regulated by a number of other whole body signalling proteins. The cytokine interleukin-6 (IL-6) has been found to activate AMPK, with AMPK signalling being suppressed in many tissues of IL-6 KO mice (Kelly et al., 2004). TNF-α has been found to decrease AMPK signalling, with a concurrent increase in the expression of PPM1A phosphatase (Steinberg et al., 2006b). The sex hormone oestrogen has been shown to directly activate AMPK in cultured muscle cells, whereas dihydrotestosterone infusion led to a decrease in AMPK activity in adipocytes (D'Eon et al., 2005; McInnes et al., 2006).

In can be seen from information provided in the above section that AMPK plays vital roles in the regulation of energy balance at a cellular and whole body level, and gaining a full understanding of the regulation AMPK is of vital importance in devising new treatments for a many diseases ranging of diabetes to cancer.
Figure 1.3 Regulation of AMPK activity
Diagram showing various cellular and extra-cellular pathways regulating AMPK activity. Green arrows indicate stimulatory effects on activity whereas red as red arrows indicate inhibitory effects on activity. Adapted from ScienceSlides online (http://www.scienceslides.com/).
1.2 PAK1

The p21-activated kinase 1 (PAK1) belongs to the PAK family of serine/threonine kinases and has a molecular mass of 68 kDa. PAK proteins are important controllers of cell polarity, actin cytoskeletal organisation, cellular morphology, migration and invasion. The PAK family consists of 6 members which are divided into two groups, with PAK1-3 belonging to group 1 and PAK 4-6 belonging to group 2 (Bokoch, 2003).

PAK1 was identified as an AMPK interacting protein in chapter 5 and its association with AMPK was characterised in chapters 5 and 6. A review of the structure, regulation and function of PAK1 is provided below.

1.2.1 Structure and regulation of PAK1

PAK1 is the best understood member of all the PAK kinases and was initially identified as a downstream effector of the Rho GTPases Rac and Cdc42. It contains a C terminal kinase domain and a N terminal regulatory domain. The regulatory domain consists of a GTPase binding domain (PBD) as well as an auto-inhibitory domain (AID). A diagram showing the primary structure of PAK1 is shown in Figure 1.4. The main distinguishing feature between group 1 and group 2 PAKs are that the group 2 PAKs lacks the auto-inhibitory domain, as a result the group 2 PAK possesses a higher basal activity compared to those of group 1. The main mode of activation of group 1 PAKs is via through direct binding to the activated forms of the GTPases Rac1, Rac2, Rac3 and Cdc42 (Knaus et al., 1998; Manser et al., 1994; Mira et al., 2000). It is also activated by less well known GTPases TC10, CHP, and Wrch-1 (Aronheim et al., 1998; Neudauer et al., 1998; Tao et al., 2001). The PAK1 protein exists as a protein dimer in cells with the auto-inhibitory domain of one PAK1 molecule pressed against the kinase domain of the other thereby inhibiting it (Parrini et al., 2002; Zhao et al., 1998). Data from structural studies reveal that upon the binding of GTPases a major structural change occurs in the inhibitory domain, thereby disrupting its interaction with the kinase domain of the dimer protein. After release of the kinase domain, PAK1 undergoes auto-phosphorylation on various sites, with
phosphorylation of Thr-423 in the kinase domain being the most important in gaining full kinase function towards its substrates, and also relief from continued auto-phosphorylation (Lei et al., 2000; Zenke et al., 1999). Other important phosphorylation sites which affect PAK1 activity include Ser-144, Ser-198/Ser199 and Ser-203 (Chong et al., 2001). All group 1 PAK kinases complex with the focal adhesion-associated proteins, known as PIX proteins. PIX proteins in turn are tightly associated with the ARF GTPase-activating protein 1 (GIT-1), and both proteins form an activation complex with PAK1 (Turner et al., 1999). GIT-1 is a ubiquitous protein localized to focal adhesions, cytoplasmic complexes and membrane protrusions. It interacts with various GTPases to help regulate focal contact assembly and cytoskeletal dynamics (Hoefen and Berk, 2006; Manser et al., 1998). PIX proteins are guanine nucleotide exchange factors (GEFs) which act to recruit and activate GTPases, which then bind to and activate PAK1. PIX together with GIT-1 direct PAK1 to focal adhesions at leading edges of motile cells, where the complex performs its actions (Manabe et al., 2002; Zegers et al., 2003).

Aside from the auto-phosphorylation caused by GTPase binding, additional mechanisms of direct regulation of PAK1 activity have also been discovered. Protease mediated digestion have been shown to activate PAK1 (Benner et al., 1995). GIT1, which associates with PAK1 and βPIX have also been observed to activate PAK1 independent of GTPases (Loo et al., 2004). Direct phosphorylation by 3-phosphoinositide-dependent kinase 1 (PDK1) at Thr-423 on PAK1 has been proven to activate the kinase (King et al., 2000). Furthermore, Akt has also been shown to activate PAK1 by direct phosphorylation on the Ser-21 residue without requiring the involvement of either Cdc42 or Rac1. Phosphorylation at the Ser-21 site decreases PAK1 binding to the adaptor protein Nck in order to modulate migration of Rat1 cells (Zhou et al., 2003). Additionally, phosphorylation of PAK1 at Ser-212 by cyclin dependent kinase 5 (CDK-5), is involved in the regulation of neurite growth (Banerjee et al., 2002). These results demonstrate the ability of PAK1 activity and interactions to be regulated by upstream kinases in addition to binding by GTPases. A diagram (Figure 1.5) illustrating the process of activation of PAK1 and its downstream effects is shown.
Interestingly, two members of the PPM phosphatase family, PPM1E and PPM1F have been shown to be effective negative regulators of PAK1, mediating the dephosphorylation the enzyme at Thr-423 (Koh et al., 2002). These phosphatases have been found in complex with the PIX proteins. The presence of these potent deactivators of PAK1 in the same complex may help explain the ability of PAK1 to be rapidly deactivated (Zhan et al., 2003). In addition to dephosphorylation, there are other mechanisms by which the enzyme is negatively regulated. The PAK interacting protein hPIP1, which shares sequence homology with the yeast PAK regulator Skb15, have been shown to interact with PAK to inhibit Cdc42/Rac mediated activation. Over-expression of hPIP in the COS-7 cell-line or mouse embryonic fibroblasts led to down regulation of PAK-mediated e-Jun N-terminal kinase and nuclear factor kappa B signalling pathways, resulting in abnormal cell morphologies (Xia et al., 2001). In 2004, a study showed that the protein nischarin, originally identified as an integrin binding protein, directly interacted with the C-terminal domain of Rac bound PAK1. This binding inhibits the ability of PAK1 to phosphorylate downstream targets resulting in decreased cell migration. Indeed RNAi based knockdown of nischarin in the phaeochromocytoma derived cell-line PC12 led to increased cell migration (Alahari et al., 2004). Similarly, the protein merlin, coded for by the Nf2 tumour suppressor gene interacted with the GTPase binding domain of PAK thereby inhibiting its activity (Kissil et al., 2003).

![Figure 1.4 Primary structure of PAK1](image)

**Figure 1.4 Primary structure of PAK1**
The p21 (Rac/Cd42)-binding domain (PBD), auto-inhibitory domain (AID) and kinase domain are shown. Additionally, the binding sites on PAK1 to the adaptor Nck and the guanine exchange factor PIX are indicated.

### 1.2.2 Upstream signals for PAK1 activation

Having discussed the direct mechanisms of PAK1 activation, below is a brief summary of the indirect upstream signals that lead to the activation of PAK1. Extracellular signals acting upon receptor tyrosine kinases and G protein coupled receptors leads to PAK1 activation (**Figure 1.5**). In general these pathways activate...
PAK1 through sequential activation of PI3K and a guanine nucleotide exchange factor (GEF) which then activates Cdc42 or Rac (Menard and Mattingly, 2003). Several examples of upstream PAK1 activators are introduced below.

In vascular smooth muscle cells, LPA, which has been suggested to mediate it pro-migratory effects through AMPK was shown to also increase PAK1 activity by over 3 fold (Kim et al., 2011a; Schmitz et al., 2002). In 2003 it was shown that basic fibroblast growth factor signalling activated PAK1 and protected cells against apoptosis (Alavi et al., 2003). In the intestine, insulin or insulin like growth factor (IGF-1) treatment led to increased PAK1 Thr-423 phosphorylation, which was abolished by the PAK1 inhibitor IPA3. This activation was found to be PI3K dependent (Sun et al., 2009; Tsakiridis et al., 1996). Furthermore, PAK1-/- mice have been found to exhibit impaired global glucose tolerance after IP or oral glucose challenges, furthermore, these mice also exhibited peripheral insulin resistance (Chiang et al., 2013; Wang et al., 2011).

1.2.3 Function and targets of PAK1

As a kinase, the main function of PAK1 is to phosphorylate other proteins. To date, Over 40 PAK1 substrates have been identified. There is a limited degree of flexibility in the peptide sequences that PAK1 is able to phosphorylate. In 2007, a study was performed using a peptide library to comprehensively characterise the consensus phosphorylation motifs of Group 1 and 2 PAKs. It was found that PAK1 and PAK2 shared virtually identical substrate recognition motifs which are distinct from the group 2 PAKs. Both PAK1/2 showed a preference for arginine at all positions from -5 to -1 from the phosphorylation site and large hydrophobic residues in positions +1 to +3 (Rennefahrt et al., 2007). In another study, the group 1 PAK proteins are also found to prefer basic amino acids at positions +2 and +3 (Tuazon et al., 1997). The reason for the discrepancy between the two studies is not clear, however the findings from the 2007 studies have been used to design specific peptide substrates for PAK1, which has been used subsequent studies (Strochlic et al., 2010). Additionally, group 1 and 2 PAKs exhibited a marked preference for serine over threonine as the phosphor-acceptor site. However when existing PAK substrates were mapped against the predicted recognition sequences generated, it was found that none of the known
substrates scored within the top 2% of the predicted sites. This may suggest that other factors such as the tertiary structure of substrates, protein interactions, subcellular localisation are perhaps more important in determining PAK substrates (Rennefahrt et al., 2007).
Figure 1.5 Activation of PAK1 and downstream targets

Signalling from receptor tyrosine kinases (e.g. insulin, EGF receptors) and G-protein-coupled receptors activates Rac1/Cdc42. Which then binds to and activates PAK1. In addition, kinases such as Akt and CDK-5 are able to directly phosphorylate and activate PAK1. PAK1 activation is enhanced and stabilised by auto-phosphorylation. Activated PAK1 then phosphorylates a range of downstream targets mediating the motility/migration properties of cells and cellular survival and proliferation.
1.2.4 PAK1 function in physiology and pathology

As stated above, PAK1 is an important regulator of cytoskeletal dynamics, cell polarity, morphology, motility and invasion (Figure 1.5). PAK1 is expressed in most tissue types, with especially high levels seen in the neuronal tissues, cardiac muscle and neutrophils (Burbelo et al., 1999; Clerk and Sugden, 1997; Dharmawardhane et al., 1999). Often, the activation of PAK1 results in its translocation from one area of the cell to another. Upon PAK1 activation in NIH3T3 cells, PAK1 redistributed from the cytosol to the cell periphery (Dharmawardhane et al., 1997; Kichina et al., 2010). Several studies have also shown PAK1 localisation to focal adhesions upon activation (Manser et al., 1997; Sells et al., 2000).

Many recent reports show that PAK1 is heavily involved in various physiological and pathological processes. Dysregulation of PAK1 activity promotes carcinogenesis, and up-regulation of PAK1 are been seen in many types of cancer as well as after stroke and other ischemic conditions. This enzyme, primarily acting downstream of the GTPases Cdc42 and Rac phosphorylates or interacts with many cytoskeletal adaptor proteins, cytoskeletal filament proteins, guanine nucleotide exchange factors, integrins, as well as other kinases and phosphatases (Bokoch, 2003; Dummler et al., 2009).

In the last two decades, PAK1 has emerged as a positive regulator of cell motility and migration. Cell migration and invasion through the extracellular matrix is a complex process, involving extensive reorganisation of the cellular actin structure, the degradation of extracellular components using proteases and the disruption of existing cell-cell junctions to facilitate movement. Hormones and growth factors such as insulin and platelet derived growth factors (PDGF) have been shown to be able to recruit PAK1 to into membrane ruffles (Sells et al., 2000). Expression of constitutively active PAK1 induced the formation of large membrane ruffles, as well as filipodia in Swiss 3T3 fibroblasts and REF52 cells (Sells et al., 1997). It is at these leading edges of cells and membrane ruffles that PAK1 acts on downstream targets to facilitate cell motility. The microinjection of constitutively active PAK1 into both Hela cell and Swiss 3T3 fibroblasts caused the loss of stress fibres along with loss of focal adhesions. These results were similar to the effect produced upon the injection
of Cdc42 or Rac into cells (Manser et al., 1997). Further studies where the PAK1 auto-inhibitory domain was over-expressed in order to induce inhibition of the enzyme produced the opposite results to those shown above (Zhao et al., 1998). The dynamics of cellular microtubules are involved in many cellular processes such as chromosomal segregation, motility and cell morphology. PAK1 directly interacts with and phosphorylates tubulin cofactor B (TCoB). This phosphorylation is essential for the proper polymerization of new microtubules (Vadlamudi et al., 2005). PAK1 has been demonstrated to be important in neuronal development, including the formation of dendritic spines (Kreis and Barnier, 2009). Pak1 has been shown to co-localise with the postsynaptic density protein 95 (PSD95) in dendritic spines. PSD95 is a membrane associated guanylate kinase (MAGUK) scaffolding protein located in neural postsynaptic densities, colocalisation of PAK1 with this protein suggests that it is involved in synaptic development (Hayashi et al., 2007).

The spatial-temporal activation pattern of PAK1 is consistent with it having a role in actin reorganisation during cell movement (Sells et al., 2000). Studies in the fibroblast cell line NIH-3T3 and in human micro-vascular endothelial cells (HMVEC) showed that the expression of constitutively active PAK1 increased myosin light chain (MLC) phosphorylation as well as cell migration (Kiosses et al., 1999; Sells et al., 1999). PAK1 and PAK2 are also strongly activated by the chemoattractant fMLP in human neutrophils (Ding et al., 1996). The chemokine CXCL1 induces Cdc42 and PAK1 activation resulting in increased chemotaxis. CXCL1 induced chemotaxis was inhibited by the expression of dominant negative PAK1 (Wang et al., 2002a).

Aside from regulation of cell motility, PAK1 has been implicated as having a role as a promoting controller of cycle progression, chromosome dynamics, and normal cell division. In the study referenced above where active forms of PAK1 were over-expressed in MCF-7 cells, the investigators also observed disruptions to normal organisation of mitotic spindles and an abnormal number of centrosomes (Vadlamudi et al., 2000). The Aurora kinases and Polo-like kinase 1 (PLK-1) are both important controllers of mitosis. During mitosis, PAK1 is recruited to centrosomes where it binds GIT1 and PIX, and is activated by GIT1 independently of Cdc42 or Rac1. Activated PAK1 have been shown to phosphorylate and activate Aurora-A and PIK-1 in two independent studies (Maroto et al., 2008; Zhao et al., 2005). The activation of
Aurora-A caused altered centrosomes formation, whereas activation of PLK1 is important in the establishment of functioning bipolar spindles. PAK1 has also been shown to co-localise with Histone H3 on condensing chromatin and phosphorylate it at Ser-10. As this phosphorylation is a required step for the induction of DNA compaction at the start of mitosis, it can be seen that PAK1 may play a pivotal role in the cell cycle (Li et al., 2002).

Another pathway PAK1 is involved in is that of cell survival, acting to down regulate pro-apoptotic signals and promoting cell survival and proliferation. PAK1 have been shown to directly phosphorylate and inactivate the pro-apoptotic protein: BCL2-antagonist of cell death (BAD), thereby preventing it from forming a complex with the anti-apoptotic proteins Bcl2 and Bcl-X. This leads to increased cell survival in the FL5.12 and NIH 3T3 cell-lines tested. Dynemin light chain (DLC1) and its interacting protein BimL (a Bcl-2 family protein) have also been reported to inhibit Bcl-2, PAK1 has also been shown to phosphorylate both these proteins leading to their inactivation and preventing BimL binding to Bcl-2 (Vadlamudi et al., 2004). The pro-proliferation transcription factor NFkappaB is activated by PAK1, however the exact mechanism by which this activation happens is still under investigation (Balasenthil et al., 2004; Dadke et al., 2003; Friedland et al., 2007). PAK1 also directly phosphorylates and inactivates the transcription factor FKHR (forkhead transcription factor, also known as Foxo1) which promotes the transcription of pro-apoptotic genes. After FKHR is phosphorylated by PAK1, it is confined to the cytosol and therefore unable to perform its actions (Mazumdar and Kumar, 2003).

1.2.5 PAK1 activation and expression in cancer

From the description in the above section about the physiological roles of PAK1 in promoting cell survival, proliferation, migration and angiogenesis, it can be seen that stimulation of the activity/expression of this kinase can promote tumour formation. PAK1 has been shown either to be overexpressed or abnormally activated in a variety of human cancers (Table 1.2). Of all types of tumours, the abnormal PAK1 signalling is best understood in breast carcinoma and neurofibromatosis, although it is also implicated in the development of other cancers such as melanomas, lung and endometrial cancers (Lu et al., 2013; Ong et al., 2011a; Ye and Field, 2012).
Of the 6 PAK isoforms, PAK1 is most often over-expressed in cancers. PAK1 overexpression has been observed in more than 50% of human breast cancers and around 70% of colorectal cancers (Table 1.2) (Carter et al., 2004). There is also a correlation between high grade breast cancers with both the protein level and kinase activity of PAK1 (Vadlamudi et al., 2000). In a study of the tumorigenic progress of MCF10A mammary epithelial cells, the level of PAK1 expression positively correlated with the stage of progression into cancer cells. This suggests that abnormal PAK1 activity/expression not only has a function in active tumours, but also has role to play in tumorigenesis (Li et al., 2008). Recently, a study of primary tissue sample from melanoma patients with the wild-type BRAF gene showed that PAK1 protein was over-expressed in 27% of samples (Ong et al., 2013). Additionally, PAK1 overexpression has also been reported in squamous non-small cell lung carcinomas (NSCLCs) and lymph node metastasis of breast cancers (Ong et al., 2011a). Two possible mechanisms that underlie the overexpression of PAK1 in cancers have been explored, the first being gene amplification and the second is micro-RNA down regulation. The PAK1 gene is located within the 11q13 genomic region, and amplification of this region has been reported in a number of cancers including breast, bladder, and ovarian carcinomas (Bekri et al., 1997; Bostner et al., 2007). MicroRNAs such as miR-7 have been shown to act as negative regulators of PAK1. Transfection of the microRNA miR-7 into breast cancer cells reduced PAK1 expression. Additionally, endogenous miR-7 levels inversely correlated with that of PAK1 (Reddy et al., 2008). Although there is much evidence of PAK1 over-expression in cancers, there is significantly less evidence for the existence of PAK1 activating mutations in tumour cells. While a mutation of PAK4 has been found in colorectal carcinoma, its effect on the activity of the enzyme is not clear (Parsons et al., 2005).

In addition to regulating cell migration in physiological tissues, PAK kinases have been implicated as pro-invasion regulators in a number of human cancers. Expression of an active form of PAK1 in MCF-7 breast cancer epithelial cells led to increased rate of cell migration (Vadlamudi et al., 2000). In another study, where siRNA was used to deplete PAK1 and PAK2 individually in T47D breast cancer cells, it was found that both PAK1 and PAK2 promoted cancer invasion (Coniglio et al., 2008). Interestingly, the two PAK enzymes seem to mediate their effects thorough different
mechanisms. For example, PAK1 knockdown had a significant inhibitory effect on the lamellipodia protrusions on the motile edge of cell, as well as the size of focal adhesions, in contrast after PAK2 depletion, lamellipodia protrusions were not as strongly inhibited and there was actually an increase in the size of focal adhesions. PAK1 depletion also caused dephosphorylation of coflin (a protein involved in actin disassembly in cytoskeletal reorganisation) whereas PAK2 knockdown did not. In another study, introduction of the microRNA miR-7 (which has been shown to negatively regulate PAK1 expression) reduced parameters such as growth, motility, invasiveness and the tumorigenic potential of highly invasive breast cancer cells (Reddy et al., 2008).

In addition to facilitating cancer cell invasion, PAK1 also plays a role in promoting proliferation of malignant cells and the growth of tumours. Over-expression of PAK1 in endometrial cell-lines increased the proliferation of those cells whereas RNAi mediated knockdown of PAK1 reduced cellular proliferation (Lu et al., 2013). Treatment of melanoma tumour xenograft models with the PAK1 inhibitor PF-3758309 resulted in 93% and 63% inhibition in tumour growth for SK-MEL23 and 537MEL xenografts respectively (Ong et al., 2013). In NSCLCs, inhibition of PAK1 led to delayed cell-cycle progression, and dual inhibition of PAK1 and X chromosome-linked inhibitor of apoptosis increased apoptosis of these cells (Ong et al., 2011a). Recently, RNAi knockdown of PAK1 has been reported to inhibit the growth, angiogenesis and migration of PC3 prostate cancer cells (Goc et al., 2013).

Neurofibromatosis types 1 and 2 are autosomal dominant inherited disorders where neurocrest derived cells (such as Schwann cells) form neurofibromas. They are caused by loss of function mutations in the NF1 and NF2 genes, which act to inhibit neural cell growth. PAK1 directly phosphorylates the NF2 gene product merlin, which inhibits its growth suppressive abilities (Kissil et al., 2002). There is also a reciprocal mechanism by which merlin regulates PAK1. Merlin is able to bind inactive PAK1, thereby preventing its activation by Rac (Kissil et al., 2003; Kreis and Barnier, 2009). It can be seen that after the loss of function of NF2 during tumorigenesis, PAK1 activity becomes less regulated. This causes increased activity of the Rac signalling pathway and leads to increased membrane ruffling and cell proliferation (Kissil et al., 2003; Pelton et al., 1998; Shaw et al., 2001).
From the roles that PAK1 play in cancer highlighted above, it can be seen that this kinase is a viable target for the development of anti-tumour agents. The development of small molecule PAK1 inhibitors is currently underway and if fruitful could help combat a number of cancers (Goc et al., 2013).

<table>
<thead>
<tr>
<th>Location of primary neoplasm</th>
<th>Type of alterations</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>Increased phosho-PAK1 levels</td>
<td>(Aoki et al., 2007)</td>
</tr>
<tr>
<td>Breast</td>
<td>Gene amplification/over-expression</td>
<td>(Balasenthil et al., 2004; Bekri et al., 1997; Holm et al., 2006)</td>
</tr>
<tr>
<td>Lung (NSCLC)</td>
<td>Protein overexpression</td>
<td>(Ong et al., 2011a)</td>
</tr>
<tr>
<td>Liver</td>
<td>Gene amplification/over-expression</td>
<td>(Ching et al., 2007)</td>
</tr>
<tr>
<td>Kidney</td>
<td>Protein over-expression and increased PAK1 activity</td>
<td>(O'Sullivan et al., 2007)</td>
</tr>
<tr>
<td>Colorectal</td>
<td>Protein over-expression</td>
<td>(Carter et al., 2004; Parsons et al., 2005)</td>
</tr>
<tr>
<td>Bladder</td>
<td>Gene amplification/over-expression</td>
<td>(Ito et al., 2007)</td>
</tr>
<tr>
<td>Ovarian</td>
<td>Gene amplification/over-expression</td>
<td>(Brown et al., 2008; Davidson et al., 2008)</td>
</tr>
<tr>
<td>T-cell lymphoma</td>
<td>Gene amplification</td>
<td>(Mao et al., 2003)</td>
</tr>
<tr>
<td>Neurofibromatosis</td>
<td>Dysregulation of activity</td>
<td>(Hirokawa et al., 2004; Kissil et al., 2003; Pelton et al., 1998; Shaw et al., 2001)</td>
</tr>
<tr>
<td>Melanoma (wild-type BRAF)</td>
<td>Overexpression</td>
<td>(Ong et al., 2013)</td>
</tr>
</tbody>
</table>

Table 1.2 Cancers with alterations in PAK1 activity/expression
This table was adapted from (Dummler et al., 2009), with additional information from other sources

1.2.6 PAK1 and AMPK

As can be seen from the above sections, AMPK and PAK1 are involved in the regulation of some very similar processes. Both enzymes regulate the survival of cells under both physiological and pathological situations (e.g. in cancer). PAK1 and AMPK both play a role in the regulation of normal cell migration and cancer cell invasion, with evidence that both enzymes are pro-migratory. With these overlapping
roles, it is possible that the AMPK and PAK1 signalling pathways may intersect at some point. However, it was only relatively recently that these new roles for AMPK have been discovered and the ongoing paradigm in the field remains that AMPK’s main role is as a regulator of metabolic processes. This perhaps explains why until recent years, little research was done to investigate any potential interplay between the AMPK and PAK signalling pathways, and only a handful of papers have been published which link the two pathways.

The first study to implicate AMPK as having a role in regulating PAK activity was conducted in 2008 (Lee et al., 2008). The researchers showed that treatment of C2C12 mouse myotubes with retinoic acid (a Vitamin A metabolite) or AICAR resulted in activation of AMPK, Rac1 and also PAK in a time dependent fashion. Pre-treatment with the AMPK inhibitor, Compound C abolished retinoic acid induced Rac1 activation, therefore suggesting that Rac1 and PAK are downstream targets of AMPK. Rather strangely, this paper does not specify which isoform of PAK was activated by AMPK, information from the material and methods sections would suggest that it is group-1 PAK (Kreis and Barnier, 2009). Furthermore, retinoic acid activation of PAK1 resulted in activation of its target cofilin (Delorme et al., 2007). This effect was blocked by pre-treating C2C12 cells with either compound C or siRNA against the AMPK α1 subunit. As cofilin is an actin binding factor primarily involved in actin assembly, the findings of this study would suggest a role for AMPK in regulating cytoskeletal arrangement by regulating the Rac1-PAK pathway. Further evidence suggesting a role for AMPK in the regulation of the Rac1-PAK1 axis was published in 2011, while work on this thesis was on going. In this publication, the researchers found AMPK activation by AICAR led to increased phosphorylation of PAK1/2 (on Thr-423 and Thr-402 respectively) and WAVE2 which are downstream effectors of Rac1. These and other effects of AMPK activation led to increases in the phagocytic ability of macrophages and neutrophils. Pre-treatment of cells with AMPK α1 siRNA, compound C or Rac inhibitors was able to abolish the increase in phagocytosis induced by AICAR (Bae et al., 2011).

It can be seen from the studies described above that AMPK is likely to have a role in regulating PAK1 activity. However, results from both these studies would suggest that this regulation is indirect acting via Rac1, as in both cases activation of Rac1
preceded PAK activation. In addition, treatment with a Rac1 inhibitor was able to reverse the effects brought about by AMPK activation.

A recent study using a novel chemical genetics approach to screen for AMPK substrates identified PAK2 as being target of AMPK. AMPK α2 directly phosphorylated PAK2 on Ser-20 (Banko et al., 2011). Further to this, elevation in the levels of myosin regulatory light chain induced (MLC) by the AMPK activator A769662 in U2OS cells was reversed by the co-expressing a non-phosphorylatable version of PAK2 (S20A). This result would suggest that the ability of AMPK to regulate MLC is at least partly mediated by PAK2. However, to our knowledge, there is currently no published evidence to implicate any direct interaction of AMPK and PAK1 and any direct mechanism by which these enzymes regulate each other.

Although perhaps not directly relevant to AMPK, both of the upstream kinases of AMPK, LKB1 and CAMKK beta have been shown to regulate PAK1 activity in vivo (Saneyoshi et al., 2008). In a 2008 study by Saneyoshi and colleagues, CaMKK beta and CaMK1 were shown to interact with the PAK1/Git1/βPIX complex which results in the phosphorylation of βPix at Ser-516 which in turn led to activation of the Rac1-PAK1 axis. This activation then led to increased formation of spines and synapses in hippocampal neurons. Inhibition of either CaMKK beta of CaMKI by pharmacological inhibitors, siRNA treatment or expression of dominant negative isoforms in hippocampal neurons resulted deceased spine formation, as did expression of the βPIX S516A mutant form. Expression of constitutively-active PAK1 on the other hand was able to reverse the inhibition of spine formation caused by the inhibition of CaMKI or the βPIX S516A mutant. While the above study revealed that CaMKK beta positively regulated PAK1 activity, LKB1 was shown to have the opposite effect. LKB1 was found to directly phosphorylate PAK1 on The-109 in the P21 binding domain which resulted in inhibition of its kinase activity. Additionally, knockdown of LKB1 in HCT116 colon cancer cells caused an increase in PAK1 activity while enhancing migration of these cells, and expression of LKB1 in LKB1 null MEFs led to both decreased PAK1 activity and PAK1 mediated cell migration. Furthermore, the non-phosphorylatable PAK1 T109E mutant was resistant to LKB1 inhibition (Deguchi et al., 2010). Taken together the results of these would suggest that while the predominant effect of interplay between the AMPK and PAK1
signalling pathways results in activation of the Rac1-PAK1 axis, LKB1 seems to exert an inhibitory effect, which is in-line with its effects as a tumour suppressor.

The AMPK related kinase SAD-A (synapses of amphids defective/aka BRSK2) has recently been shown to interact with PAK1 and directly phosphorylate it at the key activation residue Thr-423 (Nie et al., 2012). This phosphorylation leads to an elevation of glucose-stimulated insulin secretion from pancreatic β islet cells. Decreasing either PAK1 activity or expression was able to abolish the above effect.

1.3 Summary and aims

Gaining a full understanding of the function of AMPK and its regulation is very important for devising new treatments for diseases of metabolism and cancer. As outlined in this chapter, the primary way by which AMPK is activated is through phosphorylation of the Thr-172 residue within the α subunit. At the time of this study, LKB1 and CaMKKβ were identified as the upstream kinases of AMPK, whereas the protein phosphatase(s) responsible for the dephosphorylation of AMPK remain unidentified. The aim in the first part of this thesis was to identify the protein phosphatase(s) acting on AMPK using both transformed cell-lines and primary mammalian cells and investigate the physiological significance of this regulation.

In the second part of this study, quantitative mass spectrometry techniques were utilised in order to identify AMPK interacting proteins, initially with a view of identifying AMPK phosphatases. From the results generated, PAK1 was identified as a novel interactor of AMPK. Following validation of this interaction the functional and physiological significance of the association between AMPK and PAK1 was investigated.
2 Materials and Methods

2.1 Materials

2.1.1 General Reagents

Adenosine monophosphate (AMP), Adenosine diphosphate (ADP), Adenosine triphosphate (ATP), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Tris(hydroxymethyl) aminomethane (Tris), ethylene glycol tetraacetic acid (EGTA), ammonium persulphate, magnesium chloride (MgCl₂), sodium fluoride (NaF), sodium pyrophosphate (Na₄P₂O₇), dithiothreitol (DTT), N,N,N’,N’-tetramethylethylenediamine (TEMED), protein A/G-sepharose, sorbitol, β-mercaptoethanol, water soluble dexamethasone, 3,3’,5-Triiodo-L-tyronine sodium salt (T₃), BSA, agarose, N-cyclohexyl-3-aminopropanesulfonic acid (CAPS), glycine, D-mannitol, ionomycin, N,N dimethyldecyl amine N oxide (DDAO), Taq DNA polymerase, NP-40, ampicillin, perchloric acid, potassium phosphate, hydrogen peroxide, Dimethyl sulfoxide (DMSO), Triton X-100, 1-1,dimethyl biguanide hydrochloride (metformin), Calyculin A and Kodak biomax MR-1 film were obtained from Sigma (Poole, UK). Polyvinylidene difluoride (PVDF) membrane was from Perkin Elmer (Beaconsfield, UK). AICA-Riboside (AICAR) and Okadaic acid, were from Calbiochem (Nottingham, UK). Ribojuice siRNA transfection reagent was from Novagen (Nottingham, UK). Plasmid miniprep, maxiprep, polymerase chain reaction (PCR) and gel purification kits, QIAquick gel extraction kit, RNeasy columns, quantitetc primers and quantitative reverse transcription PCR (qRT-PCR) kit were from Qiagen (Crawley, UK). Cal Phos transfection kit was from Clontech (Saint-Germain-en-Laye, France). NaOH, sodium lauryl sulfate (SDS), NaCl, Tween 20, glycerol, ethylenediaminetetraacetic acid (EDTA), ethanol, methanol, KCl, KH₂PO₄, NaHCO₃, chloroform and glucose were from VWR (West Sussex, UK). Restriction enzymes were from Promega (Southampton, UK) and New England Biolabs (Hitchin, UK). 3MM chromatography paper and P81 phosphocellulose paper were obtained from Whatman (Maidstone, UK). RPMI, Neurobasal media, DMEM, non essential amino acids, Penicillin/Streptomycin, insulin, sodium pyruvate, optiMEM, and B27
supplement were from Gibco (Paisley, UK). Complete EDTA free protease inhibitor cocktail, collagenase H and Rapid DNA ligation kit were from Roche (Burgess Hill, UK). SAMS peptide (HMRSAMSGLHLVKRR) was synthesised by the MRC Peptide Synthesis Unit. Dc Protein assay kit was from Biorad (Hertfordshire, UK). [γ-32P]ATP (6000 Ci/mmol), and Amersham Hyperfilm MP film, was obtained from GE healthcare (Amersham, UK). ECL Supersignal West Femto kit, dialysis cassettes and Dharmacon siGLO Red transfection indicator, Control non-targeting siRNA and targeting siRNAs (Silencer Select siRNA) were obtained from Ambion (Invitrogen). Active PAK1 and PAKtide peptide was obtained from Stratech (UK). Myelin basic protein was obtained from Sigma. 13C arginine and 13C lysine for SILAC media supplementation was obtained from CK Gas (Leicestershire, UK).

2.1.2 Buffers

All chemicals for buffers were of experimental grade and were either bought from commercial sources or made in house:

**HST (high salt buffer)** – 20mM Tris, pH 7.4, 500mM NaCl, 0.5% (v/v) Tween 20

**Protein Sample Buffer** – 50mM Tris-HCl, pH 7.4, 2.5% (w/v) SDS, 1% (v/v) β-mercaptoethanol, 10% glycerol, 0.05% (w/v) bromophenol blue.

**SDS-PAGE Buffer** – 50mM Tris-HCl, pH 8.4, 400mM glycine, 0.1% (w/v) SDS.

**Tris/Glycine Buffer** – 25mM Tris, 192mM glycine, 10% Methanol.

**CAPS transfer buffer:** 10 mM CAPS (N-cyclohexyl-3-aminopropanesulfonic acid) powder (pH 10.5), 10% methanol

**BufferA/Homogenisation Buffer** - 50mM Tris, pH 7.4, 50mM sodium fluoride, 5mM sodium pyrophosphate, 1 mM EDTA, 250mM sucrose, 1mM dithiothreitol, 4µg/ml trypsin inhibitor, 0.1mM phenylmethylsulfonyl fluoride, 1mM benzamidine.

**Cell Lysis Buffer** - 50mM Hepes pH7.4, 50mM sodium fluoride, 5mM sodium pyrophosphate, 1mM EDTA, 10% (v/v) glycerol, 1% triton TX-100, 1mM dithiothreitol, 4µg/ml trypsin inhibitor, 0.1mM phenylmethylsulfonyl fluoride, 1mM benzamidine.
**Buffer C (HGE)** - 50mM Hepes pH 7.4, 1mM EDTA, 10% (v/v) glycerol and 1% triton TX-100, 1mM dithiothreitol, 4µg/ml trypsin inhibitor, 0.1mM phenylmethylsulfonyl fluoride, 1mM benzamidine.

**Bacterial lysis buffer**: 50 mM Hepes pH 7.4, 300 mM NaCl, 25 mM Imidazole

**Bacterial elution buffer**: 50 mM Hepes pH 7.4, 300 mM NaCl, 300 mM Imidazole

**Storage buffer**: 50 mM Hepes pH 7.4, 300 mM NaCl, 1mM Tris(2-carboxyethyl) phosphine Hydrocholride (TCEP)

**DNA-Loading Buffer** – 0.1% (m/v) Orange G, 50% glycerol

**Phosphate-Buffered saline (PBS)** – 137mM NaCl, 2.7mM KCl, 4.3mM Na₂HPO₄, 1.4mM KH₂PO₄, pH 7.4.

**TAE** – 40mM Tris, 20mM acetic acid, 1mM EDTA, pH 8.0.

**Luria Bertani (LB) Broth** – 1% (w/v) tryptone, 0.5% yeast extract, 0.5% (w/v) NaCl.

**LB agar plate**: As LB broth with 1.5% (w/v) agar and the appropriate antibiotic added

**SOC media**: 20 mM glucose, 2% (w/v) tryptone, 0.5% (w/v), yeast extract, 0.05% (w/v) NaCl

### 2.1.3 Antibodies

Primary antibodies used for immunoblotting are as follows: anti-AMPK-α₁ and anti-AMPK-α₂ (sheep) (kind gift from Professor Grahame Hardie, Dundee), anti-AMPK-β (in house, Rabbit), anti-phospho-AMPK (P-Thr-172) (Cell Signalling), anti-PPM1A (Acris), anti-PPM1B (Abcam), anti-ILKAP(Acris), anti-V5 (Invitrogen), and anti-FLAG M2 (Sigma UK), anti PP2A (Cell Signalling), anti-HA tag (C29F4) (Cell signalling), anti-total PAK1 (Cell signalling), anti-phospho-PAK1 (T423) (cell signalling), anti-phospho-PAK1 (S199) (Cell signalling), anti PSD95 (Abcam), anti-GIT1 (Cell signalling), anti-PIX (Cell Signalling). All dilutions of primary antibodies were at 1:1000, except Anti-AMPK-β which was at 1:5000.
2.1.4 Cells

**Bacterial:**
JM109 competent cells were from Promega (Southampton, UK). BL21-Condonplus (DE3) RIL cells for protein expression were from Agilent Technologies (Cheshire)

**Mammalian:**
HEK-293 cells were purchased from American Cell Type Culture Collection (Middlesex). COS-7 cells were available in house. MIN6 β cells were a gift from James Leiper (MRC). WM1361, WM1366 and WM1791c, WM266.4 cells were a gift from Richard Marais (CRUK). Primary mouse hepatocytes were isolated by Angela Woods. Primary mouse cortical neurones from both WT and AMPK α1 KO mice were isolated by Shuai Zhang and Nicola Bright in house.

2.1.5 Proteins

Recombinant AMPK complex ($\alpha_1\beta_1\gamma_1$) was expressed in Eschericha coli and purified by David Carmena or Shuai Zhang as in sections 2.2.7 and 2.2.8. Recombinant wild type and mutant PAK1 proteins were expressed in Eschericha coli and purified as described in sections 2.2.7 and 2.2.8.

2.1.6 Compounds

The AMPK activator A-769662 (a kind gift from Astrazeneca, UK) was dissolved in DMSO and kept for a maximum of 2 weeks at -20 ºC with only one freeze thawing cycle. STO-609 was obtained from Torris (Ellisville, Missouri) was dissolved in DMSO prior to treatment. Okadaic acid was obtained from Calbiochem (Subsidiary of Merck, Darmstadt, Germany) Calyculin A was obtained from Sigma. IPA-3 was obtained from Tocris.

2.1.7 Plasmids

Mammalian expression constructs were cloned into pcDNA3.1 vector (Paisley, UK). Bacterial expression constructs for PAK1 were cloned into the pETduet vector which were available in house.
2.1.8 Mice

Both wild type (AMPKα1+/+) and AMPKα1−/− mice were available in house (originally generated by Benoit Viollet, Université Paris Descartes) and has been derived as reported previously (Jørgensen et al., 2004).

2.2 Methods

2.2.1 Agarose gel electrophoresis

DNA material was resuspended in 10x Loading buffer (Invitrogen) and resolved on a 1% agarose gel at 100V. Band were visualised with UV light.

2.2.2 Polymerase chain reaction (PCR) and DNA Cloning

DNA was amplified by PCR using the high fidelity Pfu enzyme with 50ng of template and a total reaction volume of 50 μl. Qiagen gel extraction kit was used to exercise DNA from gels. The PCR products were digested with the appropriate restriction enzymes and cloned into pETduet vectors for bacterial expression and pcDNA3.1 vectors for mammalian expression.

2.2.3 Site Directed Mutagenesis

Point mutations in plasmids were generated using the QuikChange II Site-Directed mutagenesis Kit according to the manufacturer’s instructions. Briefly DNA template was mixed with forward and reverse primers containing the relevant mutation/s with the appropriate buffers after which the Pfu DNA polymerase was added to the reaction mixture. The mixture (50 μl) was subject to PCR reaction with the conditions as follows: 95 ºC for 30 seconds, 12 or 18 cycles (12 cycles for point mutations and 18 cycles for mutable amino acid changes) of 95 ºC for 30 seconds, 55 ºC for 1 minute and 68 ºC for 1 minute per kilobase of plasmid. After cycling a final extension step of 5 minutes at 68 ºC is applied. The mixture incubated with 1ul of DPN1 endonuclease at 37 ºC for 2hr to digest the methylated template DNA (which has been purified from bacteria). The digested mixture is then transformed into XL10- Gold Supercompetent cells according to manufacturer’s protocols. The DNA is then
purified using the Qiagen mini-prep kit and the mutations confirmed by DNA sequencing (MRC, CSC Sequencing Service).

### 2.2.4 Bacterial transformation and culture growth

For JM109 cells transformation 50 μl of cells were mixed with 50 ng or 100 ng of vector, the mixture was incubated on ice for 15 minutes and heat shocked at 42 °C for 90 seconds. After a further incubation of 2 minutes on ice, 750 μl of SOC media was added to the mixture and incubated at 37 °C for 30 min with shaking.

For BL21 cells a 100ul aliquot of cells were incubated with 0.2 μl β- mercaptoethanol on ice for 10 minutes. 50 μg of plasmid DNA was then added to the mixture and incubated on ice for a further 30 minutes. The cells were then heat shocked at 42 °C for 20 seconds, and mixed with 750 μl pre-heated SOC media and incubated at 37 °C with shaking.

For XL-10 Gold cells, 50ul of cells were mixed with 1μl of PCR reaction product and incubated on ice for 30 minutes. The mixture was then heat shocked at 42 °C for 45 seconds, and then incubated on ice for a further 2 minutes. 750 μl of pre-heated SOC media was then added and incubated at 37 °C with shaking.

For all cell type described above, after transformation, cells are spun at 15,000 rpm and the pellet resuspended in 100 μl of LB media and plated on LB agar plates with the appropriate antibiotic added (e.g. 100 μl/ml ampicillin and 50 μg/ml kanamycin). The plated are incubated for 24 hr at 37 °C. A single colonies were then used to inoculate LB broth with the appropriate antibiotics.
2.2.5 Determining DNA/RNA concentration and DNA sequencing

The concentration of DNA/RNA concentration was determined using the NanoDrop ND-1000 spectrometer for an absorbance at 260 nm.

Sequencing for expression plasmids were done by the MRC CSC Sequencing Service with the 3730xl DNA analyser and viewed with the DNASTar program.

2.2.6 Expression and purification of recombinant proteins in E.coli

The vectors for the expression of Wild-Type and mutant forms of His-AMPK, GST-PAK1 construct (co-expressed with untagged PP1 in the pETduet vector) (kind gift from Dr Katrin Rittinger, NIMR, MRC) were transformed into BL-21 CodonPlus (DE3)-RIL cells were done as described Chapter 2 and plated onto agar plates containing the appropriate antibiotics.

A single colony is picked from the plate and incubated in a starting culture (25 ml) of antibiotic inoculated LB media. After incubation overnight with shaking at 37 °C the starting culture was used to seed a larger culture (500 ml to 2000 ml), the size of which depends on efficiency of expression of the particular protein and the amount of protein required for experimental purposes. The culture was incubated at 37 °C with shaking until an OD600 of 0.6 – 0.8 was obtained (as blanked by fresh LB media). In the case of AMPK complexes, protein expression was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) to the mixture to a final concentration of 1 mM, and the bacterial suspension incubated for 4 hours at 25 °C. In the case of PAK1 complexes, IPTG was added to the mixture to a final concentration of 0.4 mM and the bacterial suspension incubated for over night (approximately 12 hours) at 25 °C.

After IPTG induction of expression overnight, the bacteria was pelleted by centrifugation at 6000 rpm. The pellet was washed once in PBS buffer and resuspended in bacterial lysis buffer containing protease inhibitor cocktail (EDTA free) (Roche). The mixture was sonicated for 8 cycles (1 minute on 1 minute off) on ice to release proteins. The suspension was then taken through a freeze thawing cycle at -80 °C, after which Triton TX-100 was added to the mixture to a final concentration of 1% (v/v) and incubated for 30 min at 4 °C. The suspension then was the centrifuged
at 15,000 rpm to remove the bacterial cell bodies and at 4 °C for 30 min. With the pellet discarded the supernatant was filtered with a 0.4 μm filter, and kept either at -80 °C or 4 °C.

For purification using a manual column, either nickel-Sepharose (for AMPK purification) or glutathione (for PAK1 purification) containing columns was equilibrated with bacterial lysis buffer and then incubated with the supernatant for 30 min at 4 °C for binding. The column is then washed with 3x volume of lysis buffer and the bound protein eluted with bacterial elution buffer, with fraction taken for every 1ml of the elution. A aliquot (usually 15 μl) of every eluted fraction is then visualised/analysed using SDS-PAGE electrophoresis, and eluted proteins concentrated using a VivaSpin column (Vivascience). The protein was washed into storage buffer with the spin column also to rid the sample of imidazole.

In the case of automated purification with the ACTA system (for some AMPK complexes), the nickel sepharose column was equilibrated with 6x column volumes of Bacterial Lysis buffer before protein binding. The bacterial supernatant was then ran through the column at a rate of 1ml/min, to allow protein binding. The column was then washed with 5x column volumes of lysis buffer and the protein eluted with Bacterial elution buffer (20 ml – 30 ml) into fraction of 1ml each. SDS-PAGE electrophoresis is then used to identify which fractions contain the desired protein and the protein was concentrated and buffer exchanged into storage buffer using Vivaspin columns.

In some cases the eluted proteins were then subjected to gel-filtration purification on a HiLoad Superdex 200 column (Amersham Biosciences) linked to the ACTA system. The column was first equilibrated with Storage buffer, and the eluted protein ran through this column at 1 ml/min, and fraction of 1ml were taken. The fractions containing the desired proteins were pooled and concentrated using the Vivaspin columns. The concentration of bacterially expressed proteins were identified using NanoDrop ND-1000 spectrometer for an absorbance at 280 nm (with storage buffer acting as a blank for the measurement).
2.2.7 Radio-labelling of proteins and auto-radiography

Proteins were incubated in the presence or absence of activated kinase (either active PAK1 or AMPK) in buffer containing 200 μm [γ-32P] ATP, 2.5 mM MgCl₂ for 30 min at 30 °C (for phosphorylation with active PAK1) or 37 °C (for phosphorylation with active AMPK). The reaction was terminated by the addition of protein sample buffer and resolved by SDS-PAGE. The gels were coomassie blue stained, dried and subjected to auto-radiography.

2.2.8 Activation of AMPK by CaMKKβ

Purified bacterially expressed AMPK (inactive) was incubated with CaMKKβ at a ratio of 1:200 (e.g. 1 μg CaMKKβ with 200 μg AMPK) in the presence of buffer containing 1 mM ATP and 2.5 mM MgCl₂ for either 1 hour at 37 ºC or overnight at room temperature. Titrations from the resulting mixture was taken into the SAMS peptide assays to validate activation of AMPK.

2.2.9 PAK1 activation of AMPK

Active PAK1 (200 ng) was incubated with CaMKKβ phosphorylated AMPK (2 μg) for 30 minutes at 37 ºC with shaking, this was done in buffer containing 1 mM ATP and 2.5 mM MgCl₂ for a total volume of 50 μl. After this, 2 μl of the reaction mixture was diluted to 10ul with 50 mM Hepes buffer then taken into the SAMS Peptide assay.

2.2.10 Kinase activity assays

SAMS peptide assay

The activity of AMPK was measured by the phosphorylation of the synthetic SAMS peptide (HMRSAMSGLVLKRR), by the incorporation of [γ-32P]-ATP. The SAMS peptide has a sequence derived from the AMPK phosphorylation sites on Acetyl-Co-A carboxylase. Samples were made up to 25 μl in the presence of 50 mM Hepes pH 7.4, 200 μM SAMS 10mM Hepes, 0.2 % Triton, 5 mM MgCl₂, and 200 μM ATP, [γ-32P]ATP (specific radioactivity approximately 250 cpm/pmol). This is then incubated at 37 °C for 20 or 30 minutes with shaking. The blank mixture is identical to the
reaction sample mixture with the exception that it does not contain SAMS peptide. After this 20 µl of the mixtures were then blotted onto a piece of P81 paper (approximately 1cm² in area). The P81 paper is then washed with 1% (v/v) phosphoric acid to remove unincorporated ³²P-ATP. The paper is then air-dried and placed in scintillation tubes and covered with 2 ml of scintillation fluid and placed in a Beckman scintillation counter (Beckman LS60000SC), which determines the counts per minute.

**PAK1 kinase assays (MBP and PAktide assays)**

The activity of PAK1 was measured by the phosphorylation of the synthetic PAKtide (RRRLSFAEPG), by the incorporation of [γ-³²P]-ATP. The PAKtide has a sequence derived from known PAK1 phosphorylation sites. Samples were made up to 25 µl, with either 1 mg/ml Myelin basic protein (for MBP assay) or 0.5 mg/ml PAktide (for PAktide assay) in the presence of 50 mM Heps pH 7.4, 2.5 mM glycerol phosphate, 5 mM MgCl₂, and 200 µM ATP, [γ-³²P]ATP (specific radioactivity approximately 250 cpm/pmol). This is then incubated at 30 °C for 10 or 20 minutes with shaking. The blank mixture is identical to the reaction sample mixture with the exception that it does not contain PAktide peptide. After this 20ul of the mixtures were then blotted onto a piece of P81 paper (approximately 1cm² in area). The P81 paper is then washed with 1% (v/v) phosphoric acid to remove unincorporated ³²P-ATP. The paper is then air-dried and placed in scintillation tubes and covered with 2 ml of scintillation fluid and placed in a Beckman scintillation counter (Beckman LS60000SC), which determines the counts per minute.

**2.2.11 Mammalian Cell culture**

HEK293 (Human Embryonic Kidney) and COS7 were cultured in high glucose DMEM (4.5 g/L) (Gibco-41966) supplemented with 10% Fetal Calf Serum (FCS) and N-glutamine (4mM) at 37°C and 5% CO². Cells were changed into fresh media without FCS supplementation 4 hours prior to any cell treatments.

Mouse insulinoma pancreatic cells (MIN6) was kindly provided by Dr. James Leiper (CSC MRC, UK). MIN6, WM1366 and WM1361 cells were grown and maintained in high glucose DMEM (4.5 g/L)(Gibco-41966), supplemented with 10% FCS and N-
glutamine (2 mM), 100 μM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C and 5% CO₂. For glucose concentration exchange experiments, the cell maintenance media was removed from MIN-6 cells and the cells washed once with PBS. The cells were the supplemented with fresh media as shown above (but without FCS) with either 3 or 25 mM glucose as indicated.

**Isolation of primary hepatocytes**

Isolation of primary hepatocytes was carried out as previously described (Foretz et al., 1998) with some amendments. Isolation experiments were kindly performed by Dr. Angela Woods and Dr. Alicia Barcia. Anaesthetised mice were cannulated in the inferior vena cava and the liver was perfused with Perfusion buffer at 37 °C (138 mM NaCl, 50 mM HEPES, 5.56 mM glucose, 0.5 mM EGTA, 5.4 mM KCl, 0.338 mM Na₂PO₄, 0.44 mM KH₂PO₄, 4.17 mM NaHCO₃, carboxygenated, pH 7.4). After washing with perfusion buffer for 15 minutes the liver was digested with collagenase buffer (Perfusion Buffer minus EGTA, 5 mM CaCl₂ with 0.025 % (w/v) collagenase H). Digested livers were removed and hepatocytes were released and filtered, and washed twice with ice cold Perfusion Buffer followed by washing with ice cold M199 buffer. Cells were plated onto collagen coated plates in plating medium (M199, Penicillin/Streptomycin, 10% (v/v) bovine serum albumin (BSA), Ultroser G, 100 nM T₃, 100 nM dexamethasone, 100 nM Insulin ). 4 hours later plating medium was removed and replaced with overnight medium (M199, Penicillin/Streptomycin, 100 nM dexamethasone, 1 nM Insulin) and cells were cultured for ≥ 16 hr in this medium. The following day medium was changed to M199 supplemented only with penicillin and streptomycin at least 2 hours before treatment.

**Preparation of primary neurons**

Animal use was in accordance with local rules and with the regulations and guidance issued under the Animals (Scientific Procedures) Act (1986). Primary cortical neurons were prepared from WT (wild-type) and AMPKα₁−/− E14 (embryonic day 14) animals as described previously (Lesuisse and Martin, 2002). Briefly, cortices from embryos in a single litter were dissected, meninges were removed and tissue was pooled. Cortices were roughly chopped before incubation in 0.25% trypsin/EDTA followed by trituration. Cells were pelleted by centrifugation, resuspended and plated in Neurobasal medium supplemented with B27, 100 units/ml penicillin, 100 μg/ml
streptomycin, 0.25 μg/ml amphotericin B, 300 μM glutamine and 25 μM 2-mercaptoethanol. Treatments were carried out on neurons grown for a minimum of 7 days in vitro, and all media were obtained from Invitrogen.

2.2.12 Mammalian cell treatment

HEK293, primary mouse hepatocytes (isolated by Angela Woods), primary mouse embryonic cortical neurons, WM1361 and WM1366 cells were treated with a selection of agents which activate endogenous AMPK including: AICAR (Sigma), Metformin (Sigma), hydrogen peroxide (in House), Ionomycin (Invitrogen) IPA3phenformin (Sigma), A-769662 compound (a kind gift from Astrazeneca), and M991 compound (A kind gift Astrazeneca, originally developed by Merck Sharp & Dohme Corp. and Metabasis Therapeutics, Inc.). Phosphatase inhibitors okadaic acid (Calbiochem) and calyculin A (Sigma) are also used to treat HEK293 and mouse hepatocytes. Primary mouse embryonic cortical neurons were also treated with the AMPK inhibitor compound C and the CaMKKβ inhibitor STO609. Specifically, WM1361 cells were treated with the PAK1 inhibitor IPA3 (Tocris, Bristol, UK). Specific conditions and concentrations are shown in the results section and figure legends. All reagents were dissolved in water with the exception of calyculin A, A-769662 and the M991 compounds which were dissolved in DMSO.

2.2.13 Mammalian cell transfection

For transfection of plasmid DNA into both HEK293 cells and COS7 cells, cells were seeded into 6 well plates at 5 x 10⁴ cells/cm² and transfected the following day using 0.5μg/cm² of plasmid DNA following the standard product protocols (PEI and calcium phosphate). Cells were treated and lysed 48h after transfection for analysis. For transfection of plasmid DNA into MIN6 cells, cells were seeded into 6 well plates at 1 x 10⁵ cells/cm² and transfected the following day with Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. Cells were lysed 48 hours following transfection for analysis.
2.2.14 Small interfering RNA (siRNA) silencing

HEK293 Cells were treated with either mock negative control scrambled siRNA or with 2 different siRNA oligonucleotides (Ambion) designed for the silencing of a particular target gene. Cells were transfected at 50-70% confluency with the indicated siRNA concentrations using the Ribojuice reagent according to the product protocols. Cells were treated lysed after 48hr of incubation.

2.2.15 Generation of Mammalian PPM expression constructs

Constructs for the PPM isoforms PPM1A, PPM1D, PPM1F and PPM1G were obtained from Source Bioscience. The fragment of interest was cut with the appropriate restriction enzymes and Phusion High-Fidelity DNA Polymerase (Finnzymes) was used for the PCR amplification with the conditions as follows; initial denaturing step of 98ºC for 30 seconds, followed by 35 cycles of 98ºC for 10 seconds, 55ºC for 30 seconds and 72ºC for 1--minute, and then a final extension step of 72ºC for 5--minutes. The PCR product was cut and ligated and cloned into the pcDNA3.1/v5-His-TOPO vector using the manufacturer’s protocols. The plasmids was amplified in E.coli JM109 competent cells (Stratagene). A Maxi Prep was carried out using a Qiagen kit. The cDNA was sequenced using the sequencing service at the MRC Clinical Sciences Centre.

2.2.16 Cell lysis

Cells were washed with PBS and lysed into lysis buffer (50mM Hepes pH7.4, 50mM NaF, 5mM NaPP, 1mM EDTA, 10% (v/v) glycerol and 1% triton TX-100, 1mM DTT, 4µg/ml trypsin inhibitor, 0.1mM PMSF, 1mM benzamidine). The process if known as fast lysis and was done as quickly as possible to prevent activation of AMPK. Cell lysates were centrifuged at 18000g for 15min, with the supernatant containing cytosolic proteins being retained.
2.2.17 Protein concentration determination for mammalian cell lysates

Protein concentration for both cell lysates and tissue homogenates was determined using the BioRad (Hertfordshire) Bradford protein assay system according to the manufacturers’ protocol with the absorbance at 595 nm measured using a standard computerised spectrometer device.

2.2.18 Determination of ratio of nucleotides within cells

Cells were first washed with PBS and lysed with 5% (v/v) perchloric acid. Cell lysates were centrifuged at 18000g for 15min, with the supernatant containing the nucleotides being retained. The supernatant was then washed with 10% (of the supernatant volume) of a mixture of 1:1 ratio of 1,1,2 –trichlorotrifluoroethane and tri-n-octylamine. Ion-exchange chromatography was then use to separate the different nucleotides using a SMART chromatography system (Amersham Biosciences). The nucleotides were detected by their absorbance at 254nm and compared with known standards containing nucleotides. The amount of each nucleotide is calculated by the integrated program as the area under each curve peak. The AMP/ADP:ATP ratio is calculated from the above information.

2.2.19 Western blotting

Samples were boiled in electrophoresis sample buffer and resolved on polyacrylamide (10/12%) by SDS-PAGE. Proteins were transferred PVDF membrane (Immobilon-FL, Millipore) at 30 V overnight; subsequently membrane were blocked with PBS containing 5% skimmed milk powder for 1hr.

Antibodies were diluted in 5ml High Salt Tween (HST) buffer (20mM Tris, pH 7.4, 500mM NaCl, 0.5 % Tween (v/v)), and incubated with the membrane for two hours at room temperature or overnight at 4ºC. Primary antibodies were detected using fluorescently linked secondary antibodies (Alexa Fluor, Invitrogen/LICOR IRye antibodies). These were visualised using an Odyssey Infrared Imager (Licor
Biosciences). Quantification was performed using Odyssey infrared imaging software).

2.2.20 AMPK Immunoprecipitation from cell lysates

AMPK was immunoprecipitated from cell lysates before assaying for activity after cell treatments, transfections and siRNA knockdown experiments. Lysate of 100 μg total protein was made up to a total volume of 100 μl with lysis buffer then incubated at 4 ºC for 1-2 hours with 20μl of 50% protein A slurry and 0.5 μl the pan-AMPK-β subunit antibody. The supernatant was removed and the resin was washed 2 times with Heps buffer A (50mM Heps, 1mM EDTA, 10% glycerol, 1mM DTT, 1% Triton, protease inhibitor cocktail) before use in the AMPK-activity assay.

2.2.21 RNA extraction

Cells were plated in 6 well plates and cells were either transfected with siRNA or allowed to grow for 72 hours after plating. Cells were then washed in PBS and scraped into 1ml TRIzol and incubated for 5 minutes at room temperature. Chloroform was added and the cells were inverted for 15 seconds before a further 3 minute incubation. Mixture was then spun at 12000 g for 15 minutes to separate the RNA fraction in the upper aqueous phase. Ethanol was added (0.53 % v/v) before adding this to the RNA easy column. The column was washed with Buffers RW1 and PPE before elution of the RNA in RNase free water. Samples were stored at -80ºC before use.

2.2.22 qRT-PCR

Specific Primers for each target was purchased by Sigma and diluted to the manufacturers required concentrations. RT-PCR reaction was carried out on the Opticon DNA engine 2 using the Qiagen SYBR Green kit as per manufactures instructions. Briefly, 10 ng of total RNA was incubated with SYBR green RT-PCR mastermix, RT mix and appropriate primer assay mix and subjected to reverse transcription, followed by 40 cycles of extension. A no-RT control where RNA was
subjected to a mock reverse transcription reaction was carried out as a control to ensure no genomic DNA contamination.

2.2.23 Immunoprecipitation of phosphatases and in-vitro dephosphorylation assay

Over-expressed PPM proteins were immunoprecipitated from crude cell lysate using 5ul of anti-V5 antibody (Invitrogen) and 50ul of protein G slurry made to a total volume of 1ml. Binding was allowed to take place for 6hr at 4 C; the supernatant was removed washed 2 times with Hepes buffer A (50mM Hepes, 1mM EDTA, 10% glycerol, 1mM DTT, 1% Triton, protease inhibitor cocktail). The resin was then incubated with 100ng bacterially expressed AMPK (activated using CAMKK β) (From Faith Mayer) in the presence of 2.5 mM MgCl₂ at 37 °C for 30 min, made up to a total volume of 80 µl. Positive control was provided by the incubation of active AMPK with of bacterially expressed PPM1A (from Faith Mayer) with 2.5mM MgCl₂ under the same conditions. The samples were then taken into a SAMS-peptide assay.

2.2.24 In-vivo Crosslinking and analysis

HEK293 and COS7 Cells were cultured as described above in Chapter 2 and co-transfected with AMPK expression constructs encoding α1, β1, γ1 respectively with expression proceeding for 48h. Cells are washed with PBS and covered with 2mls of 1% paraformaldehyde (per well of 6 well plate) for 5/15 minutes, the crosslinking cycle is quenched using ice cold 1.25 M glycine for 5 minutes and washed again with glycine before cell lysis as described.

AMPK was co-immunoprecipitated from 200 µg of crude cell lysate with AMPK-β subunit antibody and 50ul of protein A slurry, with the reaction allowed to proceed overnight (10hr). The resin was washed 3 times with PBS and crosslinking was reversed by heating the resin in 2x sample buffer (Sigma) to 60 °C overnight. The coimmunoprecipitated proteins were eluted from the resin (5 min at 100 °C) and the eluted proteins resolved using SDS page electrophoresis. The gel was sent for mass spectrometric analysis.
2.2.25 Stable isotope labelling by amino acids in cell culture and quantitative mass spectrometry

HEK293 cells were grown in parallel in DMEM (4.5 g/L) media containing either ‘heavy’ \(^{13}\text{C}\) or ‘light’ \(^{12}\text{C}/\text{normal}\) isotopes of the amino acids arginine and lysine \(^{13}\text{C}\) isotopes obtained from CK gas, UK) for 10 passages. After this, cells were treated as indicated in individual experiments and lysed as shown in 2.2.16 and 2.2.20. Lysates from cells grown in ‘heavy’ isotope media were subjected to immunoprecipitation with the following antibodies: AMPK pan-β-subunit antibody (in house), AMPK α subunit antibody (in house), AMPK pThr-172 antibody (Dundee University), while lysates from cells grown in ‘light’ isotope media was subjected to immunoprecipitation reactions with the corresponding pre-immune serum or non-targeting IgG from the same species. Following immunoprecipitation and washing once with Cell lysis buffer (see 2.1.2) and once with PBS, the immuno-prellets were mixed and resolved with SDS-PAGE. Protein bands were visualised by staining with Simply Blue Stain ® (Invitrogen) using the manufacturers protocol. Samples were analysed using mass spectrometry analysis (MRC proteomics unit). Briefly, samples were excised using a scalpel and processed. Proteins were digested using trypsin overnight and applied to a Thermo Scientific LTQ Orbitrap XL hybrid FTMS (Fourier Transform Mass Spectrometer) operating in positive polarity Raw data files were analysed using MaxQuant software (http://www.maxquant.org/), data were searched against the Uniprot mouse database that was current at the time.

2.2.26 Collagen based invasion assay

Cells were pre-incubated with the indicated concentration of A769662 compound for 16 hours and then resuspended in serum-free collagen I at 2.3 mg/ml to a final concentration of \(1 \times 10^3\) cells/100 μl. Aliquots of 100 μl were dispensed into 96-well ViewPlates (Perkin-Elmer) coated with bovine serum albumin. Plates were centrifuged at 300 × g and incubated at 37°C/10% CO\(_2\) for 30 min before fetal calf serum1 of 10% (in DMEM) was added. After 16 hr incubation, cells were fixed in formaldehyde (final concentration 4%) and stained with 5 μg/ml Hoechst 33258 (Molecular Probes-Invitrogen). Confocal Z slices were collected from each well at 50 μm and 3 μm (bottom of well) with an INCELL3000 high-content microscope.
Nuclear staining was quantified with INCELL3000 software with the Object Intensity module. Samples were run in quadruplicate and averaged. The invasion index was calculated as number of cells at 50 μm divided by the total number of cells. Data are presented as fold of invasion index of non-A769662 treated cells.

### 2.2.27 Transwell invasion assay

Transwell invasion assay kits (24 well format) were obtained from Millipore (Merck group) (catalogue code: ECM550). Prior to conducting the invasion assay, each invasion chamber was transferred into a 24 well plate well and equilibrated by adding 300 μl of pre-warmed serum free DMEM free media and allowing equilibration to take place for 2 hours after which this media is removed. 500 μl of media containing 20% FBS was added into the lower chamber. A suspension of WM1361 melanoma cells was prepared at a concentration of 1x10^6 cells/ml. At this point pharmacological compounds were added into this suspension. 300 μl of this cell suspension was placed into each insert (resulting in 3.3 x 10^5 cells per insert) and the assay incubated at 37°C/5% CO₂ for 48 hours to allow invasion of cells to take place.

After this, the media was removed from the top and bottom chambers and a cotton tipped swab used to remove both the ECM matrix and non-invading cells from inserts leaving only the cells which have invaded through the ECM matrix and cell-permeable membrane. Invaded cells were stained using staining solution supplied for 20 minutes, and unspecific staining removed by three washes with sterile water. A standard light microscope was used to image stained cells with 5 fields taken at 10x magnification for each insert. Cells in each field were then counted manually and averaged. Data was plotted in graph form with the average number of cell per field plotted for each different treatment.

### 2.2.28 Statistical analyses

Statistical calculations were performed in Microsoft Excel. Error bars are given as standard error of the mean. Differences between data sets were analysed by Student’s t tests and one way ANOVA where appropriate (when comparing more than one group of data). Differences were considered significant if P < 0.05.
3 Identification of AMPK phosphatase(s)

3.1 Introduction:

It is well known that the main mechanism by which AMPK activity is regulated is via the phosphorylation and dephosphorylation of the Thr-172 residue on the α subunit (Hawley et al., 1996). It can thus be appreciated that the identification of the upstream kinases and phosphatases are of utmost importance for us to gain a full picture of AMPK signalling pathway. While both LKB1 and CaMKKβ have been identified as AMPK upstream kinases, there is still a lack of consensus in the field as to the identity of the upstream phosphatase(s). The existing literature (at the time of starting this thesis) is sometimes contradictory, with almost all major classes of protein phosphatases suggested to regulate AMPK activity, (Davies et al., 1995; Gimeno-Alcaniz and Sanz, 2003; Moore et al., 1991; Samari et al., 2005; Steinberg et al., 2006b; Wu et al., 2007). The existing literature on possible AMPK phosphatases has been extensively reviewed in chapter 1.

Most of the publications referenced above are not dedicated studies specifically designed for the identification of the AMPK phosphatase. In this chapter, an approach starting from first principles and using a variety of experimental techniques, including phosphatase inhibitors and siRNA silencing, was undertaken to identify the AMPK phosphatase(s) in mammalian cell-lines.

3.2 Results:

3.2.1 Pharmacological activation of AMPK in HEK293 cells

To initially characterise the AMPK activity profile in HEK293 cells, AMPK was immunoprecipitated from HEK293 cell lysates under basal conditions using antibodies specific for either α1 or α2 subunits of AMPK. After immunoprecipitation, the resulting immune-complexes were subjected to assays using the SAMS peptide. This was done to investigate if both α1 and α2 are expressed and active in HEK293...
cells. The specific activities of AMPK immune-complexes containing $\alpha_1$ or $\alpha_2$ subunits are shown in Figure 3.1.

Having verified that both AMPK $\alpha_1$ and $\alpha_2$ are expressed and active in HEK293 cells, experiments were performed to determine the activity of LKB1 and CaMKK$\beta$ are in these cells. In this experiment, a two-step kinase assay was performed where vitro activation of recombinant AMPK by immunoprecipitated LKB1 or CaMKK$\beta$ from cell lysates of un-stimulated HEK293 cells was measured, as shown in Figure 3.2. It can be seen that both AMPK upstream kinases are active in HEK293 cells with the specific activity for LKB1 (average of 2027 U/mg) being roughly 3 times that of CaMKK$\beta$ (average of 705 U/mg) activity. Although, as different antibodies are used for the immunoprecipitation reactions, this makes direct comparison of specific activities somewhat difficult.

To establish whether AMPK activity can be modulated in HEK293 cells, they were treated with a variety of pharmacological agents previously reported to activate AMPK in various cell types. AMPK activities were then measured with the SAMS peptide assay. As shown in Figure 3.3, AMPK activity increased 6.6 fold after treatment by $\text{H}_2\text{O}_2$, by 6.4 fold after treatment by DNP (2,4-Dinitrophenol), by around 6.1 fold with phenformin, and by 2.9 fold with ionomycin. Treatment with 100 $\mu$M or 200 $\mu$M of the direct activator A-769662 resulted in a 3.2 and 5 fold elevation in AMPK respectively. On the other hand, treatment with the AMPK activators AICAR or metformin had no significant effects on AMPK activity in HEK293 cells.

As seen from the above results, AMPK $\alpha_1$ and $\alpha_2$ catalytic subunits are expressed in HEK293 cells and AMPK can be dynamically modulated by various treatments, it was decided that HEK293 cells would be an appropriate model to investigate the regulation of AMPK by protein phosphates(s).
Figure 3.1 Activity of AMPK α₁ and α₂ isoforms in HEK293 cells
Antibodies targeting either AMPK α₁ and α₂ subunits were used to immunoprecipitate AMPK complexes from soluble HEK293 lysate (50μg total protein). The SAMS peptide assay was used to determine AMPK activity. Data shown represent averages and standard errors (SEM) from 3 independent experiments and are shown as specific activity of the kinase (pmol/min/mg).

Figure 3.2 Activity of upstream kinases of AMPK in HEK293 cells
Antibodies targeting either the upstream kinases LKB1 and CaMKKβ were used to immunoprecipitate from the HEK293 protein lysates (100μg). The immune-complexes were used to phosphorylate 0.2μg - 1μg of recombinant AMPK (α₁β₁γ₁) (in the presence of calcium and calmodulin). AMPK activation by the upstream kinases was then assayed using the SAMS peptide. Activities are plotted as U/mg of lysate protein, where 1 unit is the activity of LKB1/CaMKKβ required to activate recombinant AMPK by 1 nmol/min/mg of protein. Data shown represent averages and standard errors (SEM) from 3 independent experiments.
Figure 3.3 Activation of AMPK in HEK293 cells
HEK293 cells in serum free media were treated with the following compounds: AICAR at 0.5 mM or 1.0 mM for 30 minutes, 2 mM metformin for 2 hours, 0.5 mM H2O2 for 15 minutes, 5 mM Phenformin for 30 minutes, 0.5 mM DNP for 30 minutes, 2.5 μm ionomycin for 5 minutes and A769662 at 100 μm or 200 μm for 1 hour. After treatment AMPK was immunoprecipitated using the pan-β-subunit antibody and AMPK activity determined. Results are shown as fold AMPK activity relative to the mock control treatment experiment. Data shown represent averages and standard errors (SEM) from 3 independent experiments.
3.2.2 Okadaic acid treatment of HEK293 cells

Okadaic acid is a potent inhibitor of most phospho-protein phosphatases (PPP), with IC$_{50}$ values for PP1 of around 30 nM and around 0.1 – 0.3 nM for PP2A (Swingle et al., 2007). On the other hand, this compound has no effect on the activity of PPM phosphatases and thus can be used as a tool to discriminate between different phosphatase classes of potential AMPK phosphatases. The concept behind this experiment is that inhibition of the AMPK-phosphatase should allow the constitutively active LKB1 to facilitate an increase in AMPK phosphorylation/activity.

HEK293 cells were treated for 60 minutes with increasing concentrations of okadaic acid and the cells lysed. The phosphatase responsible for dephosphorylating acetyl-CoA carboxylase (ACC) is okadaic acid sensitive (Moore et al., 1991). As such, the cell lysate was subjected to immune-blots analysis and probed for phospho-ACC (pSer-79) to reveal whether okadaic acid is having its desired effect. While treatment with 10 nM okadaic acid did not lead to significant changes in ACC phosphorylation (Figure 3.4), 50 nM and 500 nM treatments led to a 2 fold and 2.5 fold increase respectively.

Aside from phosphorylation at the Thr-172 residue, AMPK can also be allosterically activated by AMP. As okadaic acid causes inhibition of a significant proportion of the cellular phosphatase activity, it is possible that there could be an associated increase in the levels of AMP or ADP relative to ATP. Thus, nucleotide extraction was performed on control and okadaic acid treated cells with the positive control cells being treated with DNP (which has been previously seen to elicit a reduction in the ratio of ATP to AMP/ADP). The relative levels of AMP, ADP and ATP determined by ion-exchange chromatography as described in chapter 2. The amount of nucleotides are taken as the area under each nucleotide peak. In most cell types, under basal conditions, the peak for ATP would be much larger than that of ADP, which would in turn have a much larger peak than AMP. While easily detectable peaks for ATP and ADP were observed, a peak for AMP could not be detected under these conditions (although an AMP peak was visible for DNP treated cells), this is most likely due to the much lower cellular AMP concentrations compared to ADP and ATP. Thus the ATP:ADP ratio was used instead (Figure 3.5). Treatment with DNP was
used as a positive control as an agent known to cause a change in nucleotide ratios (previous work in our lab). DNP treatment caused a 78% drop in ATP:ADP ratio from ~12 to ~7. Treatment at 50 nM and 500 nM okadaic acid also caused a slight drop in ATP:ADP ratio of 9.5% and 12% respectively, although these small changes were not statistically significant.

Based on above findings, HEK293 cells were subjected to okadaic acid treatments at concentrations of up to 500 nM. From the SAMS peptide assay results, there was a small increase in both AMPK $\alpha_1$ and $\alpha_2$ activities when cells were treated with the higher concentrations okadaic acid, i.e. 18% and 23% for AMPK $\alpha_1$ and 6% and 14% for AMPK $\alpha_2$ under 50 nM and 500 nM treatments respectively. However the, levels of these changes were not statistically significant (Figure 3.6).
Figure 3.4 Inhibition of the ACC phosphatase using okadaic acid

HEK293 cells were either left untreated or treated with water soluble okadaic acid at the following concentrations: 10 nM, 50 nM and 500 nM, for 60 minutes after which cells were lysed. Cellular lysates were resolved using SDS-PAGE and subjected to western blot analysis for pACC (pS-79) and actin. Representative western blot shown above. The relative level of signal were quantitated using the Licor Odyssey software against Actin and is plotted in graphical form above. Data shown represent averages and standard errors (SEM) from 3 independent experiments.
Figure 3.5 Treatment with okadaic acid does not alter the ATP:ADP ratios in HEK293 cells

Nucleotides were extracted from HEK293 cells which were left untreated, treated with water soluble okadaic acid at the following concentrations: 10 nM, 50 nM and 500 nM, for 60 minutes or treated with DNP at 0.5 mM for 15 minutes. The cell were lysed and adenine nucleotides levels determined and ATP:ADP ratios calculated.

* Indicates a significant decrease in ADP:ATP ratios with DNP compared to control treatment (0nM Okadaic acid) as judged by one way ANOVA (P<0.05). Data shown represent averages and standard errors (SEM) from 3 independent experiments.
Figure 3.6 Okadaic acid has no significant effect on the activity of AMPK α₁ and α₂ isoforms in HEK293 cells
HEK293 cells in serum free media was treated with okadaic acid at the indicated concentrations and cells lysed. Antibodies targeting either AMPK α₁ and α₂ subunits were used to perform immunoprecipitation reaction and activity of AMPK α₁ and α₂ determined using the SAMS peptide assays. Results are shown as fold AMPK activity relative to the mock treatment control. Data shown represent averages and standard errors (SEM) from 3 independent experiments.
3.2.3 Endogenous expression of PPM family phosphatases in HEK293 cells

In order to elucidate the phosphatase acting on AMPK, a logical approach would be to alter the endogenous expression of individual PPM phosphatases in HEK293 cells by either RNAi knockdown or transient over-expression and determine if there is a change in AMPK activity. There are a total of 17 known PPM phosphatases so far identified, the results obtained with okadaic acid treatment would suggest that the AMPK phosphatase(s) is likely to belong to this family (Lu and Wang, 2008). With a view of employing RNAi for the knockdown of individual PPM phosphatases, the expression levels of these phosphatases in HEK293 cells was first profiled. Quantitative-RT-PCR was used to measure the relative mRNA level of these phosphatases. The levels of mRNA for these phosphatases were compared to the mRNA levels of ALAS1 (δ-aminolevulinate synthase) a reference gene which has previously been found to be expressed in a similar range to the PPM phosphatases (Li et al., 2009). As can be seen from Figure 3.7, mRNA expression for 16 out of the 17 PPM isoforms was detected. Expression for the T-cell activated PP2C isoform was not detected in these cells and thus was not included in the subsequent knockdown studies. From the data, the relative the mRNA levels of PPM1G was found to have the highest expression of the PPMs tested at approximately 110% of the mRNA levels of ALAS1; the next highest expressed PPM is PPM1B at approximately 80% the level. The remaining PPMs were expressed at lower levels. ALAS1 was used as the house keeping gene for all qRT-PCR experiments in this thesis.
3.2.4 Optimisation of siRNA mediated protein knockdown in HEK293 cells:

Due to lack of potent and specific PPMs inhibitors, it was decided to use siRNA to perform transient knockdowns of PPM phosphatases in HEK293 cells individually. The rational for this experiment is that the decrease in the protein level of the AMPK-phosphatase should cause a decrease in the phosphatase activity acting upon AMPK, therefore resulting in an increase in the phosphorylation and activity of AMPK.

SiGLO, a fluorescent tagged non-specific siRNA oligo-nucleotide (tagged with DY-547) was used to optimise transfection conditions. Three different transfection reagents were tested to find the most efficient agent for siRNA transfection into HEK293 cells. SiGLO was transfected using each of the agents and the transfection efficiency was determined by the percentage of cells containing the fluorescent SiGLO 24h post transfection (data not shown). Transfection with the RiboJuice reagent gave the highest percentage (over 96%) of cells containing siGLO and thus was chosen for use in future experiments (Figure 3.8).

It is important to verify that the siRNA knockdown method does not have an effect on AMPK activity at basal levels or its ability to be activated by exogenous stimuli. Non-targeting control siRNA (Ambion) (abbreviated to siControl) was transfected in HEK293 cells to a final concentration of 10 nM, 15 nM and 25 nM, these cells were either lysed while at basal conditions or after treatment with phenformin. As shown in Figure 3.9, basal AMPK activities (non-treated), or following phenformin treatment, were not significantly affected by the siControl at any of the concentrations tested.

As mRNA levels are not a direct measurement of protein levels in the cells, we obtained antibodies targeting PPM1A to optimise the concentration of siRNA for knockdown experiments and to use these conditions for all PPM knockdowns. Two different specific siRNA oligo-nucleotides targeting PPM1A were obtained, and transfected into HEK293 cells separately or together at final concentrations of 5 nM, 10 nM and 25 nM (combined concentration of both siRNA nucleotides). Cells were lysed after 72 hours and subjected to immuno-blot analysis probing for PPM1A. It was found that when both siRNA oligos were transfected together a greater level of
knockdown was achieved compared to when siRNA was transfected individually (data not shown). In light of this, it was decided to transfec two individual siRNA constructs together in future experiments. With double siRNA transfection at a total concentration of 10 nM (i.e. each siRNA oligo at 5 nM), a decrease of around 52% of PPM1A protein was achieved, compared to control siRNA transfection, while at 15 nM (i.e. each siRNA oligo at 7.5 nM) around 60% knockdown was achieved, the most efficient knockdown of around 75%, was seen with a final total siRNA concentration of 25 nM (Figure 3.10). SiRNA concentrations beyond 25 nM was not used for knockdown of individual phosphatases due to the likelihood of cell toxicity.

Next, the time-course of knockdown of PPM1A was determined. HEK293 cells were lysed either 48 h or 72 h post transfection and cell lysates subjected to immuno-blot analysis to determine the amount of PPM1A remaining after these periods. Due to the rapid growth of HEK293 cells, knockdown was not allowed to proceed longer than 72 h, due to the cells becoming over-confluent (This usually occurred around 96 h post transfection). As shown in Figure 3.11, 48 h after transfection the percentage of PPM1A protein remaining was around 25%, the most efficient knockdown of PPM1A was seen 72 h after transfection with 18% remaining. Thus, it was decided that knockdowns should proceed for 72 h in all future experiments.

3.2.5 Knockdown of PPM isoforms in HEK293 cells

siControl or siRNA targeting individual PPM isoforms were transfected into HEK293 cells, and cells were lysed into Trizol after 72 h and total cellular RNA extracted. As it was impractical to obtain specific antibodies targeting every PPM isoform, analysis using qRT-PCR was used to determine the level of mRNA remaining, allowing an indirect indication of the efficiency of knockdown to be calculated. As shown in Figure 3.12, siRNA silencing resulted in a >50% reduction in mRNA levels for all PPMs tested, with the most efficient knockdown of >75% being achieved for PPM1A, PPM1B, PPM1F, PPM1J and ILKAP, PHLPP1 PDP1 and PDP2. Knockdown efficiencies for the other PPM isoforms varied between around 58% for PPM1E and 72% for PPM1L.
To further confirm that the reductions in mRNA levels resulted in a reduction in protein levels, antibodies were obtained for three further PPM isoforms (in addition to PPM1A): PPM1B, PPM1H and ILKAP. After siRNA knockdown for 72 h, cell lysates were subjected to western-blot analysis with these antibodies (Figure 3.13). With PPM1B, there was an average of approximately 71% of depletion in protein levels, with PPM1H there was approximately 62% depletion and for ILKAP there was around 59% knockdown after siRNA transfection.

3.2.6 Effect of PPM knockdown on AMPK activity

To test if a reduction in the levels of PPM phosphatases affected AMPK activity, AMPK was immunoprecipitated from cell lysates transfected with either control siRNA or PPM siRNA using the pan-β subunit antibody, and AMPK activity in the immune complexes determined. As seen from Figure 3.14, AMPK activity after siRNA knockdown of the PPM isoforms varied from 80% (PPM1M) to 115% (PPM1F) when compared to control siRNA transfection. There was no significant alteration in AMPK activity when any of the PPMs were knocked down. However for the knockdown of isoforms PPM1F, PPM1H and PPM1L, while the average AMPK activities were not significantly higher than control samples (the average activity values were 15% - 20% higher than control but did not reach significance), there was some variation in activity data recorded for individual experiments, with the highest activity recorded in individual experiments being 151% (PPM1F), 158% (PPM1H) and, 145% (PPM1L) (data not shown).

In view of these data, and the possibility that PPM1F, PPM1H and PPM1L all contributed to the dephosphorylation of AMPK, all three phosphatases were knocked down concurrently in HEK293 cells (with a final siRNA concentration of 37.5 nM, i.e. 12.5 nM for each phosphatase). Q-RT-PCR was used to measure the remaining mRNA levels for each of these phosphatases Figure 3.15A. Due to the lower concentration of siRNA used for each PPM isoform, the knockdown achieved in this experiment was marginally lower than when individual phosphatases were silenced. Reduction in mRNA levels of between ~65% (PPM1F) and ~75% (PPM1L) were seen in these samples. AMPK activity from these knockdown samples are shown in Figure 3.15B. Although an elevation in activity of around 16% was observed in the
knockdown samples compared to control samples, this does not represent a further increase compared to individual knockdown of the phosphatases. Furthermore these results did not reach statistical significance (P value ~0.2)

Next, to determine if the AMPK α₁ and α₂ were dephosphorylated by different phosphatases, antibodies specific for the α₁ and α₂ subunits were used to immunoprecipitate AMPK from control and knockdown samples and AMPK activity assayed (Figure 3.16). No significant changes in α₁ or α₂ AMPK activities were observed when PPM phosphatases were knocked down.

Next I investigated if the depletion of phosphatases can augment the levels of AMPK activation seen when cells are treated with an AMPK activator. Experiments were performed where HEK293 cells subjected to siRNA mediated PPM knockdown for 72 h were treated with the AMPK activator phenformin. The AMPK activities in these samples were assayed and compared to phenformin activated cells subjected to control siRNA transfection. As seen from Figure 3.17, no significant difference in the levels of AMPK activation was observed between control samples and samples where PPMs have been knocked down.
Figure 3.7 Expression of PPM phosphatase isoforms in HEK293 cells
RNA was extracted from HEK293 cells and analysed using qRT-PCR for the PPM isoforms listed using specific primers, mRNA expression of PPM isoforms were quantified against the housing keeping gene ALAS1. Data shown represent averages and standard errors (SEM) from 3 different experiments.
Figure 3.8 Validation of siRNA (SiGLO) transfection in HEK293 cells
HEK293 cells were plated onto ploy-L-Lysine coverslips and transfected with SiGLO (DY-547 labelled) siRNA. Cells were fixed with formaldehyde 48 h post transfection. The Leica DMIRE2 microscope was used to obtain bright field and red channel images with 10x objective lens. The efficiency siRNA transfection was determined by dividing the total number of cells by the number of cells containing SiGLO across 10 different fields.
Figure 3.9 siControl has no effect on AMPK activity or AMPK activation

HEK293 cells were treated with 0 – 25 nM of control siRNA (siControl), 72 h post transfection, cells were treated with phenformin (30 min) or left untreated. After cell lysis, AMPK was immunoprecipitated with the pan-β-subunit antibody and activity determined using SAMS peptide assay. Data shown represent averages and standard errors (SEM) from 3 different experiments. Results are shown as fold AMPK activity relative to experiments where no siRNA was introduced.
Figure 3.10 Optimisation of siRNA concentration for PPM knockdown
HEK293 cells were transfected with increasing concentrations of siControl or siRNA targeting PPM1A, and cells lysed 72 h after transfection. Lysates were resolved using SDS-PAGE and subjected to western blot analysis for PPM1A and tubulin (representative blot shown). The relative level of protein expression are quantitated using the Licor Odyssey software and normalised with tubulin. Expression shown represent averages and standard errors (SEM) from 3 different experiments.
Figure 3.11 Optimisation of time course for PPM knockdown
HEK293 cells were transfected with 25 nM of siControl or siRNA targeting PPM1A, and cells lysed 48 hours or 72 h after transfection. Lysates were resolved using SDS-PAGE and subjected to western blot analysis for PPM1A and tubulin (representative blot shown). The relative level of protein expression after 48 h and 72 h of siRNA mediated knockdown are quantitated using the Licor Odyssey software and normalised with tubulin. Data shown represent averages and standard errors (SEM) from 3 different experiments.
HEK293 cells were transfected with either siControl or siRNA against each of the 16 PPM isoforms at a final concentration of 25 nM. Knockdown was allowed to proceed for 72 h after which the cells were lysed and RNA extracted. Levels of mRNA for the PPM isoforms were determined using qRT-PCR. Levels of knockdown was calculated by comparing mRNA expression in HEK293 cells treated with PPM siRNA with those transfected with siControl. Data shown represent averages and standard errors (SEM) from 3 independent experiments.
Figure 3.13 Validation of protein knockdown of PPM1B, PPM1H and ILKAP
HEK293 cells were transfected with either siControl or siRNA against PPM1B, PPM1H or ILKAP isoforms into at a final concentration of 25 nM. Cells were lysed after 72 h and lysates were resolved using SDS-PAGE and subjected to western blot analysis with PPM1B, PPM1H and ILKAP antibodies (representative blot shown). Tubulin was used as the loading control. The relative level of protein expression were quantitated using the Licor Odyssey software normalised with Tubulin. Data shown represent averages and standard errors (SEM) from 3 different experiments.
Knockdown of individual PPM phosphatases does not affect basal AMPK activity

Figure 3.14 Knockdown of individual PPM phosphatases does not affect basal AMPK activity

HEK293 cells were transfected with either non-targeting siRNA or siRNA against each of 16 PPM isoforms at a final concentration of 25 nm, after 72 h the cells were lysed. AMPK was immunoprecipitated using the pan-β-subunit antibody (from 50 μg total protein) and the activity of AMPK determined. Results are shown as fold AMPK activity relative to siControl transfection. Data shown represent averages and standard errors (SEM) from 3 independent experiments.
Figure 3.15 Multiplexed knockdown of PPM1F, PPM1H and PPM1L in HEK293 cells
HEK293 cells were transfected with either siControl or siRNA against PPM1F, PPM1H and PPM1L at a final concentration of 37.5 nM (12.5 nM for each siRNA oligo). A. After 72 h the cells were lysed and RNA extracted. The mRNA levels of PPM isoforms in both control and knockdown samples were determined using qRT-PCR. Data shown represent averages and standard errors (SEM) from 3 independent experiments.
B. After this cells were either lysed or treated with 5 mM phenformin for 30 minutes and then subjected to lysis. AMPK immunoprecipitated (from 50 ug total protein) and activity assayed. Results are shown as fold AMPK activity relative to the siControl transfection. Data shown represent averages and standard errors (SEM) from 3 independent experiments.
Figure 3.16 Knockdown of individual PPM phosphatases does not affect activity of AMPK $\alpha_1$ or $\alpha_2$ isoforms

HEK293 cells were transfected with either non-targeting siRNA or siRNA against each of the 16 PPM isoforms at a final concentration of 25 nm, after 72 h the cells were lysed. AMPK was immunoprecipitated with antibodies targeting either AMPK $\alpha_1$ or $\alpha_2$ subunits (from 50 $\mu$g total protein) and the activity of AMPK assayed. Results are shown as fold AMPK activity relative to siControl transfections. Data shown represent averages and standard errors (SEM) from 3 independent experiments.
Figure 3.17 Knockdown of individual PPM phosphatases does not affect the level of AMPK activation by phenformin

HEK293 cells were transfected with either siControl or siRNA against each of the 16 PPM isoforms at a final concentration of 25 nM. After 72 hrs, the cells were treated with 5 mM Phenformin for 30 minutes. After treatment cells were lysed and AMPK immunoprecipitated using the pan-β-subunit antibody (from 50 µg total protein) and AMPK activity determined. Results are shown as fold AMPK activity relative to siControl transfection.
3.2.7 Over-expression of PPM isoforms in HEK293 cells

Concurrently with the phosphatase knockdown experiments, plasmid constructs encoding for the PPM isoforms PPM1A, PPM1D, PPM1F and PPM1G were obtained (Source BioScience Limited (UK)). These phosphatases were over-expressed in HEK293 cells to see if an elevation in phosphatase activity can result in a decrease in AMPK activity. As shown by previous studies, both members of the PPP and PPM classes have been reported to dephosphorylate AMPK in vitro (Davies et al., 1995; Sanders et al., 2007b). To look at whether this dephosphorylation occurs in cells, the effect of phosphatase overexpression on AMPK activity was investigated.

PPM constructs were cloned in house (by this candidate) into the mammalian expression vector pCDNA3.1-V5 (available in house) and transfected into HEK293 cells. 48 hours post transfection the cells were lysed and immuno-blotted for phosphatases expression using an anti-V5 tag antibody. Figure 3.18 shows western blots probing for the expressed phosphatases and the control over-expression of β-galactosidase. The levels of expression of these phosphatases were variable with PPM1A giving the highest levels of expression, followed by PPM1G, PPM1F then PPM1D. The level of expression of PPM1A is around 6.6 fold higher than that of PPM1D, as analysed by western blot quantitation (data not shown).

To determine if these phosphatases are active as expressed, they were immunoprecipitated from cell lysate using the anti-V5 tag antibody and used to dephosphorylate active AMPK in vitro. As seen in Figure 3.19, all recombinant PPM phosphatases significantly decreased AMPK activity. PPM1F produced the most pronounced decrease in AMPK activity followed by PPM1A and PPM1G. The PPM1D immune-complex had the least effect on AMPK activity, lowering it to around 46% of the control immunoprecipitation. Bacterially expressed PPM1A was used as a positive control.

Next, AMPK was immunoprecipitated from cells over-expressing these phosphatases or β-galactosidase, and AMPK activity determined. As shown in Figure 3.20, no significant changes in AMPK activity were observed in immune-complexes from lysates over-expressing PPMs as compared with the controls lysates. Similarly, no
significant changes in AMPK activity were observed in cells over-expressing phosphatases that were treated with phenformin, as compared to phenformin treated control samples over-expressing β-galactosidase. These results are interesting in that they seem to suggest a level of specificity of phosphatase action in a cellular environment that does not exist *in vitro* assays.

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**Figure 3.18 Overexpression of PPM isoform constructs in HEK293 cells**

HEK293 cells were transfected with pcDNA-3.1-V5 expression constructs encoding PPM1A, PPM1D, PPM1F or PPM1G in turn. After 48 hours cells were lysed and resolved using SDS-PAGE and subjected to western blot analysis with the following antibodies: anti-V5-Tag (to detect over-expressed phosphatases), anti- β-galactosidase, and anti-actin antibodies.
Figure 3.19 Validation of the activity of overexpressed PPM phosphatase s  
HEK293 cells were transfected with pcDNA-3.1-V5 expression constructs encoding the indicated phosphatases, 48 h later cells were lysed and lysates (500 µg) were immunoprecipitated using the anti-V5 antibody. The immuno-complexes were used to dephosphorylate activated recombinant AMPK (45 min at 37 °C). A positive control is provided where bacterially expressed purified PPM1A (Labelled Bacterial PPM1A) was used to dephosphorylate active AMPK. The graph show the relative AMPK activity of samples overexpressing PPM phosphatase compared to negative controls (overexpressing β-galactosidase).
Figure 3.20 PPM overexpression does not alter basal AMPK activity or activation by phenformin
HEK293 cells were transfected with expression constructs encoding the indicated phosphatases. 48 h later cells were incubated in the presence or absence of 5 mM phenformin for 30 minutes and then lysed. AMPK was immunoprecipitated using the pan-β-subunit antibody (from 50 µg total protein) and activity determined. Results are shown as fold AMPK activity relative to that of cells expressing β-gal.
3.2.8 Okadaic acid titration in cytosolic suspensions of HEK293 cells

As described in Chapter 2, in this study, the buffer used to lyse mammalian cells normally contain phosphatases inhibitors such as sodium fluoride, sodium pyrophosphate and EDTA. However if these inhibitors are not included, action by endogenous phosphatases present in the cytosolic preparations would result in near complete dephosphorylation of AMPK (Carling et al., 1987).

Considering the inconclusive results from the experiments shown above, an experiment was devised to shed more light on the class of phosphatase that dephosphorylates AMPK in cells. HEK293 cells were lysed in ice-cold buffer with or without the usual phosphatase inhibitors and the insoluble fraction removed by centrifugation (14,000 rpm for 15 minutes). In the samples where no phosphatase inhibitors were included, okadaic acid was supplemented at various concentrations (0nM, 5nM, 40nM, 100nM, 200nM and 500nM) to inhibit the action of endogenous phosphatases. The concentrations used were determined using IC$_{50}$ values for okadaic acid found in literature (Swingle et al., 2007). The aliquots of HEK293 lysates were incubated at 37 ºC with shaking for 45 minutes to allow the dephosphorylation reaction to proceed. AMPK was then immunoprecipitated and its activities determined.

The results for this experiment are shown in Figure 3.2. At a concentration of 5 nM, there was no significant protection of phosphatase mediated decrease in AMPK activity, as compared with samples where phosphatase inhibitors were included at time of lysis. At this concentration, most PP2A activity in the cell lysate should be inhibited, whereas most of PP1 activity should remain (Swingle et al., 2007). When okadaic acid is increased to 40 nM, around the IC$_{50}$ value for PP1, 63% of AMPK activity is protected. When okadaic acid concentration is increased to 100 nM and above, AMPK activity was then fully protected as compared to samples initially lysed in the presence of phosphatase inhibitors.
Okadaic acid supplementation protects AMPK from dephosphorylation in cytosolic suspensions

HEK293 cells were lysed in the presence or absence of phosphatase inhibitors normally used in cell lysis buffer (see section 2.1.2). The bar labelled: “All PIs” are were lysed in the presence of 50mM sodium fluoride, 5mM sodium pyrophosphate and 1mM EDTA. Samples lacking phosphatase inhibitors were pre-supplemented with increasing concentrations of okadaic acid. Cytosolic suspensions were then incubated at 37 °C for 45 minutes with shaking to allow dephosphorylation to take place. AMPK was then immunoprecipitated using the pan-β-subunit antibody, and activity assayed. Results are shown as percentage AMPK activity relative to the cells lysis in the presence of sodium fluoride, sodium pyrophosphate and EDTA.
3.2.9 Calyculin-A treatment of HEK293 cells and mouse hepatocytes

As no promising candidates for the AMPK phosphatase emerged from the PPM knockdown studies, and in view of the results for cell suspension studies shown above, it was decided to carry out another phosphatase inhibitor screen with Calyculin A. Calyculin A is an inhibitor of PPP phosphatases with greater potency of inhibition than okadaic acid. (IC₅₀ values of 0.5 – 1 nM for PP2A and of ~2nM for PP1) (Swingle et al., 2007).

Firstly, HEK293 cells were subjected to Calyculin A treatment of 10 nM, 20 nM and 50 nM for 30 minutes, the resulting cell lysate was subjected to western-blot analyses for a phospho-ACC, a known PP2A target (Moore et al., 1991). As shown in Figure 3.22, there was a very considerable increase in p-ACC levels of 3.26 fold and 3.78 fold respectively for 10 nM and 20 nM treatments. These increases are larger than that see for okadaic acid treatments (Figure 3.4).

After confirming that calyculin A inhibited phosphatases in HEK293 cells, AMPK was immunoprecipitated from these samples and assayed for activity. As can be seen from Figure 3.23, treatment at both 10 nM and 20 nM were able to cause elevations in AMPK α₁ and α₂ activities by around 50%. Both of these changes were statistically significant.

As with the okadaic acid treatment, ATP:ADP ratios were also determined following calyculin A treatment. As shown in Figure 3.24, while DNP did cause a clear decrease in ATP:ADP ratio, calyculin A treatment up to 20 nM did not result in any significant change in ATP/ADP ratios. These results suggest that calyculin A does not cause any significant perturbations in cellular nucleotide levels, and therefore the elevation in AMPK activity observed is unlikely due to protection against dephosphorylation by AMP/ADP or allosteric activation.

As shown in Figure 3.3, 15 minute hydrogen peroxide treatment causes significant activation of AMPK in HEK293 cells. However, if after treatment, the cell media containing H₂O₂ is removed and replaced with fresh media without AMPK activators, AMPK activity decreases from around 3.5 – 5 fold of basal levels to around 1.2 –1.5
fold within one hour. In view of this, an experiment was performed where calyculin A was applied to the replacement media at concentrations of 10nM and 20nM after activation with H₂O₂, to see if this could prevent the decrease in AMPK activity to a significant extent. The cells were lysed after 60 minutes and AMPK activity determined. From Figure 3.25, it can be seen that when calyculin A was not supplemented, AMPK activity decreased to around 50% of the H₂O₂ activated state 60 minutes after media replacement. However at both 10 and 20nM, calyculin A caused a significant, albeit non-complete protection of AMPK activation of around 80% of the H₂O₂ activated state. These results therefore would suggest that the major AMPK phosphatase(s) is one or more members the PPP phosphatase family.

In an effort to reproduce above results in HEK293 cells in a more physiological cell type, calyculin A was used to treat primary mouse hepatocytes for one hour at concentrations of 10nM, and 20nM to see if phosphatase inhibition has any effect on AMPK activity in a primary mouse cells. In order to demonstrate that calyculin A is having the intended action, treated hepatocytes lysates were subjected to western blot analysis probing for p-ACC, as Figure 3.26 shows Calyculin A treatment at 10nM and 20 nM causes significant increased p-ACC levels.

As shown in Figure 3.27, Calyculin A treatment at 10 nM caused a ~50% elevation in AMPK activity and at 20 nM a ~55% elevation was seen. These results are similar to those obtained for HEK293 cells, and provides further evidence for the AMPK phosphatase(s) being one or more members of the PPP family. With the concentration used indicating PP1 involvement. Nucleotide extraction was also done for these cells. As with HEK293 cells, treatment with Calyculin A did not lead to any significant decrease in ATP:ADP ratios (Figure 3.28).
HEK 293 cells were either mock treated or treated with calyculin A at 10 nM or 20 nM for 30 minutes, after which cells were lysed. Cellular lysates were subjected to western blot analysis for p-ACC (pS-79) and actin. The relative level of signal were quantitated using the Licor Odyssey software against Actin. Data shown represent averages and standard errors (SEM) from 3 independent experiments.

* Indicates a significant increase in activity with calyculin A relative to control (0 nM) as judged by one way ANOVA (P<0.05).
Figure 3.23 Calyculin A treatment activates AMPK α₁ and α₂ isoforms in HEK293 cells

HEK293 cells in serum free media were treated with calyculin A for 30 minutes at the indicated concentrations and cells lysed. Antibodies targeting AMPK α₁ or α₂ subunits were used to perform immunoprecipitation reaction and activity determined.

* Indicates a significant increase in activity with calyculin A relative to control (0 nM) as judged by using an one way ANOVA (P<0.05). Data shown represent averages and standard errors (SEM) from 3 independent experiments.
Figure 3.24 Treatment with calyculin A does not alter the ATP:ADP ratios in HEK293 cells

Nucleotides were extracted from HEK293 cells which were mock treated, treated with water soluble calyculin A at: 10 nM, 20 nM, for 30 minutes or treated with DNP at 0.5 mM for 15 minutes. The cell were lysed and adenine nucleotides levels determined and ATP:ADP ratios calculated. Data shown represent averages and standard errors (SEM) from 3 independent experiments.
Figure 3.25 Protection of dephosphorylation of AMPK in HEK293 cells after H₂O₂ treatment

HEK293 cells in serum free media was treated with H₂O₂ (0.5 mM) for 15 minutes. After treatment, cells were either lysed or H₂O₂ containing media was replaced with serum free media supplemented with calyculin A and incubated for 60 minutes at 37 °C and then lysed. AMPK activity determined. Results are shown as percentage AMPK activity relative to H₂O₂ treated cells which has not undergone further incubation (Control).

* Indicates a significant difference in activity with samples treated with calyculin A compared to 0 nm calyculin treatment as judged by an one way ANOVA (P<0.05). Data shown represent averages and standard errors (SEM) from 3 independent experiments.
Mouse primary hepatocytes were either left untreated or treated with water soluble calyculin A at 10 nM and 20 nM for 30 minutes, after which cells were lysed. Proteins were resolved using SDS-PAGE and subjected to western blot analysis for pACC (pS-79) and actin. The relative level of signal was quantitated using the Licor Odyssey software against tubulin.

* Indicates a significant increase in activity with calyculin A relative to control (0 nM) as judged by one way ANOVA (P<0.05). Data shown represent averages and standard errors (SEM) from 3 independent experiments.

**Figure 3.26 Inhibition of the ACC phosphatases using calyculin A in mouse primary hepatocytes**
Figure 3.27 Calyculin A treatment activates both AMPK α₁ and α₂ isoforms in mouse primary hepatocytes
Primary mouse hepatocytes in serum free media were treated with calyculin A for 30 min at the indicated concentrations and cells lysed. Antibodies targeting either AMPK α₁ and α₂ subunits were used to perform immunoprecipitation reaction and activity determined.
* Indicates a significant increase in activity with calyculin A relative to control (0 nM) as judged by one way ANOVA (P<0.05). Data shown represent averages and standard errors (SEM) from 3 independent experiments.
Figure 3.28 Treatment with calyculin A does not alter the ATP:ADP ratios in mouse primary hepatocytes

Nucleotides were extracted from mouse primary hepatocytes which were mock treated or treated with calyculin A at: 10 nM, 20 nM, for 30 minutes or treated with DNP at 0.5 mM for 15 minutes. The cell were lysed and adenine nucleotides levels determined and ATP:ADP ratios calculated. Data shown represent averages and standard errors (SEM) from 3 independent experiments.
3.2.10 Knockdown of PPP isoforms in HEK293 cells and mouse hepatocytes

In view of the results from calyculin A treatments, the knockdown of PPP isoforms were conducted. As most of these phosphatases exist as multimeric complexes, it was decided to knockdown the catalytic subunits of PPP isoforms which should provide a rate limiting factor of phosphatase activity.

There are two different catalytic subunit isoforms for PP2A, PP2Aα and PP2Aβ, for PP1 there are 3 catalytic subunits PP1α, β, and γ. In addition siRNA for PP5 and PP6 were also obtained. The mRNA expression profiles for these subunits were determined in HEK293 cells using qRT-PCR which showed that all the above enzymes were expressed in this cell-line (data not shown). SiRNA against all three PP1 isoforms were transfected together into HEK293 cells to achieve knockdown of global PP1 protein (Figure 3.29A), both PP2A isoforms were also silenced together (Figure 3.29B). PP4 and PP5 were silenced individually as they only consisted of one isoform each (Figure 3.29C). 72 hours post-transfection, a decrease in the mRNA of PPP isoforms of between 64% (PP6) - 79% (PP1γ) was seen (Figure 3.29). Antibodies against PP2A and PP1α were also obtained to validate knockdown (Figure 3.30) which showed that the protein levels of both enzymes were significantly reduced (to 32%- 43% mRNA of remaining after knockdown).

As shown in Figure 3.31, there were no significant changes in basal AMPK activity knockdown of any PPP isoforms with or without activation by phenformin. In view of this, an additional experiment was conducted, where the highest expressing isoform of PP1 and PP2A: PP1α and PP2Aβ were knocked down together and AMPK activity determined (Figures 3.29D and 3.31). This experiment did not produce any significant alterations in the level of AMPK activity or activation by phenformin.

As calyculin A treatment caused a significant elevation in AMPK activity in mouse hepatocytes, it was decided to use siRNA to knockdown PPP family members in these cells to see if any changes in AMPK activity can be elicited. Primary mouse hepatocytes are a more physiological cell type compared to HEK293 cells, however it is much more difficult to achieve good transfection efficiencies in these primary cells.
Initially, the efficiency of siRNA transfection in hepatocytes was determined by using fluorescent tagged siRNA siGLO. Various transfection reagents (such as Ribojuice and Lipofectamine) and other conditions were tried, but no SiGLO were observed inside the cells as imaged by fluorescent microscopy (data not shown).

Having had no success with the SiGLO reagent, further attempts at siRNA transfection targeting the PP1 and PP2A catalytic subunit isoforms into hepatocytes at concentrations of up to 50nM were carried out. Cells were lysed after 24 h, 48 h and 72 h, but analysis with qRT-PCR showed no significant changes in mRNA levels of PP1 and PP2A isoforms after siRNA treatment at any time point tested (data not shown). Based on these results, it seemed unlikely to be able to introduce siRNA into these cells and achieve any significant knockdown. It was thus decided not to pursue knockdown of phosphatases in primary hepatocytes any further.
In independent experiments, HEK293 cells were transfected with either siControl or siRNA against various PPP isoforms. 72 h later cells were lysed and RNA extracted. Levels of mRNA for the PPP isoforms were determined using qRT-PCR. Levels of knockdown were calculated by comparing mRNA expression in cells treated with PPP siRNA with those transfected with siControl. All graphs shown represent averages and standard errors (SEM) from 3 independent experiments. 

A. Graph showing percentage mRNA remaining after PP1 knockdown. 

B. Graph showing percentage mRNA remaining after PP2A knockdown. 

C. Graph showing percentage mRNA remaining after PP4 or PP5 knockdown. 

D. Graph showing percentage mRNA remaining after multiplexed knockdown of PP1α and PP2Aβ.

Figure 3.29 Knockdown of PPP isoforms in HEK293 cells
Figure 3.30 Validation of protein knockdown of PP1 and PP2A
HEK293 cells were transfected with either siControl or siRNA against PP1 and PP2A isoforms (25 nM final concentration). Cells were lysed after 72 hours and lysates were subjected to western blot analysis with PP1 and PP2A antibodies (representative blots shown above). Tubulin was used as the loading control. The relative level of protein expression were quantitated using the Licor Odyssey software against tubulin. Data shown represent averages and standard errors (SEM) from 3 different experiments.
Figure 3.31 Knockdown of PPP isoforms does not affect the level of AMPK activation by phenformin

HEK293 cells were transfected with either siControl or siRNA targeting the PPP isoforms indicated. Knockdown was allowed to proceed for 72 h. Cells were then lysed or growth media were replaced with serum free media and treated with 5 mM phenformin for 30 minutes and before cell lysis. AMPK was then activity determined. Results are shown as fold AMPK activity relative to siControl transfection. Data shown represent averages and standard errors (SEM) from 3 independent experiments.
3.3 Discussion

The dephosphorylation of AMPK α at Thr-172 is of vital importance for the regulation of the enzyme, as phosphorylation of this residue is absolutely required for AMPK activity. Furthermore, as LKB1 has been determined to be constitutively active (Hawley et al., 2003; Woods et al., 2003a), this places further importance in the dephosphorylation step to regulate AMPK activity. All these factors make the identification of the AMPK phosphatase(s) of great importance to both the understanding of this signalling pathway and potentially modulating for AMPK activity for therapeutic purposes.

While two upstream kinases of AMPK have been identified as LKB1 and CaMKKβ in 2003 and 2005 respectively, and much work has been done subsequently to elucidate the finer details of their regulation of AMPK activity (Hawley et al., 2003; Woods et al., 2005; Woods et al., 2003a), the phosphatase(s) which regulates the dephosphorylation step remains relatively under investigated. At the beginning of this project, very little direct evidence was available as to the identity of the AMPK phosphatase. Several earlier studies has implicated AMPK phosphatase as belonging to the PPM/PP2C family, while further evidence gained through pharmacological inhibition of phosphatases also indicated a role for the PP2A family (Gimeno-Alcaniz and Sanz, 2003; Moore et al., 1991; Samari et al., 2005). Through the course of this study however the field has become much more contentious, with every class of protein phosphatases being suggested to regulate AMPK activity. The implications of these new results are discussed later in this section.

Characterisation of AMPK activity and activation in a model cell-line

It was important at the beginning of this study to identify a cell-line which fulfilled several criteria. First, it needs to be relatively well characterised with relatively rapid rate of division. It also needs to be easily transfectable with plasmid DNA and siRNA, enabling the expression and knockdown of specific candidate phosphatases. In addition this cell-line needs to expresses AMPK at relative high levels and the activity of endogenous AMPK should be able to be dynamically manipulated.
HEK293 was chosen cells as the model cell-line as it fulfils all the criteria laid out above. Previous studies have shown that both AMPK upstream kinases, LKB1 and CaMKKβ are expressed in HEK293 cells and AMPK activity was readily detectable (Sanders et al., 2007a). Furthermore, HEK293 cells are easily transfectable and generally expresses exogenous plasmids at high levels. Having said this however, there are many clones of HEK293 cells in circulation and therefore it was still important to characterise the particular batch of cells used in this study.

Initial characterisation of HEK293 cells involved determining the level of overall AMPK activity and the contribution by either AMPK α₁ or α₂ containing complexes respectively. In these cells, AMPK α₁ containing complexes have higher specific activities than α₂ containing complexes (Figure 3.1). However, as different antibodies are used in the immunoprecipitation of these complexes, drawing a direct comparison would be difficult. This experiment does however indicate that both AMPK α₁ and α₂ are expressed and active in these cells.

Using antibodies specific for the upstream kinases, both CaMKKβ and LKB1 was shown as being expressed and active in HEK293 cells, and that LKB1 activity was over 2 fold higher than that of CaMKKβ. However, again, as different antibodies were used for immunoprecipitation of the two upstream kinases, direct comparison would be difficult. Additionally, as CaMKKβ has not been activated in these samples while LKB1 is constitutively active, there is a possibility that when CaMKKβ is activated it may well stimulate AMPK to a greater extent than LKB1 (Collins et al., 2000; Sapkota et al., 2002).

As in changes in AMPK activity was the main readout in the experiments to identify the AMPK phosphatase, it was important to show that AMPK activity can be dynamically modulated in these cells. As HEK293 is a widely used cell-line, previous publications have shown that many AMPK activators are effective in these cells (Sanders et al., 2007a; Voss et al., 2011). In this study, indirect activators such as metformin, Phenformin were used, as well as the AICAR (which is taken up into cells and phosphorylated to form ZMP, an AMP analogue). In addition, compounds which cause a reduction in intracellular ATP levels were used (i.e. H₂O₂ and DNP), and finally the direct allosteric activator of AMPK, A769662 was also included. All
compounds used, with the exception of metformin and AICAR caused significant activation of AMPK (Figure 3.3). The lack of activation by metformin corroborates previous results by other members of our lab (data not published) and the inability of AICAR to activate AMPK in HEK293 has been reported previously (Marsin et al., 2000). While phenformin is cell permeable, HEK293 cells lack the OCT transporters necessary for the transport of metformin across the cell membrane, and ZMP has been reported not to accumulate in HEK293 cells (Kimura et al., 2005; Wang et al., 2002b).

**Phosphatase inhibition in HEK293 cells by okadaic acid**

To elucidate the identity of the AMPK phosphatase, it was decided to use protein phosphatase inhibitors which specifically target particular classes of phosphatases, narrowing down on the specific family that this enzyme belongs to, i.e. the PPP or PPM families. Although there are a number of PPP family inhibitors which are widely used in research, there are no PPM inhibitors currently available, thus the PPP inhibitor okadaic acid was used in this experiment. Okadaic acid is a marine toxin isolated from Halichondria okadai, and is a potent inhibitor or almost all PPP family phosphatases (Swingle et al., 2007). An useful feature of okadaic acid is that it has different affinities for different PPP phosphatases. The IC<sub>50</sub> values for PP2A and PP4 are around 0.1 nM, the value for PP5 is higher at approximately 3.5 nM, for PP1 on the other hand the IC<sub>50</sub> is around 15 – 50 nM. Thus, by treating cells with varying concentrations of Okadaic acid, it maybe possible to gain insights into the subclass of PPP phosphatases the AMPK phosphatase(s) belongs to.

Although there was some consensus in the field at the beginning of this study that one or more PPM members was the mostly like candidate for the AMPK phosphatase, data derived from phosphatase inhibitors have shown contradictory results. As an example, in isolated rat hepatocytes, while some sources report okadaic acid as having no effect on AMPK activity, others observe a significant increase Thr-172 phosphorylation (Moore et al., 1991; Samari et al., 2005). Due to these discrepancies in the literature, we decided to proceed from first principles in studies.

Although the IC<sub>50</sub> values of for PP2A, PP4 and PP5 are in the low nano-molar range, a higher concentration needs to be applied during cell treatment as the, intracellular
concentrations of compounds are often lower than the concentration applied to growth media. Additionally the presence of organic anion pumps (e.g. the multidrug efflux pump) cause a further decrease in intracellular concentration (Swingle et al., 2007). Therefore, we decided to treat HEK293 cells with okadaic acid concentrations of 10 nM, 50 nM and 500 nM. The 10 and 50 nM concentrations inhibit of PP2A, PP4 and PP5 (but not PP1) while the 500 nM concentration should provide inhibition of most PPP phosphatases.

To validate that okadaic acid is entering the cells and inhibiting its intended targets, phosphorylation of the known PP2A substrate of Acetyl-CoA carboxylase was used as the positive control (Moore et al., 1991). At the higher concentrations of 50 nM and 500 nM okadaic acid caused a significant increase in ACC Ser-79 phosphorylation indicating okadaic acid is indeed functional. However, at 10 nM, okadaic did not seem to have an significant effect on phospho-ACC levels. This result would support the previous statements that a higher treatment concentration than published IC50 values are needed to inhibit intracellular phosphatases (Figure 3.4).

AMPK activity was determined in lysates from okadaic acid treated HEK293 cells. Although no significant increases in AMPK activity was observed, there was a small increase in AMPK activity at 500 nM okadaic acid seen. However, this increase is unlikely to represent stimulation of AMPK due to phosphatase inhibition, as, due to the constitutively active nature of LKB1, any significant inhibition of AMPK phosphatase(s) would predictably lead to a much greater increase in AMPK activity. Secondly the small increase in AMPK activity correlated with a similarly small decrease in ATP:ADP ratios at the higher concentration of okadaic acid. Although neither of the changes were statistically significant, the decrease in ATP:ADP ratio levels could result in AMPK activation. Taken together, these results indicate that the AMPK phosphatase is okadaic acid insensitive, indicating that it is a member of the PPM family.

It should be noted here that overall, the development of phosphatase inhibitors lags behind that of kinase inhibitors. Of pressing need is for the development of a potent and specific cell permeable PPM inhibitor, of which none currently exist. Research has been conducted to find an inhibitor of PPM phosphatases, although this is still at
the validation stage, it could provide new tools for identifying phosphatases involved in AMPK regulation in the future (Aburai et al., 2010; Chuman et al., 2008; Rogers et al., 2006).

**Manipulation of PPM phosphatase expression**

Due to the lack of potent PPM inhibitors/activators, it was decided to alter the level of expression of specific PPM phosphatases and determine if there are any changes in AMPK activity as a result. This method has the advantage of assessing the function of each of the 17 PPM phosphatases individually.

Using q-RT-PCR, the expression of 16 out of the 17 known PPM phosphatases (Lu and Wang, 2008) was detected in HEK293 cells. The expression levels varied, but all 16 phosphatases were expressed in the same order of magnitude allowing the use of a single housekeeping gene. While this study was on-going, a study by Voss et al., analysed the activities of individual PPM isoforms phosphatases in HEK293 cells using phosphorylated casin as a substrate (Voss et al., 2011). Although the researchers did not measure the activities of all 17 PPM isoforms (e.g. PPM1K and PPM1M activities were not measured), the results from this study broadly matched the expression data generated in the current study. Voss et al, also reported PPM1G, PPM1B and PDP1 as having the highest activities again phosphorylated casein.

The next step in the study was to knockdown the expression of individual PPM phosphatases using siRNA transfections. Conditions for knockdown were optimised as shown in chapter 3.2.4. Due to time and resource constraints, knockdown of PPM1A was used as a proxy to optimise the siRNA concentration and time of knockdown for the other 15 PPM phosphatases. PPM1A was chosen as its mRNA expression levels is in the middle range of all 16 phosphatases (Figure 3.10) This method has potential drawbacks as the mRNA message levels and the protein half lives of the PPMs could be different, meaning the optimum siRNA concentration for one PPM may not result in the greatest levels of knockdown for others. Nevertheless, significant knockdown of mRNA expression levels for all 16 PPM isoforms was achieved, and protein knockdown verified using a number of specific PPM antibodies. In this experiment, no significant changes in AMPK activity was detected in any of the siRNA treated samples, whether in the individual knockdown of PPM isoforms...
or in the multiplex knockdown of PPM1F, PPM1H and PPM1L (Figures 3.14 and 3.15).

There have been previous reports showing differential regulation of the α1 and α2 subunits of AMPK, for example AMPK α2 show greater activation with AMP compared to α1 and the two isoforms tend to locate to different sub-cellular compartments (Salt et al., 1998). In addition, it has been reported that PP2A is involved in regulating the interaction between AMPK α2 and γ1 (Gimeno-Alcaniz and Sanz, 2003). With this in mind, AMPK was immunoprecipitated using AMPK α1 and α2 specific antibodies, in order to determine isoform specific activities. However, the kinase assays on these samples did not reveal any significant changes compared to overall AMPK activity (Figure 3.16).

It should be noted here that although small increases in AMPK activity of 10% - 20% were observed in some PPM knockdown samples, they did not reach statistical significance (Figures 3.14, 3.15 and 3.16). Furthermore, due to the observed ability of pharmacological agents to activate AMPK more than 5 fold in HEK293 cells, and considering the constitutively active nature of LKB1, it was expected that significant inhibition of AMPK phosphatase(s) expression should bring about an elevation in AMPK activity much greater than those seen.

It is possible that under conditions where AMPK activity is not stimulated, very little phosphatase activity is required to keep AMPK activity/phosphorylation at a basal level, and therefore the levels of siRNA silencing achieved in these experiments does not perturb the system to a sufficient extent to bring about a change in AMPK activity. It is also possible that during under conditions of AMPK activation, a greater quantity of AMPK phosphatase is recruited (perhaps due to a greater availability of its phosphorylated target), thereby leading to an increase in phosphatase activity under these circumstances., When phenformin was used to treat cells either with or without pre-treatment with PPM siRNAs, there was no significant differences in the level of activation of AMPK achieved (Figure 3.17).

Although much time and resources were devoted to the experiments highlighted above, it was not possible to assign the identity of the AMPK phosphatase. However
this does not prove that PPM has no role in the regulation of AMPK activity. There are several features inherent to studies that utilise siRNA knockdown that makes it somewhat difficult to draw this conclusion. Firstly, due to resource constraints and lack of availability, it was only possible to procure antibodies against four of the 16 PPM isoforms subjected to knockdown. Therefore, for the other 12 PPM isoforms, although good evidence mRNA silencing was obtained, it was not possible to verify the levels of protein knockdown. It is possible that due to differing half-lives of the phosphatase proteins, there could be substantially more protein remaining for some phosphatases after the knockdown period than the mRNA data would suggest. Furthermore, although treatment with siRNA was able to reduce mRNA/protein levels by up to 80%, there was nevertheless some protein expression remaining. It is possible that the remaining phosphatase activities are able to maintain AMPK activity at basal levels. There is also the possibility that AMPK is in fact regulated by a number of protein phosphatases, which creates a level of redundancy within the system. Although, siRNA silencing allows for a limited degree of multiplexing of knockdown targets within the same cells, it is extremely difficult to decide which combinations of phosphatases to multiplex. It is also possible that there are as yet unidentified okadaic acid insensitive phosphatases which may dephosphorylate AMPK. Additionally, although the differential regulation of AMPK α₁ and α₂ isoforms were investigated in this study, there is a possibility that different combinations of α, β and γ subunits are differentially regulated by different phosphatases. Again, the scope of this study does not allow this issue to be addressed.

While the current study was in progress, a study by Voss et al. suggested PPM1E as a AMPK phosphatase. This study was also conducted using HEK293 cells, and used shRNA mediated stable knockdown to deplete phosphatase expression (Voss et al., 2011). With PPM1E depletion, a significant elevation in AMPK phospho-Thr-172 levels was detected, which was not present when other PPM isoforms were knocked down. PPM1E depletion was also able to further increase phenformin induced AMPK activation in HEK293 cells. These findings do not match our observations. In our experiments, siRNA mediated knockdown of PPM1E did not cause any significant changes in AMPK activity. We revisited PPM1E and conducted a new knockdown experiment, achieving around 64% reduction in mRNA levels. Again no significant change in AMPK activity was seen (included as part of the final results) (Figure 3.14).
Professor Tricia Cohen (University of Dundee) kindly supplied us with the antibodies targeting PPM1E and PPM1F used in the above study so that we would be able to investigate the level of protein knockdown. However, I was unable to optimise conditions for the use of these antibodies as the blots were not of sufficient quality for phosphatase expression levels to be interpreted.

One possible reason for the discrepancy between the two studies Voss et al. used shRNA mediated stable knockdown of PPM isoforms which is able to maintain silencing of phosphatases over longer time periods. Some proteins may have relative long half-lives, therefore the treatment period of 72 hours in my experiments may not be sufficient to cause a significant reduction in the protein level of some phosphatases.

Overexpression of PPM isoforms in HEK293 cells

In other published studies which attempted to identify the AMPK phosphatase(s), experiments where phosphatases are over-expressed are generally not performed. One reason for this maybe that as PPP type phosphatases generally form multimeric complexes, there are simply too many possible combinations of catalytic and regulatory subunits for over-expression to be a feasible prospect. Over-expressing only the catalytic subunit for example may cause the other subunits to become rate limiting. In the case of PPM phosphatases, as they generally lack regulatory subunits, this would not be an issue, although over-expression could cause unspecific off targets effects.

However, as these assumptions have never tested, PPM isoforms were over-expressed in HEK293 cells. Four PPM phosphatases were over-expressed and verified in vitro to be active, but this did not cause any changes to basal AMPK activity or activation by phenformin (Figure 3.20). This data would suggest that the four phosphatases investigated in this study do not dephosphorylate AMPK in HEK293 cells. Although no positive data emerged from this experiment, it was nevertheless possible to show that despite the lack of regulatory subunits and their ability to dephosphorylate AMPK in vitro, PPMs activity seem to be tightly regulated within the cell, and unspecific dephosphorylation does not occur when phosphatases are over expressed. This result
may give researchers more confidence to conduct PPM over-expression experiments in the future.

**Revisiting inhibitor studies**

The results from the knockdown and the over-expression of PPM isoforms in HEK293 cells showed that it is possible that the AMPK phosphatase belongs to the PPP family. Therefore, it was decided to revisit the phosphatase inhibitor studies while altering the experimental setup. Initially, it was decided to apply different concentrations of okadaic acid to HEK293 cell suspensions after cell lysis, (lacking the usual phosphatase inhibitors usually present in cell-lysis buffer used) to see if this can protect AMPK from dephosphorylation. This experimental setup was chosen as in cell lysates, the limited cell permeability of okadaic acid should not be a problem, allowing it to inhibit phosphatases at concentrations close to its IC$_{50}$ values (Swingle et al., 2007).

The results generated from this experiment seem to suggest that PP1 inhibition is essential for the protection of AMPK from dephosphorylation. At 5 nM, where most PP2A activity should be inhibited (IC$_{50}$ of okadaic acid for PP2A is 0.1 – 0.3 nm), no protection from dephosphorylation was seen. Whereas at concentrations higher than 40 nM, AMPK phosphorylation/activity became protected (IC$_{50}$ of okadaic acid for PP1 is 15 – 50 nm) (Figure 3.21). Also, it would seem that inhibition of PPM type phosphatases are not required to protect AMPK activity, as okadaic not effective against this class of phosphatase. The main drawback of this experiment is its somewhat non-physiological nature. As in cytosolic suspensions, the normal regulatory mechanisms that act on phosphatases would be disrupted and the normal cellular compartmentalisation that may separate enzymes from unintended targets would be removed, thereby allowing the phosphatases enzymes to act non-specifically. Thus, inhibiting these enzymes may protect AMPK from dephosphorylation merely by virtue of inhibiting most of the phosphatase activity in cells. Nevertheless, these results do give additional hints as to how to proceed in future studies.

The results from the above experiment coupled with published studies suggesting that PP1 and PP2A being involved AMPK regulation prompted us to revisit the inhibitor
studies and use the potent PPP inhibitor calyculin A to try to elicit a change in AMPK activity (Garcia-Haro et al., 2010; Magnaudeix et al., 2012; Wang et al., 2010).

Calyculin A was able to cause a much greater increase in ACC pSer-79 levels compared to okadaic acid treatment in both HEK293 cells and primary mouse hepatocytes (Figures 3.22 and 3.26). These results suggest that calyculin A inhibited phosphatases to a greater extent than okadaic acid. In contrast to okadaic acid treatment, calyculin A caused a significant increase in AMPK activity of around 50% in both HEK293 and mouse hepatocytes cells (Figures 3.23 and 3.27). This is likely to be due to the more potent inhibition of phosphatase activity by this compound. In addition, incubation with calyculin A was able to protect against AMPK dephosphorylation seen in recovering cells after H₂O₂ treatment (Figure 3.25). Together, results from this section would indicate a role for calyculin A sensitive PPP phosphatases in the regulation of AMPK dephosphorylation. Taking into account the results from okadaic acid treatments, and the differences in their potencies against PP1 and PP2A, it makes PP1 the more likely candidate for the AMPK phosphatase compared to PP2A. Additionally, it would appear that perhaps a small amount of phosphatases are capable of maintaining AMPK activity at basal levels, and inhibition of a significant proportion of the phosphatase activity (to an extent that okadaic acid was unable to achieve in HEK293 cells) is required to observe the consequent activation of AMPK.

Knockdown of PPP isoforms

Next, it was decided to carry out siRNA knockdown of PP1 and PP2A in HEK293 cells and determine the effect on AMPK activity. Unlike the phosphatases of the PPM family, enzymes of PP1 and PP2A classes are holoenzymes made up of both catalytic, regulatory and scaffolding subunits. As there are many more regulatory and scaffolding subunits compared to catalytic subunits, it was decided to direct the siRNA knockdown towards the catalytic subunits. Substantial knockdown of the catalytic subunits should limit the rate of the dephosphorylation of its targets regardless of the level of regulatory subunit expression in the cell. The knockdown of the catalytic subunits of PPP isoforms, PP1, PP2A, PP5 and PP6 were performed.
siRNA silencing did not replicate the data gained from inhibitor studies (Figures 3.31).

While this study was in progress, a study implicated the PP1 catalytic subunit, along with its regulatory unit R6 to play important roles in the glucose induced dephosphorylation of AMPK in mouse pancreatic beta cells (MIN6) (Garcia-Haro et al., 2010). Knockdown of the α and β catalytic subunits of PP1 together or the R6 regulatory subunit was shown to prevent the decrease in AMPK phosphorylation when MIN6 cells were transferred from low to high glucose media. This effect did not depend on the activation of the upstream kinase LKB1. Additionally, knockdown of PPM1A (in the same study) had no effect. Furthermore, the R6 subunit was shown to interact physically with the AMPK β subunit through co-immunoprecipitation and yeast-two hybrid experiments. Also, PP1 was found to be the most efficient at dephosphorylating pre-activated, bacterially expressed AMPK. MIN6 cells were obtained (from James Leiper, CSC MRC) and I was able to verify the co-immunoprecipitation of AMPK and R6 in these cells (data not shown). Additionally, my initial data suggested that calyculin A was able to significantly increase basal AMPK activity and inhibit the glucose induced decrease in AMPK activity. It should be noted however, that only a 25-35% drop in AMPK activity was seen after shifting between low to high glucose media (data not shown). These results serve to back up the data shown by Garcia-Haro et al, however as the data was already in press, we did not continue with the validation process in MIN6 cells. However I was unable to replicate these results of siRNA knockdown fully in HEK293 cells. Knockdown of PP1 catalytic subunits were unable to prevent the decrease in AMPK activity caused by shifting the cells from low to high glucose media. There are several possibilities explanations for the discrepancy between my results that those of Garcia-Haro et, al. Firstly, the phosphatase that dephosphorylates AMPK may be different in HEK293 and MIN6 cell. Also there maybe a higher reserve of phosphatase activity in HEK293 cells, meaning the level of phosphatase knockdown achieved may not be sufficient to alter AMPK activity. Furthermore, MIN6 is a pancreatic beta cell-line. The AMPK signalling pathway in these cells maybe more sensitive to changes in glucose levels, as these cells are adapted in vivo to sense and respond to changes in extra-cellular glucose levels. This property may explain why HEK293 displayed a smaller drop in
AMPK activity/phosphorylation when shifted from low glucose to high glucose media. This sensitivity of the AMPK pathway to glucose levels may also mean that the system can be more easily perturbed by altering the expression of phosphatases. Additionally, it should be noted that the regulation of AMPK activity by PP1 maybe specific to the conditions of glucose deprivation, and there are other phosphatases in the maintenance of AMPK activity under basal conditions.

In addition to PP1, during my study PP2A was also proposed as negatively regulating AMPK activity. Both okadaic acid treatment (at concentrations which only inhibited PP2A) and silencing of the PP2A catalytic subunit was shown to be able to reverse heat shock induced AMPK Thr-172 dephosphorylation in HepG2 cells (Wang et al., 2010). More recently, a similar study showed both okadaic acid and PP2Ac silencing resulted in increased Thr-172 phosphorylation in cultured cortical neurons (Magnaudeix et al., 2012). It was shown that in C2C12 and primary muscle cells, chronic calcium exposure resulting from caffeine treatment leads to decreased AMPK activities, which was reversed with the application of the phosphatase inhibitor Calyculin A or PP2Ac siRNA (Park et al., 2013).

Taken together recent publications discussed in this section seem to suggests that the regulation of AMPK dephosphorylation is cell–type specific, with PPM, PP1 and PP2A all identified as AMPK phosphatases in different cell-lines. In addition, it is possible that the regulation of AMPK dephosphorylation is condition specific, with different phosphatase(s) responsible for maintaining basal AMPK activities (e.g. PPM), and other phosphatases (e.g. PP1/PP2A) becoming more important in conditions of cell-stress.

In the case of mouse hepatocytes, knockdown experiments were attempted in these cells as they are more physiological compared to HEK293 cells, with alterations in AMPK having been shown to play important roles in vivo. However, I was unable to successfully gain knockdown of any phosphatases in these cells using siRNA. Other members of our lab have also reported technical problems with siRNA transfection into hepatocytes. However, recently member of our group have been able to gain significant knockdown of cytosolic proteins in primary hepatocytes using hydrophobically-tagged siRNA oligos which do not require transfection reagents to
enter cells (Dr. Alicia Garcia, private communication). In future experiments, knockdown of phosphatases could be achieved using this method.

**Conclusions**

The result obtained in this study, suggest that the AMPK phosphatase in HEK293 cells and mouse hepatocytes lie within the phospho-protein phosphatase family, and in this family PP1 is the more likely candidate than PP2A.

While I did narrow down the enzyme class the AMPK phosphatase belongs to, we were unable to pinpoint the specific phosphatase(s) which mediate AMPK dephosphorylation. The potential reasons for this have been discussed at length in this section. The experimental results from this study and current literature both suggest that instead of a ‘master’ phosphatase, the regulation of AMPK dephosphorylation is mediated by multiple phosphatases. Indeed, one possibility is that the regulation of AMPK dephosphorylation is tissue specific, with distinct groups of phosphatases responsible for regulating AMPK activity in different cell-types. AMPK may also be dephosphorylated by distinct phosphatases under different conditions of cellular stress. Overall, the picture that is emerging for the regulation of AMPK by upstream phosphatases is rather more complicated than our current understanding of the upstream kinases. This study has shed light on some of the regulatory mechanisms at work in this important regulatory step, and is a good platform upon which future studies can be conducted.
4 Identification of AMPK interaction proteins

4.1 Introduction

After the experiments described in chapter 3 failed to pinpoint the specific AMPK phosphatase(s), a decision was made to change the direction towards identifying new AMPK interaction proteins, with a view of identifying AMPK phosphatase(s). The majority of previous AMPK binding proteins have been identified using co-immunoprecipitation reactions coupled with western blotting (Garcia-Haro et al., 2010; Kim et al., 2011b). However, this method requires the investigator to have prior knowledge about the identity of the AMPK interacting protein. Another method used by investigators to find novel AMPK interactors is the yeast two hybrid technique (Fu and Gao, 2009). However, this method has been shown to generate many false positive results (Deane et al., 2002). In addition, proteins which normally interact in mammalian cells may not do so in yeast, and the fusion proteins may actually block potential binding sites.

In this section, an approach using immunoprecipitation coupled with mass spectrometry was adopted for the identification of AMPK interacting proteins. This method does not require any prior knowledge as to the identity of the AMPK interacting protein and allows the simultaneous identification of multiple candidates.

4.2 Results

4.2.1 Over-expression of AMPK subunits in HEK293 and COS7 cells

To maximise the chance of detecting interacting proteins and to capture transient interactions, it was initially decided to chemically cross-link overexpressed AMPK (α1β1γ1) in HEK293 and COS7 cells, and use mass spectrometry techniques to identify AMPK interacting proteins. Initially, Pilot experiments to optimise the expression of
the AMPK complex in these cells and verify that the protein is active as expressed were carried out.

Mammalian expression plasmids (in pCDNA3.1) encoding for each of the \( \alpha_1 \), \( \beta_1 \), and \( \gamma_1 \) subunits of AMPK were already available in the group. These plasmids were transfected into either HEK293 or COS7 cells and AMPK expression determined by western blotting with AMPK subunit specific antibodies. The pan-\( \beta \) subunit antibody was used to immunoprecipitate AMPK from cell lysates to determine the activity of the overexpressed AMPK complex.

As can be seen in Figure 4.1, significant over-expression of AMPK \( \alpha_1 \), \( \beta_1 \), and \( \gamma_1 \) was achieved in HEK293 and COS7 cells. Quantitation of the western blots revealed that the level of over-expression achieved for the three subunits was very similar in both cell types (data not shown). AMPK activity in immune complexes showed an approximately 8-fold increase in HEK293 cells and a ~12 fold increase in COS7 cells relative to untransfected cells (Figure 4.2).

4.2.2 Chemical Cross-linking of proteins in HEK293 and COS7 cells

After confirming overexpression of AMPK in both HEK293 and COS7 cell types, experiments aimed at chemically cross-linking proteins in these cells were performed in order to provide a snapshot of protein-protein interactions.

It was decided to use paraformaldehyde as the main crosslinking reagent. Paraformaldehyde acts primarily by crosslinking lysine residues of adjacent proteins, and thereby serves to preserve transient protein interactions which may otherwise be lost during co-immunoprecipitation. Another advantage of paraformaldehyde as a chemical cross-linker is its short arm length of around 2.3–2.7 Å, and thus would be expected to only cross-link proteins that are in close proximity (Sutherland et al., 2008). Secondly, it also acts to inactivate most enzymes immediately upon addition to cells there by effectively ‘freezing’ the protein interactions in place.

Proof of principle experiments were conducted to confirm the ability of paraformaldehyde to cross-link proteins in these cells and the optimum time period of
cross-linking. For this experiment, HEK293 and COS7 cells were transfected with AMPK $\alpha_1\beta_1\gamma_1$ with expression proceeding for 48 hours, 1% paraformaldehyde was added to cells to cross-link proteins for either 5 or 15 minutes. After the reaction was terminated using glycine solution and the cells lysed.

To verify whether this method resulted in the crosslinking of AMPK, cell lysates were subjected to western-blot analysis and probed with the AMPK pan-β-subunit antibody. As seen in Figure 4.3, paraformaldehyde treatment of both cell-lines resulted in the appearance of higher molecular mass bands of AMPK β with a concurrent decrease in the intensity of the untreated band. This indicates that these subunits are being crosslinked to adjacent proteins, thereby giving rise to these higher molecular mass bands. From the above results, it was decided to use both the 5 and 15 minutes time points for cross-linking.

**4.2.3 Immunoprecipitation of AMPK and mass spectrometry analysis of cross-linked proteins**

In order to prepare the sample for mass spectrometric analysis, both HEK293 and COS7 cells over-expressing AMPK $\alpha_1\beta_1\gamma_1$ were subjected to paraformaldehyde treatment for 5 and 15 minutes and cells lysed. AMPK was immunoprecipitated from cell lysates using the pan-β subunit antibody. To verify the ability of this antibody to immunoprecipitate the cross-linked form of AMPK, the immune complex was subjected to western blot analysis probing for AMPK $\alpha_1$. As can be seen from Figure 4.4, the β subunit antibody was able to co-immunoprecipitate not only native $\alpha_1$ subunit but also the cross-linked $\alpha_1$ in HEK293 cells. Similar results were obtained for COS7 cells lysates, data not shown.

Before the proteins in the immune complexes can be identified using mass spectrometry, it was necessary to reverse the cross-linking. This was achieved by heating the immune complexes in 2X SDS-sample buffer overnight at 60 °C. To verify that this process resulted in efficient reversal of the cross-linking, the reversed and non-reversed samples were subjected to western blot analysis. Figure 4.5 shows that the cross-linking of AMPK $\alpha_1$ can indeed be reversed to a significant extent.
following this treatment. After this step, the immunoprecipitated AMPK and associated proteins were resolved using SDS-PAGE and sent for mass spectrometric analysis (MS) (CSC-Proteomics unit).

All over-expressed AMPK subunits were detected in the immune complexes as well as the endogenous AMPK α₂, β₂ and γ₂ subunits. The detection of these endogenous subunits provides an internal control which indicates the cross-linking and immunoprecipitation steps have worked as anticipated. A further 200–400 other proteins were detected in the various cross-linked samples, with the 5 min cross-linking time giving more protein hits compared to the 15 min time point. Amongst these proteins, PP2A regulatory subunits A-α and B-α were present in both HEK293 and COS7 cells subject to 5 min cross-linking. Furthermore in the 5 minute cross-linked COS7 sample, PP2A-α catalytic subunit, PP1-γ catalytic subunit, PP5 and PPM1G was detected.
Figure 4.1 Over-expression of AMPK α₁β₁γ₁ in HEK293 and COS7 cells
pCDNA3.1-HIS encoding for AMPK α₁β₁γ₁ was transfected into (A.) HEK293 or (B.) COS7 cells using calcium phosphate transfection reagent, cells were lysed after 48 h expression. Soluble cell lysates were subjected to western blot analysis probing with antibodies targeting AMPK α₁, and β and γ₁ subunits. Duplicate lanes are shown for each condition. Representative images were captured and processed using the Licor infrared system associated Odyssey software.
Figure 4.2 Over-expression of AMPK α1β1γ1 leads to increased AMPK activity in HEK293 and COS7 cells
AMPK was immunoprecipitated using the pan-β-subunit antibody (from cells transfected with AMPK α1β1γ1 or un-transfected control) and AMPK activity determined. Results are shown as fold AMPK activity relative to untransfected control experiment. Data shown represent averages and standard errors (SEM) from 3 independent experiments.
Figure 4.3 In-vivo cross-linking of proteins in COS7 and HEK293 cells

In-vivo cross-linking of proteins was performed in COS7 and HEK293 cells overexpressing AMPK α1β1γ1 using 1% paraformaldehyde with cross-linking allowed to proceed for 5 or 15 minutes before being quenched by 1.25 M glycine. Cells were lysed and subject to western blot. Representative western blot of (A.) COS7 and (B.) HEK293 cell crude lysate probed with AMPK pan-β subunit antibodies. Cross-linked AMPK-β subunit protein are highlighted by brackets.
Figure 4.4 Immunoprecipitation of cross-linked AMPK using pan-β subunit antibodies

Immunoprecipitation reactions were performed on cell lysates from HEK293 cells (uncross-linked and 5 min cross-linked, duplicate lanes are shown for each condition) using the AMPK pan-β subunit antibody. The immuno-pellet was subjected with western blot analysis with AMPK α1 antibodies. Representative images were captured and processed using the Licor infrared system associated Odyssey software.

Figure 4.5 Reversal of paraformaldehyde cross-linking

Cell lysates from COS7 and HEK293 cells which were subjected to paraformaldehyde cross-linking (5 min) were heated in 2X SDS sample buffer over night at 60 °C. Lysates were then subjected to western blot analysis and probed with AMPK α1 antibodies. Representative images were captured and processed using the Licor infrared system associated Odyssey software.
4.2.4 Stable isotope labelling by amino acids in cell culture and quantitative proteomics in HEK293 cells

At this point in the study, it became apparent that without a quantitative readout, it was very difficult to gauge which of the candidate interactors identified by MS analysis are *bona fide* physiological AMPK binding proteins and which were non-specific interactors. In order to obtain a quantitative readout for potential AMPK interactors and thereby validate specificity, it was decided to use a technique called stable isotope labelling by amino acids in cell culture (SILAC) coupled with quantitative proteomics. SILAC is a method used to achieve incorporation of labels into proteins for identification and quantification using mass spectrometry. The labels to be incorporated are either ‘heavy’ or ‘normal’ versions of amino acids (e.g. L-Lysine and L-Arginine), with the ‘heavy’ version containing substituted stable isotopic nuclei, while the ‘normal’ version contains nuclei found predominantly in nature (e.g. $^{13}$C labelled L-Lysine as the ‘heavy’ amino acid and $^{12}$C L-Lysine as the ‘normal’ version). When two identical cell populations are grown in media containing either ‘heavy’ or ‘normal’ forms of particular amino acids, all newly synthesised proteins in the two populations will contain amino acids that, while functionally identical will have different molecular masses. It only takes a few cell divisions to achieve replacement of the vast majority of the amino acids concerned with its isotopic analogue. These two cell populations can then be subjected to different conditions (e.g. one treated with a pharmacological agent and the other one untreated), and the cellular protein lysates extracted. The lysates can then be mixed and subjected to quantitative mass spectrometry, which can identify pairs of chemically identical peptides containing the ‘heavy’ or ‘normal’ isotopes. The changes in protein expression, modifications or activities caused by the differing condition(s) can then be identified in the ratio of peak intensities in the mass spectrum for the peptide/protein pairs, which is proportional to their abundance.

In the experiment to quantitatively identify AMPK interactors, due to cost and material constraints, it was decided to use only one cell-type for the initial studies. HEK293 cells was chosen because their rapid division ensures rapid incorporation of the substitute amino acids. In addition, AMPK expression and activity have been characterised extensively in these cells (see Chapter 3). Clonal HEK293 cells...
expressing endogenous AMPK were grown in media containing either ‘heavy’ or ‘normal’ versions of the amino acids L-Lysine and L-Arginine for approximately 10 cell division cycles, after which the cells were lysed.

The cell lysates (200 µg total protein) containing ‘heavy’ isotope amino acids were immunoprecipitated with antibodies targeting various AMPK subunits, whereas lysates containing ‘normal’ isotope amino acids were immunoprecipitated with pre-immune serum (as the control for non-specific interactions). The specific AMPK antibodies used along with the corresponding control sera are shown in Table 4.1. The AMPK and non-targeting immunoprecipitations were mixed and resolved together using SDS-PAGE, after which the gel was sent for MS analysis (CSC-Proteomics unit). Quantification is shown as a ratio of enrichment of the amount of a particular AMPK associated protein in immunoprecipitations with AMPK antibodies from the ‘heavy’ isotope lysate as compared to immunoprecipitations with non-targeting antibodies from the ‘normal’ isotope lysate. From these experiments, a list of interacting proteins was produced for each immunoprecipitation with an enrichment value for each protein. A threshold was set, whereby a potential interaction protein has to be enriched 2 fold or more by immunoprecipitation with AMPK pan-β antibodies compared to non-targeting antibodies. Proteins reaching this threshold were considered for further analysis.

<table>
<thead>
<tr>
<th>IP number</th>
<th>Heavy isotope IP</th>
<th>Light isotope IP</th>
<th>AMPK activation</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Pan-β Subunit antibody</td>
<td>Pre-immune serum, Rabbit</td>
<td>basal</td>
</tr>
<tr>
<td>2</td>
<td>α₁ + α₂ subunit antibodies</td>
<td>Sheep IgG</td>
<td>basal</td>
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Table 4.1 Antibodies and controls used in SILAC MS
Table showing the antibodies used and corresponding control immunoprecipitations for the SILAC MS experiments conducted in this chapter.

As can be seen from (Table 4.2), both immunoprecipitations resulted in high levels of enrichment of AMPK subunits, including the α₁, α₂, β₁, β₂, γ₁ and γ₂ subunits. The enrichment for these subunits ranged from 7.6 to 31.7 fold. In addition, the known AMPK substrate acetyl-CoA carboxylase was detected in the immunoprecipitation with both α and β subunit antibodies under basal conditions. Immunoprecipitation
with AMPK α enriched ACC protein by 4.7 fold whereas immunoprecipitation with the β subunit resulted in 2.6 fold enrichment (Table 4.2).

Analysis of the data revealed that all three members of an important cellular complex were enriched by both AMPK α and β immunoprecipitations (Table 4.2). The PAK1/GIT1/βPIX complex consists of P21-activated protein kinase 1 (PAK1), ARF GTPase-activating protein 1 (GIT-1) and Rho guanine nucleotide exchange factor 7 (βPIX). PAK1 and its associated proteins are involved in the regulation of cytoskeletal arrangement and cell motility (Ong et al., 2011b).

Due to the ability of PAK1 and its associated proteins GIT1 and βPIX to be immunoprecipitated by both AMPK α and β antibodies with large enrichment folds, this interaction is unlikely to be due to non-specific protein binding and may represent a ‘real’ interaction with physiological effects. In addition, PAK1 is an established controller of cell motility and growth. In recent years AMPK has also been implicated to regulate similar processes (Bae et al., 2011; Nakano et al., 2010; Nakano and Takashima, 2012). For these reasons the next part of this thesis was spent characterising the nature of AMPK’s interaction with the PAK1/GIT1/βPIX complex.
<table>
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<tr>
<td>Protein Name</td>
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<td>---------------</td>
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Table 4.2 Potential AMPK interactors identified using SILAC/MS
Table showing a list of protein binding proteins of AMPK identified using the SILAC/MS technique coupled with immunoprecipitations with AMPK Pan-β-subunit antibody or AMPK α₁+α₂ subunit antibodies. Proteins hits which reached the threshold of 2 fold enrichment after Pan-β-subunit immunoprecipitation are included. AMPK subunits identified are highlighted in red. Known AMPK substrates are highlighted in Green. And The PAK1/GIT1/βPIX complex are highlighted in bold. Abundant proteins which are common to most protein-interaction experiments (e.g. ribosomal subunits and tubulin) have been omitted from this list.
(Mass spec analysis performed by the MRC CSC-Proteomics unit)
4.3 Discussion:

In this Chapter, immunoprecipitation with specific AMPK antibodies coupled with mass spectrometry analysis was used to identify novel AMPK interacting proteins. A number of interesting candidates were identified including the PAK1/GIT1/βPIX complex (characterised in Chapter 5).

Due to the wide range of upstream regulators and downstream targets of AMPK, it is logical to suggest that at any one time, only a very small percentage of AMPK would be interacting with any particular binding protein. Therefore, various techniques were utilised in order to maximise the chance of detecting AMPK-interacting proteins. AMPK was over-expressed in HEK293 and COS7 cells to increase the amount of protein available for binding. In-cell cross-linking using paraformaldehyde was also employed to capture weak or transient interactions which might be lost during the course of the isolation process. There are several potential drawbacks with this approach. Firstly, the significant increase in AMPK protein levels due to over-expression may cause the enzyme to localise to cellular compartments which do not normally express endogenous AMPK and may potentially form non-physiological interactions. Secondly, the cross-linking procedure captures all molecules within a certain distance of each other (Nadeau and Carlson, 2007). Although the linker distance of paraformaldehyde has a very short arm length of 2.3–2.7 Å, any molecule close enough to AMPK will be captured, regardless of whether or not it forms a physiological relevant interaction. However, considering that the physiological relevance of all potential interactors will be validated using traditional biochemical techniques, any false positive results should be ruled out at this later stage.

By performing AMPK over-expression coupled with in vivo cross-linking and immunoprecipitations with specific AMPK antibodies, a number of potential interaction proteins were obtained using MS techniques. In all experiments, multiple isoforms of the α, β, and γ subunits of AMPK were identified, which served to confirm that both the immunoprecipitation and the mass spectrometric processes were producing expected results. However several drawbacks to the experimental setup became apparent at this point, and will be discussed below:
In relation to the technique of in cell cross linking, the number of associated proteins detected decreased with the longer cross-linking period of 15 minutes as compared with the 5 minutes time point. This result is puzzling, as one would expect that with a longer cross-linking time, an increasing number of proteins close together would be linked and therefore a greater number of associated proteins would be detected. One explanation for this anomaly is that the larger cross-linked complexes created by the longer cross-linking time point would be less able to be digested in the trypsinization step prior to loading onto the mass spectrometer (although effectors were made to reverse the cross-linking before MS analysis). As a result, these partially digested peptides may be less able to be detected by the mass spectrometer and therefore fewer associated proteins are identified. This theory would appear to suggest that although the cross-linking step may help to capture transient interactions which may otherwise to be lost, there may be difficulties involved in the detection of the cross-linked complexes using mass spectrometry, thus resulting in some potential interactors being left undetected. As a result, it was decided that in future mass spectrometric experiments, no chemical cross-linking would be employed.

Another drawback with the above set-up was that while hundreds of proteins would be identified in each immune complex, without a quantitative readout as to the amount or ratio of each associated protein relative to AMPK it is very difficult to determine which interaction is “real” and which is unspecific. It would be unfeasible and wasteful to validate all of the potential interactors identified.

Considering the points discussed above it was decided to employ a quantitative proteomics approach to better identify AMPK interactors. To this end, stable isotope labelling by amino acids in cell culture (SILAC) to achieve cellular labelling of proteins with “heavy” and “light” versions of the same amino acid in the HEK293 cells was employed. This method, first reported around 10 years ago, has proven to be an effective technique for the identification of protein interaction networks in various cellular pathways (Blagoev et al., 2003; Ong and Mann, 2005). This method coupled with quantitative mass spectrometry allowed me to obtain a figure for the fold enrichment of a particular interacting protein after immunoprecipitation with AMPK antibodies.
In these experiments, multiple AMPK subunit isoforms were greatly enriched after AMPK immunoprecipitation, and this result acted as an internal control validating the experimental approach. Furthermore, enrichment of the AMPK substrate acetyl-CoA carboxylase (ACC) in immune complexes with both AMPK α and β antibodies was seen. This is the first time our group was able to measure this interaction, and further serves to validate the usefulness of the method.

This study has identified many potential physiological AMPK interacting proteins (of which reached the set threshold of two fold enrichment with the AMPK pan-β-antibody (see Table 4.2 for full list), however due to the time and resource taken to characterising these interactions and elucidating its downstream effects, it was only possible to select a few candidates to follow up. After consideration, the next part of this thesis was dedicated to investigating the nature of interaction between AMPK and the PAK1/GIT1/βPIX complex.

There were several reasons for this decision. Firstly, as stated in the results section, all three members of the complex were highly enriched by both AMPK α and β antibodies, reducing the chance of unspecific binding of the proteins to a particular antibody. Another reason for this choice was the important role the PAK1/GIT1/βPIX complex in both normal physiology and pathology. Of the three proteins in this complex, the most tractable for study is the serine/threonine kinase PAK1. The regulation and function of PAK1 and its relevance to AMPK signalling are described in detail in the introduction section.

Although time and resource constraints limited the number of AMPK interacting proteins we could follow up. There were other interesting candidates that could form the basis of a new study. For example, the proteins mitogen-activated protein kinase kinase kinase 5/ASK1 (MAP3K5) and Mitogen-activated protein kinase kinase kinase 15 (MAP3K15) were enriched following pull-downs with both AMPK α and β antibodies (Table 4.2). Both ASK1 and MAP3K15 are part of the mitogen-activated protein kinase pathway, which phosphorylates and activates Mitogen-activated protein kinase kinase (MAP2K) which in turn activates Mitogen-activated protein kinase (MAPK). Examples of MAPKs include c-Jun N-terminal kinase (JNK) and
p38 MAPK. This pathway is activated in response to various types of stress including osmotic pressure, tumour necrosis factor-α, endoplasmic reticulum stress and reactive oxygen species (Soga et al., 2012). MAPKs, which are the effectors of this pathway, plays important roles in the regulation of apoptosis, inflammation, and cell morphogenesis.

A recent paper has proposed a potential link between AMPK and ASK1. Treatment of MCF-7 breast cancer cells with the plant flavonoid quercetin caused activation of both ASK1 and AMPK, and activation AMPK are necessary for ASK1 to mediate its apoptotic effects through stimulation of the downstream kinase p38 MAPK. The authors goes on to suggest that AMPK may be an upstream regulator of ASK1 (Lee et al., 2010). In comparison to ASK1, rather less is known about MAP3K15. In recent studies however, MAP3K15 has been shown to be increased in liver cirrhosis and knockdown of MAP3K15 protected Hela cells from stress-induced cell death. Knockout of MAP3K15 in mice have led to the appearance of a hypertensive phenotype. In fact the similarities between MAP3K15 and ASK1 has led researcher to propose ASK3 as the new name for this kinase (Kaji et al., 2010; Naguro et al., 2012). It can be seen that identification of two enzymes of the MAP3K family as AMPK interaction proteins is an interesting result and in future studies, these interaction can be investigated further and any physiological implications elucidated.
5 Investigation of AMPK regulation of PAK1

5.1 Introduction

Having identified that the PAK1/GIT1/βPIX complex interacts with AMPK, I went on to investigate the functional and physiological implications of this interaction. In this chapter, experiments were conducted both in cells (transformed and primary cells) and in cell free assays. Using these approaches, AMPK was found to both phosphorylate and activate PAK1. In addition experiments were also conducted to elucidate the physiological relevance of this activation.

5.2 The PAK1/GIT1/βPIX complex interacts with AMPK

Co-immunoprecipitation experiments were conducted to validate the MS data showing an interaction of AMPK with the PAK1/GIT1/βPIX complex. In order to detect endogenous levels of PAK1, GIT1 and βPIX, HEK293 cell lysates (20 μg total protein) were subjected to western blot analysis. All three proteins were detected in HEK293 cell lysates at molecular masses corresponding to their predicted masses (Figure 5.1). Having confirmed that available antibodies were able to detect endogenous levels of these proteins, the ability of the complex to co-immunoprecipitate with AMPK was investigated. Anti-AMPK α (α1+α2) antibody was used to immunoprecipitate AMPK from HEK293 cell lysates (300 μg total protein). The immune-complexes were subjected to SDS-PAGE and western blot analysis using antibodies against PAK1, GIT1 or βPIX. As shown in Figure 5.2, all three proteins were detected in the AMPK immune complexes. Due to cross-reaction with the IgG light chain, it was not possible to detect the β and γ subunits of AMPK in the immune-complexes by western blotting (not shown). Immunoprecipitations were also conducted with anti-PAK1 antibody and the immune-complexes subjected to western blot analysis probed with anti-AMPKα antibodies. As can be seen in Figure 5.2, AMPK α was readily detected in anti-PAK1 immune complexes.
Figure 5.1 Detection of endogenous PAK1, GIT1, βPIX in HEK293 cells
HEK293 lysates (20 µg) were subjected to western blot analysis with anti-GIT1, -PAK1 and -βPIX antibodies and detected using the Licor Odyssey system. The migration of molecular mass markers is indicated on the left and the identity of the bands is indicated on the right.

Figure 5.2 Validation of AMPK interaction with PAK1, GIT1 and βPIX
HEK293 cell lysates were subjected to immunoprecipitation analysis using anti-AMPK α antibodies (sheep IgG was used as control immunoprecipitation) and immune-complexes analysed by western blot analysis using anti-PAK1, -GIT1 and -βPIX antibodies. Anti-PAK1 antibody was also used in immunoprecipitation reactions (Protein A Sepharose as control) and the resulting western blot probed for AMPK α. (Note: For the detection of GIT1 interaction with AMPK, lanes were separated as different parts of the same gel were used for AMPK α and control immunoprecipitations). (n = 2 independent experiments)
5.3 Investigation of the effect of AMPK on PAK1

As PAK1, GIT1 and βPIX form a complex, it is likely that AMPK interacts with this complex as a whole rather than with each of these proteins individually. Due to time constraints, it was decided to concentrate on experiments to validate the effects of AMPK on PAK1, rather than focus on the nature of the AMPK/PAK1/GIT1/βPIX complex interaction. This decision was made as the function of PAK1 is relatively well understood compared to the other two proteins, and as a kinase is the main effector protein when PAK1, GIT1 and βPIX are in complex.

5.3.1 Phosphorylation of PAK1 by AMPK

In order to investigate whether AMPK directly phosphorylates PAK1, commercial, unactivatable GST-PAK protein (from Stratech) was obtained. This PAK1 isoform contains a partial (77 amino acid) deletion of its kinase domain which renders it unactivatable. PAK1 protein was incubated with $^{32}$P-ATP in the presence and absence of active AMPK ($\alpha_1\beta_1\gamma_1$) (AMPK expressed and activated as described in chapter 2). The incorporation of $^{32}$P was visualised using autoradiography. As can be seen from Figure 5.3, whereas incubation of PAK1 alone with $^{32}$P-ATP did not result in any detectable radiolabelling, incubation of PAK1 with active AMPK resulted in readily detectable $^{32}$P-phosphate incorporation. This result demonstrates that AMPK phosphorylates PAK1 in vitro. Additional labelled bands migrating at approximately 63 and 38 kDa correspond to AMPK $\alpha_1$ and $\beta_1$ subunits, respectively.

An estimation of the stoichiometry of phosphorylation (ATP incorporation) was determined from the autoradiograph this estimated by spotting serial dilutions of the $[^{32}\text{P}]$ATP onto filter paper and exposing this alongside the gel. Using this method the stoichiometry was estimated to be around 0.4 – 0.8 mole/mole for different experiments.
Figure 5.3 AMPK phosphorylates PAK1 in vitro

GST-PAK1 (1000 ng) was incubated with $[^{32}\text{P}-\gamma]\text{ATP}$ in the presence and absence of AMPK $\alpha_1\beta_1\gamma_1$ (200 ng) for 45 minutes at 37°C. Samples were resolved by SDS-PAGE and visualised by staining with Coomassie (upper panel) and radiolabelled products detected by autoradiography (lower panel). The corresponding molecular markers are indicated on the left. Identity of the protein and radiolabelled bands is indicated on the right. ($n = 3$ independent experiments)
5.3.2 Identification of AMPK phosphorylation sites on PAK1

After finding that AMPK directly phosphorylated PAK1, the next step was to identify the specific residue(s) on which this phosphorylation took place. GST-PAK1 construct (obtained via a kind gift from Katrin Rittinger (NIMR, MRC)) was used to generate kinase dead GST-PAK1 (GST-KD-PAK1) by introducing a mutation of aspartic acid residue 406 (within the ATP-binding region of the PAK1 kinase domain) to an alanine. GST-WT-PAK1 and GST-KD-PAK1 were purified from E.coli (as described in sections 2.26). A Coomassie stained gel of the purified proteins is shown in Figure 5.4. The full length GST-PAK1 migrates at approximately 95 kDa. However, as can be seen, in addition to the full-length protein, a number of degradation products were also present after protein purification. Densitometric analysis of the gel revealed that full-length PAK1 accounts for about 15% - 30% of the total protein in various purifications. All protein bands were confirmed as PAK1 by probing western blots with specific PAK1 antibodies (data not shown). The band corresponding to full-length PAK1 was excised from the gel and subjected to mass spectrometry, following digestion by trypsin. All of the peptides identified corresponded either to PAK1 or GST.

GST-KD-PAK1 was incubated with $^{32}$P-ATP in the presence and absence of AMPK and analysed by SDS-PAGE followed by autoradiography. KD-PAK1 was phosphorylated by AMPK (Figure 5.5). In a parallel incubation, GST-KD-PAK1 was incubated with AMPK and unlabelled ATP, and the band corresponding to full-length PAK1 excised, digested with trypsin and subjected to analysis by MS.

The mass spectrometry analysis achieved sequence coverage of around 65% and is shown in Figure 5.6. A total of three phosphopeptides were identified with >90% confidence, and these are highlighted in green in the sequence coverage. In two of the peptides it was possible to assign the phosphorylation sites unambiguously: SAEDYNSSNTLNVK (Ser-156 of PAK1) and LRSIVSVGDPK (Ser-258 of PAK1). In the third peptide, however, it was not possible to assign the phosphorylation site and any one of the three residues highlighted are candidates: NTSTMIGAGSK (Thr-20, Ser-21 and Thr-22 of PAK1). Despite this ambiguity, it was decided to investigate the role of phosphorylation within this peptide, since Ser-21 lies within a favorable
consensus sequence for AMPK phosphorylation and a previous publication has implicated the equivalent residue in PAK2 (Ser-20) as a potential target for phosphorylation by AMPK (Banko et al. 2011). Additionally a previous study has implicated residues in this region as being phosphorylated by protein kinases such as Akt (Banko et al., 2011; Fryer et al., 2006).

![Figure 5.4 Purification of recombinant PAK1](image)

**Figure 5.4 Purification of recombinant PAK1**
Glutathione S-transferase (GST) tagged WT or KD (D406A) PAK1 were expressed in *E. coli* and purified using glutathione-Sepharose chromatography. Proteins (5 µg) were resolved by SDS-PAGE and visualised by staining with Coomassie Blue. The migration of the molecular weight markers is indicated on the left (kDa), the identity of the bands are indicated on the right.
Figure 5.5 AMPK phosphorylation of Kinase dead PAK1
GST-KD-PAK1 was incubated with $[^{32}\text{P}-\gamma]\text{ATP}$ in the presence and absence of AMPK for 30 minutes at 37 °C. Samples were resolved by SDS-PAGE and visualised by staining with Coomassie (upper panel) and radiolabelled products detected by autoradiography (lower panel). The bands corresponding to full-length GST-PAK1 are shown. The corresponding molecular markers are indicated on the left. Identity of the protein and radiolabelled bands are indicated on the right.
**Figure 5.6 Phosphopeptide mapping of PAK1 phosphorylated by AMPK**

GST-KD-PAK1 was incubated with ATP in the absence and presence of active AMPK. The proteins were resolved using SDS-PAGE and the band corresponding to full-length GST-PAK1 was digested with trypsin and subjected to MS analysis. The sequence coverage is indicated in red with phosphorylation sites which appear after addition of AMPK shown in green.
5.3.3 Production of mutant PAK1 protein

In view of the results shown above, it was decided to produce mutant versions of PAK1 on GST-WT-PAK1 and GST-KD-PAK1 backgrounds. In the first mutant, all three residues, Thr-20, Ser-21 and Thr-22 were mutated into non-phosphorylatable alanine residues (here on referred to as the AAA-mutant). In view of the study showing Akt phosphorylating PAK1 on Ser-21 and the more recent study showing phosphorylation of PAK2 by AMPK at Ser-20 (Banko et al., 2011; Zhou et al., 2003), a second mutant where only Ser-21 was mutated to alanine (S21A-PAK1) was also generated. In total, four mutants versions of PAK1 have been expressed and visualised using SDS-PAGE (shown in Figure 5.7).

5.3.4 AMPK phosphorylation of mutant PAK1 proteins

To identify the major phosphorylation site(s) of AMPK on PAK1, active AMPK was incubated with either GST-KD-PAK1, GST-AAA-KD-PAK1 or GST-S21A-KD-PAK1 in the presence of $^{32}$P-ATP. $^{32}$P-Phosphate-incorporation was visualised by autoradiography. As shown in Figure 5.8, both the triple AAA mutation and the S21A mutation significantly reduced the ability of AMPK to phosphorylate PAK1. This data would indicate that the major AMPK phosphorylation site on PAK1 is Ser-21.
The S21A single mutation and the T20A, S21A, T22A (AAA mutant) triple mutation was introduced into GST tagged expression constructs coding for WT and KD PAK1. Proteins were expressed in *E. coli* and purified using glutathione-Sepharose chromatography. Proteins (5 μg) were resolved by SDS-PAGE and visualised with Coomassie Blue staining. The migration of the molecular mass markers is indicated on the left (kDa), and the identity of the bands are indicated on the right.

**Figure 5.8 AMPK phosphorylation of mutant PAK1**
GST-KD-PAK1, GST-KD-T20A S21A T22A (AAA) -PAK1, and GST-KD-S21A-PAK1 were incubated with $[^32P]\gamma$ATP in the presence of activated AMPK for 30 minutes at 37°C. Samples were resolved by SDS-PAGE and visualised by staining with Coomassie (upper panel) and radiolabelled products detected by autoradiography (lower panel). The bands corresponding to full-length GST-PAK1 are shown. Identity of the protein and radiolabelled bands are indicated on the right (*GST-KD-PAK1 incubation with AMPK shown in duplicate*).
5.3.5 AMPK activates PAK1 in vitro

In order to determine the functional effect of AMPK phosphorylation on PAK1, phospho-antibodies against two PAK1 activation sites were obtained, namely pSer-199 and pThr-423 (Bokoch, 2003). WT-PAK1 was incubated with ATP in the presence and absence of active AMPK. The samples were then subjected to western blot analysis using pPAK1 activation site antibodies. As shown in Figure 5.9, when PAK1 was incubated with ATP alone there was no detectable phosphorylation of either pSer-199 or pThr-423. However, following the addition of AMPK, strong signals were observed for both sites, suggesting that AMPK phosphorylation of PAK1 leads to concomitant activation.

To further confirm that AMPK activates PAK1, PAK1 activity was measured before and after phosphorylation by AMPK. The commercial PAK1 substrate PAKtide was obtained. PAKtide was synthesised according to the preferred PAK1 phosphorylation consensus sequence and thus should be a specific substrate (Rennefahrt et al., 2007; Wu and Wang, 2003; Yoshii et al., 2001). To validate the ability of activated PAK1 to phosphorylate PAKtide, Cdc-42 activated PAK1 (Stratech) was taken into radioactive kinase assays with PAKtide. As shown in Figure 5.10A, PAK1 was able to phosphorylate PAKtide. Next, varying amounts of PAK1 was used to phosphorylate PAKtide to determine linearity of this assay. As seen in Figure 5.10B, 32P-phosphate incorporation into PAKtide was directly proportional to the amount of PAK1 used, thus this substrate can be used to directly assess PAK1 activity.

In order to determine whether AMPK phosphorylated PAKtide, which could interfere with subsequent experiments, AMPK was taken into a PAKtide assay (Figure 5.11). AMPK is much less able to phosphorylate PAKtide compared to the SAMS substrate. The efficiency of phosphorylation PAKtide was 2.8% that of SAMS. These results indicate that AMPK binding to PAK1 should cause little interference in the PAK1 activation assays. Nevertheless, appropriate control experiments were included in all subsequent experiments.
WT or mutant GST-PAK1 (all produced in house) was incubated with unlabelled-ATP in the presence of active AMPK. After incubation, glutathione-Sepharose beads was used to precipitate GST-PAK1 from the mixture and the PAK1 activity assayed using PAKtide (Figure 5.12). Validation experiments conducted showed that there were no detectable unspecific binding of AMPK activity directly to the glutathione sepharose used to immunoprecipitate PAK1. In addition, to determine the basal level of PAK1 activity, un-activated GST-WT-PAK was incubated with ATP in the absence of AMPK, and then precipitated using glutathione-sepharose., PAKtide assays performed using these precipitants showed no detectable kinase activity.

As seen from Figure 5.12, co-incubation with AMPK resulted in much higher activity of WT-PAK1 compared to that of KD-PAK1 or S21A-WT-PAK1 (both mutants achieving similar specific activities of <15% compared to WT-PAK1 after incubation with AMPK). Together, these results suggest that AMPK phosphorylation of PAK1 on Ser-21 leads to PAK1 activation. To note, In our study, the specific activity of PAK1 achieved by AMPK incubation was around 25%-30% that of Cdc42 activated PAK1.
Figure 5.9 Incubation of AMPK with PAK1 results in phosphorylation of PAK1 activations sites

Purified GST-WT-PAK1 incubated with ATP in the presence or absence of activated AMPK. Western blot analysis was performed and the blot was probed with phosho-antibodies against PAK1 activation sites i.e. pT423 and pS199.
Figure 5.10 Validation of PAK1 kinase assay

A. Cdc-42 activated PAK1 (100 ng) was incubated with PAKtide for 30 minutes at 30 °C in the presence of [\(^{32}\)P-\(\gamma\)]ATP. Activities have been calculated and plotted as specific activity (nmol/min/mg).

B. Varying amounts of PAK1 (100 ng – 400 ng) were assayed as in (A) with PAKtide. Kinase activity is plotted as fold activity with the activities of 100 ng PAK1 taken to be 1 fold.
AMPK (αβγ) (100 ng) were incubated with PAKtide or SAMS peptide (as a positive control) for 30 minutes at 37°C in the presence of [³²P-γ]ATP. AMPK activity is plotted as percentage ³²P incorporation relative to that of SAMS assay for 100 ng AMPK (taken as 100%). Data shown represent averages and standard errors (SEM) from 3 independent experiments.
Figure 5.12 AMPK activates PAK1 in vitro

GST tagged WT-PAK1, KD-PAK1, S21A-WT-PAK1 was incubated with ATP in the presence of active AMPK (200 ng). PAK1 was then isolated by binding to glutathione-Sepharose. The glutathione-Sepharose beads were washed with PBS, and PAK1 activity present on the beads measured using PAKtide as substrate. Activities have been calculated and plotted as specific activity (nmol/min/mg). Data shown represent averages and standard errors (SEM) from 3 independent experiments.

* Indicates a significant difference in activity with when WT-PAK1 activities are compared to both KD-PAK1 and S21A-WT-PAK1 activities as judged by an one way ANOVA (P<0.05).
5.3.6 Activation of AMPK in HEK293 cells leads to increased PAK1 activity

Having demonstrated the ability of AMPK to activate PAK1 in vitro, experiments to determine whether this might occur in intact cells were carried out. HEK293 cells were treated with several compounds known to activate AMPK (see Figure 3.4). After treatment, the activity of endogenous PAK1 and AMPK were measured following immunoprecipitation from cell lysates.

All three treatments led to a significant activation of AMPK and a concurrent increase in PAK1 activity (Figure 5.13). In the case of PAK1, H₂O₂, phenformin and ionomycin caused increases in kinase activities of approximately 3.8 fold, 2.7 fold and 2.5 fold respectively. The result from this experiment suggests that AMPK is capable of activating PAK1 in cells, as well as in cell-free assays.

5.3.7 AMPK regulation of PAK1 activity in cortical neurons

After obtaining promising results in HEK293 cells, it was decided to conduct further experiments in a more physiological cell-type. Primary cortical neurons was used as the cell-type of choice due to its abundant expression of PAK1, and the established roles of the enzyme in regulating neuronal growth and axonal branching (Kreis and Barnier, 2009).

Primary mouse cortical neurons were isolated from embryonic day 14 C57 black-6 mice and cultured as described in Chapter 2. On day in vitro (DIV14) cells were lysed and immunoprecipitated with either AMPK or PAK1 antibodies. As shown in Figure 5.14, AMPK and PAK1 co-immunoprecipitated from the neuronal lysates.

Next, DIV14 cortical neurons were treated with three AMPK activators, Ionomycin, H₂O₂ and M991, a new novel AMPK activator (Giordanetto and Karis, 2012) (M991 corresponds to compound 9 in Figure 4 of this reference). As in HEK293 cells, both PAK1 activity and phosphorylation increased in line with AMPK phosphorylation (Figure 5.15A). H₂O₂ caused a 3.24 fold increase in AMPK phosphorylation and a 2.8 fold increase in PAK1 phosphorylation, while for ionomycin the increased
phosphorylation was 2.71 fold and 2.47 fold for AMPK and PAK1 respectively, the M991 compound was the weakest AMPK activators tested, causing increase in pT172 levels of 2.15 fold and pPAK1 of 1.79 fold. In addition, as can be seen in (Figure 5.15B), the activity of both enzymes correlate with the data obtained from western blot experiments. To note, the pPAK1 (pThr-423) antibody is also able to detect pThr-402 of PAK2. In our experiments with neuronal lysates, we picked up two bands, the top band corresponding to pPAK1 (MW 70 kDa) and a lower less pronounced band corresponding to pPAK2 (MW 62 kDa) (Figure 5.15) All quantitation was done with the top band.

In order to confirm the elevation of PAK1 activity caused by compounds shown above is indeed mediated through AMPK activation, neurons were treated with M991 in the presence and absence of the AMPK inhibitor Compound C. As can be seen from Figure 5.16, pre-treatment with compound C was able to largely abolish M991 mediated increases in PAK1 phosphorylation.

5.3.8 PAK1 phosphorylation and activity are reduced in AMPK α1 knockout cortical neurons

To further validate the results obtained above, a genetic model for AMPK depletion was sought, the AMPK α1 knockout mice was available in our lab, which have been previously verified (Jørgensen et al., 2004). AMPK α1 and α2 catalytic subunits are expressed throughout the brain, with higher expression for both subunits at embryonic stages compared to adult brains (Culmsee et al., 2001). Moreover, data from a recent study indicates that the expression of α1 in mouse cortical neurons is higher than that α2 expression (Williams et al., 2011).

Initially, we conducted experiments to measure AMPK α1 and α2 activities in wild type and α1 knockout neurons using subunit specific antibodies. As shown in Figure 5.17, AMPK α1 activity was largely abolished in the neurons of the knockout mice, being around 2-5% of the wild type neurons. On the other hand, AMPK α2 activity in the knockout neurons were found to be ~16% higher than wild type. However this difference was not statistically significant. Next, overall AMPK activity/phosphorylation (of both α1 and α2 subunits) was measured in the α1 knockout
and wild type neurons. As seen in Figure 5.18, in the α1 knockout neurons both AMPK activities (of 53%) and the phosphorylation of Thr-172 (of 43%) was much reduced compared to wild type.

Having confirmed that AMPK activity and phosphorylation is reduced in the α1 knockout neurons, experiments were conducted to see if this had any impact on PAK1 activity. Neuronal cell lysates from both wild type and α1 knockout mice were probed with the PAK1 pThr-423 antibody and also subjected to PAK1 kinase assays. PAK1 activity/phosphorylation in the α1 knockout neurons were significantly reduced compared to that of wild type (Figure 5.19). The levels of kinase activity and phosphorylation for AMPK and PAK1 were reduced to around 40% - 50% of the levels seen in wild type cells.

Next, to see if PAK1 activity can be stimulated by AMPK activators in the absence of AMPK α1, M991 was used to treat α1 knockout neurons, and the lysates then subjected to western blot analysis and kinase assays (Figure 5.20). M991 induced significant increases in the activities and phosphorylation of both AMPK and PAK1, despite the lack of AMPK α1 subunits. Increases in activities and phosphorylation of between ~0.8 fold and 1.2 fold was seen for both enzymes. These results suggest that both AMPK α1 and α2 complexes are able activate PAK1.
Figure 5.13 AMPK activation leads to elevated PAK1 activity in HEK293 cells
HEK293 cells were treated with H₂O₂ (0.5 mM 30 min), phenformin (5 mM, 30 min), or ionomycin (2.5 μM 5 min) and PAK1/AMPK were immunoprecipitated using either anti-PAK1 or anti-AMPK-pan-β antibodies respectively and their activity determined. Results are shown as fold activity of PAK1/AMPK relative to Mock treated (control) cells. Data shown represent averages and standard errors (SEM) from 3 independent experiments. The increases in AMPK/PAK1 activities as a results of all treatments shown above are statistically significant (P<0.05).

Figure 5.14 Co-immunoprecipitation of AMPK and PAK1 in mouse primary cortical neurons
Mouse cortical neurons were subjected to immunoprecipitation using antibodies against (A) AMPK α subunit (sheep IgG as the control) or (B) PAK1 (protein A sephrose as the control). Immune-complexes were subjected to western blot analysis using antibodies against either PAK1 (in A) or AMPKα (in B). Images obtained using the Licor infrared imaging system.
Figure 5.15 Stimulation of AMPK leads to PAK1 activation in mouse cortical neurons

(A) Cultured mouse cortical neurons (DIV14) were incubated with or without the AMPK activators H$_2$O$_2$ (0.5 mM 30 min), ionomycin (2.5 µM 5 min) and M991 (100 µM 60 min). After treatment, cells lysates were subjected to western blot analysis with anti-PAK1-pT423, AMPK pT172 and actin antibodies. The relative level of signals were quantified using the Licor Odyssey software using actin as a protein control. Results are shown as fold PAK1/AMPK activity relative to untreated cells. Data shown represent averages and standard errors (SEM) from 3 independent experiments.

(B) PAK1 and AMPK activity in immune complexes isolated by immunoprecipitation using anti-PAK1 or anti-AMPK-β antibodies was determined. Results are shown as fold PAK1/AMPK activity relative to untreated cells. Data shown represent averages and standard errors (SEM) from 3 independent experiments.

(The increases in AMPK/PAK1 phosphorylation/activities as a results of all treatments shown above are statistically significant, P<0.05)
Figure 5.16 Pre-treatment with the AMPK inhibitor Compound C prevents M991 induced PAK1 activation

Cultured mouse cortical neurons (DIV14) was incubated with or without M991 (100 µM 60 min), in the presence or absence of compound C (CC) (15 µM). After treatment, cells lysates were resolved using SDS-PAGE and subjected to western blot analysis with either PAK1 pT423, AMPK pT172 or actin antibodies. The relative levels of signal were quantified using the Licor Odyssey software using actin as a loading control. Data shown represent averages and standard errors (SEM) from 2 independent experiments

* Indicates a significant difference in activity/phosphorylation between M991 treated neurons and M991+CC treated neurons as judged by student’s t-test (P<0.05).
Figure 5.17 Activity of AMPK α₁ and α₂ complexes in WT and AMPK α₁ knockout cortical neurons
Antibodies targeting either AMPK α₁ and α₂ subunits were used to immunoprecipitate AMPK complexes from cell lysates (40 µg total protein) of cultured cortical neurons from either wild-type or AMPK α₁ knockout neurons (DIV14). The SAMS peptide assay was used to determine AMPK activity. Activities are plotted as specific activity (pmol/min/mg). Data shown represent averages and standard errors (SEM) from 3 independent experiments.
Figure 5.18 Overall AMPK activity/phosphorylation in AMPK WT and α₁ knockout cortical neurons

(A) Representative Western blot of total cell lysates (50 μg) from either wild-type or AMPK α₁ knockout neurons (DIV14) probed with pThr-172 and actin antibodies. The relative levels of signal were quantified using the Licor Odyssey software using actin as loading control.

(B) The pan-β subunit AMPK antibody was used to immunoprecipitate AMPK complexes from cell lysates of cultured cortical neurons from either wild-type or AMPK α₁ knockout mice (DIV14) and AMPK activity determined. Data shown represent averages and standard errors (SEM) from 3 independent experiments.

* Indicates a significant difference in activity/phosphorylation between the WT and α₁ KO neurons as judged by a student’s t-test (P<0.05).
Figure 5.19 PAK1 phosphorylation/activity is reduced in AMPK α₁ knockout neurons

(A) Representative western blot of total cell lysates (50 μg) from either WT or AMPK α₁ knockout cortical neurons (DIV14) (2 mice) probed with AMPK-pT172, PAK1-pT423, and actin antibodies. Relative levels were quantified using the Licor Odyssey software.

(B) PAK1 and AMPK was immunoprecipitated using anti-PAK1 or anti-AMPK-β antibodies respectively from either WT or AMPK α₁ knockout neurons, and PAK1 and AMPK activities assayed. Results are shown as percentage AMPK/PAK1 activity relative to the WT neurons. Data shown represent averages and standard errors (SEM) from 3 independent experiments.

* Indicates a significant difference in activity/phosphorylation between the WT and α₁ KO neurons as judged by a student’s t-test (P<0.05).
Figure 5.20 M991 treatment enhances PAK1 phosphorylation/activity in AMPK α₁ knockout cortical neurons
AMPK α₁ knockout cortical neurons (DIV14) (2 mice) were either mock treated (Control) or treated with the M991 (100 μM, 60 min) and cells lysed.

(A) Representative Western blot of total cell lysates (50 μg) were probed with AMPK-pT-172, PAK1-pT423, and actin antibodies. The relative level of signal were quantitated using the Licor Odyssey software against Actin from 3 independent experiments.

(B) PAK1 and AMPK was immunoprecipitated using anti-PAK1 or anti-AMPK-β antibodies and activities assayed. Results are shown as percentage AMPK/PAK1 activity relative to mock treated neurons. Data shown represent averages and standard errors (SEM) from 3 independent experiments.

* Indicates a significant difference in activity/phosphorylation with between the treated and non-treated neurons as judged by a student’s t-test (P<0.05).
5.3.9 Physiological implications of AMPK regulation of PAK1

PAK1 is known to be involved in axon branching and neurite formation (Zhang et al., 2005). Additionally, it has been reported that CaMKKβ facilitates neuronal spine outgrowth via positive regulation of the PAK1/GIT1/βPIX complex (Saneyoshi et al., 2008).

In view of these publications, the potential role of AMPK in regulating PAK1 mediated axonogenesis was investigated. Neurons from Wild-type or AMPK α1 knockout mice were isolated from E14 day embryos. On day in vitro 7, the neurons were either mock treated or treated for 5 days with agents which directly modulate AMPK activity, i.e. M991 and the AMPK inhibitor, Compound C. The CaMKKβ inhibitor STO-609, which has been shown to inhibit the formation of dendritic protrusions was used as a positive control (Saneyoshi et al., 2008). After treatment, neurons were fixed, permeabilized and stained with Alexa488-Phalloidin, which binds F-actin. When visualised using confocal microscopy, a good signal was obtained, with the actin filaments clearly visible (Figure 5.23). From general inspection of images, the density of axons/dendrites in each field appeared to increase with the application of AMPK activators and decrease following with treatment with either compound C or STO-609. Additionally, WT neurons seem to have a greater density of axons/dendrites compared to α1 knockout cells. Unfortunately, while axons and cell bodies were clearly visible, the fields were simply too dense to be able to identify individual dendrites or spines. Axons and dendrites of different cells were overlapping and it was not possible to follow the course of single dendrites with certainty. As a result, quantification of protrusions/spines from each dendrite was not possible, and thus it was not possible to obtain a figure for the effects of the various treatments or of knockout of AMPK α1 expression.

In an attempt to overcome these problems, in subsequent experiments neurons were plated at lower densities (from 25% - 50% of original density). However, at these lower densities, the survival of neurons in culture was greatly impaired.
Phalloidin was also applied at lower concentrations in an effort to lower the signal intensity. However, this had little effect and the field was still saturated with signal making the spines unidentifiable (data not shown).

In view of the experimental difficulties encountered, it was decided to try to obtain a more specific readout of axon/dendrite formation in place of actin-binding. To do this, quantification of postsynaptic density protein 95 (PSD95) was chosen. PSD95 specifically localises to the post-synaptic densities of dendritic spines (PSD) and has been shown to co-localise with PAK1 (Hayashi et al., 2007; Sheng and Sala, 2001).

In this experiment, wild type and AMPK α₁ knockout neurons were isolated and treated with M991, compound C or STO-609 as above. Cells were fixed and permeabilized after 4 days of treatment (DIV12). Two different PSD95 antibodies were obtained from Pierce antibodies (USA) and used to stain the treated and untreated neurons. However, after trying a variety of antibody concentrations, and other optimisation protocols, no signal could be obtained, despite being able to detect PSD95 protein by conventional western blotting (data not shown). This work is ongoing, and antibodies from Cell Signalling (Boston, USA) will be tested.
Figure 5.21 Effect of pharmacological treatment on dendritic branching of primary cortical neurons
Primary mouse cortical neurons (DIV7) were treated for 5 days with M991 (50 μM), Compound C (7.5 μM), STO-609 (20 μM). Cells were stained with Alexa488-Phalloidin and visualised under the Leica-SP5 confocal microscope. Representative images were taken with 40x objective lens and shown above.
5.3.10 AMPK enhances cancer cell invasion through PAK1 signalling

As introduced in Chapter 1, previous publications have reported that both AMPK and PAK1 may be involved in regulating the migration and invasion of a number of cancer cell types (Nagalingam et al., 2012). Recently, PAK1 has been suggested as a therapeutic target for BRAF wild-type melanoma (Ong et al., 2013). Through our collaboration with Professor Richard Marais’s group (Institute of Cancer Research, UK), we obtained two melanoma cancer cell-line, WM1361 and WM1366, to test if AMPK could modulate the invasion of these cells via activation of PAK1. Both WM1361 and WM1366 cell-lines contain the NRAS (Q61K) mutation. Expression studies performed by our collaborators have shown that PAK1 is expressed in both cell-lines.

To my knowledge, AMPK activity and activation has not been previously characterised in the cell-lines described above, and so this was established prior to further studies involving the migration/invasion assays. As can be seen, both cell types express active AMPK, with specific activities of 95 pmol/min/mg (lysate) for WM1361 cells and 58 pmol/min/mg for WM1366 (Figure 5.22). Next, the activation profile of AMPK for these cells was assayed in order to determine the most potent AMPK activator to be used in the invasion assay. Both cell-lines were treated for 16 hours with increasing concentrations of phenformin, AICAR and A769662. For both cell lines, the most pronounced AMPK activation was seen with A769662 treatment (200 μM). In WM1366 cells, A769662 (200 μM) was able to activate AMPK >5-fold. In WM1361 cells the level of activation achieved from all treatments was weaker with A769662 giving a maximal activation of ~3-fold (Figure 5.23). In view of the above results, A769662 was chosen for activating AMPK in the invasion assays.

Using the data generated above, our collaborators conducted assays to determine if AMPK activation affected the rate of invasion of melanoma cell-lines. Cells were placed at the bottom of the invasion chamber and separated from the chemo-attractant (DMEM with 10% FBS) by a layer of collagen. The cells are then given time to invade through the collagen towards the chemo-attractant, and following this the number of invading cells is quantified by confocal microscopy.
From the results, it can be seen that treatment with A769662 at 200 μM significantly \((P<0.05)\) increased the invasiveness of WM1361 and WM1366 cells by 1.96 fold and 1.65 fold respectively (Figure 5.24). At this point in the study, Professor Richard Marais’s group relocated to a different institute and were unable to continue to offer assistance in the running of the invasion assays. As a consequence, the remaining experiments were transferred in house. Due to time and resource constraints, it was decided to use only WM1361 cells in future experiments, as it showed the greatest increase in cell invasion after activation of AMPK.

Before conducting our own invasion assays, the effect of AMPK activation in WM1361 cells on PAK1 activity was determined. In addition, the effect of IPA3, an inhibitor of PAK1 (Deacon et al., 2008) was utilised in some experiments. Cells were treated for 16 hours with A769662 in the presence or absence of IPA3. After treatment, cells were lysed and AMPK/PAK1 activities determined. As shown in Figure 5.25, A769662 was able activate both AMPK and PAK1. IPA-3 had no effect on AMPK activation by A769662, while being able to largely abolish PAK1 activation by A769662. Having validated the activation of PAK1 through AMPK stimulation, and confirmed the effectiveness of IPA-3 as a specific PAK1 inhibitor, it was possible to investigate the effect of AMPK/PAK1 on invasion.

Due to the lack of a high-throughput confocal microscope for quantitation of invading cells in the Institute such as the INCELL 3000 as used by our collaborator, it was decided to alter the protocols and use a slightly different invasion assay in future experiments. The transwell invasion assay is based on the original Boyden chamber migration assay, in which cells migrate towards a chemo-attractant across a cell permeable membrane. To create an invasion assay, the porous membrane is blocked with an additional layer of extra-cellular matrix gel, through which the cells invades.

Transwell invasion assay kits were obtained from Millipore (Massachusetts, USA). In this experiment, WM1361 melanoma cells were subjected to four different conditions.  
1. Cells were pre-treated for 4 hours with A769662. Next, the pre-treated cells were washed into serum free DMEM containing A769662, and pipetted into the inserts.  
2. Cells were pre-treated with both A769662 and the PAK1 inhibitor IPA-3 and washed into serum free media containing both compounds and pipetted into inserts.  
3. Cells
were left untreated and washed into serum free DMEM and placed into inserts in 4. Cells were left untreated and placed into inserts in media containing 20% FBS to act as a no chemo-attractant control. The wells underneath all inserts conditional 1-4) were filled with media containing 20% FBS as chemo-attractant. The cells were then allowed to invade for 48 hours. After this, the ECM layer was removed along with non-invading cells. Cells which have invaded through the membrane were then stained using crystal violet dye. The number of stained cells were visualised under the microscope, with images of representative fields taken, The cells in each field are then counted and averaged (Figure 5.26).

As can be seen from Figure 5.26, treatment with A769662 causes the average number of invading cells per field to increase by 2.32 fold, from 31 cell/field to 71 cells/field (P<0.05), therefore corroborating the data from our collaborators that AMPK activation increased invasion of melanoma cells. However, with dual treatment of A769662 and IPA3, there was no significant increase in the number of invading cells beyond control.
Figure 5.22 Activity of AMPK in melanoma cell-lines
AMPK pan-β subunit antibodies was used to immunoprecipitate AMPK complexes from WM1361 and WM1366 cells (50 µg total protein) and AMPK activity determined. Activity has been calculated and plotted as specific activity (pmol/min/mg). Data shown represent averages and standard errors (SEM) from 3 independent experiments.
Figure 5.23 Activation of AMPK in melanoma cells
(A) WM1361 and (B) WM1366 cells were incubated for 16 hours in the presence or absence of either phenformin (1 mM and 2 mM), AICAR (200 μM and 400 μM), or A769662 (100 μM and 200 μM). After treatment AMPK activity was determined. Results are shown as fold AMPK activity relative to mock treated (control) cells. Data shown represent averages and standard errors (SEM) from 3 independent experiments.
Figure 5.24 AMPK activation stimulates invasion of melanoma cell-lines

WM1361 and WM1366 cells were incubated for 16 hours in the presence or absence of A769662 (200 μM) then taken into the 3D collagen invasion assay for 24 hours. Invasion index is calculated as the number of invading cells at 50 μm divided by number of cells at 3 μm. Values are expressed as invasion fold relative to mock treated (control) cells. Data shown represent averages and SEM from 3 independent experiments. * Indicates a significant increase in invasion between the treated and control cells as judged by a student’s t-test (P<0.05).

Figure 5.25 Treatment of WM1361 cells with A769662 and IPA3

WM1361 cells were incubated for 16 hours with or without either A769662 (200 μM), IPA3 (10 μM) or both A769662 and IPA3. After treatment, PAK1 and AMPK were immunoprecipitated using either anti-PAK1 or anti-AMPK-β antibodies respectively and activities determined. Results are shown as fold AMPK/PAK1 activity relative to the untreated control cells. Data shown represent averages and standard errors (SEM) from 3 independent experiments.
Figure 5.26 (A) A769662 increases invasion of WM1361 melanoma cells via PAK1 signalling

(A) Representative images of transwell invasion assays for WM1361 cells are shown (40x objective lens). Cells were placed into transwell invasion assay inserts in the presence and absence of either A769662 (200 μM), IPA3 (10 μM) or both compounds together. Cells were incubated for 48 hours at 37°C (to facilitate invasion), after which the non-invading cell were removed and invaded cells stained with crystal violet dye. The stained cells were visualised under a standard light microscope (bright field image 10X objective). With images random fields taken for each insert. (B) Graphical representation of the average number of cells per field for treated and non-treated cells (shown on the next page)
Figure 5.27 (B) A769662 induced AMPK activation increases invasion of WM1361 melaonma cells via PAK1

From images taken of treated and non-treated cells shown in Figure 5.27(A), cells in each field are counted and averaged, and the average number of cells per field for each treatment is shown on the graph above. Data shown represent averages and standard errors (SEM) from 3 independent experiments.
5.3 Discussion

In this section, the nature of the interaction between AMPK and PAK1 was investigated, extending from the initial results obtained in Chapter 4.

Validation of direct interaction between AMPK and the PAK1 complex

From the result shown in Chapter 5, immunoprecipitation with antibodies targeting either the AMPK α or β antibodies was shown to enrich for the PAK1/βPIX/GIT1 signalling complex.

Initially, experiments aimed at confirming that the PAK1/GIT1/βPIX complex interacts with AMPK in HEK293 cells were carried out. This was achieved using co-immunoprecipitation using AMPK and PAK1 antibodies, coupled with western blot analysis using reciprocal antibodies (and both AMPK and PAK1 antibodies) (Figure 5.2). The validation of AMPK/PAK1 binding using two different methods (MS analysis and western blotting) adds weight to the conclusion that this interaction does indeed occur in cells. It is well documented, that PAK1, GIT1 and βPIX are able to form a complex in vivo (Manabe et al., 2002; Manser et al., 1998), thus it is very likely that the PAK1/GIT1/βPIX signalling complex interacted with AMPK as an entire complex rather than as individual components. Unfortunately, while βPIX and GIT1 expression was readily detectable using the commercial antibodies available (Cell Signalling, USA) (and βPIX and GIT1 were detected in anti-AMPK-α immune-complexes), these antibodies did not prove successful in immunoprecipitation reactions.

Due to time and resource constraints, I was unable to fully characterise the nature of AMPK’s interaction with all three members of the PAK1/GIT1/βPIX complex. In most studies, PAK1 has been assumed to be the main effector protein in the complex. GIT1 and βPIX may be involved in the cellular localisation of the complex, in the regulation of PAK1 activation or providing protein scaffolds on which PAK1 phosphorylation of substrates can take place (Manabe et al., 2002; Saneyoshi et al., 2008; Turner et al., 1999). Therefore, I chose to concentrate my efforts on investigating the role of the interaction between AMPK and PAK1.
Phosphorylation of and activation PAK1 by AMPK in vitro

This study is the first to show direct phosphorylation of PAK1 by AMPK. In previous publications where AMPK has been suggested to activate PAK1, no direct phosphorylation was reported and it was assumed that activation occurred through Rac1 stimulation (an upstream regulator of PAK1) (Bae et al., 2011; Lee et al., 2008).

While it is accepted that the main mechanism of PAK1 activation is via binding of active Cdc42 or Rac1, studies have shown that phosphorylation of PAK1 is able to activate the enzyme. The first report of a potential upstream kinase of PAK1 came in 2000, when a study by King et al. showed phosphorylation of the critical activation site, Thr-423 in PAK1 only occurred via an intermolecular mechanism, and not through auto-phosphorylation as previously thought. Furthermore, 3-phosphoinositide-dependent kinase-1 (PDK1) was shown to directly phosphorylate and activate PAK1 at Thr-423 in vitro and expression of PDK1 in COS-7 cells led to increased PAK1 activation (Bokoch, 2003; King et al., 2000). Two separate studies have shown that both Akt and cGMP-dependent protein kinase (PKG) were able to activate PAK1 through phosphorylation of Ser-21. Furthermore, phosphorylation by Akt reduced the ability of PAK1 to bind the adapter protein Nck (Zhou et al., 2003). Additionally, in neuronal cells undergoing mitosis, cyclin-dependent kinase 5 (CDK-5) was found to phosphorylate PAK1 on Thr-212 leading to changes in the regulation of microtubule dynamics and remodelling of the axonal growth cone (Banerjee et al., 2002; Rashid et al., 2001). From these reports, it appears that PAK1 is regulated by direct phosphorylation as well as GTPase binding. While this study was in progress, a study was published which showed that AMPK was able to directly phosphorylate PAK2, another member of the Group 1 PAK1 family (Banko et al., 2011).

In light of the above publications, it was decided to investigate the possibility of AMPK acting as a PAK1 kinase. First, in-vitro phosphorylation experiments were performed where AMPK was used to phosphorylate PAK1.

Incubation of both commercial unactivatable PAK1 and KD-PAK1 (in house) with active AMPK led to PAK1 phosphorylation (Figures 5.3 and 5.5), showing that PAK1 is an AMPK substrate in vitro.
The specific residues on PAK1 phosphorylated by AMPK were identified. To eliminate auto-phosphorylation, catalytically inactive (kinase-dead) PAK1 was used in these phosphorylation experiments. Although, full length GST-PAK1 fusion protein was made, it was not possible to fully eliminate degradation of the protein. Of note, while other labs have produced less degraded PAK1 protein, expression was normally achieved in baculovirus and not E. Coli (Fryer et al., 2006; Zhou et al., 2003).

As with commercial PAK1, AMPK was also able to phosphorylate PAK1 protein produced in-house (Figure 5.5). Mass spectrometric analysis identified three AMPK phosphorylation sites. One of these sites resides in the NTSTMIGAGSK (Thr-20, Ser-21, Thr-22) peptide. While a single phosphate peak was detected, the candidate phosphorylation site could be any one of the residues highlighted in green.

Analysis with the ScanSite software showed that residues with in this peptide were potential AMPK phosphorylation sites, and Ser-21 is an already established Akt and PKG phosphorylation site (Fryer et al., 2006; Zhou et al., 2003). Furthermore, AMPK α2 has been reported to directly phosphorylate a member of the PAK family, PAK2 on Ser-20, which corresponds to the Ser-21 residue on PAK1. Using site-directed mutagenesis, Ser-21 was found to be the major AMPK phosphorylation site within PAK1 (Figure 5.8).

Having identified the major AMPK phosphorylation site in PAK1, the next logical step was to characterise its functional implications. The study by Zhou et al, has shown that phosphorylation of the Ser-21 residue by Akt was able to cause PAK1 activation and increased cell migration (Zhou et al., 2003), therefore my initial investigation focused on the effects of AMPK phosphorylation on PAK1 activity and cell migration/cancer cell invasion.

Phosphorylation by AMPK was able to significantly increase the activity of wild type PAK1, but not that of S21A-PAK1 or KD-AMPK (Figure 5.12). This result is in line with the publications described previously, where kinases such as Akt, PDK1, and CDK5 are able to phosphorylate and activate PAK1 independently of Cdc42 or Rac1. However, in previous studies as well as the present study, the level of activation by upstream kinases was less than by either Rac1 or Cdc42 (Figure 5.12). This indicates
GTPase binding may still be the most potent mechanism of PAK1 activation. PAK1 contains an auto-inhibitory domain (amino acids 70 – 149), which is released from the catalytic domain after GTPase binding (Lei et al., 2000). Ser-21 is not in the auto-inhibitory domain, so it is not surprising, perhaps, that phosphorylation of this site does not lead to full activation of PAK1 as the binding of Cdc42/Rac can achieve. It is possible that direct phosphorylation by AMPK acts to further modify the activation of PAK1 by Cdc42/Rac leading to altered/more potent effects for the PAK1 signalling pathway in vivo. In future, the interplay between AMPK and Cdc42/Rac in the regulating PAK1 activation could be investigated. It would be interesting to elucidate the effects of AMPK on the activity of PAK1 which has been pre-activated by upstream GTPases.

**Cellular models of PAK1 activation by AMPK**

In order to move the study from an in vitro basis to a more physiological environment, additional experiments in HEK293 cells were conducted. Treatment of HEK293 cells with the AMPK activators H2O2, phenformin and A769662 caused concurrent increases in both AMPK and PAK1 (Figure 5.13).

To substantiate these findings, further studies using a more physiologically relevant cell-type of primary embryonic mouse cortical neurons were carried out. This decision was made for several reasons. Firstly, PAK1 is well expressed in the brain, where it is involved in the regulation of processes such as axon generation and neurite branching (Kreis and Barnier, 2009). Secondly, neurons also exhibit substantial AMPK activity and express both AMPK α1 and α2 catalytic subunits (Bright et al, unpublished data). Additionally, the AMPK upstream kinase CaMKKβ has recently been implicated to promote neuronal spine outgrowth via activation of the PAK1/GIT1/βPIX complex (Saneyoshi et al., 2008).

As in HEK293 cells, known activators of AMPK were able to stimulate PAK1 in embryonic neurons and the trend of PAK1 activation tracked that of AMPK activation for all three activators used (Figure 5.15). Compound C was able to largely abolish the increase in both AMPK and PAK1 activities caused by M991, indicating that the M991 induced PAK1 activation was indeed acting through AMPK (Figure 5.16). In
addition to experiments using WT neurons, neurons from knockout mice, in which the AMPK activity was markedly reduced, were used (Figures 5.17 – 5.19). The reduction of PAK1 phosphorylation in α1 knockout neurons further reinforces the above results showing AMPK activation of PAK1 in cells. The ability of M991 to stimulate PAK1 activity in α1 knockout neurons suggests that stimulation of either α1 or α2 AMPK catalytic subunits is capable of activating PAK1.

The above results from HEK293 cells and primary neurons indicate that AMPK could activate PAK1 in cells as well as in vitro. Other groups have proposed links between AMPK and PAK isoforms in the past. Notably in the study by Lee et al, AMPK activation by retinoic acid and AICAR was shown to increase PAK phosphorylation (Lee et al., 2008). Another paper recently implicated AMPK activation to increase the phagocytotic ability of macrophages through stimulation of PAK1/2 activity (Bae et al., 2011). However, both these papers propose an indirect link whereby AMPK causes PAK activation via stimulation of Rac1. In the current study, evidence points to PAK1 activation via direct phosphorylation by AMPK. Additionally, the earlier reports do not specify which PAK isoforms is activated by AMPK, and the study by Lee et al did not specify the phosphorylation site their antibodies targeted.

The phospho-PAK1 antibodies used in my study cross react with PAK2 protein phosphorylated on Thr-402. Activation of AMPK in cortical neurons led to increased phosphorylation of PAK2. This result corroborates data from the recent study by Banko et al which shows AMPK-mediated phosphorylation and activation of PAK2 both in vitro and in cells, and suggests a more general role for AMPK in the regulation of the group 1 PAKs (Banko et al., 2011).
Physiological implications of PAK activation by AMPK

Having shown the ability of AMPK to regulate PAK1 activity through experiments conducted *in vitro*, in transformed cell-lines and primary cells, it was important to elucidate the functional significance of this regulation.

Many papers have previously implicated a role for PAK1 in the regulation of neuron morphology, polarity and the correct formation of axons and dendrites (Jacobs et al., 2007; Nikolic, 2008). Expression of constitutively active PAK1 has been shown to increase the number of dendrites and to encourage neurite outgrowth (Hayashi et al., 2007; Hayashi et al., 2002; Li et al., 2013). ShRNA mediated knockdown of PAK1 caused an almost global inhibition of neurite growth (Jacobs et al., 2007). Furthermore, expression of a mutant form of PAK1 resulted in formation of abnormal dendritic spines (Hayashi et al., 2007). In 2005, Rac was suggested to form a signalling module with the GIT1/βPIX/PAK complex which acts to phosphorylate MLRC, leading to increased dendritic spine formation (Zhang et al., 2005). Correct formation of dendritic spines is very important for cognitive development, and several genetic and non-genetic forms of mental retardation exhibit decreased spine densities (Kaufmann and Moser, 2000; van Galen and Ramakers, 2005). Inhibition of group 1 PAK isoforms resulted in impaired performance of memory tasks in a mouse model. Following on from the results showing memory impairment, activities and expressions of group 1 PAKs is reduced in neurodegenerative Alzheimer disease leading to the downstream loss of spine actin-regulatory protein drebrin. Additionally PAK inhibition in adult mice led to drebrin loss and memory impairment, strongly suggesting a role for PAKs in the development of the disease (Nguyen et al., 2008; Zhao et al., 2006). Also, PAK1 expression was seen to be up-regulated in brain cancers, its activities are associated with a poorer prognosis (Kumar et al., 2006).

In 2008 a study by Saneyoshi et al showed that CaMKKβ was involved in the regulation of the PAK1/GIT1/βPIX complex via activation of CAMKI. This led to PAK1 activation which results in increased formation of dendritic protrusions and increased frequency of mini-excitatory-postsynaptic-potentials (mEPSC). Specifically, CaMKKβ inhibition did not affect the development of initial projections called filapodia, which are long and thin in shape, while it did reduce the number of mature mushroom-shaped dendritic spines which form from the filapodia (Saneyoshi et al.,
As CaMKKβ is one of two major upstream kinases of AMPK, it is possible AMPK activation of PAK1 in these cells could lead to similar effects as that of CaMKKβ. In view of this, I modified the experiments performed by Saneyoshi et al, to determine if there were any differences in the formation of dendritic spines between wild type and AMPK α1 knockout neurons. In the 2008 study, neurons were transfected with RFP-tagged β-actin, which allowed the visualisation of the dendrites and associated protrusions under confocal microscopy. To avoid the technical difficulty of transfecting primary neurons, Alexa-488-phalloidin was used to stain for F-actin to visualise dendritic protrusions. Wild type or α1 knockout neurons were either left un-treated or treated with AMPK activators/inhibitors then stained with Alexa-488-phalloidin.

While good staining of actin filaments was achieved, there did seem to be a decrease in the density of dendritic protrusions on general inspection, although the field of cells was too dense to allow accurate quantitation of spines or filapodia. It is possible that the use of phalloidin to stain for actin filaments instead of expressing RFP-tagged β-actin caused this drawback. Whereas β-actin preferentially co-localised to the expanding edge of cells, phalloidin stains all F-actin structures, leading to bright staining of the neuronal cell body, axons and all dendrites which could interfere with the visualisation of small structures such as neuronal spines (Lodish et al., 2007). Plating primary neurons at lower densities resulted in significant impairment of dendrite branching and greatly increased death of cells. The reason for this occurrence is not clear, but one possibility could be that neurons in culture require a sufficient level of local paracrine secretion, the reduction in the concentration of these factors due to lower cell density may have impaired cell survival and dendritic branching. In future experiments, it would be prudent to optimise plasmid transfection into primary neurons so that the experiments conducted by Saneyoshi et al can be repeated in wild type versus AMPK α1 knockout neurons.

In view of the experimental difficulties encountered, a different readout of axon/dendrite protrusions than actin staining was attempted. PSD95, which specifically localises to the postsynaptic densities of dendritic spines and forms a lattice immediately under the postsynaptic membrane, emerged as an interesting candidate. This protein is involved in the anchoring of synaptic proteins such as...
potassium channels, NMDA and AMPA receptors (Sheng and Sala, 2001). Due to its localisation at the tips of dendritic spines, the formation of which PAK1 has been shown to positively regulate, PSD95 was chosen as the readout for altered PAK1 function downstream of AMPK (Deo et al., 2013; Hayashi et al., 2007; Zhang et al., 2005). In addition, PAK1 has been shown previously to co-localise with PSD95 in dendritic spines, thereby regulating spine formation and maintenance (Hayashi et al., 2007). This work is on-going, and preliminary data have indicated a decrease in PSD95 protein level in AMPK α1 knockout neurons (data not shown).

**The role of AMPK regulation of PAK1 in cancer invasion**

In parallel with the study of AMPK regulation of PAK1 in neurons, another line of investigation was initiated to look at possible links between the two enzymes in controlling the migration of normal cells as well as invasion of cancer cells. As a regulator of cytoskeletal dynamics and cellular motility, PAK1 has a recognised role in promoting migration of cells (Eswaran et al., 2012a; Whale et al., 2011). In recent years, AMPK has also been implicated in as a controller of these processes. Chapter 1 gives a detailed introduction of this topic.

This research was conducted with the help of our collaborators from Richard Marais’s group at the Institute of Cancer Research, who specialise in research into melanoma cancers. After discussion, we obtained two melanoma cell-lines which express both AMPK and PAK1. Several studies have previously shown that both PAK1 and AMPK play a role in the growth and migration of melanoma cells (Martin et al., 2012; Ong et al., 2011a; Ong et al., 2013). The cell-lines, WM1361 and WM1366 carry mutations in the small GTPase NRAS, which was shown to be important in neoplastic development (Colombino et al., 2012; Kelleher and McArthur, 2012).

As part of our collaboration, Professor Marais’s group members performed invasion assays using melanoma cells. This invasion assay was originally developed by Professor Chris Marshall’s group (ICR, UK) (Ahn et al., 2012; Sanz-Moreno et al., 2008). Results from this experiment showed that pre-treating WM1361 and WM1366 cells with A769662 significantly increased the ability of these cells to invade through collagen. Unfortunately, at this point Professor Marais’s lab relocated from London to
Manchester and his group were unable to assist further, so the study was transferred to our group. The time constraints of my PhD meant I concentrated my experiments using the WM1361 cell-line which showed the strongest increase in invasion after AMPK activation. Additionally, the lack of a fully automated confocal microscope prevented me from fully replicating the collagen invasion assays. After some time, it was decided to use a different setup in the form of the transwell invasion assay to conduct further experiments.

This assay shares similarities with the collagen invasion assay. Both involve the cell invading through an extra-cellular matrix-like substance towards a set chemo-attractant. The transwell invasion assay has been described in detail in a 2011 review by Marshall (Marshall, 2011), however a brief description will be included here. As stated before, this assay is a modification of the Boyden chamber migration assay, the difference being that a layer of matrix gel is placed over the porous membrane of the Boyden chamber to simulate the extra-cellular matrix for cells to invade through (Chen, 2005). Pre-made invasion chambers are available commercially (Millipore, USA), and these invasion chambers act as inserts which fit into multi-well plates. Cells are placed into the inserts without chemo-attractant, while the media containing chemo-attractant is placed into the multi-well plate (which sits under and surrounds the inserts). The cells are then left to invade and the outcome can be visualised under microscope by counting the number of cells which has invaded through the ECM layer and migrated through the membrane. Like the collagen invasion assays, A769662 was able to significantly promote invasion of WM1361 cells in the transwell assay, and this effect was abolished by treatment with the specific PAK1 inhibitor IPA3 (Figure 5.26).

As stated before, AMPK has been shown to promote the migration of normal cells and to increase the invasion of cancer cells (Frigo et al., 2011; Kanellis et al., 2006; Kim et al., 2011a; Nagata et al., 2003). Although no papers yet directly link AMPK and PAK1 in cell migration or invasion, there have been hints that the Rac1/PAK pathways may have some role to play in this process. A recent paper implicated AMPK activation in macrophages as partially mediating increased migration and phagocytosis via activation of Rac1 and PAK1/2 (Bae et al., 2011). In a 2011 study Kim et al, found AMPK activation mediated by LPA induced migration of ovarian
cancer cells. In addition to AMPK, LPA treatment also resulted in the activation of the GTPases RhoA and Rac1. However, siRNA mediated knockdown of AMPK α₁ only attenuated LPA induced RhoA activation and not that of Rac1 (Kim et al., 2011a). As can be seen from the limited evidence presented above, the effectors downstream of AMPK in the control of cell migration and invasion are not well understood. The results in this section helps to fill the gaps in our knowledge by indicating that activation of AMPK has the ability to enhance invasion of melanoma cells and that this effect is mediated through direct phosphorylation of PAK1.

In 2003, Akt was found to phosphorylate PAK1 on Ser-21 leading to activation of PAK1 and a reduction in its association with the adaptor protein Nck (Zhou et al., 2003). The same study also found that Akt phosphorylation caused release of PAK1 from focal adhesions. These effects led to stimulation of this pathway and caused an increased transwell migration (the transwell invasion assay was not used) of Rat1 cells (Zhou et al., 2003). Since AMPK also phosphorylates PAK1 on Ser-21, it is likely that PAK1/Nck binding is also affected, which could lead to relocation of PAK1 and thereby to the increased invasion of cancer cells observed.

It is clear that further study is needed to gain the full picture of all the players involved in this pathway. It would be very useful to find the sites of interaction on AMPK and PAK1 respectively and whether AMPK binding to PAK1 affects its auto-inhibition at all. The contribution of GIT1 and βPIX would in this pathway also needs to be investigated, as they tend to form a signalling complex with PAK1 (Mayhew et al., 2006).

Conclusions
In conclusion, several novel results are presented in this section, firstly, that AMPK is able to directly phosphorylate PAK1 thereby leading to activation of the enzyme. It is possible that this process acts to augment/supplement PAK1 activation mediated by Cdc42/Rac binding. Next the role of AMPK in the regulation of PAK1 signalling was investigated in both cortical neurons and melanoma cells and AMPK was shown to positively regulate both neuronal PAK1 activity and melanoma cell invasion through PAK1 signalling. Further work is going to unravel the finer details of this regulation.
6 Investigating PAK1 regulation of AMPK

6.1 Introduction

Having established in Chapter 5 that AMPK phosphorylation of PAK1 resulted in its activation, I went on to investigate if PAK1 is able to regulate AMPK activity. Like AMPK, PAK1 is also a serine/threonine protein kinase, thus whether PAK1 is able to phosphorylate AMPK was examined.

There have been no previous publications which implicates any member of the Rac/PAK signalling pathway in the regulation of AMPK. In this study, the ability of PAK1 to phosphorylate and regulate the activity of AMPK was investigated.

6.2 Results

6.2.1 PAK1 phosphorylates AMPK in vitro

PAK1 pre-activated by co-expression with Cdc42 in baculovirus was obtained from Stratech (also used in chapter 5), and the kinase dead (KD) (D157A mutant) forms of AMPK α1β1γ1 and α2β1γ1 complexes (KD-AMPK) were expressed in E. Coli using the protocol set out in chapter 2. To confirm that PAK1 is indeed active as described, it was incubated with myelin basic protein (MBP), a known PAK substrate in the presence of $^{32}$P-ATP and the radiolabelled proteins subjected to analysis using SDS-PAGE and autoradiography. As seen on Figure 6.1, the autoradiograph gave two radiolabelled bands corresponding to both PAK1 and MBP. This indicates that PAK1 is active and was capable of both auto-phosphorylation and phosphorylation of MBP.

Having established that the PAK1 was active, it was then incubated with and without KD-AMPK ($\alpha_1\beta_1\gamma_1$ and $\alpha_2\beta_1\gamma_1$) for increasing time periods in the presence of $^{32}$P-ATP. The incorporation of $^{32}$P-PO$_4$ into proteins was visualised using autoradiography. As can be seen from Figure 6.2, whereas incubation of KD-AMPK alone with $^{32}$P-ATP did not result in any detectable signal, phosphorylation of KD-AMPK with active
PAK1 caused the appearance of two radiolabelled protein bands, corresponding to PAK1 and the AMPK \( \alpha_1 \) and \( \alpha_2 \) subunits respectively. There was a marked, time dependent increase in labelling, showing PAK1 phosphorylates AMPK in a time dependent manner (Figure 6.2). In this experiment no radioactive signal was detected for AMPK \( \beta_1 \) or \( \gamma_1 \), indicating AMPK \( \alpha_1/\alpha_2 \) to be Pak1’s preferred substrate. An estimation of the stoichiometry of phosphorylation was determined from the autoradiograph. The radioactivity of the ATP was estimated by spotting serial dilutions of the \([^{32}\text{P}-\gamma]\text{ATP}\) onto filter paper and exposing this alongside the gel. Using this method the stoichiometry was estimated to be around 1.5 – 2.0 mole/mole.
Figure 6.1 Validation of PAK1 activity
Cdc42 activated PAK1 was incubated with myelin basic protein (MBP) in the presence of \([^{32}P-\gamma]ATP.\) Proteins were resolved by SDS-PAGE and visualised by staining by Coomassie (left panel) and radiolabelled products detected by autoradiography (right panel). The corresponding molecular markers are indicated on the left. Identity of the protein and radiolabelled bands are indicated on the right.
Figure 6.2 PAK1 phosphorylates kinase dead AMPK α₁ and α₂ in vitro
Activated PAK1 was incubated with [³²P-γ]ATP in the presence and absence of kinase dead (KD) AMPK α₁β₁γ₁ (left panel) or α₂β₁γ₁, (right panel) at 30 ºC for the indicated times. Samples were resolved by SDS-PAGE and visualised by staining by Coomassie (upper panel) and radiolabelled products detected by autoradiography (lower panel). The corresponding molecular markers are indicated on the left. Identity of the protein and radiolabelled bands are indicated on the right.
6.2.2 The effect of PAK1 on activity of AMPK

Having established that the α subunit of AMPK is a PAK1 substrate in vitro, the next step was to see if this phosphorylation had any effect on AMPK activity. To this end, an experiment was designed where active PAK1 was used to phosphorylate either wild type AMPK α₁β₁γ₁ or α₂β₁γ₁ complexes in the presence of ATP, which was then taken into an AMPK kinase assay.

In order to validate whether PAK1 directly phosphorylated the SAMS peptide, which is used to assay AMPK’s activity, active PAK1 was taken into SAMS assays. It can be seen from Figure 6.3, while active PAK1 was able to phosphorylate PAKtide, the phosphorylation of SAMS was virtually undetectable. The PAKtide assays gave an average specific activity of 106 nmol/min/mg, while for the SAMS assay a specific activity of 0.42 nmol/min/mg was achieved (very low CPM counts were observed, close to background levels). This makes the PAK1’s ability to phosphorylate SAMS around less than 0.5% as efficient as its ability to phosphorylate PAKtide. Thus for the purposes of on-going experiments it can be assumed that PAK1 has no capacity to phosphorylate the SAMS peptide.

Next, increasing amounts of active PAK1 (100ng - 200ng) were incubated with unactivated wild-type AMPK α₁β₁γ₁ or α₂β₁γ₁ complexes in the presence of ATP (non-radioactive) for 30 minutes. An aliquot of these mixtures were then taken into SAMS peptide assays to measure the activity of AMPK. As a positive control, CaMKKβ was co-incubated with AMPK in the presence of ATP. The results for this experiment are shown in Figure 6.4, as can be seen, incubation with CaMKKβ caused a dramatic activation of AMPK, to 420 nmol/min/mg for the α₁β₁γ₁ complex and to 555 nmol/min/mg for the α₂β₁γ₁ complex. In contrast incubation with PAK1 caused very little elevation of AMPK activity from basal/unactivated levels.

6.2.3 The effect of PAK1 on activity of pre-phosphorylated AMPK

Having established that PAK1 incubation had no significant effect on AMPK activity at basal levels, it was decided to determine it can affect the activity of active AMPK.
To this end, both AMPK $\alpha_1\beta_1\gamma_1$ and $\alpha_2\beta_1\gamma_1$ isoforms produced in *E. coli* were activated by co-incubation with CaMKK$\beta$ overnight.

To confirm that AMPK has been activated, the activities of both $\alpha_1\beta_1\gamma_1$ and $\alpha_2\beta_1\gamma_1$ complexes (100ng) which have or have not been incubated with CaMKK$\beta$ were compared. For $\alpha_1\beta_1\gamma_1$ complex, overnight CaMKK$\beta$ activation resulted in an increase in kinase activity of around 409 fold from 2.91 nmol/min/mg to 1193 nmol/min/mg. For the $\alpha_2\beta_1\gamma_1$ complex the increase was around 393 fold from 2.12 to 833 nmol/min/mg (*Figure 6.5*). Next, to determine the optimum amount of AMPK to use in the PAK1 phosphorylation assay, titrations of both AMPK complexes (12.5ng to 800ng of AMPK) were assayed with the SAMS peptide. From the results *Figure 6.6* it was decided to assay the activities of 50 ng both $\alpha_1$ and $\alpha_2$ after phosphorylation by PAK1, as this was in the linear range of activation for both isoforms of AMPK.

In a similar protocol to the previous experiment, 1000 ng of active AMPK ($\alpha_1\beta_1\gamma_1$ and $\alpha_2\beta_1\gamma_1$) was incubated for 30 minutes in the absence and presence of 200 ng of active PAK1. After this, 5% of these incubation mixtures (equivalent to 50ng of AMPK protein) was assayed for AMPK activity using the SAMS peptide. As shown in *Figure 6.7*, incubation of AMPK with active PAK1 caused a significant increase in the activity of AMPK. For the $\alpha_1\beta_1\gamma_1$ complex, PAK1 caused a 4.3 fold increase in AMPK activity, while for AMPK $\alpha_2\beta_1\gamma_1$ a 4.1 fold increase was seen. Two negative control experiments were also conducted. Firstly, co-incubation of kinase dead PAK1 with activated AMPK did not cause any further increase in AMPK activity. Secondly, co-incubation of active PAK1 with CaMKK$\beta$ phosphorylated kinase dead AMPK did not give significant activity when assayed with SAMS peptide (data not shown).

Taken together, these results presented above show that while PAK1 was unable to alter the activity of non-activated AMPK, it is able to significantly increase the activity of AMPK which has been pre-phosphorylated by CaMKK.
Figure 6.3 Active PAK1 does not phosphorylate the SAMS peptide
Activated PAK1 (100 ng) was incubated with $[^{32}\text{P}}\gamma\text{ATP}$ in the presence of either PAKtide or SAMS peptide for 30 minutes at 30 ºC, and activities determined. Activities have been calculated and plotted as specific activity (nmol/min/mg). Data shown represent averages and standard errors (SEM) from 3 independent experiments.

Figure 6.4 PAK1 does not activate non-activated AMPK in vitro
Activated PAK1 (100 ng or 200 ng) or CAMKKβ was incubated in the presence of either AMPK $\alpha_1\beta_1\gamma_1$ or $\alpha_2\beta_1\gamma_1$ (1000 ng) at 30 ºC for 30 minutes with ATP. After phosphorylation an aliquot of the reaction was taken and AMPK activity determined. Activities have been calculated and plotted as specific activity (nmol/min/mg). Data shown represent averages and standard errors (SEM) from 3 independent experiments.
Figure 6.5 CaMKKβ activation of AMPK $\alpha_1\beta_1\gamma_1$ or $\alpha_2\beta_1\gamma_1$ complexes
CaMKKβ was incubated in the presence of $[^{32}\text{P}]\gamma\text{ATP}$ with $\alpha_1\beta_1\gamma_1$ or $\alpha_2\beta_1\gamma_1$ overnight at room temperature. An aliquot (50 ng) of these reactions was used in a SAMS assays. Activities are plotted as specific activity (nmol/min/mg).

Figure 6.6 Titration of AMPK in the SAMS peptide assay
Increasing amounts of CAMKKβ activated AMPK $\alpha_1\beta_1\gamma_1$ or $\alpha_2\beta_1\gamma_1$ (12.5 ng – 800 ng) were assayed using the SAMS peptide. Activities are plotted as (background corrected) counts per minute against nanograms of AMPK used.
Figure 6.7 PAK1 causes further activation of CaMKKβ phosphorylated AMPK

CaMKKβ phosphorylated AMPK α₁β₁γ₁ or α₂β₁γ₁ (1000 ng) was incubated in the presence and absence of activated PAK1 (200 ng) at 30 ºC for 30 minutes with ATP. After phosphorylation a aliquot was taken into a AMPK activity assay (50 ng AMPK). Data shown represent averages and standard errors (SEM) from 3 independent experiments. * Indicates a significant increase in AMPK activation between the assays performed in the presence and absence of PAK1 as judged by student’s t-tests (P<0.05).
6.2.4 PAK1 phosphorylation sites on AMPK α

Having determined that PAK1 further activated CaMKKβ phosphorylated AMPK, the next step was to determine the mechanism that underlies this activation. As seen from Figure 6.2, AMPK is phosphorylated by PAK1 on the α subunits. It is logical to suggest that this phosphorylation of AMPK is linked to its activation.

The effect of PAK1 on complexes containing either AMPK α₁ or α₂ subunits were similar, thus it was decided to focus future experiments on complexes containing the α₁β₁γ₁ subunits only. In order to identify the specific sites on AMPK phosphorylated by PAK1, KD-AMPK α₁β₁γ₁ complex was incubated in the presence and absence of active PAK1 with ATP. After phosphorylation of AMPK, the mixture was resolved SDS-PAGE and Coomassie stained to visualise the proteins. The band corresponding to AMPK α₁ was excised from the gel and subjected to tandem mass spectrometry analysis to identify phosphorylated peptides.

The mass spectrometric analyses achieved sequence coverages of 77% and 81% for AMPK α₁, two phospho-peptides were identified which only appeared following phosphorylation by PAK1. The first peptide identified is SQSRPNDIMAEVCR. The PAK1 phosphorylation site is either the serine residue at position 1 or position 3 in this peptide (highlighted in red) which corresponds to Ser-403 or Ser-405 in rat AMPK α₁ used. Unfortunately, the mass spec analysis did not provide enough information to resolve the exact location of the phospho-residue. The second phosphorylated residue corresponds to Thr-377 on AMPK α₁. Thr-377 was previously identified as an AMPK auto-phosphorylation site (Xiao et al., 2011). Of note, Thr-377 was only phosphorylated after PAK1 incubation of 30 minutes, whereas the Ser-403/Ser-405 site was phosphorylated after a 15 minute co-incubation. The sequence coverages with the phospho-sites highlighted are shown in Figure 6.8.

As KD-AMPK was used in this experiment, the phosphorylation sites identified should not be as a result of AMPK auto-phosphorylation.
AMPK Alone
Sequence coverage 76%

1  MATAEQRHID GRVKIGHYL GDTLGVOTFG RRVGHRHETL GRHVAVKILN RQRILSDVY
121 LPPQLSGVD YCHLMVVRQR DVRFENVLAD AIMNARKIAADF GLSNMMSDGE PLRTSCSGPN
181 YAAPEVISOR LYAGPEVDWI SSGVILYALL CGTLFEDDDH VPTLKFKICD GIFYTPQYLN
241 PSVIIKLMH LQVDPMPKAT IRDIREHEWF RQDLPRYLFP EDFYSSTEMI DDEALKEVEC
301 RFECSEEEVL SCLYRNEQD PLAVHLLII DRNRRIMNEAK DFYLATSPFD SFLDDHHLTR
361 PEPERVPLV AETPRARHFL DELNPQKSSK QCVRKRKWHL GIRSQQRPND TMAEV CraIHK
421 QLQYEWKVRN PYYLLRVRKRN PVSTFSYKMS LQILYQVDSRT YLLDPRSSDD EITEAKSGTA
481 TPQRSGSISN YRSCQRSDSD AEAOGKPSEV SLTSSVTSLD SSEPVDAEPR GSHTIEEFFEM
541 CANLIKILAQ

PAK1+AMPK
Sequence coverage 82%

1  MATAEQRHID GRVKIGHYL GDTLGVOTFG RRVGHRHETL GRHVAVKILN RQRILSDVY
121 LPPQLSGVD YCHLMVVRQR DVRFENVLAD AIMNARKIAADF GLSNMMSDGE PLRTSCSGPN
181 YAAPEVISOR LYAGPEVDWI SSGVILYALL CGTLFEDDDH VPTLKFKICD GIFYTPQYLN
241 PSVIIKLMH LQVDPMPKAT IRDIREHEWF RQDLPRYLFP EDFYSSTEMI DDEALKEVEC
301 RFECSEEEVL SCLYRNEQD PLAVHLLII DRNRRIMNEAK DFYLATSPFD SFLDDHHLTR
361 PEPERVPLV AETPRARHFL DELNPQKSSK QCVRKRKWHL GIRSQQRPND TMAEV CraIHK
421 QLQYEWKVRN PYYLLRVRKRN PVSTFSYKMS LQILYQVDSRT YLLDPRSSDD EITEAKSGTA
481 TPQRSGSISN YRSCQRSDSD AEAOGKPSEV SLTSSVTSLD SSEPVDAEPR GSHTIEEFFEM
541 CANLIKILAQ

Figure 6.8 Phospho-peptide mass spectrometry analysis of PAK1 phosphorylated AMPK
Kinase dead (D157A) AMPK α₁β₁γ₁ was incubated in the absence and presence of active PAK1 at 30 °C for 15/30 minutes with ATP. The proteins were resolved using SDS-PAGE and detected using Coomassie staining. The band corresponding to AMPK α₁ was subjected to Tandem mass spectrometry analysis (CSC proteomics unit). The sequence coverage is indicated in red with identified phosphopeptide shown in green.
6.2.5 Mutation of PAK1 phosphorylation sites in AMPK

In order to confirm that the sites identified on AMPK α₁ are indeed PAK1 phosphorylation sites, and to see if this phosphorylation mediates PAK1’s effect on AMPK activity. These residues are replaced by the non-phosphorylatable alanine amino acids. Since the specific site which PAK1 phosphorylates in the SQSRPNDIMAEVCR peptide is ambiguous, a double amino acid mutation was generated where both serine(s) at positions 1 and 3 (highlighted in red) are converted into non-phosphorylatable alanines (i.e. SQS-AQA).

To produce the mutant AMPK α₁ described above, the corresponding S403A/S405A double mutant (to be called the AQA mutant), and T377A mutant were introduced into the tricistronic vectors containing either WT or KD version of the AMPK α₁β₁γ₁ complex using specific primers (see Appendix 2). All mutations were generated using site-directed mutagenesis methods and confirmed by DNA sequencing. After expressing, these proteins are analysed using SDS-PAGE and shown in Figure 6.9.

6.2.6 Phosphorylation of AMPK mutants by PAK1

To determine the ability of PAK1 to phosphorylate AMPK α₁ at the sites identified above, active PAK1 was incubated in the presence of ³²P-ATP with various mutants of AMPK (α₁β₁γ₁ complex), i.e. KD-AMPK, KD-AQA-AMPK and KD-377A-AMPK. The incorporation of ³²P into proteins were visualised using autoradiography. As can be seen in Figure 6.10, PAK1 was able to phosphorylate KD-AMPK to a much greater extent compared to KD-AQA-AMPK and KD-377A-AMPK. It appears the ability of PAK1 to phosphorylate the AQA and T377A mutants was reduced to a similar extent, although quantitative analysis of the radio-labelled bands for AMPK α₁ shows that ³²P-PO₄ incorporation into the AQA mutant is slightly less (around 20% less) than that into the T377A mutant (data not shown).
6.2.7 The effect of PAK1 phosphorylation on the activity of mutant AMPK

To test if these mutants have any effect on the ability of PAK1 to further activate pre-phosphorylated AMPK. CaMKKβ phosphorylated AMPK complexes were used $\left(\alpha_\beta_1\gamma_1\right)$. Figure 6.11 shows results of the activity of the different AMPK complexes. All three forms of AMPK are able to be activated by CaMKKβ, with WT-AMPK activity of 880 nmol/min/mg, AQA-AMPK activity of 530 nmol/min/mg and T377A-AMPK activity of 1090 nmol/min/mg.

The experiment to determine the effect of PAK1 phosphorylation of activity of these AMPK mutants was conducted in the same way as in section 6.2.3, active PAK1 was incubated with $\alpha$ of the WT and mutant forms of AMPK in the presence of ATP, with a fraction (5%) of the mixture then taken into the SAMS peptide assay.

The results from this experiment are shown in Figure 6.12. Of note, the degree of activation of WT-AMPK in this experiment is much greater than recorded in the previous experiment (Figure 6.7), this is perhaps due the fact that a new batch of active PAK1 was used with a much greater specific activity compared to that used previous experiments (Data not shown). Phosphorylation by PAK1 induced a 10.3 fold activation of AMPK, while for the S403A/S405A and T377A mutants the activation was much more modest 1.5 fold and 2.9 fold respectively.

The data shown above would suggest that it is the phosphorylation of AMPK by PAK1 on the residues in the $\alpha$ subunit that lead to its activation.

6.2.8 Expression of the AMPK AAA triple mutant and its effects on PAK1 induced phosphorylation

Although both the AQA and the T377A mutants were much less able to be phosphorylated and activated by PAK1, the activation/phosphorylation was not entirely abolished. In order to see if the phosphorylation of AMPK can be completely abolished, a T377A/S403A/S405A triple mutant (from here on referred to as the AAA Triple mutant) was created (by inserting the T377A mutation into the vectors
containing the AQA mutation). All three mutations were verified as present using DNA sequencing (Data not shown).

The triple mutant in the KD background was incubated with and without of PAK1 (Figure 6.13). In this experiment the AQA and T377A mutants were also included for comparison. KD-AMPK is a much better substrate for PAK1 compared all three mutants. PAK1 phosphorylation of AMPK was almost completely abolished in the KD-triple mutant (Figure 6.13), thereby indicating that the Ser-403/405 and Thr-377 are the predominant PAK1 phosphorylation sites in AMPK.

In can be seen that in these experiments, PAK1 seems to phosphorylate the T377A mutant to a greater extent compared to the AQA mutant, although $^{32}$P-ATP incorporation into both mutants is still much less than that of KD-AMPK. This result is somewhat different to that shown in Figure 6.10, where the ability of PAK1 phosphorylation of the T377A mutant was only slightly greater than the AQA mutant. In addition, with PAK1 incubation, there is also a small amount of phosphorylation of AMPK β/γ subunits. Due to the proximity of the β1 and γ1 bands, we were unable to distinguish which subunit this phosphorylation corresponds to.

After determining that the T377A/S403A/S405A triple mutant essentially abolished the ability PAK1’s ability to phosphorylate AMPK, experiments were planned to see what its effects are on PAK1 induced activation. These experiments are currently in progress.
Figure 6.9 Expression of mutant AMPK complexes

Wild type (WT) or kinase dead (KD) AMPK α1β1γ1 complexes with or without α subunit point mutations were expressed in *E. Coli* and purified by nickel-sephrose chromatography and gel filtration. Proteins were resolved using SDS-PAGE and Coomassie staining. The migration of the molecular weight markers are indicated on the left and the identity of the proteins are indicated on the right.
Figure 6.10 PAK1 phosphorylation of KD AMPK mutants
KD-AMPK $\alpha_1\beta_1\gamma_1$complexes harbouring the AQA and T377A was incubated with $[^{32}\text{P}}\gamma$ATP in the presence and absence PAK1 (200 ng), at 30 °C for 30 minutes. Samples were resolved by SDS-PAGE and visualised by Coomassie staining (upper panel) and radiolabelled products detected by autoradiography (lower panel). The corresponding molecular markers are indicated on the left. Identity of the protein and radiolabelled bands are indicated on the right.
Figure 6.11 CaMKKβ activation of AMPK complexes
WT AMPK α₁β₁γ₁ complex or complexes harbouring the AQA and T377A mutations was incubated with CaMKKβ overnight at room temperature in the presence of ATP. An aliquot (50 ng) of these reactions was used in a SAMS assays. Activities are plotted as specific activity (nmol/min/mg). Data shown represent averages and standard errors (SEM) from 4 independent experiments.

Figure 6.12 PAK1 activation of pre-phosphorylated WT and mutant AMPK
CaMKKβ phosphorylated WT or mutant (AQA and T377A) AMPK α₁β₁γ₁ complexes were incubated in the absence and presence of PAK1 (200 ng) at 30 °C for 30 minutes with ATP. After phosphorylation an aliquot of this reaction was taken into an AMPK activity assay. Data shown represent averages and standard errors (SEM) from 3 independent experiments.
* Indicates a significant change in activity from WT (P<0.05) as judges by one way ANOVA.
**Figure 6.13 PAK1 phosphorylation of KD AMPK mutants**

KD-AMPK αιβγ complex or complexes harbouring the AQA (S403A+S405A), T377A, and AAA (T377A+ S403A+S405A) mutations were incubated with $[^{32}P-\gamma]ATP$ in the presence and absence of PAK1 for 30 min at 30 ºC. Samples were resolved by SDS-PAGE and visualised by staining by Coomassie (upper panel) and radiolabelled products detected by autoradiography (lower panel). The corresponding molecular markers are indicated on the left. Identity of the protein and radiolabelled bands are indicated on the right. Representative image from 2 independent experiments.
6.3 Discussion:

In chapter 5, the effects of AMPK phosphorylation of PAK1 were investigated, and it was concluded that AMPK activates PAK1 and mediates its downstream effects. In the current chapter, the goal was to investigate any potential effects PAK1 might have on AMPK. Like AMPK, PAK1 is also a protein kinase and exerts its effects through phosphorylation of downstream targets. Consequently, it is possible for PAK1 to regulate AMPK through direct phosphorylation.

Currently there is no evidence to suggest any of the PAK family members regulates any component of the AMPK signalling pathway or any AMPK related kinase. The work presented in this chapter shows for the first time that PAK1 is able to phosphorylate AMPK at two sites and causes further activation of CaMKKβ phosphorylated AMPK.

**PAK1 phosphorylation of AMPK**

Like many other protein kinases, AMPK has an obligate requirement for phosphorylation on a Threonine residue in the activation loop in the kinase domain for its activation (Hawley et al., 1996). Three upstream kinases have been suggested to phosphorylate AMPK at this residue. The two established AMPK upstream kinases are LKB1 and CaMKKβ, both of which were identified as AMPK regulators around 10 years ago (Hawley et al., 2003; Shaw et al., 2004; Woods et al., 2005; Woods et al., 2003a). Since then the only new AMPK kinase that has been suggested was the TAK1. Indeed the role of this enzyme in AMPK regulation is still unclear (Steinberg and Kemp, 2009). In the current study, PAK1 is shown to phosphorylate the α subunit of AMPK on sites other than the Thr-172 residue and enhancing its activity as a result.

In addition to determining the ability of PAK1 to phosphorylate AMPK, it was critical to determine how this effects AMPK function. Phosphorylation of AMPK with active PAK1 had no significant effect on AMPK activity (Figure 6.4). This is logical, as from the phosphorylation data, it can be seen that PAK1 does not phosphorylate AMPK in the Thr-172 residue, which is absolutely required for activity of the AMPK molecule (Hawley et al., 1996). Therefore, AMPK will have to be pre-phosphorylated...
by its upstream kinases before any stimulatory effects of PAK1 phosphorylation on it can be seen.

Additional phosphorylation sites have been shown to be able to further modulate AMPK activity secondary to Thr-172 phosphorylation (Steinberg and Kemp, 2009). Mitchelhill et al., were the first to identify α1 Ser-485 as being phosphorylated in rat livers, and this was confirmed by experiments conducted with bacterially expressed AMPK (Mitchelhill et al., 1997; Woods et al., 2003b). More recently, the α1 Ser-485 site has been suggested to be involved in cAMP mediated AMPK inhibition and Ser485 phosphorylation of α1 was necessary for forskolin to inhibit AMPK activity (Hurley et al., 2006). In another study, Akt has been shown to directly phosphorylate α1 Ser-485 as part of the insulin signalling cascade which leads to reduced Thr-172 phosphorylation by LKB1 (Horman et al., 2006). Recently, p70S6 kinase has been shown to phosphorylate hypothalamic AMPK α2 on Ser-491 (which corresponds to α1 Ser-485) and thereby inhibiting its activity, leading to reduced food intake (Dagon et al., 2012). Although the effects of Ser-485 phosphorylation have been the subject of numerous studies, how the signal is transmitted from the C-terminus (where the Ser-485 sites lies) of α1 to the catalytic domain is unknown. In addition to Ser-485, studies have shown that PKA mediated phosphorylation at Ser-173 reduces the ability of LKB1 to phosphorylate Thr-172, thereby leading to reduced AMPK activity (Djouder et al., 2010). Additionally, Thr259, Thr-373, Thr-379, Thr-481, Ser-499, Ser-514, Ser-515 and Thr-517 have also been identified as AMPK α1 phosphorylation sites, the physiological roles of most of the sites identified remains unexplored (Horman et al., 2006; Hurley et al., 2006; Steinberg and Kemp, 2009; Villen et al., 2007; Woods et al., 2003b). It is significant that many of the additional phosphorylation sites identified in AMPK α subunit are close to the C terminus, which is involved in binding to other AMPK subunits.

Thus, in the next stage of this study, the ability of PAK1 to modulate the activity of activated AMPK was investigated. While PAK1 was unable to directly activate AMPK in the basal/un-activated state, it was able to cause further activation by >4-5 fold of both AMPK α1β1γ1 and α2β1γ1 which were pre-phosphorylated by CaMKKβ. This suggests that the PAK1 phosphorylation sites on AMPK are secondary regulatory sites which are able to further modulate AMPK activity. Of the three
residues identified as potential PAK1 phosphorylation sites, T377 is not present in the AMPK α₂ isoform. Serine residues equivalent to S403 and S405 (in α₁) are present in α₂. However, inspection of the crystal structures of truncated AMPK α₁β₂γ₁ and full-length α₂β₁γ₁ do not reveal any obvious mechanistic insight into how these sites might affect AMPK activity. (Xiao et al., 2011).

The underlying mechanism of AMPK activation by PAK1

The most likely method by which PAK1 activates AMPK is through direct phosphorylation. To identify the specific residues within the AMPK α subunit which PAK1 phosphorylated, tandem mass spectrometry analysis was performed on AMPK α₁ subunit protein after PAK1 phosphorylation. The time and resource pressures of this project prohibited the analysis of PAK1 phosphorylation sites on AMPK α₂. As there was little difference between the ability of PAK1 to phosphorylate and further activate AMPK α₁ or α₂, perhaps there is little difference in the regulation of the two subunits by PAK1.

Mass spectrometry analysis showed PAK1 phosphorylated AMPK α₁ on two sites. From the data, it was seen that the Ser-403/Ser-405 site (mass spec analysis could not resolve the specific site of phosphorylation) was phosphorylated after 15 minutes incubation with PAK1 while the Thr-377 site did not become phosphorylated until after 30 minutes. This result would suggest that the Ser-403/Ser-405 site is the preferential site for PAK1 compared to Thr-377 and may a more significant role in regulating the activity of AMPK.

According to current knowledge, the phosphorylation of Ser-403/Ser-405 identified in this study has not been shown as a phosphorylation site of AMPK in previous studies. AMPK Thr-377 (α₁) on the other hand, have been shown to be phosphorylated in vivo, in both insect cells and mouse liver (Steinberg and Kemp, 2009; Villen et al., 2007). Although it was suggested to be an auto-phosphorylation site of AMPK, the researchers did not explore the function or physiological implication of phosphorylation at this site.
As can be seen in Figure 6.10, compared to PAK1’s phosphorylation of KD-AMPK α1, phosphorylation of both the AQA and the T377A mutants was much reduced. This data suggests that both Ser-403/Ser-405 and the Thr-377 identified by mass spectrometry analysis are indeed phosphorylated by PAK1. Additionally, from the data it appears that PAK1 showed some preference towards Ser-403/Ser-405 site compared to the Thr-377 site. This inference was born out in later experiment where PAK1 phosphorylation of above two AMPK mutants and the triple mutant was compared to KD-AMPK phosphorylation (Figure 6.13). In this experiment, which was performed with a new batch of commercial PAK1 protein which was more active than the batch used for previous experiments, showed that although the phosphorylation of both AQA and T377A mutants were reduced compared to KD-AMPK, there is clearly more substantial T377A phosphorylation compared to AQA. Importantly, PAK1 phosphorylation of AMPK was almost completely abolished in triple mutant, this result showed that these sites corresponds to the major PAK1 phosphorylation sites in AMPK.

The data generated from SAMS assays showed that both AQA and T377A mutations were able to greatly attenuate the ability of PAK1 to enhance the activity of pre-phosphorylated AMPK (Figure 6.12). Taken together, PAK1’s ability to phosphorylate and activate AMPK suggests that this enzyme may be a novel upstream kinase for AMPK. In addition, this study is the first to show the activity of Thr-172 phosphorylated AMPK can be further enhanced by phosphorylation at additional sites. This hints at an additional layer of AMPK regulation previously unknown and opens the possibility that activation of AMPK is not all or nothing but in fact consists of several states of increasing activation modulated by different upstream kinases.

Further experiments are needed to validate these observations in a more physiological in vivo system, this work is currently on going. Experiments are currently being conducted to determine if PAK1 activation of AMPK can be completely abolished in the AAA mutant.

**Potential relevance of PAK activation of AMPK in physiology and pathology**

This is the first study which establishes direct phosphorylation of AMPK by PAK1 and the enhancement in AMPK activation that results from this. With further research,
the finer details of this activation and its physiological roles should become clear. At this stage, we are nevertheless able to make some logical inferences about the functional relevance of this activation, and points in normal physiology and pathology where the paths of these two enzymes could converge.

Both AMPK and PAK1 are protein kinases, with AMPK mainly involved in the control of cellular energy state and PAK1 involved in the regulation of actin organisation, cell polarity and cell migration. In recent years, new evidence has emerged which may help us link the function of these two enzymes.

Some of the most important roles that PAK1 plays is the regulation of cytoskeletal dynamics, cell polarity and motility (Bokoch, 2003; Kreis and Barnier, 2009). In recent years, AMPK has also been shown to be involved in these processes. AMPK activity has been found to be important for maintaining normal cell polarity especially under conditions of energy stress (Brenman, 2007). In a drosophila model system where AMPK activity was abolished, severe abnormalities in cell polarity was seen, and in epithelial cells were seen to lose their normal cell polarity upon the expression either LKB1 or AMPK mutants (Lee et al., 2007; Mirouse et al., 2007). In canine kidney cells, treated with the AMPK activator A769662 caused shortening of actin stress fibres which led to cytoskeletal reorganisation (Miranda et al., 2010).

PAK1 has also been implicated as an important regulator of the function of myosin regulatory light chain (MLC), a component of both myosin II (Park et al., 2011). Expression of constitutively active PAK1 in NIH-3T3 cells caused increased MLC phosphorylation (Sells et al., 1999). PAK1 has also been suggested to exert an inhibitory effect on myosin phosphatase targeting protein 1 (MYPT1) which leads to increased MLC phosphorylation (Takizawa et al., 2002). Indeed, PAK1 has been shown to directly phosphorylate MLC in in-vitro preparations (Chew et al., 1998). The field is controversial however, with other studies implicating PAK1 as having an inhibitory effect on MLC phosphorylation through an inhibitory phosphorylation of MLCK (Sanders et al., 1999; Wirth et al., 2003). A recently publication has put forward an explanation for this apparent discrepancy, by suggesting that while PAK1 enhances MLC phosphorylation under physiological conditions, it acts to inhibit its actions under pathological situations (Chu et al., 2013). Interestingly, AMPK has also
been shown to regulate MLC. Activation of AMPK increased the phosphorylation of cofillin and myosin light chain. Additionally, AMPK has also been shown to directly phosphorylate MLC on the key regulation site at Ser-19 (Lee et al., 2007; Miranda et al., 2010). Taken together the regulation of MLC phosphorylation could present another point of convergence of PAK1 and AMPK. It is possible post PAK1 activation, AMPK acts as the effector protein to regulate in MLC phosphorylation. This could be achieved either through direct phosphorylation or indirectly by either activation of MLCK or the inhibition of MYPT (Bultot et al., 2009).

As discussed in Chapter1, activation of PAK1 has been shown to promote tumour cell survival through multiple signalling pathways (Eswaran et al., 2012b). It is logical to suggest that in these tumour cells, especially within large solid tumours, have relatively low energy stores which will make preserving adequate cellular ATP levels a priority; it is then rational to assume that activation of AMPK will be helpful in circumstances in maintaining survival of cells. Traditionally, activation of AMPK was seen to restrain cancer cell growth through the inhibition of protein and fatty acid synthesis. However, more recently, AMPK has been suggested to have a pro-tumourogenic role through the promotion of cell survival under states of energy stress. For example, in prostate cancer cells, inhibition of AMPK activity/expression of AMPK led to decreased cancer cell proliferation and increased cell death (Park et al., 2009). In another study, expression of the AMPK β1 subunit was found to be essential for tumour cell survival (Ros et al., 2012). Furthermore, in brain tumour models, AMPK has been found to be highly activated during the early stages of tumourgenesis, and AMPK α1α2 double knockout MEFs have a severely impaired ability to form tumours in vivo (Jang et al., 2011; Laderoute et al., 2006). From the above evidence, it can be seen that a potential link between PAK1 and AMPK exists in cancer, whereby the ability of PAK1 to further activate AMPK in energy deprived cancer cells could help it mediate effects in the enhancement of cancer cell survival.

Another major role PAK1 plays in cancer formation is its ability to promote the migration and invasion of cancer cells (Vadlamudi et al., 2000). Likewise, in recent years, AMPK activation has been shown to enhance the migration and invasion and cancer cells (Frigo et al., 2011; Kim et al., 2011a). This could provide another
potential linking point for these two enzymes, where part of the effect of PAK1 on cell migration and cancer cell invasion is mediated through AMPK.

**Conclusions**

The results presented here have are novel and suggest a novel role for PAK1 in the regulation of AMPK activity. Further studies are needed to clarify the mechanistic details of this activation and its physiological implications. We are currently in the process of planning/conducting experiments in model cell-lines to see if activation of PAK1 results to stimulation of AMPK activities.
7 Conclusions and Future perspectives

As a central regulator of the cellular energy state, AMPK plays both positive and negative roles in a wide range of disease states. AMPK has emerged as a potential drug target for conditions such as type 2 diabetes, cardiovascular diseases and cancer (Steinberg and Kemp, 2009). Gaining a fuller understanding of both the regulation of AMPK activity and its downstream effects will aid the design of new treatments which modulate this important enzyme.

In Chapter 3, experiments were conducted with the aim of investigating the regulation of AMPK activity by protein phosphatase and to identify the specific phosphatase(s) which act to dephosphorylate AMPK on the critical Thr-172 residue. HEK293 cells identified as the cell-line of choice due to its relative high level of AMPK expression and the ability of endogenous AMPK activity to be dynamically modulated by pharmacological activators and manipulation of expression (e.g. of upstream kinases). Primary mouse hepatocytes were also used for selected experiments to provide a more physiological counterpart to HEK293 cells.

Both pharmacological inhibition of phosphatase and siRNA mediated knockdown were used to identify candidate AMPK phosphatases. Knockdown of individual PPM phosphatases was able to significantly decrease the expression of these proteins in HEK293 cells. However this did not lead to significant elevation of AMPK activity either at basal levels or pre-activated by phenformin. Treatment of both HEK293 cells and primary hepatocytes with the phosphatase inhibitor Calyculin A caused significant elevations in AMPK activity. These results suggest that the AMPK phosphatase lies within the PPP family, and judging by the concentrations of inhibitor used, the candidate is more likely to be PP1 than PP2A. Knockdown of individual or combinations of PPP enzymes, PP1, PP2A, PP5 and PP6 did not produce positive results. Several inferences can be drawn from the results shown in this chapter. The discrepancy between the results from inhibitor studies and those obtained from siRNA knockdown suggest two things, either a very low level of phosphatase activity/expression is required to maintain AMPK activity under basal and activated conditions, or that AMPK is regulated by a number of phosphatase simultaneously,
and inhibition of all or most of these are required to generate a significant disturbance in AMPK activity. Either way, there does seem to be a substantial level of reserve/redundancy built into the system. This is not overly surprising, due to the wide ranging effects of AMPK, its activity needs to be tightly controlled when cells are not undergoing stressful conditions. Having a large pool of AMPK phosphatases in reserve to prevent aberrant activation of AMPK at basal levels may be helpful.

While knockdown of PPM phosphatases did not produce any positive results, it does not mean that these phosphatase are not involved in AMPK regulation. Future studies are likely to greatly benefit from new pharmacological inhibitors of these phosphatases, the use of which will hopefully give us a definitive answer as to their involvement in AMPK dephosphorylation.

Results from recent studies have suggested that different phosphatases maybe involved the dephosphorylation of AMPK under different conditions. For example, the PP1-R6 complex have been suggested to inactivate AMPK in response to increase glucose levels in the MIN6 pancreatic β cells. On the other hand siRNA knockdown of PP2A catalytic subunit was shown to be able to reverse heat shock induced AMPK Thr-172 dephosphorylation in HepG2 cells (Garcia-Haro et al., 2010; Wang et al., 2010). With this in mind, coupled with the indications drawn from the results of this study, it is perhaps wise for future studies to focus on specific conditions of cellular stress in relevant cell-types, instead of trying to identify a ‘master’ AMPK phosphatase.

Results from this and other studies have indicated PPP family members to be involved in AMPK dephosphorylation. As described in chapter 1, PPP family phosphatases such as PP1 and PP2A are mostly multimeric enzymes, where a limited number of catalytic subunits isoforms combine with a much larger number of regulatory subunit isoforms to confer substrate and conditional specificity. It is therefore possible that a particular PPP phosphatase could combine with different regulatory subunits to exert control on AMPK activity under different conditions. For example, the regulatory subunit that helps regulate AMPK activity under basal conditions could be replaced with another when cells undergo stressful conditions, which would help fine-tune AMPK activity under those conditions. Future experiments could perhaps help shed
more light on this process by identifying the full range of regulatory subunits which assists in AMPK dephosphorylation under different conditions and in different tissue-types.

In Chapter 4, SILAC coupled with quantitative mass spectrometry was utilised to identify novel AMPK interacting proteins. To my knowledge, this is the first time that such a technique has been used for AMPK. Over 60 potential AMPK interacting proteins were identified, which fitted with our selection protocols, of which the PAK1/GIT1/βPIX complex was chosen for further investigation. However, the list of interactors generated contains other interesting candidates, such as ASK1 and MAP3K15, and future work can be carried out to investigate the physiological significance of these interactions.

Following the successful application this SILAC/mass spectrometry in HEK293 cells, other members of our group have begun utilising this technique to screen for AMPK interacting proteins in more physiological cell types such as primary cortical neurons. In future experiments perhaps more cell-types can be included in these studies. In addition to identifying novel interaction proteins, any variations in the AMPK interacome in different cells would also be revealed by this method.

In Chapter 5, the functional relevance of AMPK’s interaction with PAK1 was investigated. AMPK was found to directly phosphorylate PAK1 on the Ser-21 which activated the enzyme independent of GTPase binding. Furthermore, it was shown that activation of AMPK in number of cell-lines (including HEK293 cells, primary cortical neurons and melanoma cells) led to increased PAK1 phosphorylation/activity. Importantly, PAK1 phosphorylation and activity was found to be reduced in AMPK α₁ knockout neurons which serves to back up the results obtained using pharmacological agents. As PAK1 has been shown to be involved in neuronal branching and dendrite formation, it is likely that such as sustained reduction in PAK1 activity will have implications on these structures in the developing brains of AMPK α₁ knockout mice. Although, due to technical difficulties, my experiments were not able to pinpoint the nature of these structural disturbances. Future experiments could follow on this work to identify if there are any differences in the post-synaptic structures of wild-type and AMPK α₁ knockout mice.
Interestingly, the Ser-21 site has also previously been identified as an Akt phosphorylation site, which has also been demonstrated to activate PAK1 (Zhou et al., 2003). Phosphorylation of this site was reported to reduce binding of PAK1 to Nck which led to increases in cell migration. In the current study, AMPK activation of PAK1 in melanoma cell-lines led to increases in the invasion of these cells. These data suggest that both AMPK and Akt are able to increase cell motility by the similar mechanisms. In the current study, time constraints meant that the effects of AMPK activation on PAK1 interaction with Nck and other PAK1 binding proteins were not investigated, and this could form a part of a future study.

Recently, AMPK has been implicated in the regulation of cell migration and invasion. The results of this study shows that activation of AMPK leads to increased invasion of melanoma cancer cells through PAK1 signalling. Our group is currently in the process of validating the results shown in chapter 5 by repeating invasion experiments with M991 (as opposed to A769662), a new and more potent specific activator of AMPK. Additional studies aimed at verifying these results with an additional PAK1 inhibitor, such as the PF-3758309 compound used by Ong et al to investigate melanoma tumour signalling (Ong et al., 2013) are currently being planned. RNAi mediated knockdown of AMPK and PAK1 can also be used to validate the proposed pathway. Although at this point the involvement of Rac1 in stimulating cancer cell invasion after AMPK activation cannot be excluded, we plan to conduct experiments using specific Rac1 inhibitors (e.g. the NSC23766 compound) to reveal these effects.

In the future, the downstream effects of this novel signalling pathway on cell migration/invasion could be tested in other cancer cells as well as normal non-neoplastic cell-lines. This signalling cascade could contain attractive drug targets, the modulation of which can be used to reduce the invasive and metastatic ability of cancer cells. We await future experiments to shed more light on this possibility.

As PAK1 is also a serine/threonine kinase, the possibility that it may be capable of phosphorylating AMPK was investigated in Chapter 6. PAK1 was able to directly phosphorylate the AMPK α subunit on the Thr-377 and Ser-403/405 residues. This phosphorylation did not result in activation of non-activated AMPK. This is not
surprising as PAK1 did not phosphorylate the critical Thr-172 residue, which is absolutely required for AMPK activity. However, PAK1 was able to significantly increase the activity of CaMKKβ phosphorylated AMPK by over four fold. Mutation of the PAK1 phosphorylation sites on AMPK α₁ to alanine abolished this activation.

To our knowledge, this study presents the first report that phosphorylation of additional sites on AMPK is able to further increase the activity of Thr-172 phosphorylated AMPK. This opens up the possibility that there may be different grades of AMPK activation and in some states active AMPK requires further stimulation by PAK1 before exerting its downstream effects. There may be other members of this ‘second layer’ upstream kinase family which are able to regulate the activity of Thr-172 phosphorylated AMPK.

We are currently in the process of planning/conducting experiments to further validate the PAK1’s effects on AMPK. The next step would be to use cellular models in order to reveal the physiological implications of this regulation. RNAi techniques coupled with pharmacological agents will be used to manipulate PAK1 activity/expression with the resulting effects on AMPK analysed. By phosphorylating AMPK, it is possible that PAK1 has previously unknown effects on cellular metabolism. In future experiments, this possibility could be investigated by the using model cell-lines and perhaps tissue specific knock-out models where PAK1 expression is selectively silenced.

Additionally, due to time constraints, the interaction between the PAK1 associated proteins GIT1 and PIX was not fully investigated during this study. Future work could focus on these two proteins to reveal the physiological relevance of these interactions.

In conclusion, the work presented in this thesis goes someway in furthering our understanding of the regulation of AMPK and its downstream effects. Much work still needs to be conducted in order to fully reveal the implications of AMPK’s interaction with PAK1 and its associated proteins. Further experiments are currently on going.
References


Parrini, M.C., Lei, M., Harrison, S.C., and Mayer, B.J. (2002). Pak1 kinase homodimers are autoinhibited in trans and dissociated upon activation by Cdc42 and Rac1. Mol Cell 9, 73-83.


kinases and study of their roles by site-directed mutagenesis. J Biol Chem 278, 28434-28442.


Appendices

Appendix 1
Table showing the siRNA sequences used for knockdown of protein phosphatases in Chapter 3. (In some instances the siRNA sequences were not supplied).

<table>
<thead>
<tr>
<th>Target</th>
<th>Direction</th>
<th>Sequence (siRNA 1)</th>
<th>Sequence (siRNA 2)</th>
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<td>PPM1A</td>
<td>Sense</td>
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<td>GACUUUGAAGUGCAUGAUGAtt</td>
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### Appendix 1

The following oligonucleotides were used in this study to introduce mutations into various proteins.

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### Appendix 2

The following oligonucleotides were used in this study to introduce mutations into various proteins.

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<td>Reverse</td>
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<td>PAK1</td>
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<td>GAGGCTACTCTTTGTGCGGCTGATACTAAGCTCGGC</td>
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<td>PAK1</td>
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251
Appendix 3
Antibodies.
A table of the non-commercial antibodies and the antigen that they were raised against.

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<td>Rabbit</td>
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